

## REFERENCES

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## **APPENDIX**

## APPENDIX A Approval of Animal Ethics Clearances Naresuan University Animal Ethics Committee



เอกสารรับรองโครงการวิจัยในสัตว์ทดลอง  
คณะกรรมการจริยธรรมการใช้สัตว์ทดลอง มหาวิทยาลัยนเรศวร

ชื่อโครงการ                      การใช้โปรตีโอมิกส์ในการวิเคราะห์การเปลี่ยนแปลงของโปรตีนในตับหนูจากผล  
ของสมุนไพรศีกุฎ  
The Proteomic analyses of rat livers affected by Trkatu

ชื่อหัวหน้าโครงการ          รศ.ดร. สุทธิกิจ ยะโสธรศรีกุล

เลขที่โครงการ/รหัส          62-04-0017

สังกัดหน่วยงาน/คณะ        วิทยาศาสตร์การแพทย์

การรับรอง                      ขอรับรองโครงการวิจัยดังกล่าวข้างต้นนี้ ได้ผ่านการพิจารณาและรับรอง  
จากคณะกรรมการจริยธรรมการใช้สัตว์ มหาวิทยาลัยนเรศวร  
ในการประชุมครั้งที่ 3/2552 วันที่ 21 ตุลาคม 2552

ลงนาม

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## APPENDIX B Chemical reagents preparation

### 1. Reagent for determination of the serum AST and ALT activity (Guidelines on Standard Operating Procedures for CLINICAL CHEMISTRY, WHO)

#### 1.1 AST substrate 100 ml

|                         |               |
|-------------------------|---------------|
| DL- aspartic acid       | 2.66 g        |
| $\alpha$ -ketoglutarate | 30 mg         |
| 1M NaOH                 | 20.5 ml       |
| Phosphate buffer        | add to 100 ml |

Adjust the pH to 7.4 by adding 1 M NAOH drop wise while stirring. Add 1 ml of chloroform as preservative that stable for 2 months when stored at 2-8° C. Discard if it becomes turbid.

#### 1.2 ALT substrate 100 ml

|                         |               |
|-------------------------|---------------|
| DL-alanine              | 1.78 g        |
| $\alpha$ -ketoglutarate | 30 mg         |
| 0.4 M NaOH              | 1.25 ml       |
| Phosphate buffer        | add to 100 ml |

Adjust the pH to 7.4 by adding 1 M NAOH drop wise while stirring. Add 1 ml of chloroform as preservative that stable for 2 months when stored at 2-8° C. Discard if it becomes turbid.

#### 1.3 Color reagent 1 litre

|                                   |            |
|-----------------------------------|------------|
| 2,4 dinitrophenylhydrazine (DNPH) | 200 mg     |
| 1M HCl                            | add to 1 L |

Stable for 6 months when stored at 2-8°C.

#### 1.4 Sodium hydroxide (0.4M) 1 litre

|                                  |            |
|----------------------------------|------------|
| NaOH                             | 16 g       |
| Deionized water (18 M $\Omega$ ) | add to 1 L |

Store in a polyethylene container at 25-35°C that stable for 6 months.

**1.5 Pyruvate standard (2 mM/ml) 100 ml**

|                  |                |
|------------------|----------------|
| Sodium pyruvate  | 220 mg         |
| Phosphate buffer | add to 100 ml. |

Dilute 10 ml of this solution to 100 ml with phosphate buffer to obtain the working standard containing 2 mM pyruvate per ml. The remaining 90 ml of the first solution should be discarded. The working standard should be stored in small aliquots of 2 ml in the freezer. One aliquot of working standard should be used for preparing a calibration graph. Discard the leftover standard in the vial.

**1.6 Phosphate buffer (pH 7.4) 1 litre**

|                                |           |
|--------------------------------|-----------|
| Disodium hydrogen phosphate    | 14.9 g    |
| Potassium dihydrogen phosphate | 2.2g      |
| Deionized water (18 MΩ)        | add to 1L |

Stable for 3 months when stored at 2-8° C.

