

CHAPTER II

LITERATURE REVIEW

One of the most famous complementary and alternative medicines for health promotion and treatment has been the use of single or combined natural products or herbal therapies (Muazu and Kaita, 2008). In many countries, the folk medicine has been taken from beliefs, cultures or societies (de Fátima Agra *et al.*, 2007; Jorim *et al.*, 2012). World Health Organization (WHO, 2009) reported the medicinal plants were used for basic health care especially in developed countries about 70-80% of the time because of the history and culture of folk medicine. It is still a choice although modern medicine is more advanced (de Fátima Agra *et al.*, 2008; Liu *et al.*, 2013; Chan, 2003). Therefore, folk medicines need attention to identification, description and scientific investigation (WHO, 2009).

In Thailand, many medicinal plants have been used in daily living for a long time. They could be applied via food ingredients, juice, alcoholic drinks, and traditional medicine, but the safety has not been a concern. Many people have had no guidelines and used overdoses. They assumed these plants are safe because they are herbal and natural products. In this chapter, details of the medicinal plants, *Boesenbergia rotunda*, their properties and the toxicity test by using micronucleus assay is the focus.



Figure 1 *Boesenbergia rotunda* (L.) Mansf. (A) and their fresh rhizomes (B)

2.1 *Boesenbergia rotunda* (L.) Mansf.

Boesenbergia rotunda (L.) Mansf. or Krachai in Thai name belonging to the ginger family (Zingiberaceae) (Larsen, 1996). It is a small herbaceous plant, 60 -90 cm high, the fresh roots are cylindrical to spindle shape, 6-10 cm long, yellow to brown outside and yellow inside. The shape of rhizome resembles that of fingers growing out of a center piece, this feature has been called fingerroot and it was rhizomatous herbs. The leaves are fairly large, 10-30 cm long, 5-10 cm wide, blade or oval shape and 2-7 leaves are alternately arranged. The flowers bloom in terminal or between leaf pairs, reddish to purple in color (Wutythamaweck, 1997).

The fresh rhizomes of *Boesenbergia rotunda* have a characteristic aroma and slightly pungent taste. It is indigenous in India, Sri Lanka, Southern China and Southeast Asia (Eng-Chong *et al.*, 2012). In Thailand, the aromatic rhizome are used both as a spicy condiments in Thai food and as traditional medicine for health promotion, treatment of colic disorders, oral diseases, abdominal pain, anti-flatulent,

leucorrhea and considered to be an aphrodisiac (Wutythamaweche, 1997). In 2006, *Boesenbergia rotunda* had been specified as one component of a traditional herbal prescription of Thailand, Yapasakanplu remedies for flatulence, and was approved by National List of Essential Medicines (NLEM, 2006). Later in 2011, *Boesenbergia rotunda* was one component of Yahomthip-osot, a remedy for dizziness, and added into traditional herbal prescriptions of NLEM. Recently 2012, Yaphasomphetsangkhat remedy for hemorrhoids, Yalueadngam remedy for leucorrhoea and Yakasaisen treatment for stiff muscles were added to the list of the same National institute of Thailand and had *Boesenbergia rotunda* (NLEM, 2012). In the chemical studies, several chalcones and flavanones such as boesenbergin A, boesenbergin B, panduratin A, pinostrobin, and pinocembrin could be isolated and identified from *Boesenbergia rotunda* rhizomes (bin Jantan *et al.*, 2001).

Their several biological properties were reported such as antibacterial (Voravuthikunchai *et al.*, 2005; Tewtrakul *et al.*, 2009; Bhamarapavat *et al.*, 2006), antimutagenicity (Trakoontivakorn *et al.*, 2001; Nakahara *et al.*, 2002), anti-inflammatory (Tewtrakul *et al.*, 2009), anticancer (Kirana *et al.*, 2007), and anti-oxidant (Shindo *et al.*, 2006). Boesenbergin A, in isolation from *Boesenbergia rotunda*, was found to have cytotoxicity and induce apoptosis towards human T4-lymphoblastoid cell line (CEMss) (Ng *et al.*, 2013), induce apoptosis in human non-small cell lung cancer (A549) cells (Isa *et al.*, 2013) and may inhibit the activity of human acetylcholinesterase for treatment of neurological disorder, especially Alzheimer's disease (Abdelwahab, 2013). In animal studies, the extract of *Boesenbergia rotunda* was found to accelerate wound healing (Mahmood *et al.*, 2010) and take an anti-ulcerogenic (Abdelwahab *et al.*, 2011). The aphrodisiac enhancing

property was focused to study in male rats and was found that *Boesenbergia rotunda* extract could increase seminiferous tubules diameter and testis and seminal vesicle weights (Sudwan *et al.*, 2007). The recent study of *Boesenbergia rotunda* juice could increase progressive movement of sperm motility, normal sperm morphology and stages VII to VIII of seminiferous epithelium in mature male rats (Yotarlai *et al.*, 2011).

2.2 Studies of medicinal plants for male reproductive enhancing

Erectile dysfunction (ED), prostate disease and lower urinary tract symptoms are men's reproductive health problems and most frequently occur in older groups (Holden *et al.*, 2005; Lindau *et al.*, 2007). In particular, ED has been proposed as an indicator of men's health and was found in 10% to 20% of men in many countries (Corona *et al.*, 2009). This problem affects the quality of men's life and well being (Gonzales *et al.*, 2002). There are several ways that can improve ED such as the use of medical, psychological and pharmacological therapies (Holden *et al.*, 2005). Folk medicines in many countries are widely used for hundreds of years in food ingredients, dietary supplements and sexual enhancers for males (Chaturapanich *et al.*, 2008). Some of these plants were used to treat and relieve illness in various forms such as extractions and juices. The studies showed that they could induce the expression of sexual behavior: for example *Pedaliium murex*, *Myristica fragrans* and *Syzygium aromaticum*, *Bulbine natalensis*, Black tea brews of *Camellia sinensis*, *Fadogia agrestis*, *Phoenix dactylifer*, *Montanooa tomentosa*, *Tricholepis qlaberrima*, *Massularia acuminate*, *Anacyclus pyrethrum*, *Curculigo orchioides*, *Paederia foetida* and a crystalline ketone obtained from essential oils of *Cinnamomum camphora* or

