

BRINE SHRIMP TERATOGENIC SCREENING TEST ON SOME SELECTED PLANTS

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Received: 03 April 2017; Revised and Accepted: 24 April 2017

ABSTRACT

Objectives: The aims of this research were to analyze teratogenic properties on some selected plants which often used for woman's health were pomegranate (*Punica granatum* L.) pericarp, coriander (*Coriandrum sativum* L.) seeds, liman (*Elephantopus scaber* L.) leaves, fennel (*Foeniculum vulgare* M.) seeds, and kaempferia (*Kaempferia galanga* L.) rhizomes using brine shrimp teratogenic screening test (BSTST) method.

Methods: *Artemia* cysts were hatched into first stage nauplii then taken and put into seawater medium which contain test substance and kept alive until the second stage, third stage, and fourth stage and then observed number of deaths, morphological abnormalities, body length, and retarded of development for each stage.

Results: Hatchability of cysts in fennel seeds compared extracts 1 and 0.5 mg/ml, coriander seeds extract 1 mg/ml, pomegranate rinds extract 6 mg/ml, and liman leaves extracts 6, 3, and 1.5 mg/ml were significantly different to control ($p < 0.05$). Survival nauplii in fennel seeds extracts 1 and 0.5 mg/ml and liman leaves extracts 6 and 3 mg/ml were significantly different to control ($p < 0.05$). The morphological abnormalities were exposed on coriander seeds extract 1 mg/ml, liman leaves extract 6 mg/ml, and pomegranate rinds extract 3 mg/ml. Nauplii with retarded development were presented on fennel seeds extracts 1 and 0.5 mg/ml, coriander seeds extracts 1 and 0.25 mg/ml, pomegranate rinds extracts 6, 3, and 1.5 mg/ml, and liman leaves extracts 3 and 1.5 mg/ml. Nauplii body length on plants extract showed no significantly difference compared to control.

Conclusion: Based on BSTST, fennel seed and pomegranate rind extracts had no teratogenic effect, kaempferia rhizomes act as larvicide its analog in mamalia as embryo lethal, while coriander seeds and liman leaves extracts potentially had teratogenic properties.

Keywords: Brine shrimp test, Teratogen, Fennel, Coriander, Pomegranate, Liman, Kaempferia.

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INTRODUCTION

The use of traditional medicine has been known long time, for curing and also as preventive and health care. Around 30.4% households in Indonesia used traditional health services, and 49.0% of them used jamu which contained 1-5 of the crude drug [1]. The use and knowledge regarding traditional medicine are very diverse, and some of traditional medicines were used by women to cure diseases and health care.

Pomegranate (*Punica granatum* L.), coriander (*Coriandrum sativum* L.), liman (*Elephantopus scaber* L.), fennel (*Foeniculum vulgare* M.), and kaempferia (*Kaempferia galanga* L.) were plants that often used as traditional medicine for women's health care, such as during pregnancy, menstrual disorders, aphrodisiacs, contraceptives, body treatments, and postnatal [2-4]. Several studies revealed that these plants also had antifertility, anti-implantation [5] and inhibit proliferation effects [6-9]. The use of these five plants by women, especially, during conception and pregnancy was feared could affect the growth and development of embryo or fetus; therefore, it is very necessary for testing teratogenic on these plants.

Teratogenicity test is a test designed to observe the special effects of a substance on a fetus that includes death, morphological abnormality, retarded development, and functional disorders [10]. Some teratogenic testings have been developed lately in hopes of finding a reliable method, rapid testing, as well as cost [11]. One teratogenic testing method, namely, brine shrimp test has been developed, which used *Artemia salina* L. belongs to crustacean [12].

Teratogenic test using brine shrimp has been conducted by Kerster and Schaeffer [13], Sleet and Brendel [14] then developed and modified in the previous research [12]. This method is an alternative method for screening teratogenic. It does not require complex skills, inexpensive and can provide rapid result [12-14], therefore, this method can be selected to evaluate teratogenic effect on five medicinal plants that are often used by the public for women's health.

The purposes of this study were to analyze any teratogenic properties on extracts of fennel seeds, coriander seeds, liman leaves, pomegranate pericarp, and kaempferia rhizomes as a medicinal plant for women's health and are expected to reveal useful information for safety usages of those plants during pregnancy.

MATERIALS AND METHODS

Materials

Animals were *Artemia salina* L. cysts which were produced by Ocean Star International Inc., Utah, USA. Pomegranate (*P. granatum* L.) pericarp, coriander (*C. sativum* L.) seeds, liman (*E. scaber* L.) leaves, fennel (*F. vulgare* M.) seeds, and kaempferia (*K. galanga* L.) rhizomes were collected from Lembang, West Java, Indonesia.

Sample preparation

Pomegranate (*P. granatum* L.), coriander (*C. sativum* L.), liman (*E. scaber* L.), fennel (*F. vulgare* M.), and kaempferia (*K. galanga* L.) were determined at Herbarium of School of Life Sciences and Technology, Bandung Institute of Technology. All samples were thoroughly washed with tap water, sorted while wet, cut, dried, and grinded into powder.