**2. Reagent for Protein extraction and precipitation****2.1 Phosphate buffered saline (PBS) (1X solution) 1 litre**

|                                |            |
|--------------------------------|------------|
| Sodium chloride                | 8g         |
| Potassium chloride             | 0.2g       |
| Disodium hydrogen phosphate    | 1.44g      |
| Potassium dihydrogen phosphate | 0.24g      |
| Distilled water(18 MΩ)         | add to 1 L |

Adjust pH to 7.4 and sterilize by autoclaving

**2.2 Extraction media 100 ml**

|                         |               |
|-------------------------|---------------|
| 1.5 MTris-HCl, pH 8.8   | 11.66 ml      |
| SDS                     | 5 g           |
| 87% glycerol            | 17.24 ml      |
| DTT                     | 0.046 g       |
| Deionized water (18 MΩ) | add to 100 ml |

**2.3 80% acetone 1 litre**

|                         |            |
|-------------------------|------------|
| 100% Acetone            | 800 ml     |
| Deionized water (18 MΩ) | add to 1 L |



## 2.4 IEF extraction solution 100 ml

|                         |               |
|-------------------------|---------------|
| Urea (m.w. 60.06)       | 48.05 g       |
| Thiourea (m.w. 76.12)   | 15.23 g       |
| CHAPS                   | 2 g           |
| Triton X-100            | 2 ml          |
| DTT                     | 0.77 g        |
| Deionized water (18 MΩ) | add to 100 ml |

## 2.5 Trichloroacetic acid (72%) 10 ml

|                         |              |
|-------------------------|--------------|
| TCA                     | 7.2 g        |
| Deionized water (18 MΩ) | add to 10 ml |

## 2.6 Deoxycholate (0.15%) 10 ml

|                         |              |
|-------------------------|--------------|
| DOC                     | 15 mg        |
| Deionized water (18 MΩ) | add to 10 ml |

## 2.7 SDS (0.5%) 10 ml

|                         |              |
|-------------------------|--------------|
| 10% SDS                 | 0.5 ml       |
| Deionized water (18 MΩ) | add to 10 ml |

# 3. Reagent for Protein determination by Lowry assay

## 3.1 Alkaline copper solution

### 3.1.1 Solution A (10 ml)

|                                      |              |
|--------------------------------------|--------------|
| CuSO <sub>4</sub> ·7H <sub>2</sub> O | 40 mg        |
| Sodium citrate dehydrate             | 100 mg       |
| Deionized water (18 MΩ)              | add to 10 ml |

### 3.1.2 Solution B (100 ml)

|                         |               |
|-------------------------|---------------|
| Sodium carbonate        | 2 g           |
| Sodium hydroxide        | 0.4 g         |
| Deionized water (18 MΩ) | add to 100 ml |

### 3.1.3 Solution C (51 ml)

|            |      |
|------------|------|
| Solution A | 1ml  |
| Solution B | 50ml |

**3.2 Folin-Ciocalteu phenol reagent (20%) 20 ml**

|                                |       |
|--------------------------------|-------|
| Folin-Ciocalteu phenol reagent | 10 ml |
| Deionized water (18 MΩ)        | 10 ml |

**4. Reagent preparation for SDS-PAGE**

**4.1 SDS loading buffer (5X stock) 25 ml**

|                         |         |
|-------------------------|---------|
| 1 M Tris-HCl, pH 6.8    | 3.25 ml |
| SDS                     | 2.5 g   |
| 87% glycerol            | 12.5 ml |
| DTT (M.W. = 154.25)     | 1.925 g |
| Deionized water (18 MΩ) | 8.75 ml |
| Bromophenol blue        | 0.006 g |

**4.2 electrophoresisbuffer (10X stock) 100 ml**

|                        |              |
|------------------------|--------------|
| Tris-base              | 3 g          |
| Glycine                | 14 g         |
| 10% SDS                | 10 ml        |
| Deionized water(18 MΩ) | add to100 ml |

**4.3 separating gel (12% polyacrylamide gel) 10 ml**

|                           |         |
|---------------------------|---------|
| 1.5 M Tris-HCl, pH 8.8    | 2.5 ml  |
| 40% Acrylamide            | 3.0 ml  |
| 10% SDS                   | 125 µl  |
| Deionized water (18MΩ)    | 4.35 ml |
| Ammonium persulfate (APS) | 50 µl   |
| TEMED                     | 5µl     |

**4.4 stacking gel (4% polyacrylamide gel) 3 ml**

|                           |         |
|---------------------------|---------|
| 0.5 M Tris-HCl, pH 6.8    | 0.75 ml |
| 40% Acrylamide            | 0.3 ml  |
| 10% SDS                   | 30 µl   |
| Deionized water (18MΩ)    | 1.82 ml |
| Ammonium persulfate (APS) | 50 µl   |
| TEMED                     | 5µl     |