camphor (Sharma *et al.*, 2012; Tajuddin *et al.*, 2003; Yakubu and Afolayan, 2008; Yakubu and Afolayan, 2009; Ratnasooriya and Fernando, 2008; Yakubu *et al.*, 2005; Abedi *et al.*, 2013; Carro-Juarez *et al.*, 2004; Padashetty and Mishra, 2007; Yakubu *et al.*, 2011; Sharma *et al.*, 2009; Chauhan *et al.*, 2007; Soni *et al.*, 2012; Jamshidzadeha *et al.*, 2006). The various plant species could increase testosterone levels such as *Pedaliium murex*, *Bulbine natalensis*, Black tea brews of *Camellia sinensis*, *Fadogia agrestis*, *Phoenix dactylifer*, *Massularia acuminata* and *Tribulus alatus* (Sharma *et al.*, 2012; Yakubu and Afolayan, 2008; Ratnasooriya and Fernando, 2008; Yakubu *et al.*, 2005; Abedi *et al.*, 2013; Yakubu *et al.*, 2011; El-Tantawy *et al.*, 2007). *Bulbine natalensis* could increase luteinizing hormone concentration (Yakubu and Afolayan, 2008; Yakubu and Afolayan, 2009), *Phoenix dactylifer* and *Massularia acuminata* could increase estradiol and progesterone and follicle stimulating hormone level (Abedi *et al.*, 2013; Yakubu *et al.*, 2011). *Pedaliium murex* could increase the serum level of protein and cholesterol (Sharma *et al.*, 2012). Some studies showed that *Phoenix dactylifer* could enhance sperm motility and sperm count (Bahmanpour *et al.*, 2006), *Lepidium meyenii* could enhance sperm count in vas deferens and epididymis (Gasco *et al.*, 2007; Chung *et al.*, 2005) and *Anacyclus pyrethrum* could enhance sperm count and fructose levels in seminal vesicle (Sharma *et al.*, 2009). Many studies were found *Bulbine natalensis* and *Phoenix dactylifer* could increase the weight of testis and epididymis (Yakubu and Afolayan, 2008; Yakubu and Afolayan, 2009; Bahmanpour *et al.*, 2006) and *Curculigo orchioides* could increased penile erection index and the weight of prostate gland and seminal vesicle (Chauhan *et al.*, 2007) and *Paederia foetida* Linn. enhanced the length of stage VIII of seminiferous tubules (Soni *et al.*, 2012).

In Thailand, several medicinal plants are used for aphrodisiacs. *Kaempferia parviflora* or Krachaidum extract could increase the weights of epididymis, seminal vesicles, prostate gland and levator ani muscle when concurrent with exercise training (Chaturapanich *et al.*, 2008) and increase sperm density and the activities of Sertoli cell in male rats (Sudwan *et al.*, 2006). *Eurycoma longifolia* (Tongkat Ali) is popularly used in Southern of Thailand which could increase testosterone concentration, sperm vitality (Solomon *et al.*, 2013), sperm count and motility (Solomon *et al.*, 2013; Wahab *et al.*, 2010) and could increase the number of spermatocytes and round spermatids at stage VII of spermatogenesis cycle, spermatozoa production rate and the number of Leydig's cells, the fertility index, fecundity index and the pup litter size delivered from female after mating with male treated rats, increased plasma of testosterone, luteinizing and follicle stimulating hormone level and testicular testosterone level (Low *et al.*, 2013). *Butea superba* increased the percentage weight ratio of testis and sperm count in male rats (Manosroi *et al.*, 2006). *Elephantopus scaber* increased mounting frequencies, sperm count and weight of ischiocavernosus and bulbospongiosus muscle as well as increasing female/male offspring sex ratio (Chaidichoe and Srikhao, 2003).

For many years, various traditional medicine products have been used in both Eastern and Western culture (Liu *et al.*, 2013). Today, natural products are rapidly growing, because of their inexpensive cost and effective bioactive compounds (Dike *et al.*, 2012). The scientific studies could support and enhance traditional knowledge and increase the value of local medicinal plants.

2.3 Seminiferous epithelium

Within testes, the seminiferous tubule consists of a central lumen lined by a specialized stratified epithelium containing two distinct cell populations, the Sertoli cells and the spermatogenic cells (spermatogonia, spermatocytes and spermatids) (Figure 2) (Gartner and Hiatt, 2001). The seminiferous epithelium is encircled by a basement membrane and a wall formed by collagenous fibers, fibroblast and myoid cells. The myoid cells have a role for the rhythmic contractile activity that propels the nonmotile sperm to the rete testis. Sperm acquired forward motility after they have passed through the epididymal duct. The space between the seminiferous tubules is occupied by abundant blood vessels and lymphatic channels encircling each seminiferous tubule and aggregates of the interstitial cells or Leydig cells. Leydig cells are androgen-producing in close proximity to the lymphatic and blood circulation (Hess and Renato de Franca, 2008; Kierszenbaum and Tres, 2012).

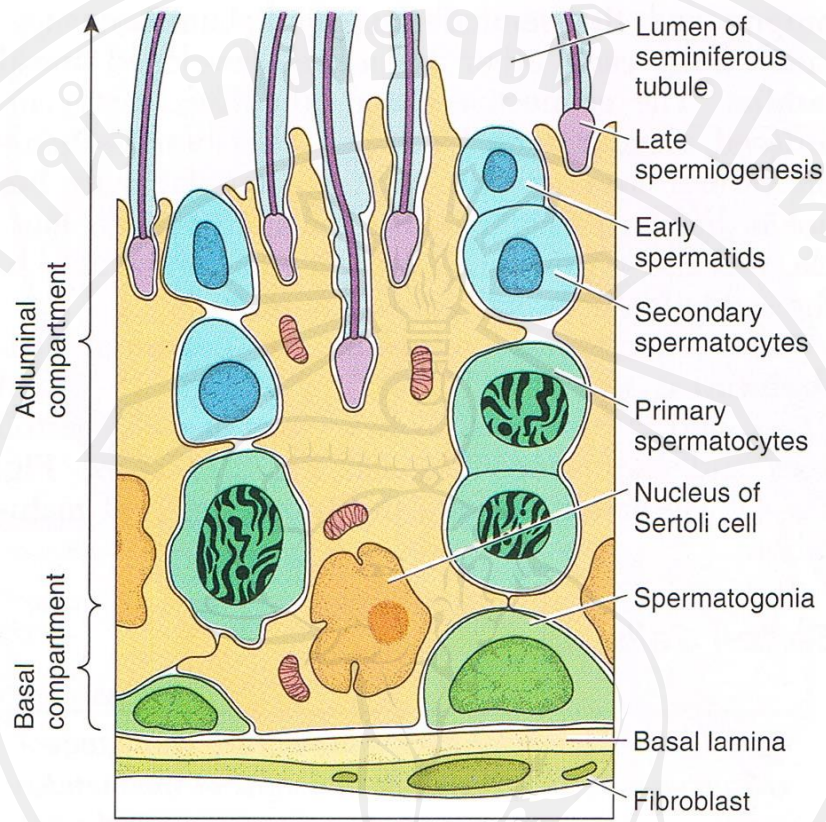


Figure 2 Diagram of the seminiferous epithelium that shows spermatogenic cells and Sertoli cells. The lateral parts of Sertoli cells adjacent form junctional complex are divide the epithelium into basal and adluminal compartments.

