CHAPTER IV
DEVELOPMENT OF EASTERN BLOTTING FOR DETERMINATION OF PSEUDOJUJUBOGENIN GLYCOSIDES USING ANTI-BACOPASIDE I MONOCLONAL ANTIBODY

1. Introduction

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.

Due to the interested compound of our study is pseudojujubogenin glycosides, PES membrane was selected as the stationary phase to separated individual pseudojujubogenin glycosides. The proper mobile phase was selected by the criteria of membrane resistance, the separate efficiency. Morinaga et al. (2005a,b; 2006a,b) studied the mobile phase for separated of many glycosides by vary the ratio of ACN, H_2O and MeOH, H_2O. Base on the previous report of Phrompittayarat et al. (2007a) on HPLC mobile phase (ACN: H_2O: phosphoric acid ; 35:65:0.2 by volume), this system was selected for preliminary experiment of eastern blotting using PES membrane for determination of pseudojujubogenin glycosides contents.

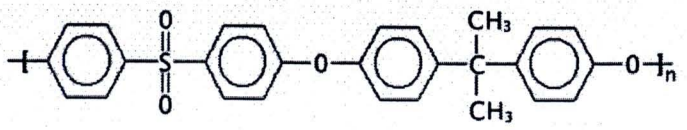


Figure 20 Chemical structure of polyethersulphone (PES) membrane

At first, the pseudojujubogenin glycosides in the sample was fixed on the PES membrane by conjugation of pseudojujubogenin glycosides and gelatin using periodate oxidation reaction as follow in figure 21.

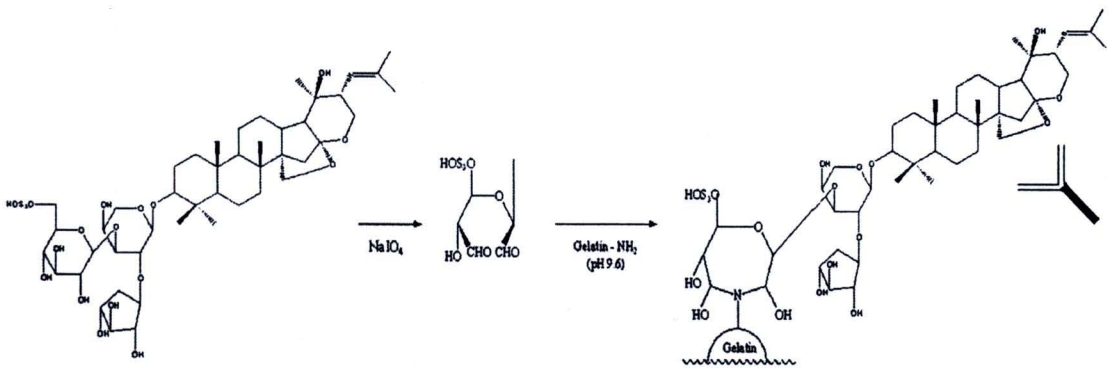


Figure 21 Fixing of bacopaside I-gelatin conjugate on PES membrane by periodate oxidation reaction

2. Methodology

2.1 Chemicals and immunochemicals

Bacopaside I was perchased from Chromadex (Irvine, CA, USA). PES membrane was purchased from Pall Corporation (USA). BSA and HSA were provided from Fluka (Buchs, Switzerland). All other chemicals were standard commercials 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*.

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 Visual detection of pseudojujubogenin glycosides by on-membrane immunoassay using an eastern blotting technique

Eastern blotting was performed as reported previously (Tanaka *et al.*, 1997) except for separation by TLC plate and transfer to a polyvinylidene difluoride (PVDF) membrane as follows. Standard bacopaside I and plants (*Bacopa monnieri*, *B. diffusus*, *B. cordifolia*, *Zizyphus jujuba and Z. cambodiana*) extracts were applied onto a PES membrane. After drying, this membrane was hung in the tank, immersed and developed by acetonitrile-water-phosphoric acid (35-65-0.2 by volume). The developed PES membrane was dried and dipped into water containing $NaIO_4$ (10 $mg.ml^{-1}$) for 1 hr. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing gelatin (1%) was added and was stirred at room temperature for 5 hour or overnight. After washing twice with TPBS for 5 min, the membrane was immersed in anti-bacopaside I MAbs (hybridoma-supernatant was used: Phrompittayarat et al., 2007a) and stirred in ice box for 2 hr. After washing twice with TPBS for 5 min, a 1:1,000 dilution of peroxidase-labeled goat anti-mouse IgG was added and stirred in ice box for 1 hr. And washing twice with TPBS for 5 min then the mixture of 4-chloro-1 naphthol in MeOH (10 $mg.ml^{-1}$)-PBS (1-9 by volume) and 0.01% H_2O_2 was added and stirred for 15 min at room temperature. The reaction was stopped by washing with water, and the immunostained PES membrane was allowed to dry. The intensity of the dark blue spots on PES membrane was analyzed using Gene tool analysis software. Pseudojujubogenin glycosides content was correlated to the intensity of the dark blue spot. In this study, standard bacopaside I was used as the standard so that pseudojujubogenin glycosides contents were reported equal to bacopaside I content.

