CHAPTER II
LITERATURE REVIEWS

1. Botanical data of *Bacopa monnieri* (L.) Wettst.

Figure 2 *Bacopa monnieri* (Brahmi)

Bacopa monnieri (L.) Wettst., a medicinal plant belonging to Family Scrophulariaceae, is known as Brahmi or Bacopa. *B. monnieri* is a creepy and succulent herb. The leaf mostly 5-25 mm long and arise from creeping stems that from roots at the nodes. The leaves are narrowly spatulate, ovate-oblong, sessile, opposite, obtuse apex approximately 2x1 cm with entire margin. Flowers are pale blue, purple or white, solitarily on long pedicles in the leaf axils. The corolla is 5-lobe. The fruit is an up to 5 mm capsule, which develops in the persistent calyx.

B. monnieri is found as weeds in rice fields and found growing abundantly in the marshy and distributed throughout the tropical regions of India, United States and Asia (Barrett, Strother, 1978).

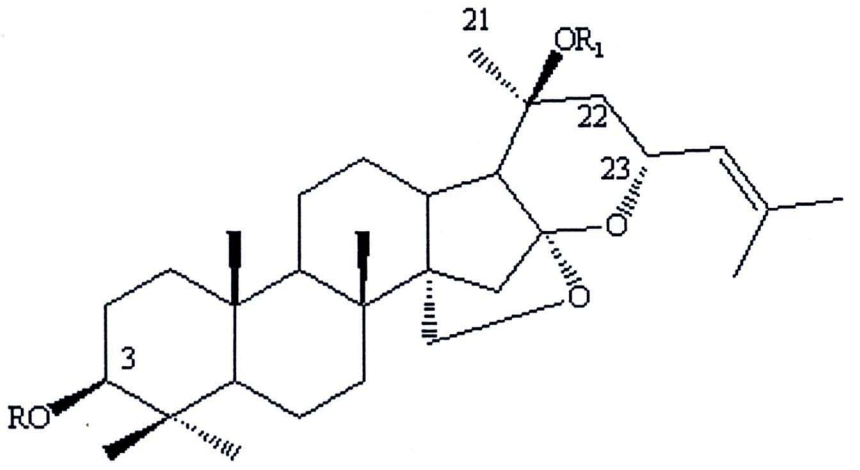
B. monnieri is a medicinal plant used for centuries in the Ayurvedic system of medicine to improve intelligence, memory and revival of sensory organs (Singh, Dhawan, 1997). Recent studies revealed the therapeutic potential of this plant in treatment or prevention of neurological diseases and improvement of cognitive processes (Singh, Dhawan, 1997; Vohara et al., 2000; Stough et al., 2001; Das et al., 2002; Sairam et al., 2002; Russo et al., 2003). Dammarane-type triterpenoid saponins classified as pseudojujubogenin and jujubogenin glycosides were reported to be responsible for the cognitive enhancing activity in this plant (Singh et al., 1988).

2. Triterpenoid saponins in *B. monnieri*

Triterpenoid saponins are a group of secondary metabolites. They are characterized by the surfactant properties, soap-like foams in aqueous solution. Saponins are complex molecules consisting of non-sugar aglycone coupled to sugar units.

The major and active compounds in *B. monnieri* are dammarane type of triterpenoid saponins. Two groups of dammarane-typed saponins in *B. monnieri* are classified as pseudojujubogenin and jujubogenin glycosides. They are different at the position of isoprene side chain at C-22 or C-23. Jujubogenin glycosides in *B. monnieri* are bacoside A_1 (Jain, Kulshreshtha, 1993), bacopaside III-IV (Chakravarty et al., 2003), bacopasaponin A (Garai et al., 1996a), bacopasaponin E-F (Mahato et al.,2000) and bacopasaponin G (Hou et al., 2002) where as pseudojujubogenin glycosides in *B. monnieri* are bacoside A_2 (Rastogi, Kulshreshtha, 1999), bacopaside I-II (Chakravarty et al., 2001), bacopaside V (Chakravarty et al., 2003), bacopasaponin B-C (Garai et al., 1996a) and bacopasaponin D (Garai et al., 1996b) as shown in figure 3 and 4.

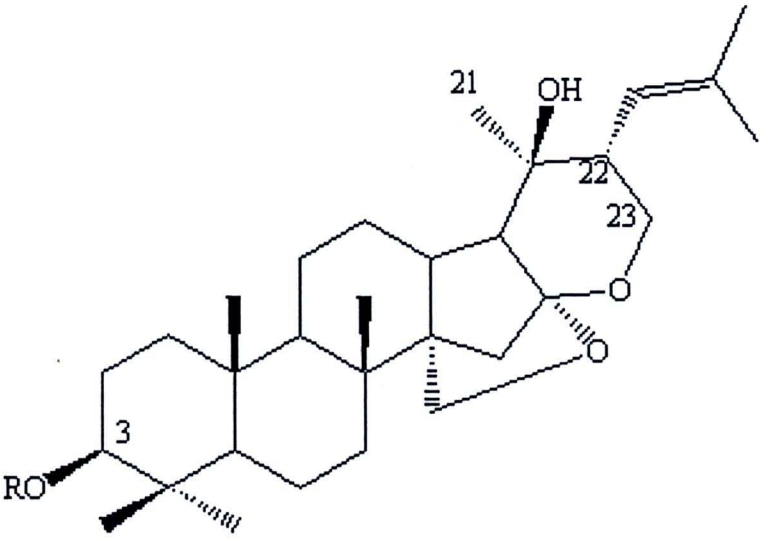
Many studies were reported that the biological effects of Brahmi attributed to two saponins called bacoside A and bacoside B (Bhattacharya et al., 2000; Singh et al., 1988). It has been believed that bacoside A was a single compound and bacoside B was bacoside A isomer. Deepak et al. (2005) has recently reported that bacoside A is in fact mixture of bacoside A_3, bacopaside II, bacopasaponin C and bacopasaponin C isomer. However, the chemical constituents in bacoside B are still unrevealed.



