Conference Paper

Skeletal Malformation on Balb-C Foetal Mice (*Mus musculus*) Administered by Immature Pineapple Fruits Extract

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Abstract

Nutrients for pregnant woman affect the development of foetus in the womb. Pineapple has bromelain enzyme which benefit in medicinal uses, but it can be considered as teratogenic agents due to its proteolytic activities. The period of organogenesis is the critical period in foetal development. Examination of foetal anatomy is still a fundamental part of teratological studies despites of modern molecular techniques. Molecular techniques can be done by conducting the skeletal proteome analysis to evaluate the teratogenic effects on the foetal skeleton. This research was performed to examine the effect of immature pineapple fruits extract on the occurrence of skeletal malformation and identify the protein profiles of skeleton on Balb-C foetal mice. Pregnant mice were administered by immature pineapple fruits extract orally by gavage with doses 0%, 20%, 40%, 60% and 80% at day 6 d to 15 d of gestation. Skeletal malformation observed after Alizarin red-Alcian blue staining, and SDS-PAGE was conducted to identify the protein profiles of foetal skeleton. Immature pineapple fruits extract caused the decreasing of weight and length of litter, causing hemorrhage, ossification retardation on sternebra, metacarpal, metatarsal, caudal vertebrae, undulated costae and asymmetric sternebra. Protein profiling analysis showed that skeletal proteins at molecular weight of 32 kDa, 35 kDa, 42 kDa and 49 kDa did not found in foetal mice administered by immature pineapple fruits extract.

Keywords: bromelain; immature pineapple fruits; skeletal malformation; skeletal proteome analysis.

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1. Introduction

Nutrients for pregnant woman affect the development of foetus in the womb. The period of organogenesis is the critical period in foetal development [1], especially toward the placental substances. Pineapple has bromelain enzyme which benefit in medicinal uses, as an emmenagogue, abortifacient, antiamoebic and vermifuge and for the correction of stomachal disorders, inhibition of platelet aggregation, fibrinolytic

action, anti-inflammatory processes, and skin debridement and enhancement of drug absorption [2, 3]. The proteolytic activities of bromelain enzymes also causes teratogenic effects so it can be considered as teratogenic agents [4]. Examination of foetal anatomy is still a fundamental part of teratological studies despites of modern molecular techniques [5]. Molecular techniques can be done by conducting the skeletal proteome analysis to evaluate the teratogenic effects on the foetal skeleton. Proteome analysis gives the information about mechanisms of physiological/pathological process in bone tissues [6]. This research was performed to examine the effect of the immature pineapple fruits extract on the occurrence of skeletal malformation and identify the protein profiles of skeleton on Balb-C foetal mice.

2. Materials and Methods

2.1. Immature pineapple fruits extract

Immature pineapple fruits were obtained from a plantation in Kediri, East Java. Immature pineapple fruits were macerated within methanol 90% for 72 h and filtered until solvent produced. Solvent evaporated using rotary vacuum evaporator until extract of immature pineapple fruits produced. Those extracts were diluted in distilled water with doses 0% (control group), 20%, 40%, 60% and 80%.

2.2. Animals

The twenty Balb-C female mice 8 wk to 10 wk old were observed for their estrous cycle using lavage methods. Classification of estrous cycle based on vaginal cytology or cell morphology in vaginal smear [7]. Female mice in estrous phase were placed in a cage with fertile male mice in similar strains in the evening. In the next morning they were observed for the presence of vaginal plug in the female and that day was the day 0 of gestation if there is vaginal plug, and tomorrow was determined by the first day of gestation.

The twenty pregnant female mice were grouped into five, administered by immature pineapple fruits extract at doses of 0%, 20%, 40%, 60% and 80%. It was administered orally by gavage 0.2 mL/animal/day, at the 6 d to 15 d of gestation. At the 18 d of gestation, caesarean was performed for foetal necropsy.

