CHAPTER V
IMMUNOAFFINITY COLUMN FOR SEPARATION OF PSEUDOJUJUBOGENIN GLYCOSIDES

1. Introduction

The method for separation or quantification of pseudojujubogenin glycosides have been reported, such as, HPLC (Ganzera et al. 2004; Deepak et al., 2005). However, the methods are far from satisfactory for the analytical purpose in terms of high sensistivity, reproducibility, large amounts of extraction solvents and time-consuming factors. Previously chapter of this thesis, we developed the rapid method for detection of pseudojujubogenin glycosides such as immunochromatographic strip and eastern blotting for quantify each and total pseudojujubogenin glycosides content in plant samples. As an extension of this approach, one-step immunoaffinity chromatography for separation of pseudojujubogenin glycosides using anti-bacopaside I polyclonal antibody (anti-bacopaside I PAb) with detection by means of an enzyme-linked immunosorbent assay (ELISA) has been established. Total pseudojujubogenin glycosides were separated directly from the crude extract of selected sample by established immunoaffinity column. Due to the results from immunochromatographic strip and ELISA, *Z. jujuba* that contained pseudojujubogenin glycosides was selected to separate pseudojujubogenin glycosides by immunoaffinity column.

2. Methodology

2.1 Chemicals and immunochemicals

Bacopaside I was perchased from Chromadex (Irvine, CA, USA). Hydrazide gel was purchased from Bio-Rad (Hercules, CA, USA). BSA and HSA were provided from Fluka (Buchs, Switzerland). All other chemicals were standard commercials products of analytical grade.

2.2 Plant materials

The leaf part of *Zizyphus jujuba.*

2.3 Extraction of plant samples

Dried samples (50 mg) of plant sample 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. The sample extracts was evaporated and re-dissolved in washing buffer before analyzed by immunoaffinity column.

2.4 Preparation of immunoaffinity column using anti-bacopasides I PAb

Purified anti-bacopaside I PAb (4 mg) was dialyzed against coupling buffer, pH 5.5, overnight at 4°C and after that anti-bacopaside I PAb was oxidized with $NaIO_4$. Performed the anti-bacopaside I PAb oxidation in container cover with aluminium foil and then mixed gently for 1 hr at room temperature. Immediately after oxidation, glycerol was added at a final concentration of 20 mM, mixed for 10 min and dialyzed against coupling buffer, pH 5.5 at 4 °C. Oxidized anti-bacopaside I PAb was added to a slurry of Affi-Gel hydrazide gel (5 ml gel volume, Bio-Rad®) in coupling buffer and coupled by stirring at room temperature for 24 hr. The eluant and washing solution (20 mM phosphate buffer, 0.5 M NaCl, pH 7.0) were combined. The immunoaffinity gel was washed with PBS and packed in plastic mini-column. The gel was equilibrated with PBS which contained 0.02 % sodium azide and stored at 4°C.