Jujubogenin glycosides

	R	R_1
Bacoside A_1	α-L-arabinofuranosyl-(1→3)-α-L-arabinopyranosyl	H
Bacoside A_3	β-D-glucopyranosyl-(1→3)-O-[α-L-arabinofuranosyl-(1→2)-O-β-D-glucopyranosyl]oxy	H
Bacopaside III	α-L-arabinofuranosyl-(1→2)-β-D-glucopyranosyl	H
Bacopaside IV	β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl	H
Bacopasaponin A	α-L-arabinopyranosyl	α-L-arabinopyranosyl
Bacopasaponin E	β-D-glucopyranosyl-(1→3)-α-L-arabinofuranosyl-(1→2)-α-L-arabinopyranosyl	α-L-arabinopyranosyl
Bacopasaponin F	β-D-glucopyranosyl-(1→3)-α-L-arabinofuranosyl-(1→2)-β-D-glucopyranosyl	α-L-arabinopyranosyl
Bacopasaponin G	α-L-arabinofuranosyl-(1→2)-α-L-arabinopyranosyl	H

Figure 3 Chemical structures of jujubogenin glycosides found in *B. monnieri*



Pseudojujubogenin glycosides

	R
Bacoside A_2	α-L-arabinofuranosyl-(1→5)-α-L-arabinopyranosyl-(1→6)-α-D-glucopyranosyl
Bacopaside I	α-L-arabinofuranosyl-(1→2)-[6-O-sulphonyl-β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranosyl
Bacopaside II	α-L-arabinofuranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl
Bacopaside V	β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl
Bacopasaponin B	α-L-arabinofuranosyl-(1→2)-α-L-arabinopyranosyl
Bacopasaponin C	β-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→2)]- α-L-arabinopyranosyl
Bacopasaponin D	α-L-arabinofuranosyl-(1→2)-β-D-glucopyranosyl

Figure 4 Chemical structures of pseudojujubogenin glycosides found in *B. monnieri*

3. Biological activity of *B. monnieri*

B. monnieri has demonstrated mental function activity, cognition-enhancing, antioxidant, anti-inflammatory, anxiolytic, relaxing, brochodilatory and other pharmacological effects. Most of studies were conducted using the extract from aerial part of *B. monnieri*. The summary of its biological activities was shown as follow.

3.1 Neuropharmacological activity

3.1.1 Learning and memory enhancing activity

Singh et al. (1988) suggested that bacoside induces membrane dephosphorylation, with a concomitant increase in protein and RNA turnover in specific brain areas. Singh, Dhawan (1997) reported that *B. monnieri* enhances protein (5-hydroxytryptamine) kinase activity in the hippocampus which may also contribute to its nootropic action. Beside, Bhattacharya et al. (2000) informed that an enriched bacoside *B. monnieri* extract could reverse cognitive deficit induced by colchicine and ibetonic acid in rats.

Standard extract of *B. monnieri* and *G. biloba* was used to evaluate the antidementic and anticholinesterase activities in adult male Swiss mice. Antidementic activity was tested against scopolamine induced deficits in passive avoidance test. In passive avoidance test, increased transfer latency time (TLT) and no transfer response (NTR) were taken as criteria for learning. *B. monnieri* and *G. biloba*-treated groups produced significant increase in TLT and NTR on second trial (40-80%) after scopolamine treatment. However, *in vitro* study showed that *B. monnieri* extract had very low inhibitory effect on acetylcholinesterase (AChE) activity (Das et al., 2002).

3.1.2 Neuroprotective effect and antioxidant activity

The neuroprotective effect and antioxidant activity of *B. monnieri* extract have been reported in different mechanism as follow. The high concentration of nitric oxide (NO), generated by activated astrocytes, might be involved in a variety of neurodegenerative diseases, such as Alzheimer's disease, ischemia and epilepsy. It has recently been suggested that glial cells may produce NO under superoxide radical stimulation by enzyme independent mechanism. Russo et al. (2003) examined the effect of a methanolic extract of *B. monnieri* on toxicity induced by the nitric oxide donor, S-nitro-N-acetyl-penicillamine (SNAP), in culture of

purified rat astrocytes. The resulted indicated that, after 18 hour of treatment, SNAP induced an increase in the production of reactive species. *B. monnieri* extract significantly inhibited the formation of reactive species and DNA damage in a dose dependent manner.

Vohara et al. (2000) evaluated *B. monnieri* extract alone and in combination with phenytoin on passive avoidance test (PA) task, maximal electroshock seizure and locomotor activity in mice. Phenytoin (25 $mg.kg^{-1}$ PO, 14 days) adversely affected cognitive function in the PA task. *B. monnieri* (40 $mg.kg^{-1}$ PO, 7 days), given along with phenytoin in second week of two-week regimen, significantly reversed phenytoin-induced impairment. The result showed that *B. monnieri* extract was corrected a cognitive deficit associated with phenytoin therapy.

Anbarasai et al. (2006) evaluated the antioxidant role of bacoside A against chronic cigarette smoking induced oxidative damage in rat brain. Adult male albino rats were exposed to cigarette smoke for a period of 12 weeks and simultaneously administered with bacoside A (10 $mg.kg^{-1}.day^{-1}$, PO). Antioxidant status of the brain was assessed from the levels of reduced glutathione, vitamin C, vitamin E and vitamin A and the activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. The level of copper, iron, zinc and selenium in brain and serum ceruloplasmin activity were also measured. Oxidative stress was evident from the diminished levels of both enzymatic and non-enzymatic antioxidants. Alterations in the levels of trace elements with accumulation of copper and iron, and the depletion of zinc and selenium were also observed. Bacoside A administration improved the antioxidant status and maintained the level of trace elements. These results suggested the chronic cigarette smoke exposure enhanced oxidative stress thereby disturbing the tissue defense system and bacoside A protected the brain from the oxidative damage through its antioxidant potential.