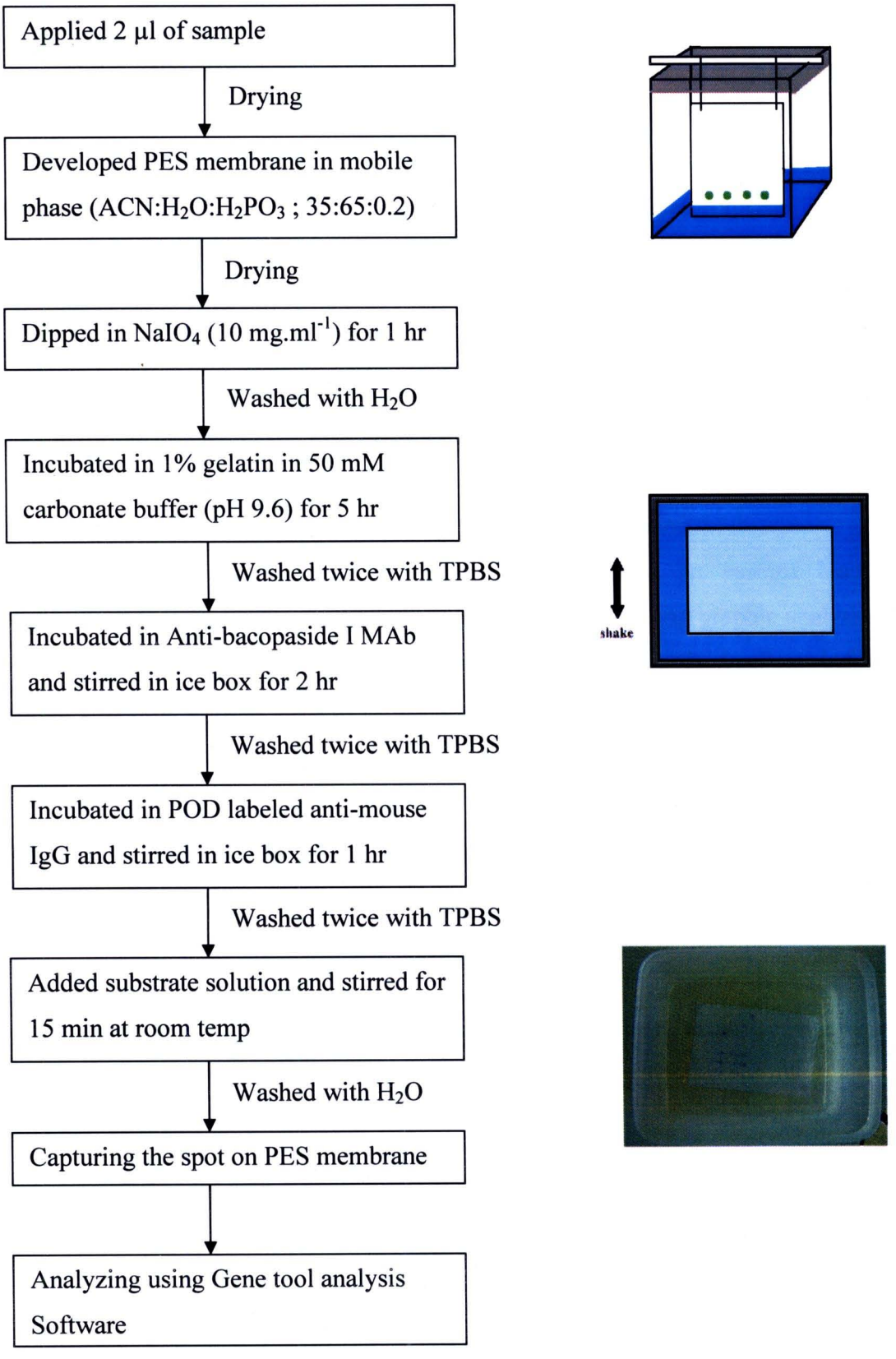


Figure 22 Protocol of eastern blotting using PES membrane

3. Results and discussion

3.1 Development of eastern blotting for detection of standard pseudojujubogenin glycosides

For the visual detection of pseudojujubogenin, we demonstrated a chromatographic separation of pseudojujubogenin glycosides utilizing a PES membrane and their immunostaining by using the eastern blotting technique. The sugar moieties of pseudojujubogenin glycosides were cut off randomly by $NaIO_4$ to create aldehyde groups. At alkaline condition, the opened sugar moieties conjugated with NH_2 group of gelatin to form shiff-base of antigen. Therefore, it is clear that binding on the surface of the PES membrane and the conjugation with carrier protein are dependent on functions of sugar moieties in this method. On the other hand, an aglycone part functions as epitope in the antigen-antibody reaction.

We applied the chromatographic resolution in eastern blotting technique to the quantitative immunoassay for bacopaside I using graphic analysis of Gene tool analysis