CHAPTER III
IMMUNOCHROMATOGRAPHIC STRIP FOR DETECTION OF PSEUDOJUJUBOGENIN GLYCOSIDES

1. Introduction

Bacopa monnieri (L.) Wettst. (Scrophulariaceae) is a medicinal plant that has been used for centuries in the Ayurvedic system of medicine to improve intelligence and memory (Stough et al., 2001; Roodenrys et al., 2002) and to revitalise the sensory organs (Singh, Dhawan, 1997). These pharmacological activities have been primarily attributed to the saponin compounds present in the alcoholic extract of the plant. The major chemical constituents that have been isolated and characterised from *B. monnieri* are all dammarane-type triterpenoid saponins and include pseudojujubogenin glycosides and jujubogenin glycosides (Garai et al., 1996; Hou et al., 2002; Chakravarty et al., 2003).

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 the determination of saponin glycosides and pseudojujubogenin glycosides in *B. monnieri* are HPLC (Ganzera et al., 2004; Deepak et al., 2005) and an enzyme-linked immunosorbent assay (ELISA) that used both polyclonal and monoclonal antibodies (Phrompittayarat et al., 2007a,b).

Immunoassays using monoclonal antibodies (MAbs) are highly specific and are, therefore, useful for both quantitative and qualitative analyses. Recently MAbs against bacopaside I have been produced and an ELISA method has been developed for the determination of pseudojujubogenin glycosides in *B. monnieri* plant extracts (Phrompittayarat et al., 2007a).

A variety of immunochromatographic assays have been reported for the detection of *Staphylococcus aureus* antigen (Huang et al., 2007), sulfadiazine (Wang et al., 2007) ginsenosides Rb1 and Rg1 (Putalun et al., 2004a) and glycyrrhizin (Putalun et al., 2005). Such immunochromatographic assays are based on competitive

immunoassays that utilise the antigen-antibody binding properties and provide rapid and sensitive detection of analytes. In the present study, we have developed an immunochromatographic strip test for the detection of pseudojujubogenin glycosides using an anti-bacopaside I monoclonal antibody. The method development and applications are discussed in this chapter.

The immunochromatographic test strip of pseudojujubogenin glycosides was based on a competitive immunoassay methodology using an anti-bacopaside I MAb as the detection reagent (figure 13). Pseudojujubogenin glycosides in the sample competed for binding to the limited amount of antibodies in the detection reagent with the immobilized bacopaside I-HSA conjugates on the membrane. The test material solution was applied to the sample pad, and pseudojujubogenin glycosides in solution were bound to the detection reagent (containing colloidal gold conjugated of anti-bacopaside I MAb) present in the conjugate pad. Free pseudojujubogenin glycosides or the respective free detection reagent migrated up the strip and passed over the capture reagents (bacopaside I-HSA) where any detection reagent that was free of analytes would bind to the appropriate capture reagent at the respective capture zone whilst the control reagent (anti-goat to mouse IgG) would bind to the anti-bacopaside I MAb at the control zone. Color appeared at both the capture and control zones if the sample contained no pseudojujubogenin glycosides (negative sample; fig. 13, lane C), while no color developed at the capture spot zone when the sample contained pseudojujubogenin glycosides (positive sample; fig. 13, lane B).