3.1.3 Anti-stress (Adaptogenic) activity

Stress represents reaction of body to stimuli that tend to disturb its normal physiological equilibrium or homeostatis and has been defined as nonspecific response of the body to any demand imposed on it. Rai et al. (2003) reported that standardized extract of *B. monnieri* possessed a potent adaptogenic

activity by the investigations on the adaptogenin property of a standardized extract of *B. monnieri* against acute (AS) and chronic stress (CS) models in rats. *Panax uinquefolium* root was taken as standard. The rats were exposed to immobilization stress for 150 min once only for AS and for seven consecutive days in CS. Prior to each exposure of stress 45 min, rats were fed with *B. monnieri* or *P. ginseng* root powder daily for 3 days in AS and for 7 days in CS. AS exposure significantly increased the ulcer index, adrenal gland weight, plasma glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine kinase (CK) but significantly decreased the spleen weight. Pretreatment with *B. monnieri* at 40 $mg.kg^{-1}$ PO significantly reduced the AS-induced increase in the ulcer index, adrenal gland weight, plasma glucose, AST and CK. A dose of 80 $mg.kg^{-1}$ PO significantly reversed the AS-induced changes in adrenal gland weight, spleen weight, plasma glucose, ALT and AST. Panax root powder, 100 $mg.kg^{-1}$ PO, significantly reversed the AS-induced changes in spleen weight, plasma ALT, AST and CK. CS exposure resulted in a significant increase in the ulcer index adrenal gland weight, plasma AST and CK with a significant decrease in the thymus and spleen weight, plasma triglyceride and clolesterol. Pretreatment with low dose of *B. monnieri* at 40 $mg.kg^{-1}$ significantly reversed changes in ulcer index and plasma AST only, whereas the pretreatment with higher dose significantly reversed CS-induced changes in ulcer index, adrenal gland weight, CK and AST. Panax root powder significantly reversed CS-induced increase in ulcer index, adrenal gland weight, CK and AST.

3.1.4 Antidepressant

The standardized methanolic extract of *B. monnieri* was investigated for potential antidepressant activity in rodent models of depression. The effect was compared with the standard antidepressant drug imipramine (15 $mg.kg^{-1}$, ip). The extract when given in the dose of 20 and 40 $mg.kg^{-1}$, orally once daily for 5 days was found to have significant antidepressant activity in forced swim and learned helplessness models of depression and was comparable to that of imipramine (Sairam et al., 2002).

3.2 Other biological activity

3.2.1 Anti-inflammatory

The methanolic extract of *B. monnieri* was investigated the anti-inflammotory effect in rat (*in vivo*) using rat paw model. In carrageenan-induced rat paw edema, *B. monnieri* extract brought about 82% edema inhibition at a dose of 100 $mg.kg^{-1}$ ip when compared to indomethacin (3 $mg.kg^{-1}$) that showed 70% edema inhibition (Viji, Helen, 2008). The ethanol extract of *B. monnieri* exhibited marked anti-inflammatory activity against carrageenan-induced paw edema in mice and rats, an acute inflammatory model. To assess the possible mechanism of anti-inflammatory action against carrageenan, the ethanol extract was treated with chemical mediators (histamine, serotonin, bradykinin, prostaglandin E2 and arachidonic acid)-induced edema in rats. The extract selectively inhibited prostaglandin E2-induced inflammation. Thus, it may be inferred that *B. monnieri* possesses significant anti-inflammatory activity that may well be relevant for its effectiveness in the healing of various inflammatory conditions in traditional medicine (Channa et al., 2006).

3.2.2 Broncho-vasodilatory activity

Channa et al. (2003) has reported the various fractions and sub-fractions isolated from *B. monnieri* produced significant inhibition of carbachol-induced bronchoconstriction, hypotension and bradycardia in anaesthetized rats which is attributed mainly to inhibition of calcium ions.

As mentioned above, the preclinical studies of *B. monnieri* extract mostly involved neuropharmacological effects. Moreover, Russo, Borrelli (2005) reported the clinical studies of *B. monnieri* extract as follow (Table 1).

Table 1 Clinical studies of *Bacopa monnieri*

Substances/treatments	Subjects/patients	Results/comments	References
Brahmi crude extract; chronically for 4 weeks	Patients with anxiety neurosis	Enhancing the memory	Singh, Singh (1980)
BM extract; chronically for 12 weeks	Children	Enhancing memory and learning	Sharma et al. (1978)
BM standardized extract	Mentally retarded children	Effective in enhancing learning and in controlling abnormal behavior	Dave et al. (1993)
BM standardized extract (300 mg); chronically for 12 weeks	Healthy adult subjects	Improving early information processing, verbal learning and memory consolidation	Stough et al. (2001)
BM standardized extract (300 mg); acute treatment for 2 hour	Healthy adult subjects	No significant changes were found	Nathan et al. (2001)
BM standardized extract (300 mg) combined with *Ginkgo biloba* extract (120 mg); acute treatment for 90 and 180 minute	Healthy adult subjects	No acute effect on cognitive was found on any of the tests	Maher et al. (2002)
BM standardized extract (300 mg); one trial after chronic treatment for 3 months and an other trial for 6 weeks after the completion of trial	Healthy adult subjects	Significant effect on a test for retention of new information. Tasks assessing attention, verbal and visual short term memory and the retrieval of pre-experiment knowledge were unaffected	Roodenrys et al. (2002)

(Russo, Borrelli, 2005)

4. Analytical method of saponin glycosides in Brahmi

Due to the fact that triterpenoid saponins in *B. monnieri* are a mixture of structurally related forms with the very similar polarities, their separation still remain a challenge. Previously, procedures for the determination of "Bacopaside A", the mixture of active saponins in *B. monnieri* were performed using various analytical methods such as ultraviolet-visible spectroscopy (Pal, Sarin, 1992) and HPTLC (Gupta et al., 1998). However, the accuracy and sensitivity of the methods could still be improved.

Recently, HPLC techniques were reported to determine individual saponin glycosides in *B. monnieri*. Renukappa et al. (1999) determined 3 compounds as bacoside A_3, 3-β-[*O*-β-D-glucopyranosyl(1→3)-*O*-[α-L-arabinofuranosyl-(1→2)]-*O*-β-D-arabinopyranosyl) oxy] jujubogenin and bacopasaponin C. The separation was performed by using a RP C-18 HPLC column 18 and a gradient mobile phase composed of acetonitrile (ACN) and water. The solvent program used was an isocratic run with 30% aqueous ACN for 20 min followed by an increase to 80% aqueous ACN at a flow-rate of 0.8 $ml.min^{-1}$. Moreover, Ganzera et al. (2004) separated bacoside A_3, bacopaside II, IV-V and bacopasaponin C by using 3 μm C-8 column material (Luna C-8) and a mobile phase comprising of water and methanol. The developed HPLC method gave baseline separation of seven major saponins within less than 30 min. Flow-rate, detection wavelength and temperature were adjusted to 0.5 $ml.min^{-1}$, 205 nm and 40°C, respectively.