2.3. Skeletal malformation

Foetal necropsy done by observed feotal morphology include life foetus, dead foetus, weight and length of foetus, and the occurrence of hemorrhage. Skeletal malformation observed include the skeletal structure and the completeness of sternebra, metacarpal, metatarsal and caudal vertebrae. Alizarin red-Alcian blue staining were used to determine skeletal malformation. Foetus from pregnant mice 18 d of gestation were eviscerated and the skeleton were double stained for cartilage and bone with Alcian blue and Alizarin red. All carcasses were fixed in ethanol 95% for 24 h. Samples were placed in staining solution for 24 h to 48 h for cartilage and bone staining followed by a 95% ethanol washed for 8 h. Staining solution containing (i) the one volume of 0.1% Alizarin red in 95% ethanol, (ii) the one volume of 0.3% Alcian blue in 70% ethanol, (iii) the one volume of glacial acetic acid and (iv) the 17 volume of 70% ethanol. Samples then macerated in 1% KOH for ±7 d until the muscles cleared. Samples were cleared more in 20%, 50%, and 80% glycerin in 1% KOH for 24 h in each step, then sample stored in glycerin 100%. Samples were observed using stereo microscope to identify the skeletal malformation [8, 9]. Mice foetal morphology and the occurrence of skeletal malformation are analyzed statistically using One Way of Anova and Duncan's Multiple Range Test (DMRT) for significancy test.

2.4. Protein profiling of foetal skeleton

Proteome analysis was done to identify the protein profiles of foetal skeleton administered by immature pineapple fruits extract. Protein extraction were conducted by the following methods: (i) The foetal skeleton of mice were trimmed free of soft tissue and washed to remove contaminants with phosphate buffer saline (PBS), pH 7.4, containing PMSF as protease inhibitor, overnight at 4°C, with ratio PBS:PMSF were 9:1; (ii) Samples were demineralized by incubated them in 1.2 M HCl (0.2 g samples/mL of solution) overnight at 4°C; (iii) Samples (demineralized bone tissue) were centrifuged in 1 000 rpm at 4°C for 20 min (1 rpm = 1/60 Hz) and supernatant separated from residue; (iv) The residue was washed with lysis buffer comprises of 8 M urea, 100 mM Tris, pH 7.4 containing PMSF as protease inhibitor at 4°C for 72 h, with ratio urea:Tris:PMSF were 5:4:1; (v) Supernatant was separated from residue after centrifugation in 1 000 rpm at 4°C for 20 min and residue incubated in lysis buffer containing 8 M urea, 100 mM Tris, 0.5 M EDTA, pH 7.4 at 4°C for 72 h, with ratio urea:Tris:EDTA were 4:1:5; (vi) Supernatant was separated from residue after centrifugation in 1 000 rpm at 4°C for 20 min and residue incubated in 6 M HCl at 4°C overnight; (7) Supernatant was collected after centrifugation in 1 000 rpm at 4°C for 20 min and it was protein isolate. (8)

Group (%)	Parameter Observed (means ± SD)				
	Life foetus (individu)	Dead foetus (individu)	Weight of foetus (g)	Length of foetus (cm)	Hemorrhage (individu)
0 (control)	8.000 ± 1.632^{a}	0.000 ± 0.000^{a}	1.217 ± 0.024^{a}	2.322 ± 0.066^{a}	0.000 ± 0.000^{a}
20	7.500 ± 1.290^{a}	0.000 ± 0.000^{a}	1.050 ± 0.049^{b}	1.990 ± 0.148^{b}	0.750 ± 1.500^{ab}
40	7.500 ± 1.290^{a}	0.000 ± 0.000^{a}	0.930 ± 0.069^{c}	1.890 ± 0.088^{bc}	0.750 ± 0.957^{ab}
60	6.750 ± 0.957^{a}	0.500 ± 0.577^{b}	0.740 ± 0.099^{d}	1.790 ± 0.029^{cd}	1.000 ± 0.000^{ab}
80	6.750 ± 0.957^{a}	0.000 ± 0.000^{a}	0.730 ± 0.294^{d}	1.750 ± 0.037^{d}	2.250 ± 1.707^{b}

Notes: A letter after the number in a column shows significance ($p < 0.05$), analized using Anova and DMRT.