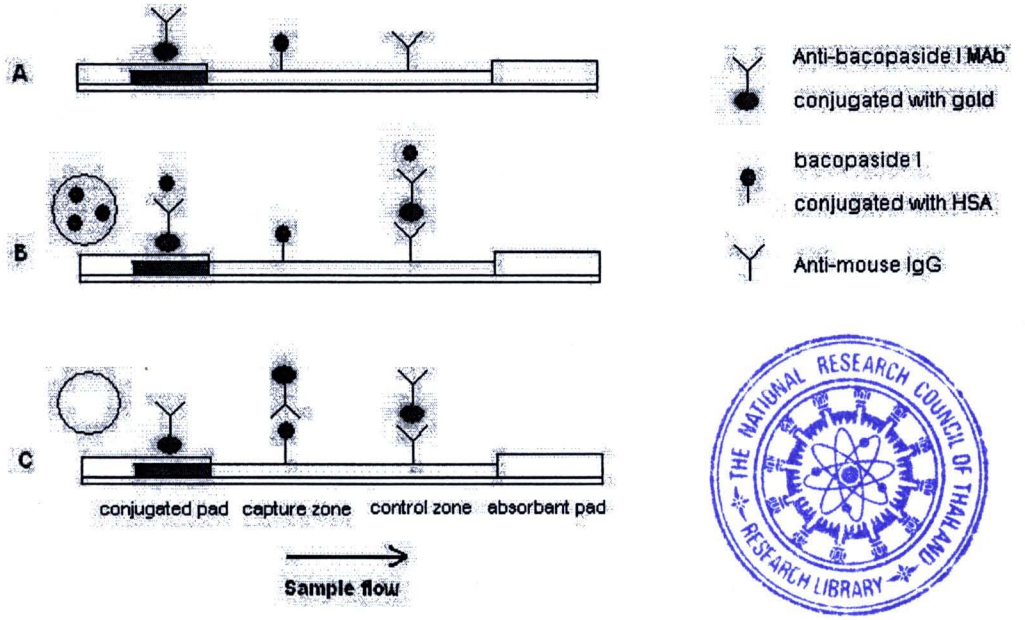


Figure 13 The strip test is based on a competitive immunoassay methodology using anti-bacopaside I MAb as a detection reagent (A). Color only appears in the control zone if a sample is positive for pseudojujubogenin glycosides (B), whilst color appears in both the capture zone and the control zone if a sample contains no pseudojujubogenin glycosides (C)

2. Methodology

2.1 Chemicals and immunochemicals

Bacopaside I were perchased from Chromadex (Irvine, CA, USA). Colloidal gold with an average particle diameter of 10 nm was purchased from Sigma (St Louis, MO, USA). BSA and HSA were provided from Fluka (Buchs, Switzerland). All other chemicals were standard commercial products of analytical grade.

2.2 Plant materials

Different plants part (Top, Stem, Root, Shoot from TDZ0.1, Regenerated plants from TDZ0.1 and CallusD0.5K1) of *B. monnieri*. *B. diffusus*, *B. cordifolia*, *Zizyphus jujuba* and *Z. cambodiana* were collected from Khon Kaen, Thailand. The identification of the plants was confirmed by Dr. Tripetch

Kanchanapoom, Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. Voucher specimens (NI-PSKKU 001-005) are deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand.

2.3 Extraction of plant samples

Dried samples (50 mg) of plant samples were powdered extracted five times with 500 µl of methanol under sonication for 15 min and centrifuge at 3,000 rpm for 1 min. The extracts were evaporated at 60 °C until dryness, and the dried extracts were dissolved in 1 ml of MeOH for analysis using Eastern blotting and ELISA.

2.4 Preparation of antibody-colloidal gold

Colloidal gold solution was adjusted to pH 9.0 with 2% potassium carbonate solution. Bacopaside I-MAb (125 µg) was added to the colloidal solution (1 ml) and stirred gently at room temperature (about 25°C) for 10 min. The conjugate was stabilized with BSA in 0.1 M Tris-hydrochloride (pH 8.0) and adjusted to a final concentration of 1% BSA. The mixture was incubated for 1 hour at room temperature and centrifuged at 14,000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 1% BSA in 0.1 M Tris-hydrochloride (pH 8.0). The concentrated MAb-gold conjugate in 1% BSA was stored at 4°C until required for use. The detection reagent contained 10 µl of anti-bacopaside I MAb conjugated colloidal gold, 2 µl of 10% sucrose in water, 0.4 µl of 1% Tween 20 in water and 0.6 µl of water. An aliquot (13 µl) of the detection reagent was applied to the glass fiber conjugate pad (Millipore Temecula, CA, USA) and the pad was dried for 2 hr at room temperature prior to assembly onto the strip test.