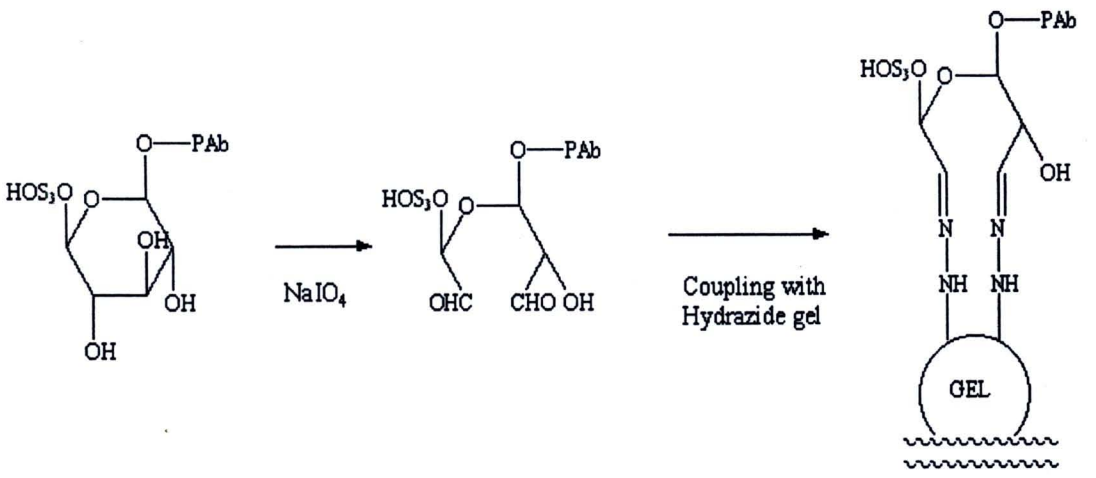

Figure 33 The coupling of anti-bacopaside I PAb with hydrazide gel

2.5 Protocol of immunoaffinity column

The procedure was carried out at room temperature, except incubation. The immnuaffinity column was washed with phosphate buffer before used. A sample dissolved in phosphate buffer was loaded onto the immunoaffinity column. The loaded column was incubated at 4°C for 2 hr with the flow stopped, and the washed with phosphate buffer (50 ml). The column was eluted with 40% methanol in phosphate buffer (30 ml). After elution of pseudojujubogenin glycosides, the immunoaffinity column was washed with phosphate buffer, equilibrated with phosphate buffer containing 0.02% of sodium azide, and then stored at 4°C until subsequent use.

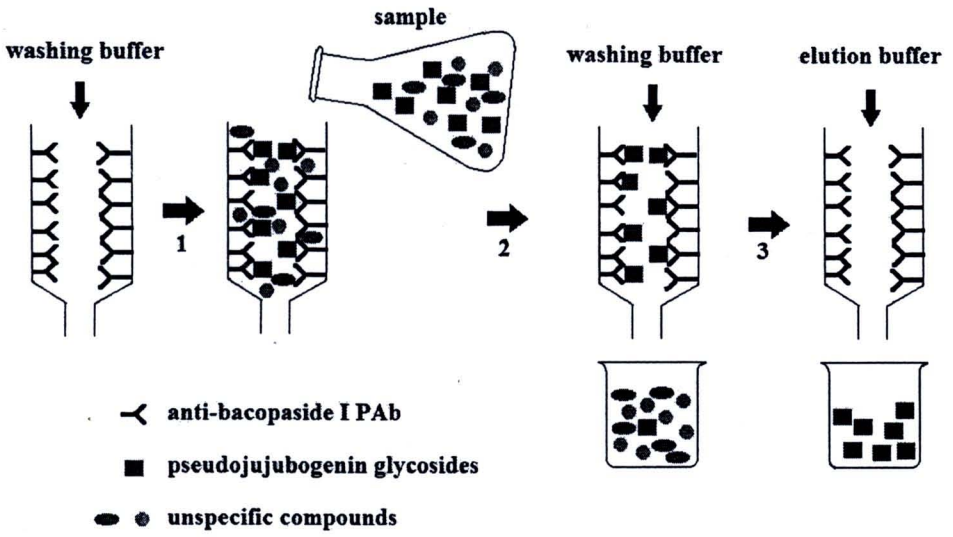

Figure 34 The flow chart of Immunoaffinity column procedure

The immunoaffinity column, using hydrazide gel as the stationary phase, was equilibrated by circulate the washing buffer (10 column volume = 50 ml) before used. The sample solution was loaded onto the immunoaffinity column, circulated and then incubated at 4°C for 2 hr. The column was washed with phosphate buffer, after that eluted pseudojujubogenin glycosides with 40% MeOH in phosphate buffer. After elution of pseudojujubogenin glycosides, the immunoaffinity column was washed with phosphate buffer, equilibrated with phosphate buffer containing 0.02% of sodium azide, and then stored at 4°C until subsequent use.

2.6 Purification of pseudojujubogenin glycosides by immunoaffinity column

The dried sample of *Z. jujuba* was extracted with methanol (0.5 ml) 5 times using ultrasonic bath 15 min. After filtered with 0.45 μm filter, filtrated was dried. The residue was redissolved with methanol and diluted with phosphate buffer. The solution was loaded on the immunoaffinity column and stood at 4°C for 2 hr. The column was washed with the washing solution. After pseudojujubogenin glycosides had disappeared, the column was eluted with 40% methanol in phosphate buffer at 0.1 $ml.min^{-1}$. The total pseudojujubogenin glycosides concentration was assayed by competitive ELISA.