Crude drug was extracted using 96% ethanol by reflux method and evaporated by rotary evaporator. Each extract was prepared in three different concentrations. The fennel seed and coriander seed were 1, 0.5 and 0.25 mg/ml, whereas liman leaves, pomegranate pericarp, and kaempferia rhizomes were 6, 3, and 1.5 mg/ml.

Hatching ability

As much as 10 cysts were hatched in vial which contain 5 ml of seawater (with salinity 9 mg/ml) and extract, at temperature of 25-28°C, aerated and illuminated. After 36 hrs, hatching ability of cysts in each group were analyzed and the replication was performed 5 times.

Death of nauplii, morphological abnormalities, retarded development, and body length

As much as 1 g cysts were hatched in a beaker which contain 400 ml of seawater (salinity 9 mg/ml) and test substance, at temperature of 25-28°C, aerated and illuminated. After hatching, the first stage of *Artemia* nauplii (1-2 hrs after hatching) was sampled out then tested in each group which contains seawater and extract. In Group 1, nauplii were keep alive until second stage, Group 2 keep alive until third stage, and Group 3 keep alive until fourth stage. Nauplii that were taken from each group then put into Bouin solution for fixating. Observation was carried out using a microscope with a magnification of 100 times, with 5 times replication, included death of nauplii, morphological abnormalities, retarded development, and body length of nauplii.

RESULTS

Hatching ability of cyst

A number of cysts were hatched in medium seawater which contain extract with various concentrations in each group. Cysts cannot hatched in all concentrations of kaempferia rhizome extracts and liman leaves extract with a dose of 6 mg/ml. Hatching ability of cyst in fennel seeds extracts 1 and 0.5 mg/ml, coriander seed extract 1 mg/ml, and pomegranate pericarp extract 6 mg/ml were significantly different compared to the control group ($p < 0.05$) and can be seen in Fig. 1.

The results also showed relationship between the concentration and the hatching ability of cysts, where a high concentration tends to depress hatching ability of cyst larger than low concentration.

Death of nauplii

It can be seen in Table 1, Nauplii in all concentrations of kaempferia rhizomes extract cannot survive. All nauplii died since the second stage (19 hrs). At the second stage, liman leaves 6 mg/ml gave the highest

mortality of nauplii. Kaempferia rhizomes extract and liman leaves extract had significantly different compared to control group ($p < 0.05$).

At the third stage, all of nauplii were dead in liman leaves extract 6 mg/ml, while fennel seeds extracts 1 and 0.5 mg/ml presented significantly different compared to control group ($p < 0.05$).

At the fourth stage, 28% of nauplii died in control group and 100% died in fennel seeds extracts at all concentrations, pomegranate pericarp extract 3 mg/ml, and liman leaves extracts 6 and 1.5 mg/ml, while in the other groups nauplii could survive only <10%.

Morphological abnormality

Observations have been carried out on all groups both the control and extract to see morphological abnormalities. Each group was done 5 times replications. At the second stage, there was morphological abnormality in liman leaves extract 6 mg/ml, while the nauplii without antenna and there were arches at the thoracopod body part (Fig. 2).

The third stage found two morphological abnormalities, which were nauplii with shorter bilateral *gnatobasten* in coriander seeds extract 0.5 mg/ml and nauplii with an arch in the lateral portion of the tail in pomegranate pericarp extract 3 mg/ml (Fig. 3).

Retarded development

At the second stage, liman leaves extract 3 mg/ml expressed the greatest number of nauplii with retarded development (7.14%). At the third stage, the highest retarded development of nauplii was given by fennel seeds extract 1 mg/ml, whereas at the fourth stage only coriander seed extract 0.5 mg/ml showed nauplii with retarded development (Table 2).