Source: Gartner and Hiatt, 2001

2.3.1 Sertoli cells

Sertoli cells are columnar cells extending from basal lamina to the lumen of the seminiferous tubule. The apical and lateral plasma membrane has an irregular outline because they provide crypts to house the 30-50 developing spermatogenic cells at the various stages. The nucleus displays indentations and large nucleolus with heterochromatin masses. The cytoplasm contains smooth and rough endoplasmic

reticulum, mitochondria, lysosomes, lipid droplets, an extensive Golgi apparatus and a rich cytoskeleton (Kierszenbaum and Tres, 2012).

At their basolateral domain, Sertoli cells are bound together from tight junctions that divide the seminiferous epithelium into a basal compartment and adluminal compartment, called blood-testes barrier, which protects developing spermatocytes and spermatids from autoimmune reactions (Dym and Fawcett, 1970; Gartner and Hiatt, 2001). The functions of Sertoli cells are (1) to support, protect and nourish developing spermatogenic cells, (2) to eliminate by phagocytosis the excess cell portions, called residual bodies that are discarded by spermatids at the end of spermiogenesis, (3) to facilitate the release of mature spermatids into the lumen of the seminiferous tubule by actin-mediated contraction, called spermiation, (4) to secrete a fluid-rich protein and ions into the seminiferous tubular lumen, and (5) to secrete androgen-binding protein (ABP), which concentrates testosterone to a level required for spermiogenesis. In an endocrine manner, Sertoli cells release inhibin and activin subunits (α and β subunits). Inhibin exerts a negative feedback on gonadotropin-releasing factor and FSH release by the hypothalamus and anterior hypophysis. Activin exerts a positive feedback on the release of FSH (Dym and Fawcett, 1970, Mruk and Cheng, 2004).

Sertoli cells are the predominant cell type of the seminiferous epithelium until puberty. After puberty, they represent about 10% of the cells lining seminiferous tubules. In the elderly men, when the population of spermatogenic cells decreases, Sertoli cells again become the major component of the epithelium (Kierszenbaum and Tres, 2012).

2.3.2 Spermatogenic cells

The thick seminiferous epithelium is composed with most of the spermatogenic cells in various stages of maturation (Hess, 1990). Spermatogonia are located in the basal compartment with most of developing cells; primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa occupy the adluminal compartment (Kierszenbaum and Tres, 2012). Between both compartments, spermatogenic cells are surrounded by process extension of Sertoli cells, which act as a nurse between developing (Dym and Fawcett, 1970). These details of spermatogenic cells are following.

Spermatogonia

Spermatogonia are diploid spermatogenic cells directly in contact with the basal lamina in the basal compartment and located below the Sertoli cell junctional complex and therefore outside the blood-testes barriers. They are derived from the spermatogonial stem cells and undergo successive rounds of mitotic cell divisions starting at puberty which is influenced by testosterone. There are three major morphologic spermatogonial cell types.

1. Dark type A spermatogonia are small (12 μm in diameter), dome-shaped cells, flattened, oval nuclei with abundant heterochromatin. Dark type A spermatogonia are reserve cells that have not entered the cell cycle but may do so, once they undergo mitosis for increased dark and pale type A spermatogonia (Gartner and Hiatt, 2001).
2. Pale type A spermatogonia are identical similar to the dark type A cells but their nuclei have shown differences. They are abundant, in euchromatin, which give them present in a pale appearance. These cells have only a few organelles including

mitochondria, a limited Golgi complex, rER and numerous free ribosomes. These cells are induced by testosterone to proliferate and give rise by mitosis to additional pale type A spermatogonia and to type B spermatogonia (Gartner and Hiatt, 2001).

3. Type B spermatogonia resemble pale type A spermatogonia but their nuclei are usually round. These cells also divide mitotically to give rise to primary spermatocytes (Gartner and Hiatt, 2001).

Primary spermatocyte

Primary spermatocytes are from each type B spermatogonia that grow in size and spherical cells with euchromatic nuclei that enter meiotic prophase immediately after completing the last S phase (DNA synthesis). The last round of major DNA synthetic activity in the lifetime of spermatogenic cells determines that a primary spermatocyte starting the first meiotic prophase will have two times the amount of DNA of a spermatogonium. The primary spermatocyte has 46 (44+XY) chromosomes which are diploid numbers and has 4N of DNA content (N=the haploid number of chromosomes or the amount of DNA in this set) (Gartner and Hiatt, 2001). After their formation, these cells enter the first meiotic prophase which lasts about 22 days. The primary spermatocytes are the largest cells of the spermatogenic lineage and are characterized by the presence of partially condensed chromosomes in various stages of synapsis and recombination. The prophase substages of the first meiotic division are the leptotene (theadlike), zygotene (pairing), pachytene (thickening), diplotene (appearing double) and diakinesis (moving apart) stage. After this prolonged prophase, pairs of sister chromatids pass through metaphase, anaphase and telophase and are separated into secondary spermatocytes (Hess and Renato de Franca, 2008; Kierszenbaum and Tres, 2012).

Secondary spermatocyte

Primary spermatocytes are separate in the first meiotic division which produce smaller cells called secondary spermatocytes with only 23 chromosome ($22+X$ or $22+Y$) and each still consists of two chromatids that amount of DNA is $2N$. Secondary spermatocytes are rare in testis sections because they are short-lived cells that remain in interphase and quickly undergo the second meiotic division and are separate sister chromatids and produce two haploid cells called spermatids (Mescher, 2010; Kierszenbaum and Tres, 2012).