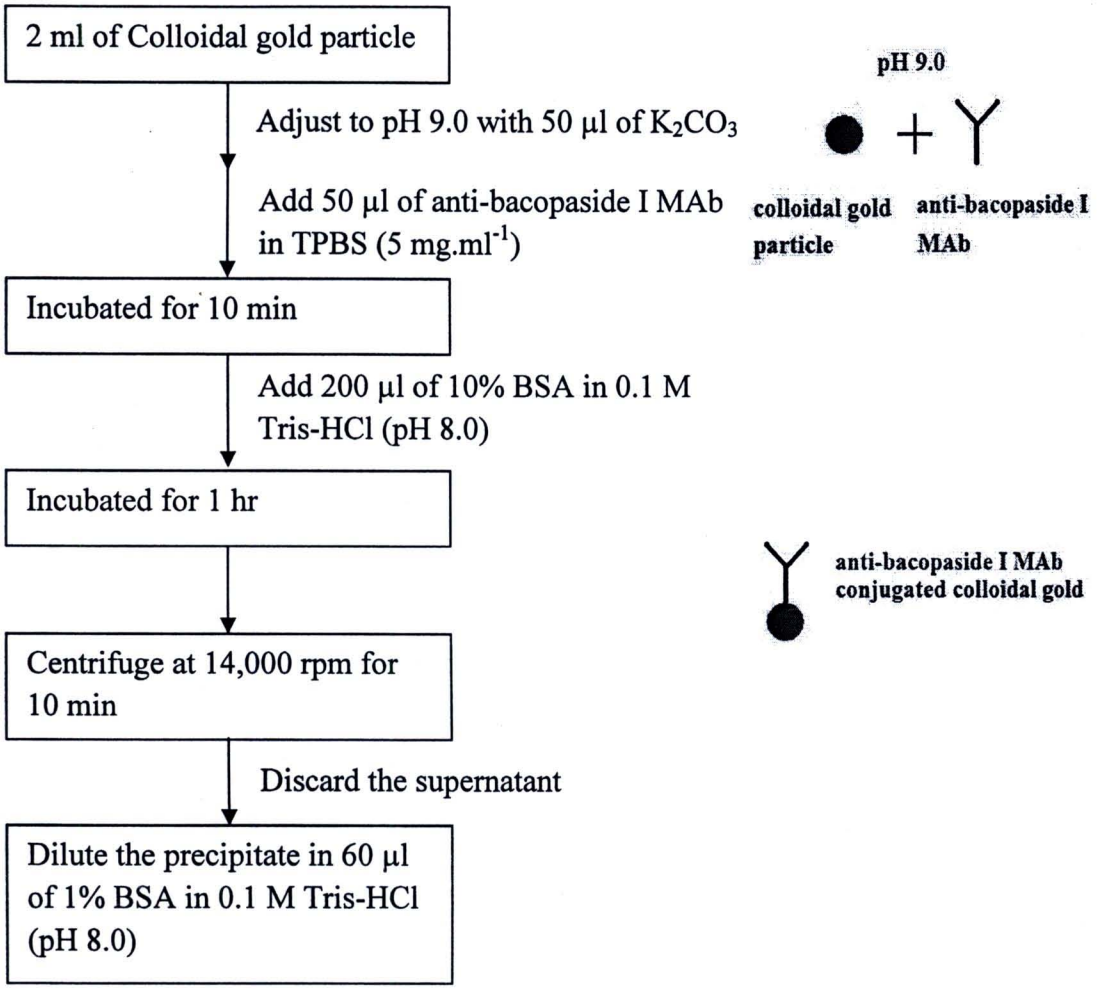


Figure 14 The conjugation of colloidal gold particle and anti-bacopaside I MAb

2.5 Preparation of capture reagent

Bacopaside I-HSA conjugate was synthesized by a peroxidase oxidation method (Shan *et al.*, 2001). Bacopaside I (4 mg) in 0.5 ml of methanol was added drop wise to 0.5 ml of sodium iodate solution (4 $mg.ml^{-1}$) and then stirred at room temperature for 1 hour. Subsequently, HSA (4 mg) in 1 ml potassium carbonate buffer (50 mM, pH 9.6) was added and stirred for 5 hr. The reaction mixture was dialyzed against water five times and then lyophilized to yield bacopaside I-HSA conjugate (6.8 mg).