5. Application of immunological assay in phytochemical analysis

Immunoassay using monoclonal (MAb) or polyclonal antibodies (PAb) against low molecular weight is becoming a promising tool in determination of natural bioactive compounds in complex mixtures. The great advantage of this method is its high specificity and sensitivity. Therefore, it is suitable for rapid screening high numbers of samples with small amount. Enzyme-linked immunosorbant assay (ELISA) based on MAb/PAbs is in many cases more sensitive than conventional HPLC methods (Shoyama et al., 1999). Numbers of MAb/PAbs against saponins such as glycyrrhizin (Shan et al., 2001) and saikosaponins (Zhu et al., 2006) were developed. The high selectivity and sensitivity were achieved.

Further, development of the application of MAbs and PAbs on natural product analysis such as eastern blotting (Shan et al., 2001) and immunochromatographic strip (Putalun et al., 2004a,b) was also studied.

5.1 Enzyme-linked immunosorbent assay (ELISA)

Competitive ELISA is appropriate for detection of total pseudojujubogenin glycosides with high sensitivity, rapid and high-throughput method. The used of ELISA for analyzing of total pseudojujubogenin glycosides using monoclonal and polyclonal antibodies against bacopaside I were reported by Phrompittayarat et al. (2007a,b). The properties of these anti-bacopaside antibodies were described below.

Table 2 Cross reactivities (CRs) of the anti-bacopaside I PAbs against some naturally occurring compounds

Compound	Classification	CRs (%)
Bacopaside I	Triterpenoid saponins (pseudojujubogenin glycosides)	100.00
Bacopaside II	Triterpenoid saponins (pseudojujubogenin glycosides)	99.84±0.31
Bacopasaponin C	Triterpenoid saponins (pseudojujubogenin glycosides)	102.68±17.16
Bacopaside V	Triterpenoid saponins (pseudojujubogenin glycosides)	58.38±5.62
Bacoside A_3	Triterpenoid saponins (jujubogenin glycosides)	0.77±0.29
Bacopasaponin C isomer	Triterpenoid saponins (jujubogenin glycosides)	1.75±1.12
Bacopaside IV	Triterpenoid saponins (jujubogenin glycosides)	0.83±0.66
Ginsenoside Rg1	Triterpenoid saponins	<0.01
Ginsenoside Rb1	Triterpenoid saponins	<0.01

Table 2 Cross reactivities (CRs) of the anti-bacopaside I PAbs against some naturally occurring compounds (Cont.)

Compound	Classification	CRs (%)
Glycyrrhizin	Triterpenoid saponins	<0.01
Saikosaponin a	Triterpenoid saponins	<0.01
Quilaja saponin	Triterpenoid saponins	<0.01
α-Amyrin	Triterpenoids	<0.01
Diosgenin	Steroids	<0.01
Prednisolone	Steroids	<0.01
Digitonin	Cardiac glycosides	<0.01
Caffeic acid	Purine alkaloids	<0.01
Solanine	Steroidal alkaloids	<0.01
Swertiamarin	Iridoid glycosides	<0.01
Geniposide	Iridoid glycosides	<0.01
Sennoside A	Anthraquinones	<0.01
Aesculetin	Coumarins	<0.01
Griseofulvin	Coumarins	<0.01
Hesperidin	Flavonoids	<0.01
Camphor	Ketones	<0.01
Cinnamic acid	Phenyl propanes	<0.01

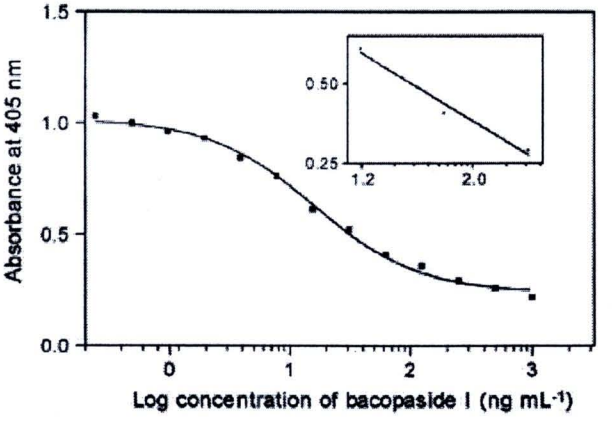


Figure 5 Dose-response curve of bacopaside I detected by competitive ELISA using anti-bacopaside I PAb at 405 nm

Figure 5 show the standard curve of standard curve of bacopaside I by plotting absorbance at 405 nm against the logarithm of bacopaside I concentrations. Under these conditions, the full linear range of the assay was extended from 1.95-62.5 $ng.ml^{-1}$ as indicated in figure 5. Limit of detection of ELISA technique using anti-bacopaside I PAb is 0.1 $ng.ml^{-1}$.

Table 3 Cross reactivities (CRs) of the anti-bacopaside I MAb against some naturally occurring compounds

Compound	Classification	CRs (%)
Bacopaside I	Triterpenoid saponins (pseudojujubogenin glycosides)	100.00
Bacopaside II	Triterpenoid saponins (pseudojujubogenin glycosides)	299.33±122.74
Bacopasaponin C	Triterpenoid saponins (pseudojujubogenin glycosides)	64.05±4.11
Bacopaside V	Triterpenoid saponins (pseudojujubogenin glycosides)	94.40±5.60
Bacoside A_3	Triterpenoid saponins (jujubogenin glycosides)	<0.01
Bacopasaponin C isomer	Triterpenoid saponins (jujubogenin glycosides)	0.49±0.18
Bacopaside IV	Triterpenoid saponins (jujubogenin glycosides)	<0.01
Ginsenoside Rg1	Triterpenoid saponins	<0.01
Ginsenoside Rb1	Triterpenoid saponins	<0.01
Glycyrrhizin	Triterpenoid saponins	<0.01
Saikosaponin a	Triterpenoid saponins	<0.01
Quilaja saponin	Triterpenoid saponins	<0.01
α-Amyrin	Triterpenoids	<0.01
Camphor	Ketones	<0.01
Prednisolone	Steroids	<0.01

Table 3 Cross reactivities (CRs) of the anti-bacopaside I MAb against some naturally occurring compounds (Cont.)