software because the newly established method reflected direct sample amounts without transfer efficiency. Figure 23 show the standard curve of standard bacopaside I by plotting raw volume of the spot against the logarithm of bacopaside I concentrations. Under these conditions, the full linear range of the assay was extended from 3.125 to 100 ng as indicated in figure 23.

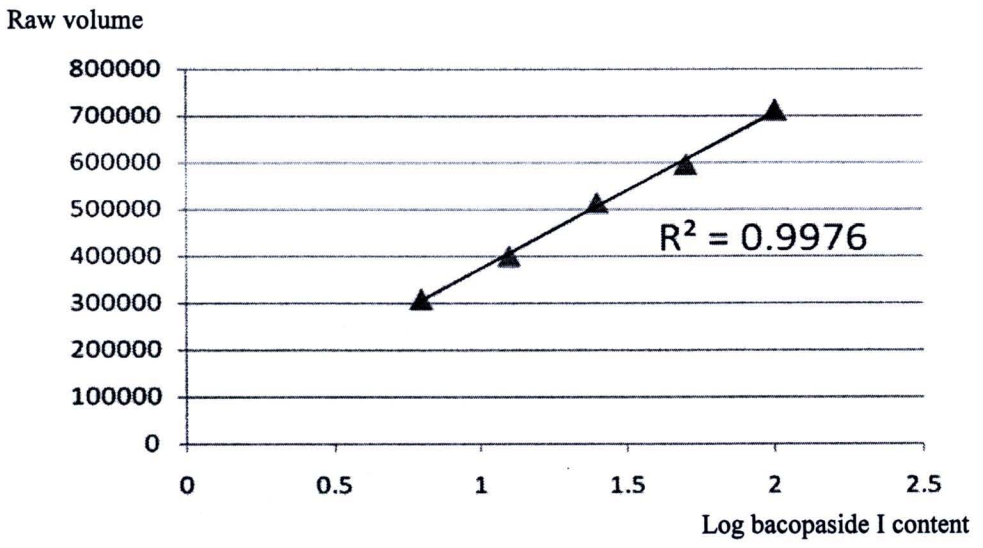
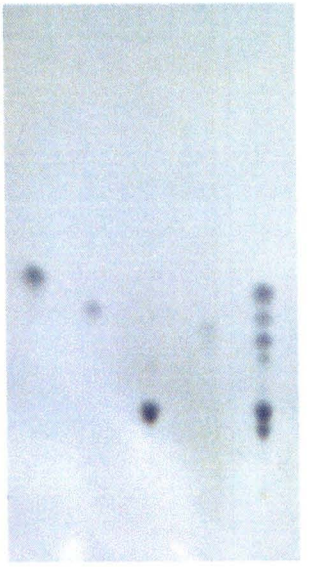


Figure 23 Standard curve of bacopaside I detected by Eastern blotting



1 2 3 4 5

Figure 24 Eastern blotting assay for standard pseudojujubogenin glycosides and brahmi extract. Lane 1, standard bacopaside II; lane 2, standard bacopasaponin C; lane 3, standard bacopaside I; lane 4, standard bacopaside V; and lane 5, *B. monnieri* extract

In this study, four standard pseudojujubogenin glycosides including bacopaside II (fig. 24, lane 1), bacopasaponin C (fig. 24, lane 2), bacopaside I (fig. 24, lane 3) and bacopaside V (fig. 24, lane 4) were used as shown in the figure 24. The right spot is Brahmi extract (fig. 24, lane 5).