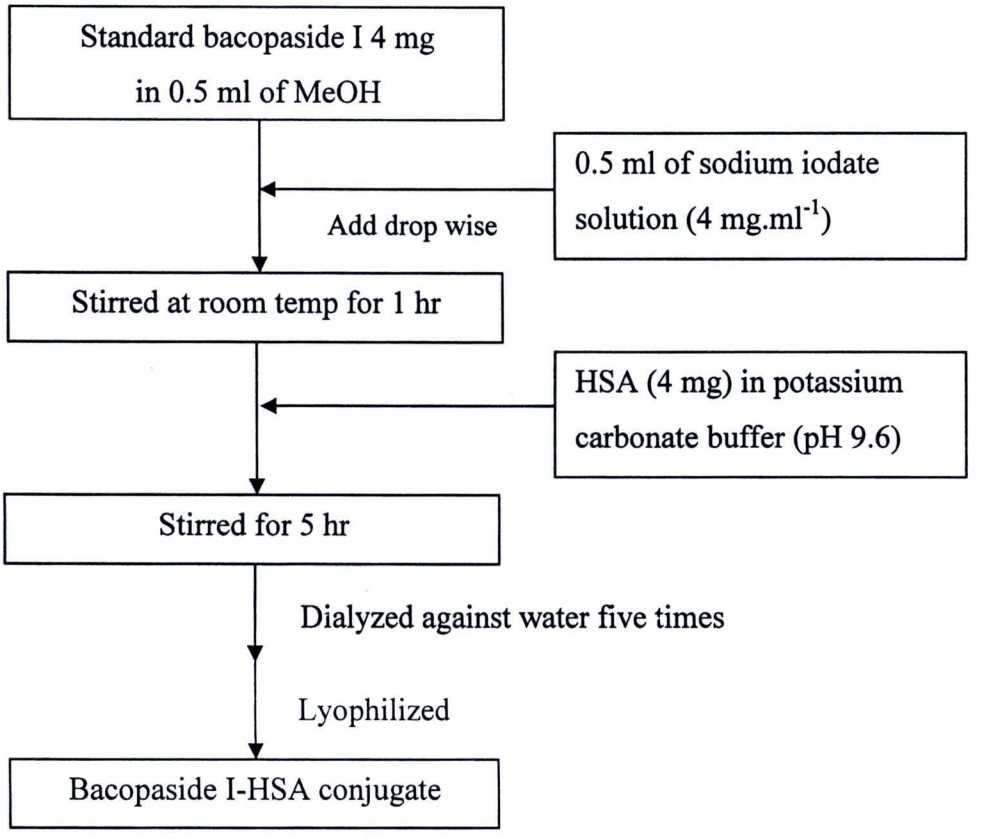


Figure 15 The flow chart of capture reagent preparation

2.6 Preparation of chromatographic strip

Control (anti-mouse IgG; 1 μg) and test capture (bacopaside I-HSA; 2 μg) reagents were applied to a strip of nitrocellulose membrane (Millipore Temecula, CA, USA). After drying at room temperature for 1 hr, the membrane was immersed in phosphate-buffered saline (PBS) containing 1% BSA and incubated with stirring at room temperature for 2 hr. The membrane was washed twice with PBS containing 0.05% Tween 20 (TPBS) for 10 min. After drying the membrane was cut into single test strips. Each test strip of the nitrocellulose membrane contained an adsorbent pad, the detection reagent in the conjugate pad and a sample pad. The sample solution (250 μl) was transferred to a tube into which the lower edge of the test strip was dipped. The sample migrated upwards and the results of test were read after 15 min.