Compound	Classification	CRs (%)
Diosgenin	Steroids	<0.01
Digitonin	Cardiac glycosides	<0.01
Solanine	Steroidal alkaloids	<0.01
Swertiamarin	Iridoid glycosides	<0.01
Geniposide	Iridoid glycosides	<0.01
Sennoside A	Anthraquinones	<0.01
Aesculetin	Coumarins	<0.01
Griseofulvin	Coumarins	<0.01
Hesperidin	Flavonoids	<0.01
Cinnamic acid	Phenyl propanes	<0.01

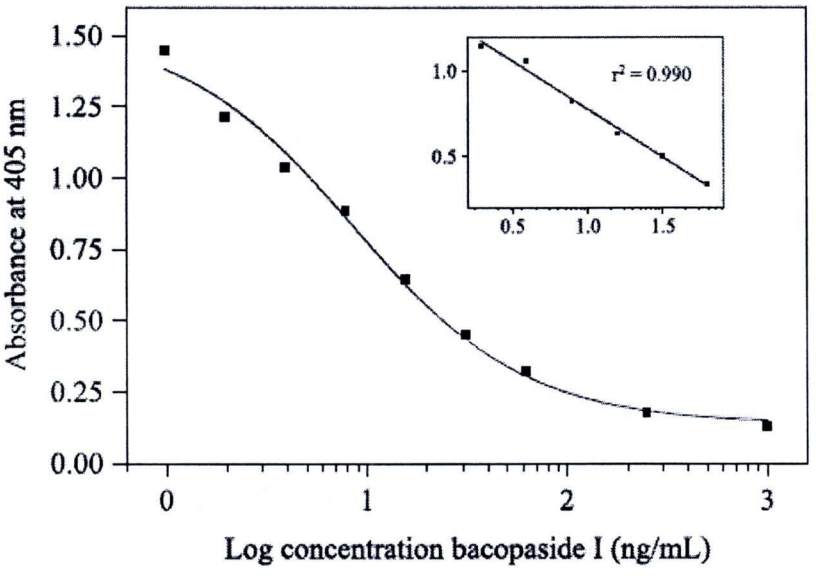


Figure 6 Dose-response curve of bacopaside I detected by competitive ELISA using anti-bacopaside I MAb at 405 nm

Figure 6 show the standard curve of standard curve of bacopaside I by plotting absorbance at 405 nm against the logarithm of bacopaside I concentrations. Under these conditions, the full linear range of the assay was extended from 1.95-62.5 $ng.ml^{-1}$ as indicated in figure 6. Limit of detection of ELISA technique using anti-bacopaside I MAb is 0.5 $ng.ml^{-1}$.

Pseudojujubogenin glycosides contents in the sample using ELISA technique was reported as total pseudojujubogenin glycosides. The sensitivity of this method is high, represented low limit of detection, as mentioned above.

5.2 Immunochromatographic strip

In order to screen large numbers of plant samples for the presence of pseudojujubogenin glycosides, a rapid and simple assay system is required for application to small quantities of test materials. The reported methods used for determination of saponin glycosides and pseudodjujubogenin glycosides in *B. monnieri* are HPLC (Ganzera et al., 2004; Deepak et al., 2005) and an enzyme-linked immunosorbent assay (ELISA) using both polyclonal and monoclonal antibodies (Phrompittayarat et al., 2007a,b).

The major component of immunochromatogrphic strip including sample pad, conjugate pad, membrane and absorbent pad as shown in the figure 7.

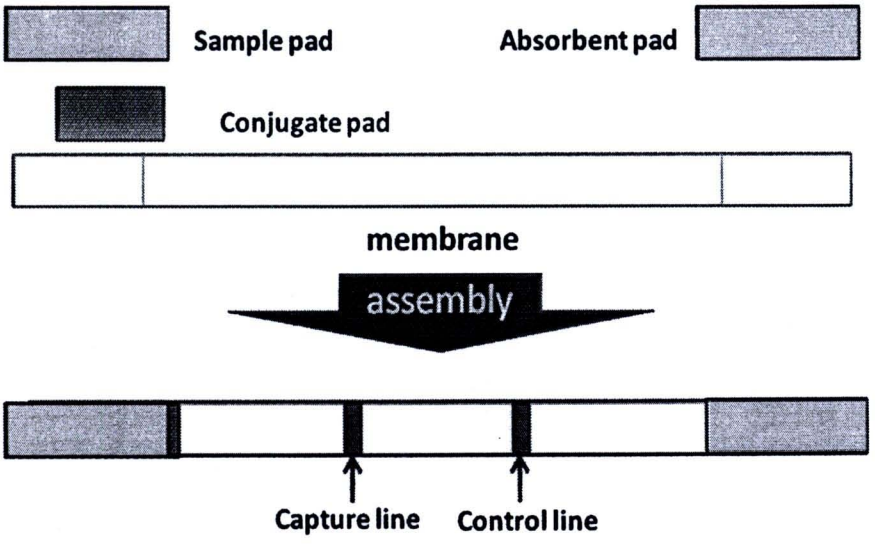


Figure 7 Immumnochromatographic strip component

5.2.1 Immunochromatographic strip components (Anonymous, 1999)

5.2.1.1 Sample pad

Which is to promote the even distribution of the sample volume into the conjugate pad, the sample pad helps to spread out the sample volume so that liquid enters the conjugated pad in an even and controlled manner. The sample pad may also control the rate at which liquid enters the conjugate pad so that flooding of the device is prevented. In some instances, the sample pad may also perform a much wider range of functions. By pre-treating the sample pad with components such as proteins, detergents, viscosity enhancers and buffer salts, the sample pad can also be used to increase sample viscosity, enhance the ability of the sample to solubilize the detector reagent and prevent the conjugate (and analyte) from binding non-specifically to any of the down-stream materials.

5.2.1.2 Conjugate pad

The most important of which is acting as a reagent delivery vehicle for the assay detector reagent. The detector reagent is deposited into the pad material often in conjunction with some combination of reagents, detergents, resolubilization promoters and stabilizers.

5.2.1.3 Absorbent pad

Absorbent pads, when used, are placed at the far end of the immunochromatographic strip. The major advantage of using an absorbent pad is that the total volume of sample that enters the test can be increased. This increased volume can be use to wash away unbound detector reagent from the nitrocellulose membrane. The net result is that the assay readout zone will be lower background and assay sensitivity can be enhanced.