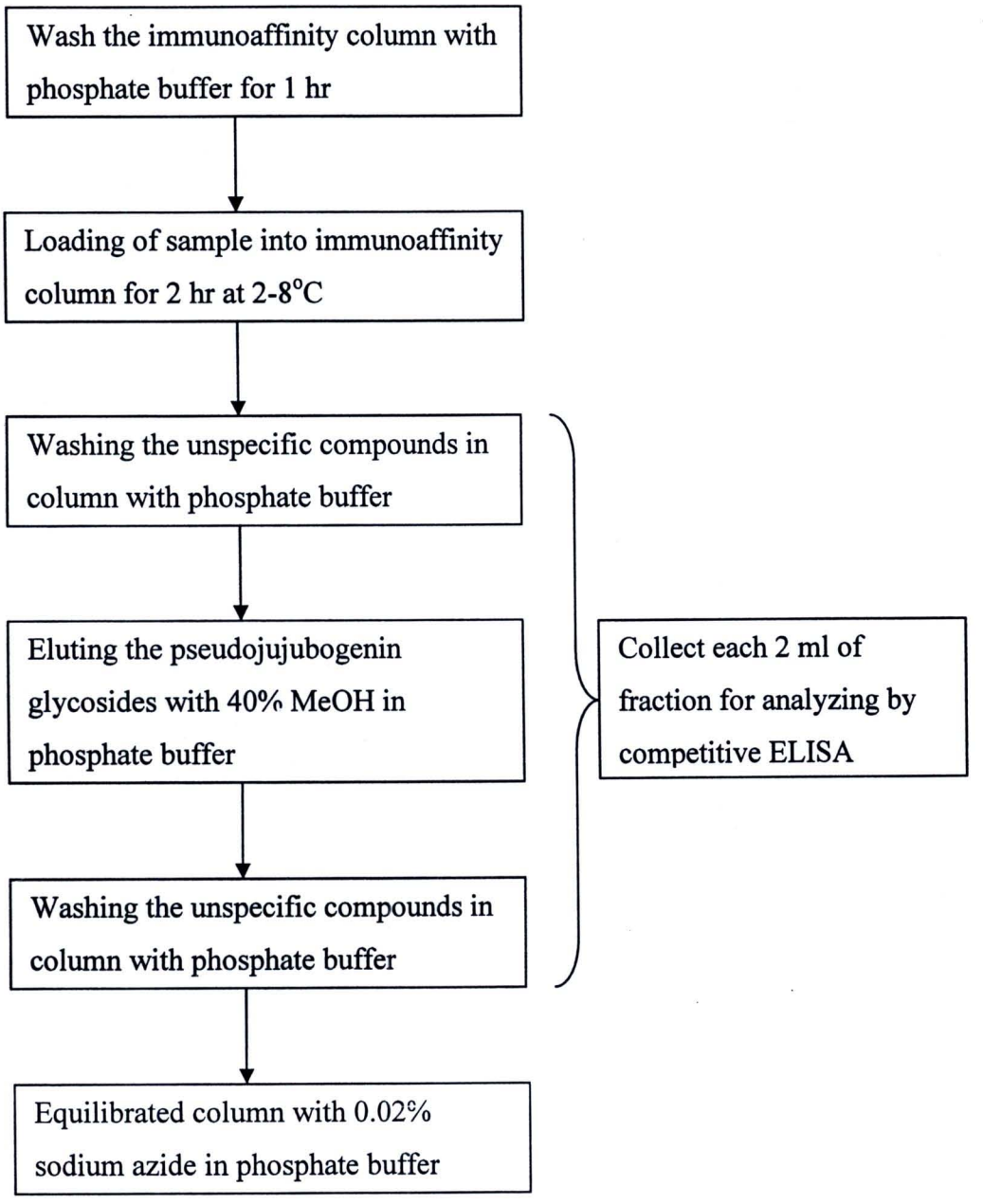

Figure 35 The flow chart of immunoaffinity column for separate pseudojujubogenin glycosides

2.7 Confirmation of pseudojujubogenenin glycosides eluted from immunoaffinity column by Thin layer chromatography

The fraction from immunoaffinity column contained pseudojujubogenin glycosides were analyzed by thin layer chromatography (TLC) to confirm that immunoaffinity chromatography could purify/isolate pseudojujubogenin glycosides from plant extract.