Table 1: The Effect of Immature Pineapple Fruits Extract on Morphology of Foetus.

The protein isolate was precipitated in acetone at -20°C overnight, then precipitated protein samples were redissolved in a buffer containing 8 M urea, 100 mM Tris, pH 8.1 and protein concentration was determined by nanodrop spectrophotometer [vi]. Samples of proteins isolate then analyzed by Sodium Dodecyl Sulfate Polyacrilamide Gel Electrophoresis (SDS-PAGE) following the standard procedure.

3. Result and Discussion

Immature pineapple fruits extract causes the decreasing of weight and length of litter, causing hemorrhage (Table 1), ossification retardation (Table 2) on sternebra (Figure 1a–1c), metacarpal, metatarsal, caudal vertebrae (Figure 2), undulated costae (Figure 1d) and asymmetric sternebra (Figure 1). Proteome analysis of foetal skeleton shows that molecular weight (MW) 35 kDa and 49 kDa of skeletal proteins did not found in foetal mice administered by immature pineapple fruits extract dose 60% and 80% (Figure 3a), MW 32 kDa did not found in foetal mice administered by immature pineapple fruits extract doses 40% to 80%, while MW 42 kDa did not found in foetal mice administered by immature pineapple fruits extract doses 20% to 80% (Figure 3b).

Major structural components of bone matrix is type I collagen and other types, such as type X and XXIV and also noncollagenous proteins like Bone Morphogenic Proteins (BMP) having function in growth and mineralization of bone. Collagen fibers and other proteins could be degraded by proteinases or proteolytic resulting collagen fragments [10]. Proteolytic activities of bromelain degrade type I, X and XXIV collagen causing ossification retardation so the weight and length of foetus decreased. Degradation of proteins shown by the difference of protein profiles shown by Figure

Group (%)	Parameter Observed (means ± SD)				
	Sternebra	Metacarpal	Metatarsal	Caudal Vertebrae	Asymetrical sternebra
0 (control)	6.000 ± 0.000^{a}	4.000 ± 0.000^{a}	4.000 ± 0.000^{a}	4.850 ± 0.131^{a}	0.000 ± 0.000^{a}
20	6.000 ± 0.000^{a}	3.880 ± 0.094^{b}	3.865 ± 0.091^{b}	4.552 ± 0.041^{b}	1.000 ± 0.816^{ab}
40	5.420 ± 0.033^{b}	3.352 ± 0.041^{c}	3.460 ± 0.042^{d}	3.407 ± 0.009^{c}	3.000 ± 2.160^{b}
60	5.145 ± 0.041^{c}	3.265 ± 0.044^{d}	3.730 ± 0.046^{c}	2.937 ± 0.043^{d}	6.000 ± 1.632^{c}
80	4.225 ± 0.150^{d}	2.515 ± 0.543^{e}	2.757 ± 0.419^{e}	2.452 ± 0.045^{e}	10.750 ± 0.957^{d}
Notes: A letter after the number in a column shows significance ($p < 0.05$), analized using Anova and DMRT.					

Table 2: The Effect of Immature Pineapple Fruits Extract on Skeletal Malformation.