As shown in the figure 24, brahmi extract gave six spots of pseudojujubogenin glycosides compared to the standard pseudojujubogenin glycosides. From these results, brahmi extract contained six pseudojujubogenin glycosides including bacopaside II, bacopasaponin C, bacopaside I and three unknown compounds.

1 2 3 4 5

Figure 25 Eastern blotting assay for standard bacopaside I, jujubogenin glycosides and Brahmi extract. Lane 1, standard bacopaside I; lane 2, *B. monnieri* extract; lane 3, standard bacoside A_3; lane 4, standard bacopasaponin C isomer; and lane 5, standard bacopaside IV

The result from figure 25 represented only pseudojujubogenin glycosides could be detected by eastern blotting technique due to high cross reactivity of anti-bacopaside I MAb against pseudojujubogenin glycosides. Jujubogenin glycosides (fig. 25, lane 3-5) could not be detected by eastern blotting because of low cross reactivity of anti-bacopaside I MAb against jujubogenin glycosides.

3.2 The limit of detection

At first, standard bacopaside I was selected to be the representative of pseudojujubogenin glycosides for evaluation the limit of detection of this method. The standard bacopaside I was diluted 2-fold dilution to give various concentrations for assayed of the detection limited. In these results, the lowest content of standard bacopaside I as 3.125 ng level give the positive result (fig. 26, lane 5) which presented the detection of the immunochromatographic strip.

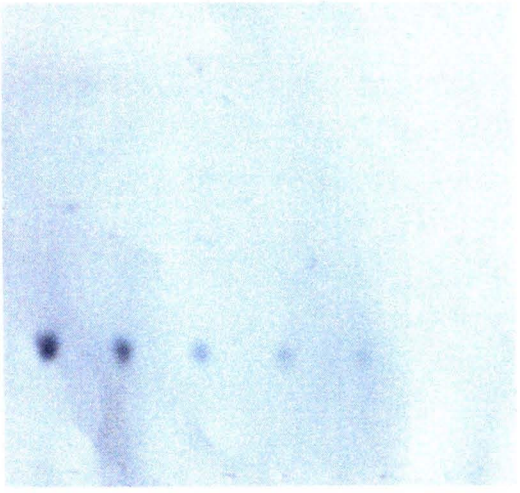


Figure 26 The limit of detection of pseudojujubogenin glycosides by eastern blotting analysis using standard bacopaside I. Lane 1; 50 ng; lane 2; 25 ng; lane 3; 12.5 ng; lane 4; 6.25 ng; lane 5; 3.125 ng and lane 6; 1.5625 ng

Table 10 The limit of detection of pseudojujubogenin glycosides by eastern blotting analysis using standard bacopaside I

Standard bacopaside I content (ng)	Result on PES membrane
50	positive
25	positive
12.5	positive
6.25	positive
3.125	positive
1.625	negative

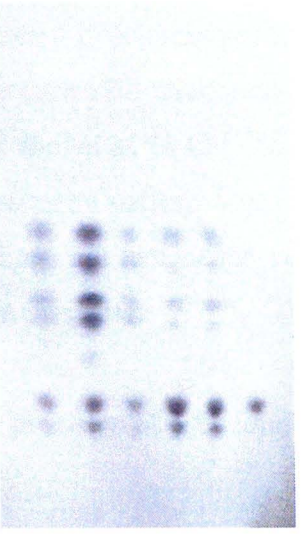


Figure 27 Eastern blotting profile of brahmi extracts and standard bacopaside I. Lane 1, *B. monnieri* (callus D0.5K1); lane 2, *B. monnieri* (regenerated plants from TDZ0.1); lane 3, *B. monnieri* (root); lane 4, *B. monnieri* (stem); lane 5, *B. monnieri* (top) and lane 6, standard bacopaside I

From the result above, only *B. monnieri* gave a positive result that could be determined each pseudojujubogenin glycosides. The different part of *B. monnieri* including callus of brahmi (fig. 27, lane 1), whole plant of regenerated brahmi from TDZ0.1 (fig. 27, lane 2) , root (fig. 27 , lane 3), stem (fig.27, lane 4) and top (fig. 27, lane 5) were detected comparing with standard bacopaside I (fig. 27, lane 6) as shown in figure 27.