Pseudojujubogenin glycosides from various fractions were developed by TLC to ensuring the pseudojujubogenin glycosides could be separated by immunoaffinity column. The concentrated various fractions, collected from immunoaffinity column, were spotted on TLC plate and immersed in appropriate mobile phase ethyl acetate (EtOAc) : MeOH : H_2O in the ratio 75:15:10 by volume (Sivaramakrishna *et al.*, 2005). After that, sprayed with 10% sulfuric acid (H_2SO_4) in 50% EtOH solution then heated at 120°C to give the TLC band.

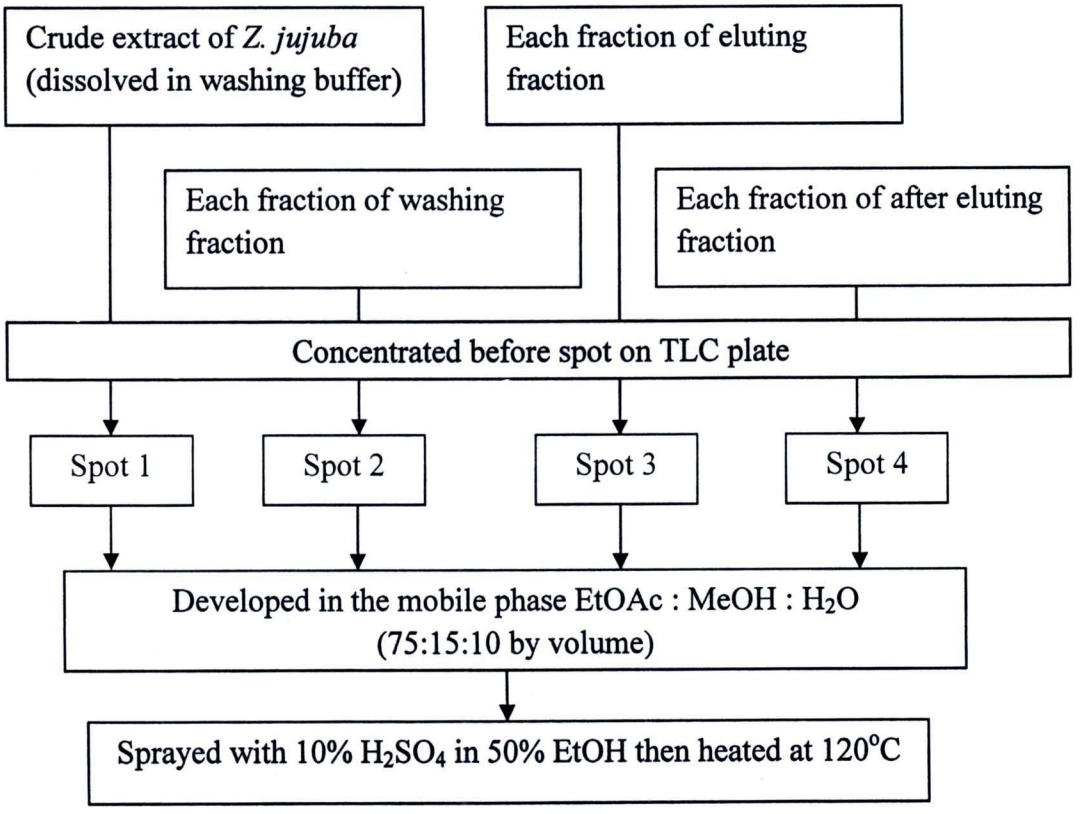

Figure 36 The procedure of TLC for confirmation of pseudojujubogenin glycosides separated by immunoaffinity column

3. Results and discussion

3.1 Column capacity

At first, the immunoaffinity column was determined the capacity for pseudojujubogenin glycosides separation. Standard bacopaside I was used to determined the capacity of column in this study.