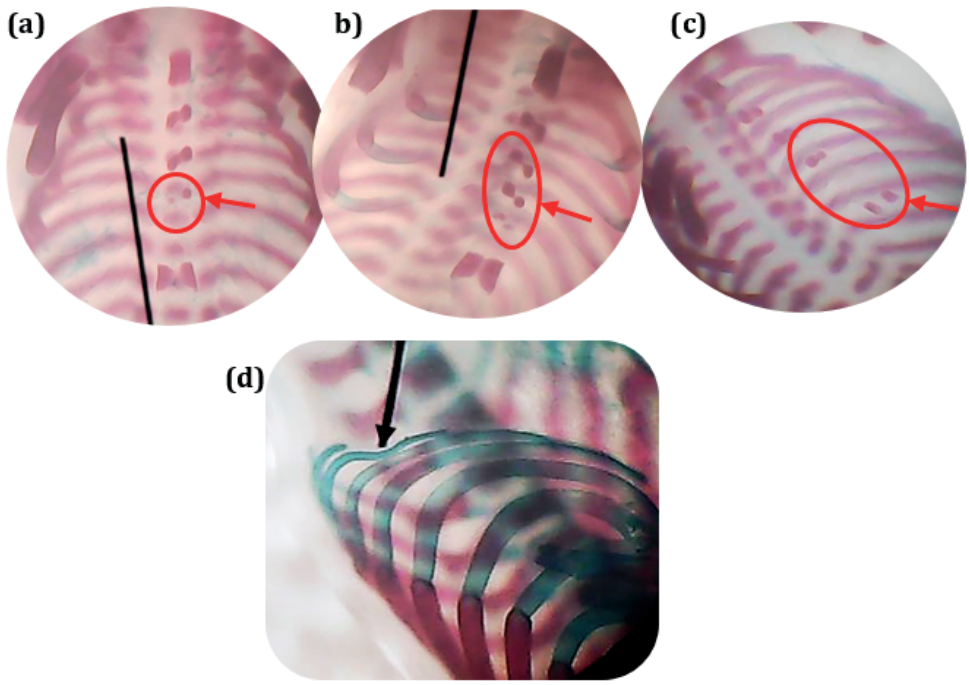

Figure 1: Malformation of mice foetal skeleton administered by immature pineapple fruits extract. The fifth sternebra of (a) and (c) did not ossified. The third sternebra of (b) and the fourth of (a), (b), and (c) had incomplete ossified so they looked asymmetric. (a), (b) and (c) were sternum from treatment dose 80% of immature pineapple fruits extract. (d) was convoluted costae from foetal mice administered by immature pineapple fruits extract dose 40%.

3, between normal foetal skeleton and administered by immature pineapple fruits extracts.

Immature pineapple fruits extract causes hemorrhage in mice foetus. Hemorrhage is bleeding or ruptured of blood vessels from the internal organs but there is no sign of bleeding outside the body. Type III collagen gives flexibility of blood vessels [10]. Degradation of type III collagen resulting of rupture of vessels and internal organ

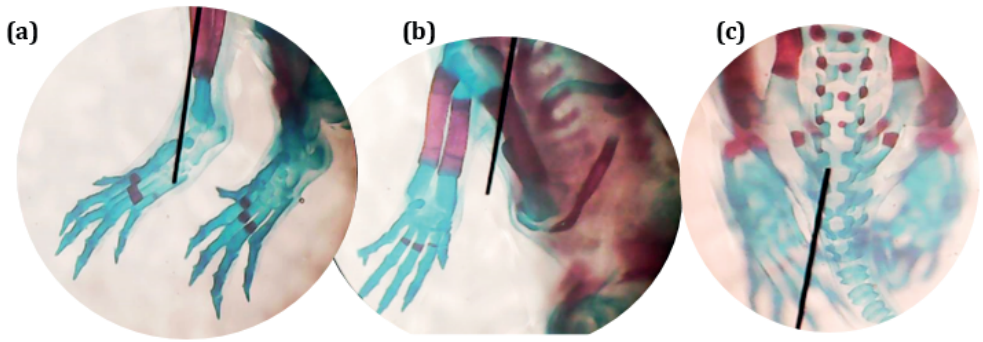

Figure 2: Malformation of mice foetal skeleton (metacarpal, metatarsal, caudal vertebrae) administered by immature pineapple fruits extract. (a) and (b) were ossification retardation on metacarpal, metatarsal and phalanges. (c) was ossification retardation on caudal vertebrae.