Spermatid

Spermatids contain 23 chromosomes because no S phase (DNA replication) occurs between the first and second meiotic division. The amount of DNA per cell is reduced by half when the chromatids separate and the cells formed are haploid ($1N$). Haploid spermatids are located in the adluminal compartment. There are two major types: (1) round or early spermatids; housed in niches in the cytoplasm of Sertoli cells and (2) elongated or late spermatids; housed in crypts and deep invaginations in Sertoli cells apical cytoplasm. Spermatids are engaged in highly differentiated cell processes designating spermiogenesis which is the last phase of spermatogenesis (Gartner and Hiatt, 2001; Kierszenbaum and Tres, 2012).

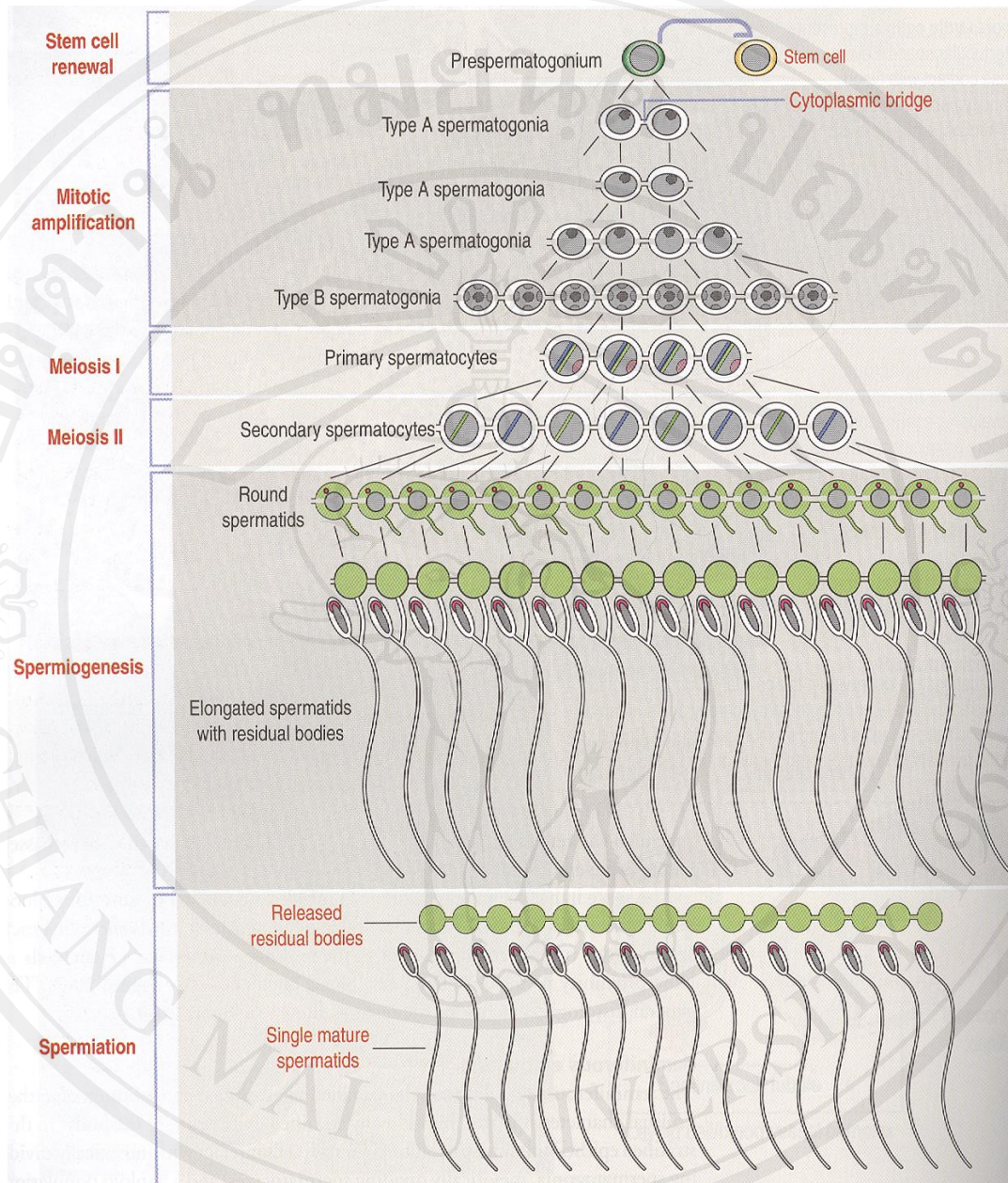


Figure 3 Diagram of spermatogenesis which occur in seminiferous epithelium, showing the cytoplasmic bridge that maintains syncytium spermatogenic cells conjoined to each other during differentiation and maturation. The mitotic amplification of spermatogonia, first meiotic division of primary spermatocytes, secondary meiotic division of secondary spermatocytes, spermiogenesis of spermatids and spermiation of mature spermatid. Source: Kierszenbaum and Tres, 2012

2.4 Spermatogenesis

Spermatogenesis is the process of development of spermatogonia into spermatozoa which occurs in seminiferous tubules under the influence of levels of pituitary gonadotropin releasing-hormones and many other factors. Spermatogenesis starts in puberty when spermatogonial stem cells undergo mitotic cell division to produce two daughter cells. One daughter cell is dark type A spermatogonia as a stem cell, the other is pale type A spermatogonia, initiates a mitotic amplification sequence two or three more times, then differentiates as type B spermatogonia.

Type B spermatogonia completes the S phase (DNA synthesis) of their cell cycle and advances to G₂, then translocates to the adluminal compartment and initiates meiosis I and becomes primary spermatocytes (4N). Primary spermatocytes produce smaller cells called secondary spermatocytes (2N) that reduce the chromosome number and the amount of DNA. Secondary spermatocytes very quickly undergo second meiotic division and produce two haploid cells called spermatid (n). Finally, spermatids are transformed into spermatozoa which occur during spermiogenesis (Gartner and Hiatt, 2001; Hess and Renato de Franca, 2008; Mescher, 2010).