From our study, the standard bacopaside I was loaded 4 μg on the immunoaffinity column against anti-bacopaside I polyclonal antibody then washed with phosphate buffer after that eluted with 40% methanol in phosphate buffer to collect bacopaside I that bounded with anti-bacopaside I PAbs in the column. The standard bacopaside I was detected in all fractions only 2,978.52 ng equal to 74.46 %. The elution profile of standard bacopaside I, as shown in figure 37, presented two peaks. First peak (fraction 1-24) is the excess bacopaside I that could not bounded with anti-bacopaside I PAbs in the column equal to 2,141.35 ng and the second peak (fraction 25-40) is the standard bacopaside I which bounded with anti-bacopaside I PAbs, was eluted by 40% methanol in phosphate buffer equal to 847.07 ng bacopaside I resulted 21.18%.

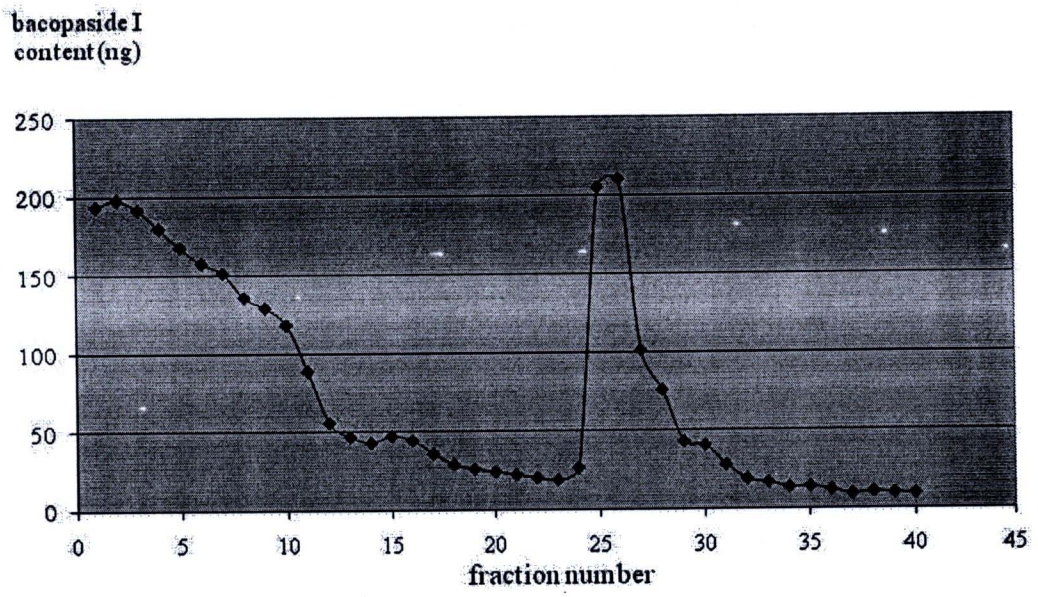

Figure 37 The elution profile of bacopaside I by immunoaffinity column using anti-bacopaside I polyclonal antibody.

3.2 Separation of pseudojujubogenin glycosides from *Zizyphus jujuba*

In addition, *Z. jujuba* was selected to analyze by immunoaffinity column using anti-bacopaside I PAbs due to the highest content of pseudojujubogenin glycosides in all samples except brahmi.

Z. jujuba extract was evaporated and dissolved in phosphate buffer then filtered through 0.45 μm diameter filter before loading to immunoaffinity column. These extract was circulated in the immunoaffinity column (2 hr) for pseudojujubogenin glycosides and anti-bacopaside I PAbs coupling. After that, unspecific binding to anti-bacopaside I PAbs was washed with phosphate buffer and then eluted pseudojujubogenin glycosides bounded to anti-bacopaside I PAbs with 40% methanol in phosphate buffer. From figure 38, the first peak (fraction 1-6) contained pseudojujubogenin glycosides 605.87 ng that presented excess pseudojujubogenin glycosides. The second peak (fraction 16-21) presented pseudojujubogenin glycosides that coupled to anti-bacopaside I PAbs which was eluted with 40 % methanol in phosphate buffer equal to 457.02 ng pseudojujuboenin glycosides.