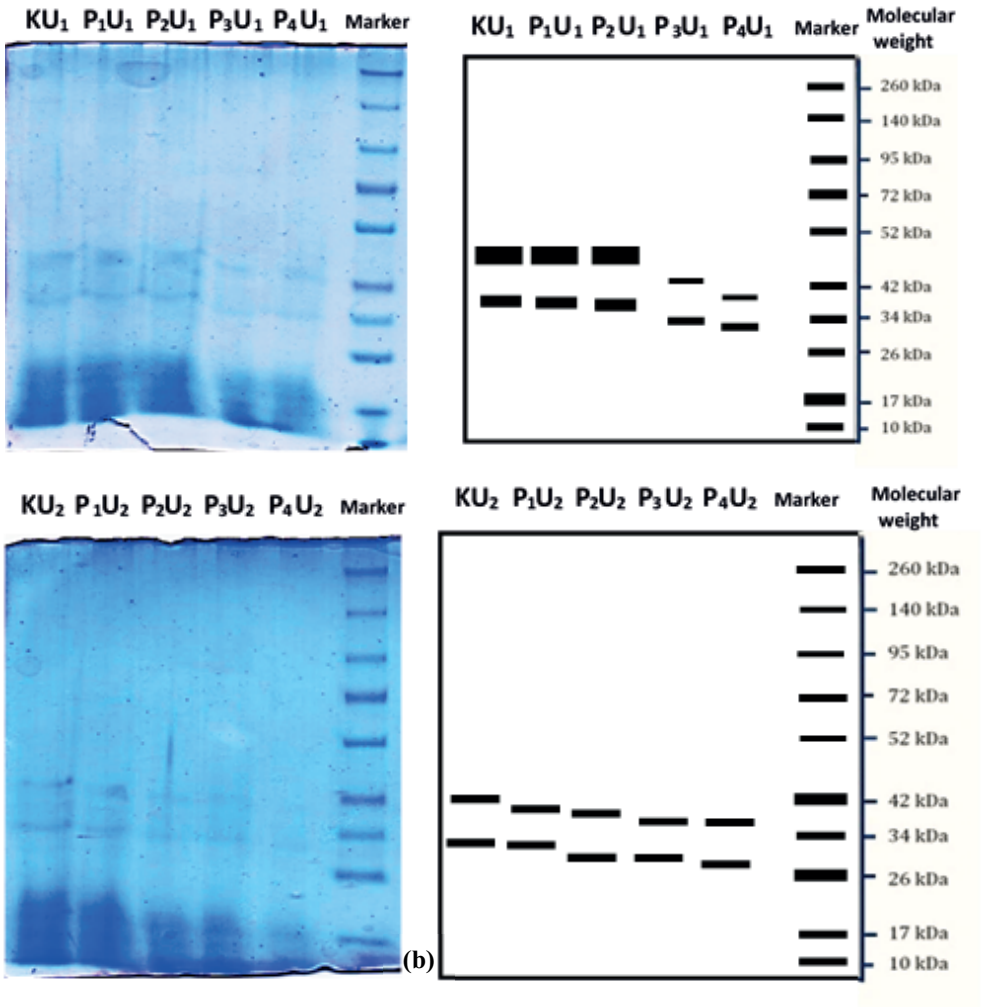

Figure 3: Differentially expressed skeletal proteins between normal foetal mice and administered by immature pineapple fruits extract. (a) SDS-PAGE profile of skeletal proteins from skull, sternum, ribs, vertebrae (thoracic, cervical and lumbar). (b) SDS-PAGE profile of skeletal proteins from forelimb and hindlimb, sacrum and caudal vertebrae. K: 0%, P_1: 20%, P_2: 40%, P_3: 60%, P_4: 80% doses of immature pineapple fruits extract.

so hemorrhage occurred. Degradation of type III collagen in foetus administered by immature pineapple fruits extract, caused by the proteolytic activities of bromelain.

4. Conclusions

Immature pineapple fruits extract effect on the occurrence of skeletal malformation and the protein profiles of skeleton on Balb-C foetal mice are different between normal and admininistered by Immature pineapple fruits extract. Immature pineapple fruits extract causes the decreasing of weight and length of litter, causing hemorrhage, ossification retardation on sternebra, metacarpal, metatarsal, caudal vertebrae, undulated costae and asymmetric sternebra. Proteomic analysis shows that molecular weight 32 kDa, 35 kDa , 42 kDa and 49 kDa of skeletal proteins did not found in foetal mice administered by immature pineapple fruits extract.

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