A characteristic feature of spermatogenesis is incomplete cytokinesis, in which cells are conjoined to each other by cytoplasmic bridges, a condition that persists until the completion of spermatogenesis and are finally loose as the haploid spermatids complete their differentiation into sperm. During differentiation, each spermatid sheds excess cytoplasm as residual bodies, then phagocytosed by Sertoli cells (Gartner and Hiatt, 2001; Kierszenbaum and Tres, 2012).

2.5 Spermatogenesis influencing factors

The process of spermatogenesis depends on many factors, especially the hormonal control that is the result of hypothalamo-pituitary-gonadal axis (HPGA). The hypothalamus produces gonadotropin-releasing hormones (GnRH) and stimulates the anterior part of pituitary gland to produce luteinizing hormones (LH) and follicle-stimulating hormones (FSH). In a male, the Leydig cell is stimulated to synthesize male sex hormone, testosterone, by LH. In the case of testosterone level in the blood which is not sufficient to initiate and maintain spermatogenesis, FSH stimulates Sertoli cells to synthesize and release androgen-binding protein (ABP) to binds testosterone for prevention of the hormone leaving the seminiferous tubule and enhancing the testosterone level for spermatogenesis (Holdcraft and Braun, 2004; Sofikitis *et al.*, 2008).

Release of LH is inhibited by enhancing the level of testosterone and its more active form, dihydrotestosterone, whereas FSH is inhibited by inhibin, which is produced by Sertoli cells. This is the negative feedback to regulate the production and secretion of GnRH, LH and FSH in the hypothalamic-pituitary unit (Holdcraft and Braun, 2004; Sofikitis *et al.*, 2008). Moreover, the process of spermatogenesis is highly sensitive to the environment, particularly temperature (Jung *et al.*, 2005; Jung *et al.*, 2008; Sharpe, 2010), because the core body temperature is 37.8°C but testicular temperature is about -2 °C below normal. Consequently, the testes are located outside the body in the scrotum and away from the heat of the body held by the cremasteric muscle and the dartos smooth muscle in the scrotum. In addition, a rich venous plexus surrounded in each testicular artery regulates the blood flow and forms a counter current of heat-exchange system (Kierszenbaum and Tres, 2012). Dietary

deficiencies such as vitamin A, B and E, x-ray exposure, alcoholism, metal such as cadmium and lead, and also infectious disease will also effect to the rate of spermatogenesis (Sharpe, 2000; Sharpe, 2010).

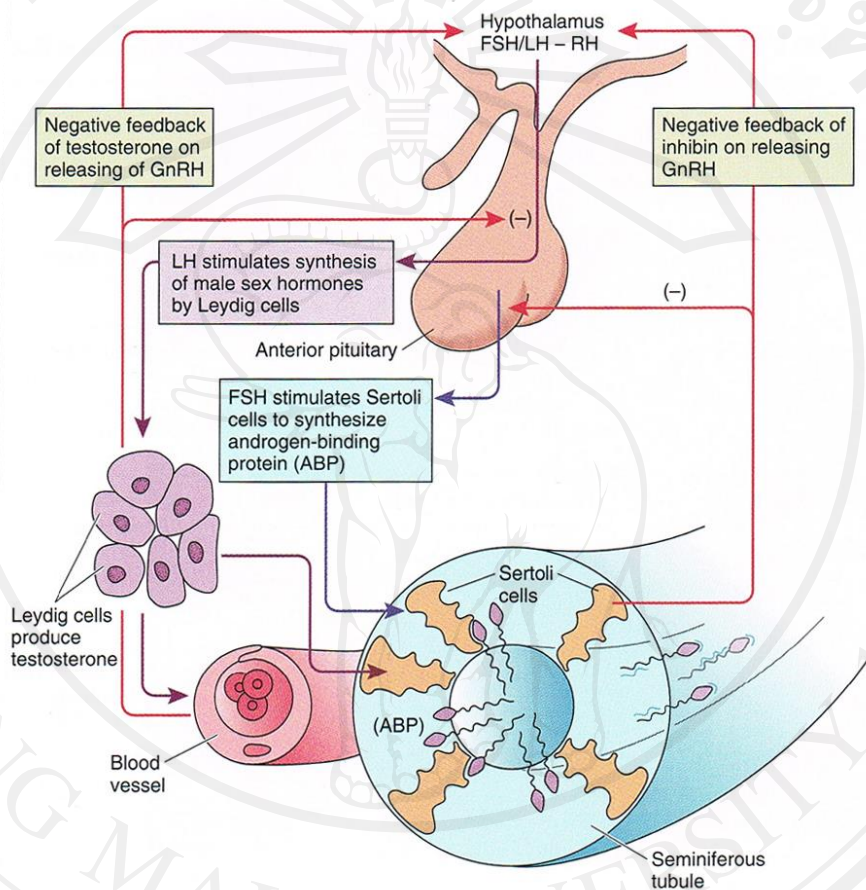


Figure 4 Diagram of the hormonal control of spermatogenesis. Follicle- stimulating hormone (FSH); Gonadotropin-releasing hormone (GnRH); Luteinizing hormone-releasing hormone (LH-RH).

Source: Gartner and Hiatt, 2001

2.6 Spermatogenic cycle length of male rats

In the rat's seminiferous epithelium, the stages of spermatogenic cycle consisted of 14 stages (Figure 5) based on the development of the acrosomic system and the nuclear morphology change (Hess, 1990; Hess and Renato de Franca, 2008). The stages are repeated in consecutive order along the length of the folded seminiferous tubules (Segatelli *et al.*, 2004). The mean duration of each seminiferous epithelium cycle was approximately 12 days (Hess, 1990). Each stage had one pattern of cell association appearance and occupies on the length of the seminiferous tubule (Figure 6, 7). The identification of the seminiferous epithelium stages followed Hess's diagram (Hess, 1990).

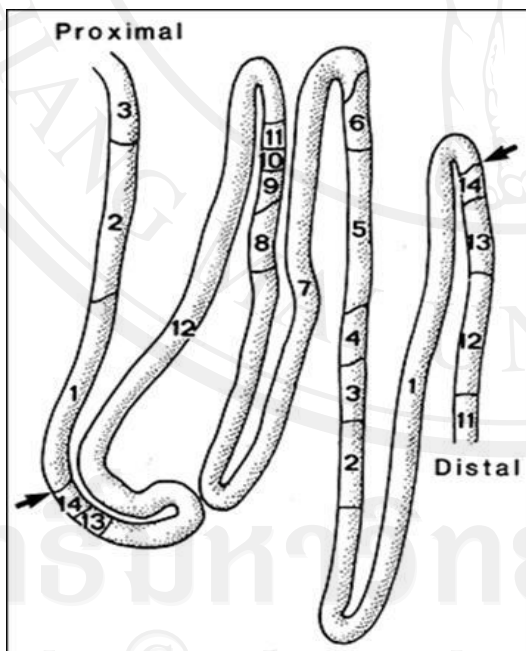


Figure 5 Diagram of seminiferous tubule showing the pattern of the stages of spermatogenic cycle (I-XIV) in consecutive order along the length of the folded seminiferous tubules. The arrow represents one cycle of the folded seminiferous tubule.