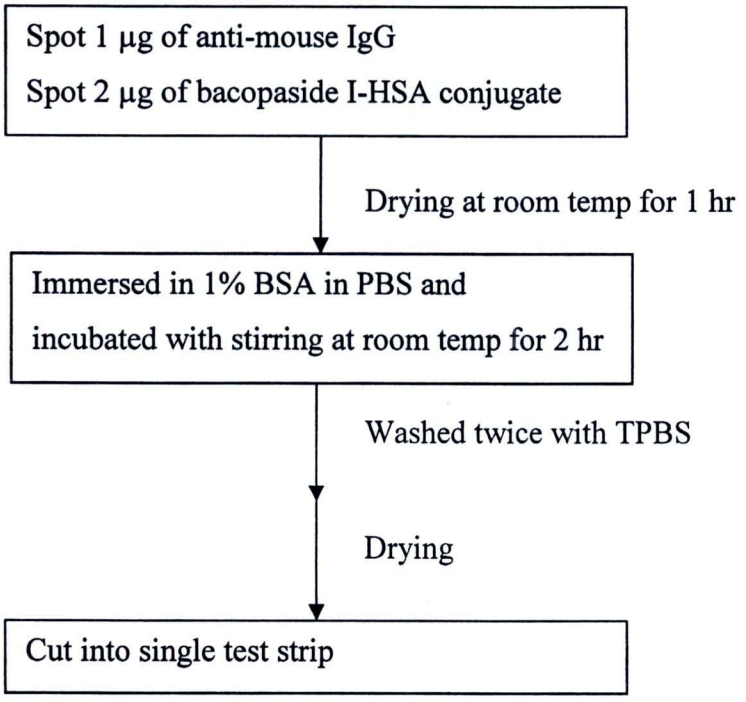


Figure 16 The flow chart of immunochromatogrphic membrane preparation

3. Results and discussion

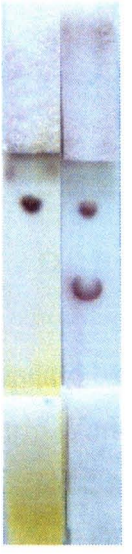
The immunochromatographic strip test (figure 13), was based on a competitive immunoassay using anti-bacopaside I MAb as detector antibodies. A sample solution was applied to the sample pad, and pseudojujubogenin glycosides in the sample were bound by the detection reagent in the conjugate pad. This pad contain detection reagent, anti-bacopaside I MAb colloidal gold conjugates.

Pseudojujubogenin glycosides were bound to the detection reagents, and free pseudojujubogenin glycosides, and detection reagent migrated up the strip with the sample. We used 1% BSA in PBS as a blocking solution to reduce non-specific adsorption and to immobilize the capture reagents on the nitrocellulose membrane. Any non adsorbed blocking agent was removed by washing the membrane with TPBS to promote uniform re-wetting of the membrane. BSA was used as a stabilizer for the anti-bacopaside I MAb colloidal gold conjugates. Sucrose was added to increase the solubility of the detection reagent. The addition of Tween 20 in the detection reagent promoted solubilization, as did pretreatment of the sample pad with TPBS.

When the sample solution passed over the capture reagent (bacopaside I-HSA), the detection reagent that was free of analyte bound the capture reagent at capture zone whereas the control capture reagent (anti-mouse IgG) bound to the anti-bacopaside I MAb held to the detection at the control zone. When pseudojujubogenin glycosides were present in the sample, they competed with the immobilized bacopaside I conjugate with HSA on the membrane for the limited amount of antibody of the detection reagent. Thus, the immobilized capture reagent was prevented from binding with detection reagent on the membrane when sufficient amounts of pseudojujubogenin glycosides were present in the sample. Therefore, a positive sample produced no visible test spot in the test capture zone and the control test spot was always visible. Color appeared at capture spot if the sample contained no pseudojujubogenin glycosides.

Anti-bacopaside I MAb is highly cross-reactive against pseudojujubogenin glycosides, whereas the cross-reactivity of MAb described here against jujubogenin glycosides was very low. These result indicated that the aglycone part of the saponin glycosides is essential for reactivity of anti-bacopaside. I MAb. Therefore, an

immunochromatographic strip test based on an immunoassay system with an anti-bacopaside I MAb was developed specifically to detect pseudojujubogenin glycosides with high sensitivity and specificity.