5.2.2 Type of immunochromatographic strip

Immunochromatographic strip could be separated by the antigen-antibody reaction as direct reaction and competitive reaction.

5.2.2.1 Direct reaction

The detector reagent on conjugate pad is antibody-signal reagent conjugate. The reagent on the capture line of the membrane is antibody against interested compound whilst immunoglobulin G (IgG) on control line.

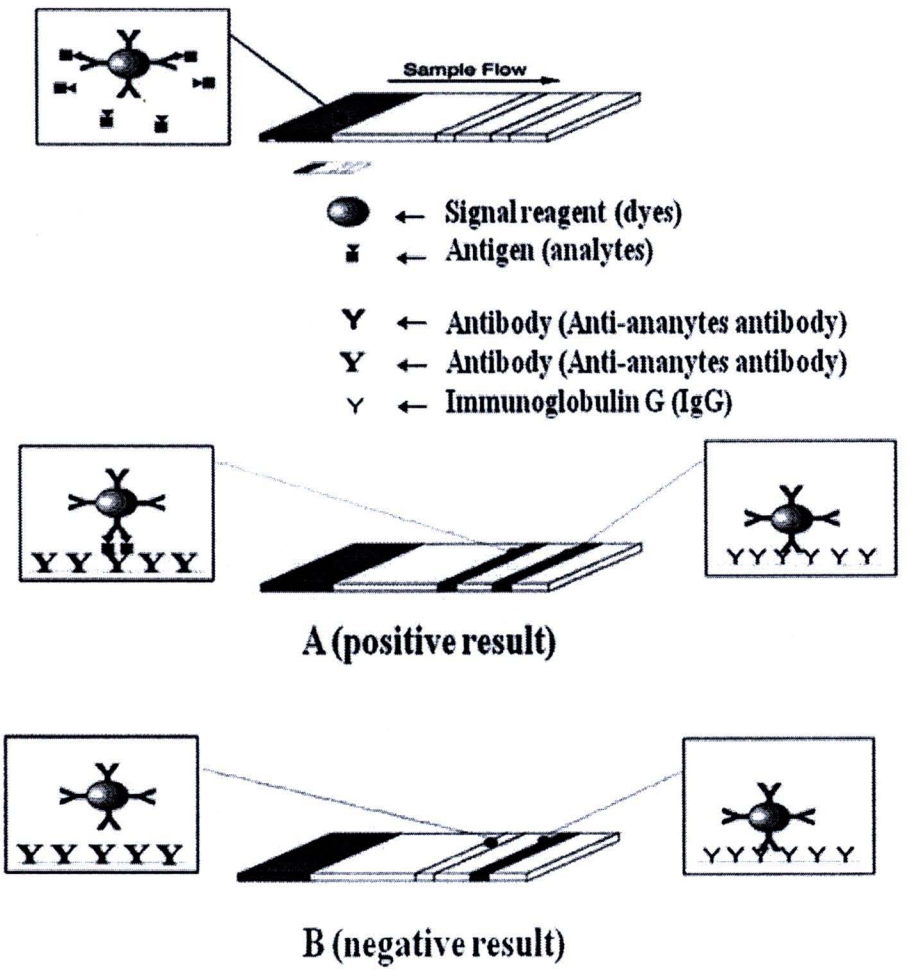


Figure 8 Principle of immunochromatographic strip based on direct reaction of antigen-antibody

5.2.2.2 Competitive reaction

The detector reagent of the immunochromatographic strip using competitive reaction still the same detector reagent of direct reaction. The difference part of these two immunochromatographic strip is the reagent at capture

line on the membrane. In this case, the reagent on the capture line of the membrane is antigen or interested compound conjugated with protein, commonly human serum albumin (HSA) or bovine serum albumin (BSA), which binding the membrane.

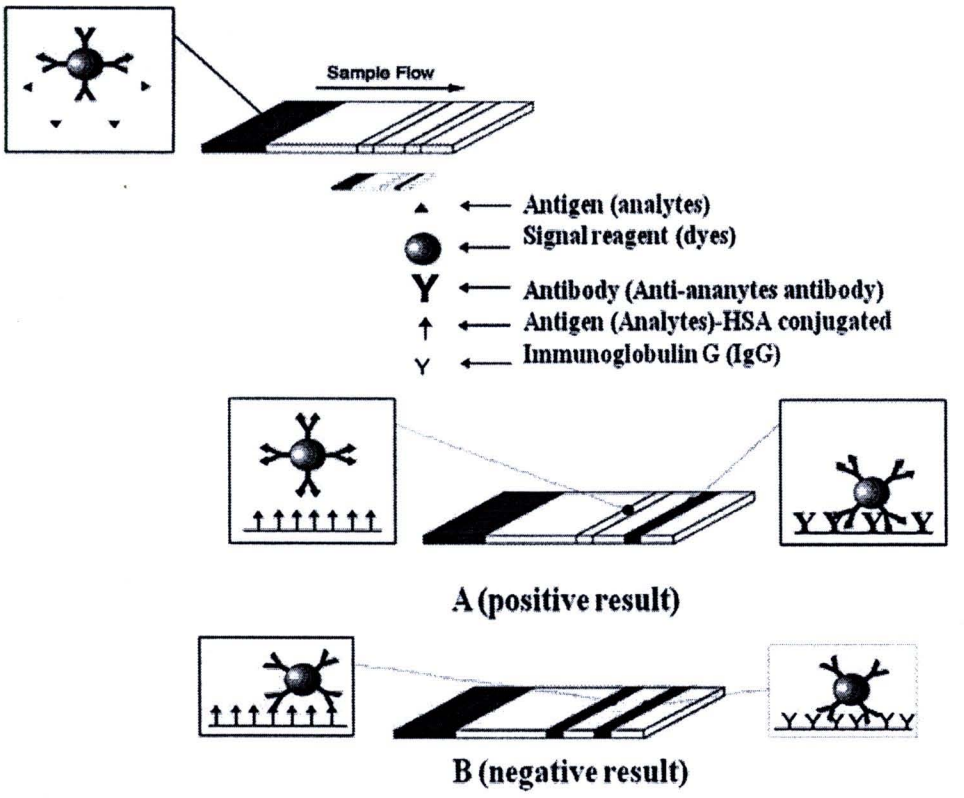


Figure 9 Principle of immunochromatographic strip based on competitive reaction of antigen-antibody

Immunoassays using monoclonal antibodies (MAbs) are highly specific and are, therefore, useful for both quantitative and qualitative analyses. Immunochromatographic assays are based on competitive immunoassays that utilize the antigen-antibody binding properties and provide rapid and sensitive detection of analytes. A variety of immunochromatographic strip assays have been reported for the detection of *Staphylococcus aureus* antigen (Huang et al., 2007), sulfadiazine residues (Wang et al., 2007), ginsenoside Rb1, Rg1 (Putalun et al., 2004a) and glycyrrhizin (Putalun et al., 2005) as describe in the table below.