Source: (de Kretser, 2013)

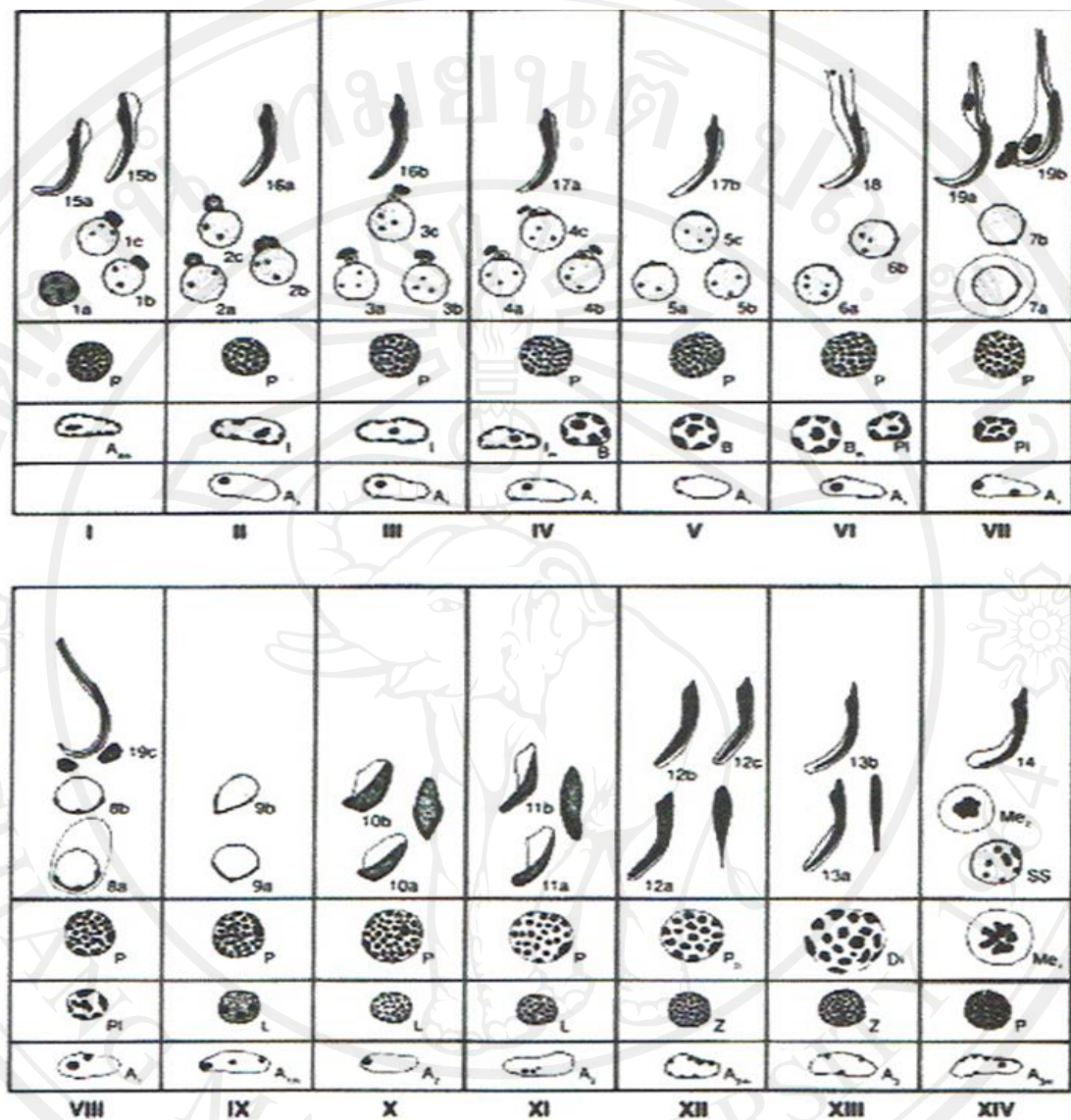


Figure 6 Diagram of morphological characteristic of spermatogenic cells in 14 stages of seminiferous epithelium (I-XIV). Each column appears in one stage of the epithelium. Spermatogenic cells are labeled by mitosis (m); Meiosis (Me₁ or Me₂); Type-A spermatogonia (A); Type-B spermatogonia (B); Preleptotene spermatocyte (Pl); Leptotene spermatocyte (L); Zygotene spermatocyte (Z); Pachytene spermatocyte (P); Dipotene spermatocyte (D); Diakinetik spermatocyte (Di); Secondary spermatocyte (SS); Round spermatids (1-8); Elongating Spermatids (9-19). Source: (Hess, 1990)