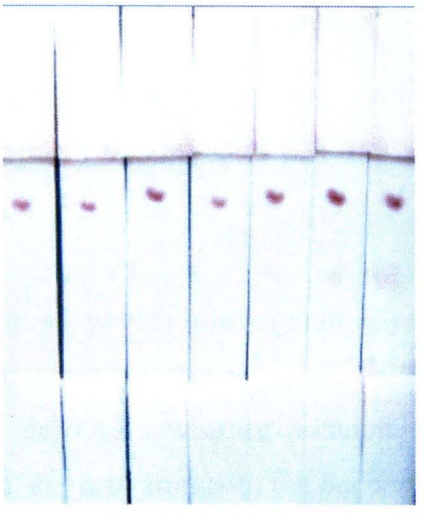


A B

Figure 17 The positive result of immunochromatographic strip (lane A) and the negative result of immunochromatographic strip (lane B)

3.1 The detection limit of the immunochromatographic strip of standard bacopaside I

In this study, standard bacopaside I was selected to representative of pseudojujubogenin glycosides for validation the detection limit of immunochromatographic strip to pseudojujubogenin glycosides. Standard bacopaside I were diluted 2-fold dilution to be a test for the detection limit of this strip.



A B C D E F G

Figure 18 The limit of detection of standard bacopaside I using immunochromatographic strip. Various concentration of standard bacopaside I were diluted 2-fold dilution. Lane A; 2 $\mu g.ml^{-1}$, lane B; 1 $\mu g.ml^{-1}$, lane C; 0.5 $\mu g.ml^{-1}$, lane D; 0.25 $\mu g.ml^{-1}$, lane E; 0.125 $\mu g.ml^{-1}$, lane F; 0.0625 $\mu g.ml^{-1}$ and lane G; 0.03125 $\mu g.ml^{-1}$

From the figure 18, we could summarize the result of the detection limit of this strip as shown in the table 7 below.

Table 7 The limit of detection of strip test for pseudojujubogenin glycosides

Standard bacopaside I concentration ($\mu g.ml^{-1}$)	Result of strip test
2	positive
1	positive
0.5	positive
0.25	positive
0.125	positive
0.0625	negative
0.03125	negative

The detection limit for pseudojujubogenin glycosides was calculated to be 125 $ng.ml^{-1}$. The appropriate volume size was 250 µl and the assay could be performed in about 10-15 min.

3.2 Determining of pseudojujubogenin glycosides in plant samples using immunochromatographic strip

In order to determine whether pseudojujubogenin glycosides were present in the studies plants, extracts from both the *Bacopa* and *Zizyphus* species were diluted five times with water, because the test strip membrane was destroyed by the application of undiluted sample. All *B. monnieri* samples (fig. 19, lane 1-5) contained pseudojujubogenin glycosides. In contrast, the pseudojujubogenin glycosides levels in extracts of *B. diffusus*, *B. cordifolia* and *Zizyphus cambodiana* were below the detection limits of the assay (fig. 19, lane 6-7 and 9). The results from figure 19 (lanes 1-3) indicated that various parts of *B. monnieri* were positive for pseudojujubogenin glycosides. Interestingly, a light pink color at the capture zone in the test conducted using *Z. jujuba* extract indicated that the sample was positive for pseudojujubogenin glycosides (fig. 19, lane 8). These results were confirmed by a competitive ELISA (Table 8). The leaf of *Z. jujuba* contained pseudojujubogenin glycosides (0.1060 ± 0.0138 $\mu g.mg^{-1}$ dry weight). Similar to a previous report, pseudojujubogenin glycosides were isolated from the leaf of *Z. glabrata* (Ganapathy et al., 2006). According to the immunochromatographic strip test and the ELISA analysis, the presence of pseudojujubogenin glycosides in *Z. jujuba* could be confirmed. The immunochromatographic strip test and the ELISA assay were more sensitive than HPLC method; the detection limits determined by ELISA and HPLC were 0.5 $ng.ml^{-1}$ and 0.31 $\mu g.ml^{-1}$, respectively (Phrompittayarat et al., 2007a). Therefore, the combination assay system involving immunochromatographic analysis and ELISA is suitable for the qualitative and quantitative analysis of pseudojujubogenin glycosides in plant extracts.