Table 4 The studies of immunochramatographic strip

Interested compound	Detector reagent	Capture zone	Control zone	Reaction	Researcher
Ginsenosides Rb1 and Rg1	Gold nanoparicles conjugated with anti-ginsenoside Rb1 and Rg1 MAb	Ginsenoside Rb1-HSA and Ginsenoside Rg1-HSA conjugated	Anti-mouse IgG	Competitive	Putalun et al., 2004a
Glycyrrhizin	Gold nanoparicles conjugated with anti-glycyrrhizin MAb	Glycyrrhizin-HSA conjugated	Anti-mouse IgG	Competitive	Putalun et al., 2005
Staphylococcus aureus (Protein A)	Gold nanoparticles conjugated with anti-protein A IgG	Anti-protein A IgG	Goat anti-rabbit IgG	Direct	Huang et al, 2007
Sulfadiazine	Gold nanoparicles conjugated with anti-sulfadiazine MAb	Sulfadiazine-BSA conjugated	Goat anti-mouse IgG	Competitive	Wang et al., 2007

5.3 Eastern blotting

In this thesis, eastern blotting using polyethersulphone membrane (PES membrame) was developed. Shan et al., (2001) reported the eastern blotting technique which, make it possible to visualize small molecule compounds on a polyvinylidene difluoride (PVDF) membrane from a TLC plate developed by solvent system to a PVDF membrane and separated the glucoside molecule into two functional parts, the epitope and sugar parts. The sugar parts in glucosides were oxidatively clevaged to give aldehyde groups which were conjugated with carrier protein to fix on a PVDF membrane. However, because the transfer efficiency was not efficient, the method could not be applied for the quantitative immunoassay. Therefore, the direct development of glucosides by solvent system without transfer from a TLC plate was needed. Morinaga et al., (2005b) reported the eastern blotting using PES membrane without transfer from a TLC plate.

Morinaga et al. (2005b) studied the property of membrane for eliminate the transfer efficiency effect from TLC plate though PVDF membrane that shown in table below.

Table 5 The study of a variable membrane for Eastern blotting technique

Membrane	Eastern blotting	Physical property	Physical strength
nitrocellulose	poor	hydrophilic	weak
positive charged PVDF	good	hydrophobic	strong
uncharged PVDF	fair	hydrophobic	strong
positive charged nylon	poor	hydrophilic	strong
uncharged nylon	poor	hydrophilic	strong
positive charged PES	good	hydrophilic	strong
uncharged PES	poor	hydrophilic	strong

As shown in the table 5, Morinaga et al (2005b) studied the seven membranes property to find the proper membrane for quantitative analysis of

ginsenoside Rb1, Rc and Rd using Eastern blotting technique. Positive-charged PES membrane was selected to determine the amount of ginsenoside Rb1, Rc and Rd. Moreover, many studies of Eastern blotting using PES membrane were described in the table 6.

Table 6 The quantitative analysis of interested compound in plant samples using eastern blotting

Interested compound	LOD	The content	Researcher
Ginsenoside Rb1 (G-Rb1), G-Rc, G-Rd	62.5 ng	0.125-2.0 μg	Morinaga et al., 2005b
Glycyrrhicin	0.5 μg	1.0-8.0 μg	Morinaga et al., 2005a
Ginsenoside Re	125 ng	0.25-4.0 μg	Morinaga et al., 2006a
Saikosaponin a (SSa), SSc, and SSd	62.5 ng	62.5 ng-1.0 μg	Morinaga et al., 2006b

From the above table, the studies of Morinaga et al. (2005a,b; 2006a,b) represented the PES membrane could be used for quantitative analysis of many glycosides.

A variety of eastern blotting using PES membrane have been reported for the detection of ginsenoside Rb1, Rc and Rd in crude extracts of various ginsengs (Morinaga et al., 2005b), glycyrrhizin in licorice roots and traditional Chinese medicines (Morinaga et al., 2005a) and saikosaponins in *Bupleuri radix* (Morinaga et al., 2006b). Such eastern blotting is based on direct immunoassays that utilize the antigen-antibody binding properties and provides specific detection only pseudojujubogenin glycosides. The advantage of the new approach over the HPLC method are mainly its saving cost-performance (e.g. organic solvents and analytical equipments), speed and ease of use, which are useful if large numbers of smaller samples are to be analyzed.

5.4 Immunoaffinity column

Immunoaffinity column were developed for separation or purification of pseudojujubogenin glycosides in the sample. This technique have been reported for separation of solasodine glycosides in plant samples (Putalun et al., 1999) and glycyrrhizin in plant samples (Xu et al., 2007). Due to the high sensitivity of ELISA and the published document by Phrompittayarat et al. (2007a,b), ELISA assay was used as the reference result.

As mentioned above, application of antibodies for immunological assays were developed due to variety of their advantages such as rapid method for screening of interested compound like immunochromatographic strip, ELISA for analyzed numerous samples and immunoaffinity column for separation of small amount of sample.