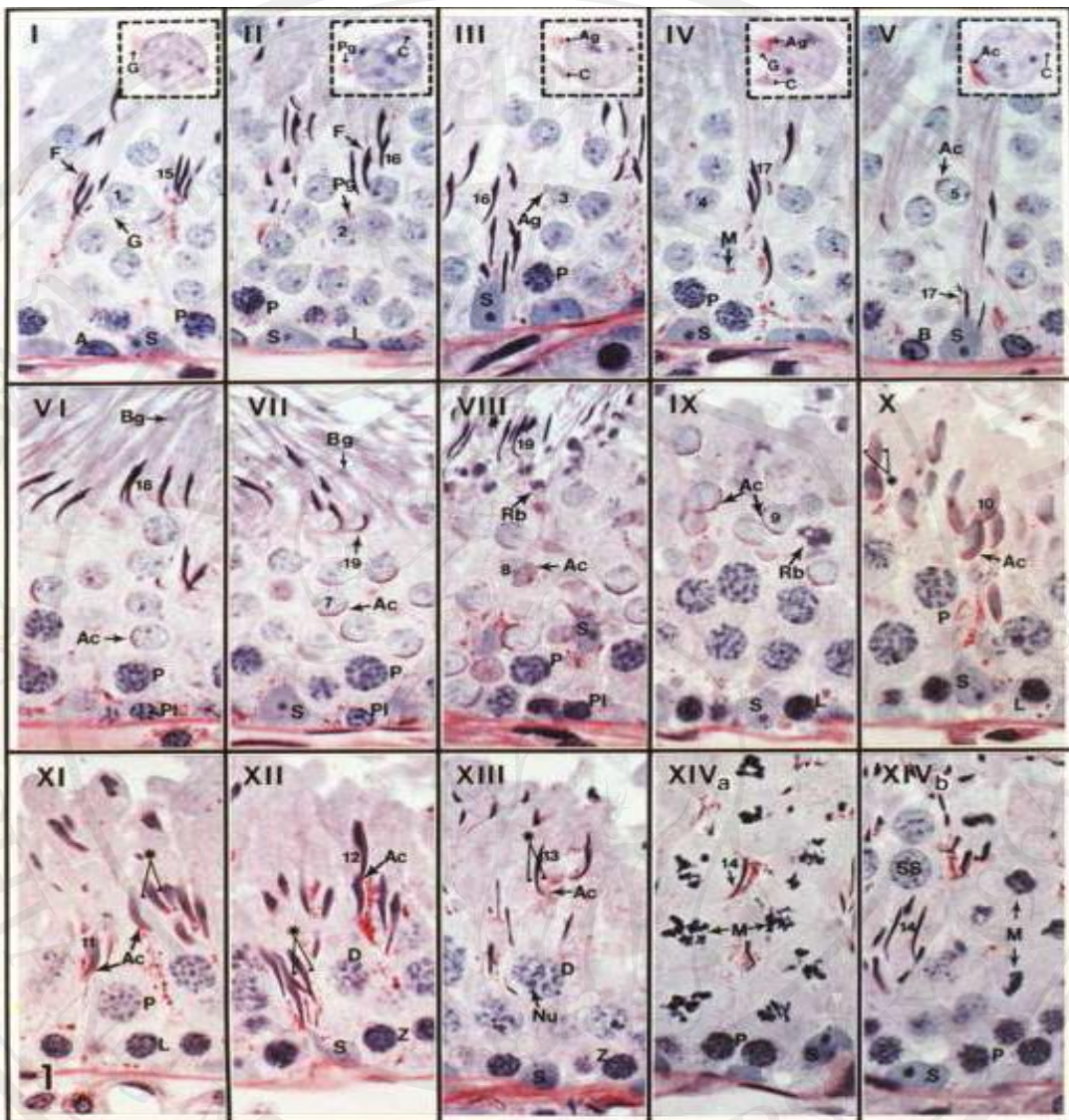


Figure 7 Photograph of glycol methacrylate section (1.5-2 μ m) of the seminiferous epithelium stained by periodic acid-Schiff reaction and hematoxylin. Nucleus of Sertoli cell (S); Type-A spermatogonia (A); Type-B spermatogonia (B); Intermediate spermatogonia (I); Preleptotene spermatocyte (Pl); Leptotene spermatocyte (L); Zygotene spermatocyte (Z); Pachytene spermatocyte (P); Dipotene spermatocyte (D); Diakinetik spermatocyte (Di); Secondary spermatocyte (SS). The identification of stages of the seminiferous described as follows; (Hess, 1990)

Stage I: This stage composes step 1 of spermatid, in which appears Golgi apparatus (G) without dense granules. Step 15 of spermatid present a dorsal acrosomic fin (F).

Stage II: Small proacrosomeal granules (Pg) and chromatid body (C) appear in the step 2 of spermatid. Reduction of dorsal acrosomic fin (F) in the step 16 of spermatid was found.

Stage III: This stage is showing step 16 and step 3 of spermatid, which a single acrosomal granules (Ag) and chromatid body (C).

Stage IV: Extension of acrosomal head cap forming at the pattern margin (M) occurs in late step 4 of spermatids. The Golgi apparatus (G), chromatid body (C) and step 17 of spermatid are found.

Stage V: Acrosome (Ac) begins extension to cover over nucleus of step 5 spermatid. It is also found in step 17 of spermatid, which is deeply embed adjacent to nucleus of Sertoli cell (S). Chromatid body (C).

Stage VI: Acrosome (Ac) covers approximately one-quarter of the nucleus of the step 6 spermatid. Step 18 spermatids present small basophilic granules (Bg) in the distal cytoplasm which migrate from Sertoli cell nucleus toward the lumen.

Stage VII: Acrosome (Ac) covers one-half of the nucleus of the step 7 spermatid. Step 19 of spermatid presented hook-shaped head and large basophilic granule (Bg) in their cytoplasm, which is located near the nucleus.

Stage VIII: In this stage step 19 of spermatid appears with prominent residual bodies (Rb), which lie between mature sperm and round spermatid. Step 8 of spermatid presented acrosome (Ac) and nucleus at the opposite to the cytoplasm.

Stage IX: The nucleus of spermatid in step 9 begins slightly elongate as well acrosome (Ac). Residual bodies (Rb) appear within the epithelium.

Stage X: Acrosome (Ac) and nucleus of step 10 spermatid are extended to the caudal region of nucleus, forming a V-shaped structure (*) of the nucleus. Condensation of the nucleus is also seen.

Stage XI: Acrosome (Ac) and nucleus of step 11 spermatid protrude to the caudal region of the nucleus, forming Acrosomal-nuclear angle (*).

Stage XII: The nucleus of the late step 12 spermatid appear long and straight-shape head; and narrow width at the acrosomal-nuclear junction (*) characteristic. Acrosome (Ac) extends to form a blunt tip of the spermatid nucleus.

Stage XIII: Acrosome (Ac) of the late step 13 spermatid extends beyond the nuclear apex and forms a bulge on the ventral side of nucleus. Acrosomal-nuclear junction (*).

Stage XIVa: The nucleus of step 14 spermatid is thin and curved shape. The feature of the first meiotic division (M) presents clumps of chromatin forming scattered patterns in the center of the spermatocyte nucleus.

Stage XIVb: In the second meiotic division appears dense clusters of chromatin (M). Acrosome extends to forms a prominent ventral bulge. Secondary spermatocyte (SS).