Various parts of *B. monnieri* were analyzed and pseudojujubogenin glycosides were detected by the chromatographic resolution of pseudojujubogenin glycosides in eastern blotting technique (figure 27). The intensity of coloring spots on this membrane were calculated using graphic analysis of Gene tool analysis software. Regenerated plants from TDZ0.1 media contained high levels of pseudojujubogenin glycosides. These results were in a good agreement with those from ELISA technique.

3.3 Intra-assay and inter-assay

Precision (intra- and inter-assay) of the assay was verified by analyzing 3 replicates of six samples of brahmi extract. Each sample solution was spotted in

triplicate (intra-assay) on the same PES membrane. For inter-assay analysis, each sample was spotted on the difference PES membrane and assayed at difference time. The precision of the assay was shown as % CV. The result showed that % CV not higher than 4 was found for intra-assay analysis but, for intra-assay, % CV was higher than 10 for two sample including root and callus D0.5K1 due to small amount of pseudojujubogenin glycosides in these part.

Table 11 Intra- and inter-assay precision of pseudojujubogenin glycosides analyzed by eastern blotting

Sample	% CV intra-assay (n=3)	% CV inter-assay (n=3)
TOP	0.303	7.866
STEM	1.687	4.198
ROOT	2.144	29.473
Shoot from TDZ0.1	3.672	7.387
Regenerated plants from TDZ0.1	2.324	6.479
Callus D0.5K1	1.650	13.230

It is typical that intra-assay variations are generally lower than inter-assay. Although many factors such as applying spot, multichannel pipette, edge effect due to chromatographic resolution, uneven temperature during incubation, and day-to-day variation in the preparation of reagents affected variations. However, the variation might be reduced when a new standard curve is prepared each time.

3.4 Correlation of pseudojujubogenin glycosides content in brahmi extracts measured by eastern blotting and ELISA

Pseudojujubogenin glycosides analysis by eastern blotting was more advantage than ELISA due to the available to determine each pseudojujubogenin

glycosides. Based on antigen-antibody reaction principle from both eastern blotting and ELISA, hence results from both methods should be correlated.

Table 12 Total pseudojujubogenin glycosides in Brahmi extracts determined by Eastern blotting and ELISA using MAb against bacopaside I

Sample	Total pseudojujubogenin glycosides content ($\mu g.mg^{-1}$ dry weight)	
	Eastern blotting	ELISA
TOP	13.95 ± 1.09	16.76 ± 3.14
STEM	12.17 ± 0.51	12.33 ± 3.74
ROOT	4.44 ± 1.31	4.23 ± 1.35
Shoot from TDZ0.1	10.07 ± 0.74	13.09 ± 2.72
Regenerated plants from TDZ0.1	23.07 ± 1.49	27.95 ± 1.20
CallusD0.5K1	10.36 ± 1.38	10.12 ± 0.97

Recently, ELISA system for measurement of total pseudojujubogenin glycosides using a specific MAb against bacopaside I has been reported (Phrompittayarat et al., 2007a). However, it is difficult to gain each amount of pseudojujubogenin glycosides by competitive ELISA method. Fortunately, we found a new fact that the positive-charged PES membrane was suitable for the immunodetection of pseudojujubogenin glycosides by using a dot blot analysis. We noticed its intrinsic hydrophilicity and strong physical property against organic solvents may make it possible to separate pseudojujubogenin glycosides chromatographically on this membrane. As mentioned above, resulting that eastern blotting can be detected each pseudojujubogenin glycosides.