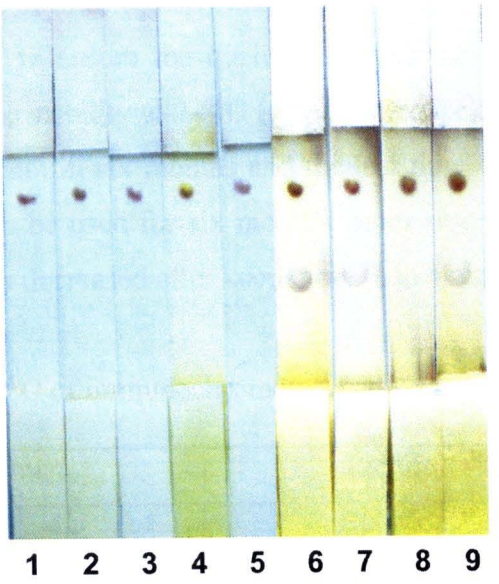


Figure 19 Immunochromatographic strip test assays for pseudojujubogenin glycosides in *Bacopa* and *Zizyphus* species. Lane 1, *Bacopa monnieri* (top); lane 2, *B. monnieri* (stem); lane 3, *B. monnieri* (root); lane 4, *B. monnieri* (*in vitro* plant); lane 5, *B. monnieri* (callus); lane 6, *B. diffusus* (leaf); lane 7, *B. cordifolia* (leaf); lane 8, *Zizyphus jujuba* (leaf); and lane 9, *Z. cambodiana* (leaf)

Table 8 Pseudojujubogenin glycosides content in *Bacopa* species and *Zizyphus* species as determined by the immunochromatographic strip test and ELISA.

Sample	Pseudojujubogenin glycosides content	
	Strip test	ELISA ($\mu g.mg^{-1}$ dry wt)
Bacopa monnieri (Top)	positive	16.75±3.14
Bacopa monnieri (Stem)	positive	12.33±3.74
Bacopa monnieri (Root)	positive	4.23±1.35
Bacopa monnieri (*In vitro* Plant)	positive	27.94±1.19
Bacopa monnieri (Callus)	positive	10.12±0.97
Bacopa diffusus (Leaf)	negative	0.0124±0.0035
Bacopa cordifolia (Leaf)	negative	0.0106±0.0012
Zizyphus jujuba (Leaf)	positive	0.1060±0.0138
Zizyphus cambodiana (Leaf)	negative	0.0710±0.0015

3.3 The stability of immunochromatographic strip

Furthermore, to ensure the quality of immunochromatographic strip, the strip test was assayed the stability at 2-8°C (refrigerator) for 1 year. At first, these strips were tested every month in six months and the last time on 1 year. The result showed that these strips can be used for six months under stored at 2-8°C (table 9). The activity of antibody was decreased after keep more than 6 months.

Table 9 The stability at 2-8°C of immunochromatographic strip

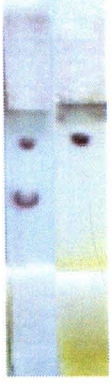
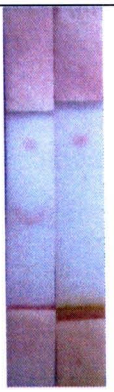
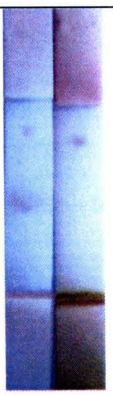
Time	Picture
1 month	
2 month	
3 month	

Table 9 The stability at 2-8°C of immunochromatographic strip (Cont.)

Time	Picture
4 month	
5 month	
6 month	
1 year