2.7. Toxicity testing of chemicals

In recent decades, toxicity testing of chemical substances addressed area of biological science. It has looked at human risk assessment from chemical exposure such as pollution (Chen and Liao, 2006), pesticide residues in agricultural commodity and food (Boobis *et al.*, 2008) and pharmaceutical chemicals (Stuer-Lauridsen *et al.*, 2000). Most effects usually result from the several complex and intact organisms (Chapman *et al.*, 2010). The primary objective of toxicology is human protection. Current toxicity testing relies on the *in vitro* and *in vivo*. The results of blood and tissue investigation under specific exposure conditions were applied to predict human health; particularly the observation of adverse health responses in laboratory animals treated with the study agents and then to human (Andersen and Krewski, 2009). The U.S. Environmental Protection Agency (EPA) and the U.S. National Institute of Environmental Health Sciences (NIEHS) asked the U.S. National Research Council (NRC) to provide guidance on new directions in toxicity testing and incorporate new technologies such as genomics and molecular epidemiology (Perera, 1996). The recent report of the toxicity testing committee for a modern approach to toxicity testing was written (NRC, 2007). The key elements in this proposal are related to toxicity testing, which include the types of *in vitro* tests and short-term *in vivo* tests which provide the requisite tools for interpret toxicity testing and assessing human health risk (MacDonald and Robertson, 2009).

2.8 Micronucleus test

A micronucleus test is one that is short term and has been widely used in toxicological screening to detect genotoxicity after exposure to carcinogenic chemicals and ionizing radiation causing genetic damage (Shin *et al.*, 2012) which can result in the formation of a smaller micronucleus apart from the main nucleus. Micronucleus was proposed by Schmid and Heddle and also known as Howell-Jolly bodies to hematologists (Krishna and Hayashi, 2000). These micronuclei may originate from chromosome breaks or whole chromosomes from the damage to spindle apparatus that lag behind in the anaphase of cell division and may not become integrated into the daughter nuclei and eventually form micronucleus (Hayashi *et al.*, 2000). A fragment or whole chromosomes were seen in the cytoplasm of dividing cell populations because the nucleus is extruded from the erythroblast after the last cell division (Ha *et al.*, 2011). The *in vivo* study normally uses rodent bone marrow or peripheral blood (Fenech, 2000). The processes of erythropoiesis in adult rodents occur in bone marrow and spleen. During proliferation, the cells continue to divide and mature as well as an erythroblast developing into polychromatic erythrocyte (PCE). A young erythrocyte still contains RNA and stains light blue or blue gray with Giemsa. After that, the PCE are lost RNA, contain primary hemoglobin and become normochromatic erythrocyte (NCE), which is smaller than PCE. With Giemsa, NCE stains light orange or orange-pink. This staining could differentiate between two types of erythrocyte in bone marrow, blood and spleen (Krishna and Hayashi, 2000; Hayashi *et al.*, 2000).

Micronuclei are generally round or almond shape, however the ring shape occasionally occurs. They have a sharp border, the diameters about 5-20% of PCE's size, may occur in either PCE or NCE and could appear in more than one micronucleus per cell. In the laboratory, the frequency of micronucleus in PCE (MNPCE) was determined in a total of 2000 PCE for clastogenic effect of chemical agent testing in each animal and the ratio of PCE to total erythrocytes (PCE+NCE) could provide a cytotoxic index which scores at least 200 and 1000 erythrocytes in bone marrow and peripheral blood assays, respectively (Krishna and Hayashi, 2000).

Micronucleus testing is popularly used for genetic toxicology studies because it is the cheapest, simplest, saves time and is more sensitive than clastogenic agents (Yener and Dikmenli, 2009). The conventional micronucleus assay with erythrocytes such as bone marrow and peripheral blood is simple and highly sensitive to clastogenic agents that reach the target tissue (Styles *et al.*, 2001). However, the detection at target tissue may be suitable for the effects and mechanistic studies of clastogens. Micronucleus assays could be used in various tissues such as buccal epithelial cells (Bonassi *et al.*, 2011, Holland *et al.*, 2008), urothelial cells (Warner *et al.*, 1994), nasal epithelial cells (Burgaz *et al.*, 2001), hepatocytes (Charoensin *et al.*, 2010), lymphocytes (Speit *et al.*, 2012) and amniocytes (de la Chica *et al.*, 2005). Moreover, micronucleus assay can be integrated into routine toxicity studies (Krishna and Hayashi, 2000).

2.9 Safety assessment of medicinal plants by using micronucleus test

Medicinal plants are popularly use for the prevention and therapeutic treatment of disease and will likely increase. However, lack of quality control and safety including toxicity of medicinal plants should be of concern and require some scientific support. The following researches reported the information on its toxicity study by using micronucleus test. In Korea, the genotoxicity evaluation in the bone marrow with micronucleus assay of medicinal plants and herbal prescription such as *Mahwangyounpae-tang* (Seo *et al.*, 2008), *Yukmijihwang-tang* (Ha *et al.*, 2011) and *Gumiganghwal-tang* (Shin *et al.*, 2012) did not find the increased of MNPCE and the PCE/PCE+NCE ratio in ICR mice. The use of *Annona crassiflora* leave extract (Vilar *et al.*, 2008), *Orthosiphon stamineus* extract (Muhammad *et al.*, 2011), triterpenoid isolated from *Caulis bamfusae* (Zhang *et al.*, 2004), Mangiferin and Vimang isolated from *Mangifera indica* (Rodeiro *et al.*, 2012), *Prunus mume* extract (Lu *et al.*, 2009), *Phaseolus vulgaris* extract (Azevedo *et al.*, 2003) did not significantly increase the number of MNPCE and PCE/NCE ratio in bone marrow of mice and *Labisa pumila* extract (Zaizuhana *et al.*, 2006), *Mucuna collettii* extract (Cherdshewasart *et al.*, 2008) and *Butea superba* extract (Cherdshewasart *et al.*, 2010) did not significantly increase of MNPCE and PCE/NCE ratio in bone marrow of rats. The study of Magnolol-containing kampo medicine, the traditional medicine of Japan by using micronucleus test in blood showed high level of medicine had significant clastogenic inhibition (Saito *et al.*, 2009). Thus, the toxicity study by using micronucleus test should be applied to confirm for safety use in a variety of medicinal plants. The new information will support the use of confidence in the consumption in folk medicines.