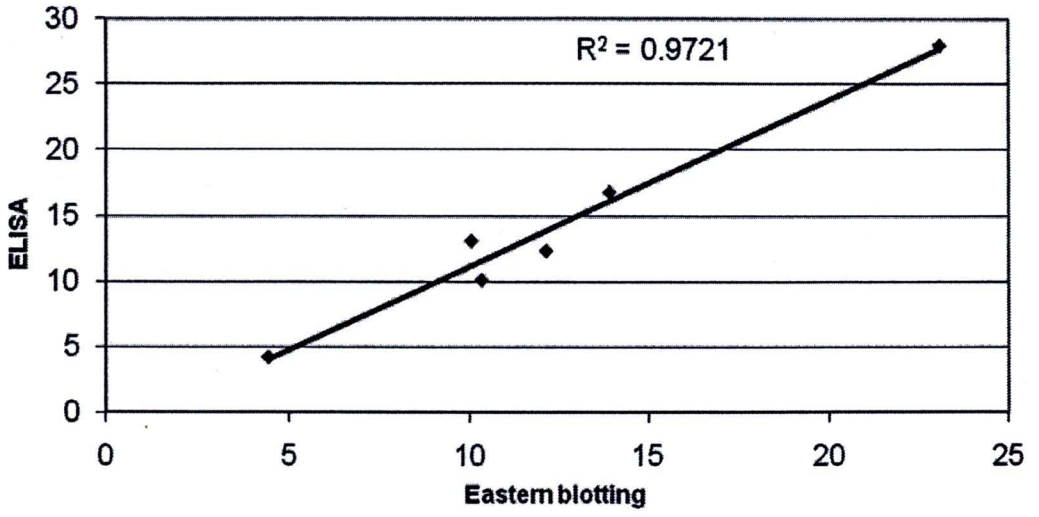


Figure 28 Correlation of pseudojujubogenin glycosides content measured by Eastern blotting and ELISA assay

Linearity with a correlation coefficient of 0.9721 could be obtained from eastern blotting and ELISA comparable. As mentioned above, eastern blotting technique could effective to analyze pseudojujubogenin glycosides like ELISA. Moreover, eastern blotting has more advantage than ELISA for quantitative analysis of each pseudojujubogenin glycosides.

Moreover, beside *Bacopa monnieri*, *B. diffusus, B. cordifolia, Zizyphus cambodiana* and *Z. jujuba* were selected to determine by eastern blotting. Among various plants, only *Bacopa monnieri* gave a positive result on the membrane. Another sample gave a negative result because the concentration is under the limit of detection of this method. The established eastern blotting method could be detected each pseudojujubogenin glycosides from brahmi extract that has more advantage than ELISA.

As shown in the figure 29, *Bacopa* species except *B. monnieri* gave a negative result that represented the pseudojujubogenin glycosides contents in these samples were lower than the limit of detection using eastern blotting. Beside *bacopa* species, *Zizyphus* species including *Z. cambodiana* (fig. 30, lane 2) and *Z. jujuba* (fig. 30, lane 3) also gave a negative result.

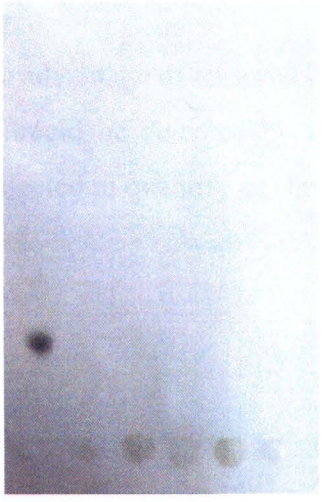


Figure 29 Eastern blotting profile of standard bacopaside I and *Bacopa* species. Lane 1, standard bacopaside I; lane 2, *B. diffusus* extract (stem); lane 3, *B. diffusus* extract (leaf); lane 4, *B. cordifolia* (stem); land 5, *B. cordifolia* (leaf) and lane 6, *B. cordifolia* (flower)

Figure 30 Eastern blotting profile of standard bacopaside I and *Zizyphus* species. Lane 1, standard bacopaside I; lane 2, *Z. cambodiana* extract and lane 3, *Z. jujuba* extract

3.5 Quantitative analysis of each pseudojujubogenin glycosides using eastern blotting.

According to the advantage of eastern blotting, each pseudojujubogenin glycosides could be detected by this method. In this study, six samples of *B. monnieri* were selected to quantify as shown in the table 11.

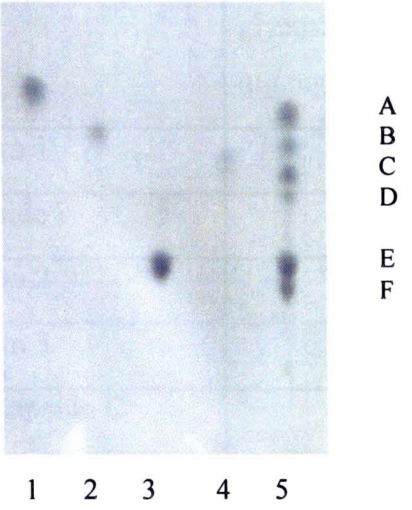


Figure 31 Standard pseudojujubogenin glycosides and brahmi extract

Brahmi extract (fig. 31, lane 5) gave six spots of pseudojujubogennin glycosides which were compared to standard pseudojujubogenin glycosides. From the bottom to the top spot are unknown I (fig. 31, lane 5F), bacopaside I (fig. 31, lane 5E), unknown II (fig. 31, lane 5D), unknown III (fig. 31, lane 5C), bacopasaponin C (fig. 31, lane 5B) and bacopaside II (fig. 31, lane 5A). Eastern blotting could provide both each pseudojujubogenin glycosides and total pseudojujubogenin glycosides.