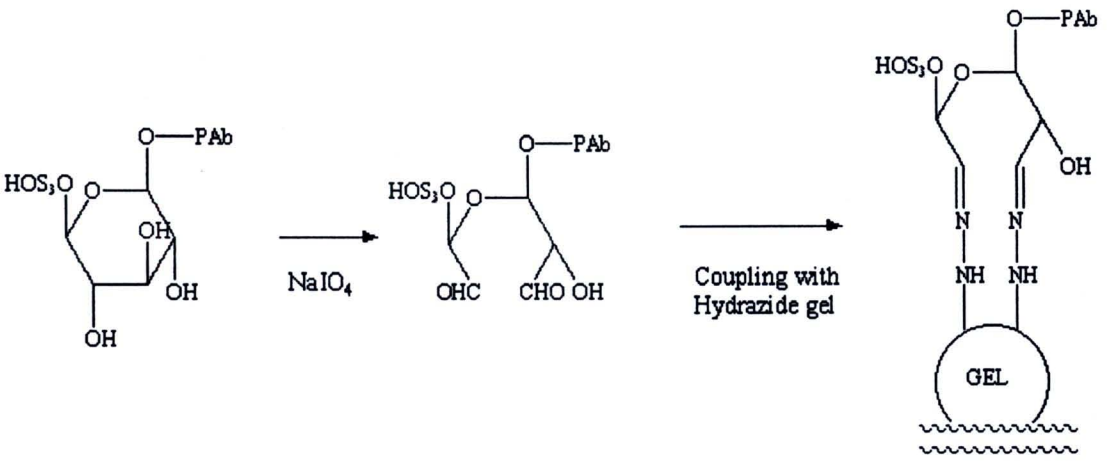


Figure 10 The coupling of antibody with hydrazide gel

Immunoaffinity chromatography is one such method that takes advantage of the specific and reversible binding interaction of antibody with its antigen, which can often result in separation or purification of the target compound in a single step. Hydrazide gel is an agarose support which reacts with the aldehydes of oxidized carbohydrates to form stable, covalent hydrazone bonds. Immunoglobulin G, antibody, is a glycoprotein which contains approximately 3% carbohydrate localized

on the Fc region (heavy chain) of the antibody. Periodate oxidation of vicinal hydroxyls of the sugars of these carbohydrates forms aldehyde groups for specific coupling to hydrazide gel. This coupling through the carbohydrate eliminates the loss of antibody activity experienced in primary amino coupling at or near the antigen binding site by allowing the correct orientation of the antibody.

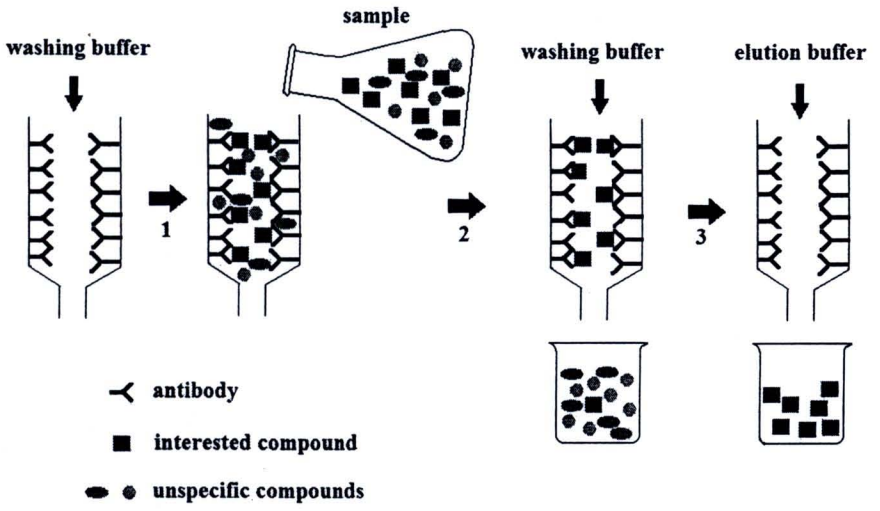


Figure 11 The separation of interested compound using immunoaffinity column

Figure 11 shows the flow for separate the interested compound from the immunoaffinity column. After completely coupling of antibody to hydrazide gel, washing buffer was used to equilibrate the immunoaffinity column. Then loaded and circulated the sample into this column to take a time for binding of interested compound to antibody in column. After that, the washing buffer was used to eliminate unspecific compounds from the column. Finally, the interested compound was eluted from the immuaffinity column by elution buffer. All fractions from washing and eluting method were collected to determine the interested compound contents.

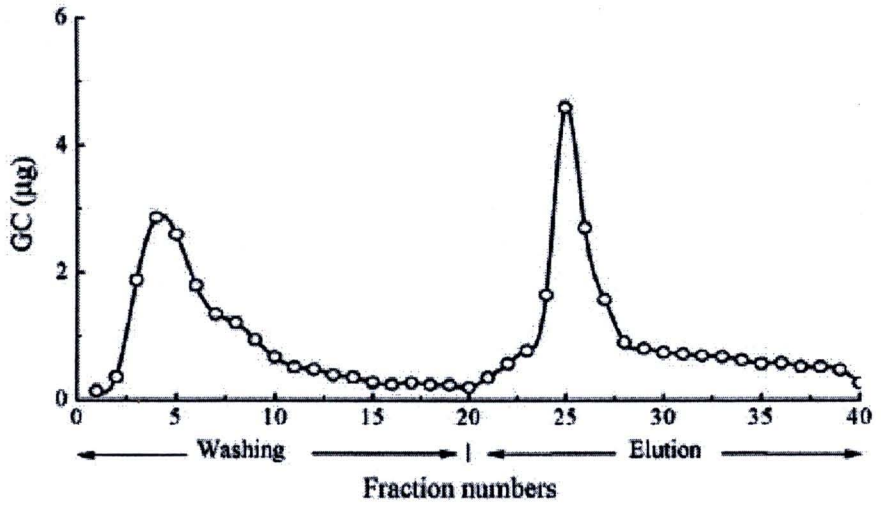


Figure 12 Elution profile of glycyrrhizin from the crude extracts of *Shakuyaku kanzo to* (*Shaoyao gancao tang*) with an immunoaffinity column coupled with the anti-glycyrrhizin-MAb

The elution profile from figure 12 represented that the excess glycyrrhizin, which not bind to anti-glycyrrhizin MAb coupled with hydrazide gel, was washed with washing buffer as same as the unspecified compound at the first peak of elution profile. After washing the immunoaffinity column then elute the glycyrrhizin with elution buffer to eluted glycyrrhizin that bind to anti-glycyrrhizin MAb coupled with hydrazide gel. The fraction from washing and eluting procedure were collected to analyzed the glycyrrhizin content by ELISA (Xu et al., 2007).

Immunoaffinity column was proper for separation of interested compound due to rapid. However, the capacity of immunoaffinity column is necessary for separate efficacy of interested compound. The factors including column volume, antibody property, washing buffer and elution buffer were affected column capacity.