## 5. Silver staining processes

### 5.1 Fixing solution 200 ml

|                         |               |
|-------------------------|---------------|
| 99.8% Methanol          | 100 ml        |
| 100% Acetic acid        | 24 ml         |
| 37% Formaldehyde        | 100 µl        |
| Deionized water (18 MΩ) | add to 200 ml |

### 5.2 Washing solution 200 ml

|                         |               |
|-------------------------|---------------|
| 96% Ethanol             | 73 ml         |
| Deionized water (18 MΩ) | add to 200 ml |

### 5.3 Sensitizing solution 200 ml

|                         |               |
|-------------------------|---------------|
| Sodium thiosulfate      | 0.04 g        |
| Deionized water (18 MΩ) | add to 200 ml |

### 5.4 Staining solution 200 ml

|                         |               |
|-------------------------|---------------|
| Silver nitrate          | 0.4 g         |
| Deionized water (18 MΩ) | add to 200 ml |

### 5.5 Developing solution 200 ml

|                          |               |
|--------------------------|---------------|
| Sodium carbonate         | 12 g          |
| 37% Formaldehyde         | 100 µl        |
| 0.02% sodium thiosulfate | 4 ml          |
| Deionized water (18 MΩ)  | add to 200 ml |

### 5.6 Stopping solution 100 ml

|                         |               |
|-------------------------|---------------|
| Disodium, EDTA          | 1.4 g         |
| Deionized water (18 MΩ) | add to 100 ml |

**Table 9 Gel staining protocol**

| Step | Processes | Reagent              | time                                           |
|------|-----------|----------------------|------------------------------------------------|
| 1    | Fix       | fixing solution      | 30 min.                                        |
| 2    | Wash      | washing solution     | 2 X 5 min.                                     |
| 3    | Sensitize | sensitizing solution | 2 min.                                         |
| 4    | wash      | deionized water      | 2 X 5 min.                                     |
| 5    | Stain     | staining solution    | 20 min.                                        |
| 6    | wash      | deionized water      | 2 X 1 min.                                     |
|      |           |                      | 15 min. (max.) or                              |
| 7    | Develop   | developing solution  | completed visual band<br>marker protein on gel |
| 8    | Stop      | stopping solution    | 20 min.                                        |
| 9    | wash      | deionized water      | 3 X 5 min.                                     |

Gel can be stored in 5% acetic acid solution at 4°C for several weeks prior to in-gel digestion.

**6. Reagent for In-gel trypsin digestion**

**6.1 Ammonium bicarbonate (10mM) 50 ml**

|                                   |              |
|-----------------------------------|--------------|
| Ammonium bicarbonate (m.w.79.056) | 39.5 mg      |
| Deionized water (18 MΩ)           | add to 50 ml |

**6.2 Reducing solution 10 ml**

|                           |              |
|---------------------------|--------------|
| DTT                       | 15.42 mg     |
| 10 mMAmmonium bicarbonate | add to 10 ml |

**6.3 Alkylating solution 10 ml**

|                             |              |
|-----------------------------|--------------|
| Iodoacetamide (m.w. 184.96) | 185 mg       |
| 10 mMAmmonium bicarbonate   | add to 10 ml |

**6.4 50% acetonitrile /10mM ammonium bicarbonate 10 ml**

|                           |      |
|---------------------------|------|
| 100% acetonitrile         | 5 ml |
| 10mM ammonium bicarbonate | 5 ml |

**6.5 Trypsin solution (10 ng/μl) 1 ml**

|                                             |       |
|---------------------------------------------|-------|
| Trypsin                                     | 10 mg |
| 50% acetonitrile /10mM ammonium bicarbonate | 1 ml  |

**6.6 Acetonitrile (30%) 10 ml**

|                         |      |
|-------------------------|------|
| 100% Acetonitrile       | 3 ml |
| Deionized water (18 MΩ) | 7 ml |

**6.7 Acetonitrile (50%) 10 ml**

|                         |      |
|-------------------------|------|
| 100% Acetonitrile       | 5 ml |
| Deionized water (18 MΩ) | 5 ml |

**6.8 Formic acid (0.1%) 5 ml**

|                         |             |
|-------------------------|-------------|
| Formic acid             | 5 μl        |
| Deionized water (18 MΩ) | add to 5 ml |

**6.9 Peptide extracts solution 10 ml**

|                   |      |
|-------------------|------|
| 0.1 % Formic acid | 5 ml |
| 100% Acetonitrile | 5 ml |

## **BIOGRAPHY**



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