This is apparently the first report of chromatographic resolution of pseudojujubogenin glycosides in eastern blotting technique on positive-charged PES membrane and its application. Although it has been believed difficult to determine the concentrations of small molecular compounds by eastern blotting, and the methodology described here may open a wide field of comparable studies with other families of carbohydrates containing compounds of low molecular weight such as saponin glycosides, glucuronides, aminosugar conjugated and/or glycolipid, and glycophingolipids.

Table 13 Each pseudojujubogenin glycosides content in *B. monnieri* extract using eastern blotting.

Bacopa monnieri (part)	Pseudojujubogenin glycosides	The amount of pseudojujubogenin glycosides ($\mu g.mg^{-1}$ dry weight)	Bacopaside II : bacopaside I ratio
Top	Unknown 1	0.5234	2.09
	Bacopaside I	3.0125	
	Unknown 2	0.5381	
	Unknown 3	1.3846	
	Bacopasaponin C	1.8485	
	Bacopaside II	6.6447	
Stem	Unknown 1	0.5929	1.60
	Bacopaside I	3.4749	
	Unknown 2	0.4540	
	Unknown 3	1.1894	
	Bacopasaponin C	0.9762	
	Bacopaside II	5.4880	
Root	Unknown 1	0.4103	1.16
	Bacopaside I	1.2428	
	Unknown 2	0.3577	
	Unknown 3	0.5128	
	Bacopasaponin C	0.4996	
	Bacopaside II	1.4213	

Table 13 Each pseudojujubogenin glycosides content in *B. monnieri* extract using eastern blotting (Cont.)

Bacopa monnieri (part)	Pseudojujubogenin glycosides	The amount of pseudojujubogenin glycosides ($\mu g.mg^{-1}$ dry weight)	Bacopaside II : bacopaside I ratio
Shoot from TDZ0.1	Unknown 1	0.3880	4.00
	Bacopaside I	1.2803	
	Unknown 2	0.8030	
	Unknown 3	1.3611	
	Bacopasaponin C	1.1213	
	Bacopaside II	5.1185	
Regenerated plants from TDZ0.1	Unknown 1	0.4210	9.22
	Bacopaside I	1.4540	
	Unknown 2	0.8826	
	Unknown 3	2.0774	
	Bacopasaponin C	4.8130	
	Bacopaside II	13.4201	
Callus D0.5 K1	Unknown 1	0.1060	7.12
	Bacopaside I	1.0481	
	Unknown 2	0.1284	
	Unknown 3	0.3237	
	Bacopasaponin C	1.5366	
	Bacopaside II	7.2170	

Each pseudojujubogenin glycosides in six parts of brahmi was determined by eastern blotting. As shown in table 13, all part of natural brahmi including top, stem and root gave a bacopaside II : bacopaside I ratio at 2 time or lower. Brahmi regenerated from hormone (TDZ) and callus gave higher bacopaside II : bacopaside I ratio from 4-9 time, represented hormone in the medium and plant age influence the production of pseudojujubogenin glycosides in brahmi.

Moreover, the aerial part of *B. monnieri* was selected to direct stuffed on PES membrane due to high contents of pseudojujubogenin glycosides for eastern blotting assay as follow.

3.6 Eastern blotting profile of *B. monnieri*

Figure 32 The eastern blotting profile of the aerial part of *B. monnieri*

As shown in the figure 32, *B. monnieri* was stuffed on PES membrane about 10 min to analyze the pseudojujubogenin glycosides by eastern blotting. Pseudojujubogenin glycosides in *B. monnieri* could be detected by eastern blotting so we may conclude that pseudojujubogenin glycosides content in *B. monnieri* is reached or over the detection limit. *B. monnieri* gave a good yield of pseudojujubogenin glycoside used as a major source of this compound.