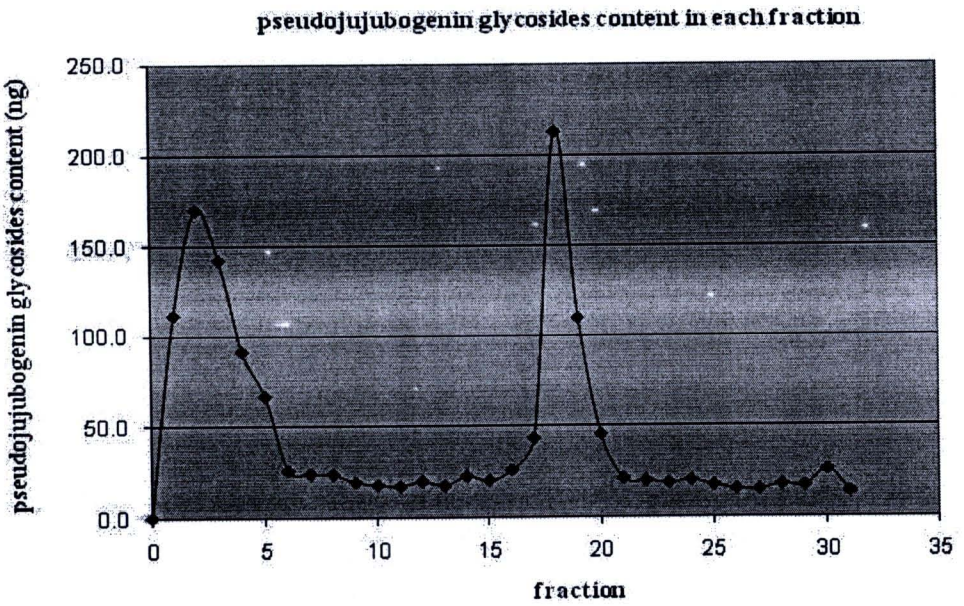

Figure 38 The elution profile of pseudojujubogenin glycosides content in *Z. jujuba* extract.

3.3 Confirmation of pseudojujubogenin glycosides separated by immunoaffinity column by TLC

ELISA technique was used to determine the pseudojujubogenin glycosides content. Furthermore, TLC was selected to confirm that immunoaffinity chromatography could be separated pseudojujubogenin glycosides from the sample to ensure the pseudojujubogenin glycosides in eluting fraction.

From the figure 39, TLC could not detect the pseudojujubogenin glycosides in eluting fraction even if this fraction was concentrated before spotted on TLC plate. We may conclude that TLC is not proper for proving the pseudojujubogenin glycosides in the sample especially low content of pseudojujubogenin glycosides in the sample. However, the crude extract and washing fraction gave a similar chromatogram, resulting that phosphate buffer can wash non-specific compound or unbounded compound from the *Z. jujuba* extract. Although we could not detected pseudojujubogenin glycosides by immunoaffinity column, we could estimate that only pseudojujubogenin glycosides could bounded with anti-bacopaside I PAb in affinity column which were eluted with 40% methanol in phosphate buffer.

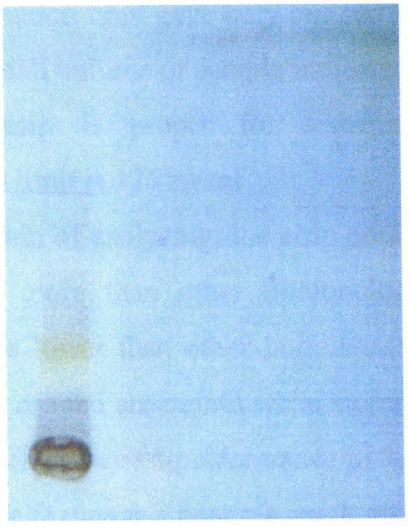

Figure 39 The chromatogram of the represented fractions from immunoaffinity column using TLC. Lane 1, *Z. jujuba* extract; lane 2, Washing fraction; lane 3, Eluting fraction and lane 4, After eluting fraction