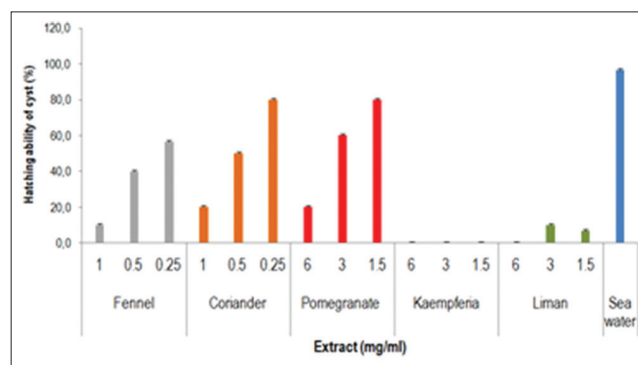


Fig. 1: Hatching ability of cyst in extracts

Table 1: Death of nauplii in extracts

Sample	Death nauplii (%)		
	Second stage	Third stage	Fourth stage
Seawater	0.00±0.00	0.00±0.00	28.00±2.31
Methotrexate	0.00±0.00	0.00±0.00	100.00±0.00*
Fennel 1 mg/ml	50.00±1.53	96.00±0.58*	100.00±0.00*
Fennel 0.5 mg/ml	46.00±1.00	78.00±3.21*	100.00±0.00*
Fennel 0.25 mg/ml	12.00±0.00	82.00±3.61	100.00±0.00*
Coriander 1 mg/ml	40.00±0.00	40.00±0.00	98.00±0.58*
Coriander 0.5 mg/ml	40.00±0.00	24.00±0.00	92.00±0.58*
Coriander 0.25 mg/ml	0.00±0.00	4.00±0.00	90.00±1.53
Pomegranate 6 mg/ml	26.00±1.15	76.00±5.77	92.00±2.31*
Pomegranate 3 mg/ml	2.00±0.00	14.00±4.04	100.00±0.00*
Pomegranate 1.5 mg/ml	2.00±0.58	6.00±1.73	98.00±0.58*
Kaempferia 6 mg/ml	100.00±0.00*	100.00±0.00	100.00±0.00*
Kaempferia 3 mg/ml	100.00±0.00*	100.00±0.00*	100.00±0.00*
Kaempferia 1.5 mg/ml	100.00±0.00*	100.00±0.00*	100.00±0.00*
Liman 6 mg/ml	96.00±0.58*	100.00±0.00*	100.00±0.00*
Liman 3 mg/ml	72.00±5.03*	80.00±5.77	98.00±0.58*
Liman 1.5 mg/ml	64.00±3.46	78.00±5.51	100.00±0.00*

Replication is performed 5 times, n=50. *Significantly different compared to control ($p < 0.05$)

Body length

In Table 3, it can be seen that the shortest body length of nauplii was given by liman leaves extract 6 mg/ml at the second stage while the third stage shown by fennel seeds extract 1 mg/ml which was significant different to the other groups. At the fourth stage, pomegranate pericarp extract 1.5 mg/ml represented the shortest body length although the difference was not statistically significant.

DISCUSSION

In the present study, *Artemia* cysts hatched within 24-36 hrs. It was similar to research by Sorgeloos *et al.* [15] and Lestari *et al.* [12]. It can also be seen that the relationship between concentration and number of cysts hatch, where in higher concentration, could depress the hatchability of cyst and give the lower number of cyst.

In medium which contained extract, only around 2-8% nauplii still lived at the fourth stage. In some extracts such as liman leaves extract and pomegranate pericarp extract, it can due to increasing in density, while extract which contain oil such as coriander seed extract and

kaempferia rhizomes extract, it can also be caused by increasing level in turbidity. Density and level of turbidity of sample in medium can directly affect survival of nauplii. pH below 5 or higher than 10 could kill *Artemia* [12,15]. All of nauplii in kaempferia rhizomes extracts were died since the second stage, so there was no nauplii progressed to the further stage. Hence, it can be predicted that kaempferia rhizome extract had larvicide effect for *Artemia's* nauplii its analog in mamalia as embryo lethal. It was similar to the previous research which claimed that the kaempferia rhizome extract gave larvicide properties for *Culex quinquefasciatus* [16] and also *Aedes aegypti* [17].

Morphological abnormalities which were found in this study were the arch at the thoracopod body part, shorter *gnatobasten*, and antenna did not grow. In adult *Artemia*, there are 11 pairs of thoracopod which acts as a means of motion, gills, and foragers [18]. Abnormalities in any part of the nauplii's body that would later become thoracopod may cause disturbances in the respiratory system, transport, digestion, and reproduction in adult *Artemia*. *Gnatobasten* on *Artemia* acts as a means of motion [18], so the shorter *gnatobasten* might cause disruption of transportation in nauplii. Antenna on adult *Artemia* serves as a sensory, and on male *Artemia*, its act as a hook tool when copulation [18];

Table 2: The retarded development of nauplii in extracts

Sample	Retarded development (%)		
	Second Stage	Third Stage	Fourth Stage
Seawater	-	-	-
Methotrexate 15 µg/ml	3.33±0.58	20.00±1.73	-
Fennel 1 mg/ml	-	50.00±0.58	-
Fennel 0.5 mg/ml	3.70±0.57	-	-
Fennel 0.25 mg/ml	-	-	-
Coriander 1 mg/ml	3.33±0.57	16.67±2.89	-
Coriander 0.5 mg/ml	-	-	4.76±0.58
Coriander 0.25 mg/ml	3.33±0.57	3.33±0.58	-
Pomegranate 6 mg/ml	-	10.00±0.58	-
Pomegranate 3 mg/ml	3.45±0.57	13.04±0.00	-
Pomegranate 1.5 mg/ml	-	18.52±2.89	-
Kaempferia 6 mg/ml	-	-	-
Kaempferia 3 mg/ml	-	-	-
Kaempferia 1.5 mg/ml	-	-	-
Liman 6 mg/ml	-	-	-
Liman 3 mg/ml	7.14±0.57*	10.00±0.58	-
Liman 1.5 mg/ml	-	36.36±1.53	-

Replication is performed 5 times, n=50. *Significantly different compared to control (p<0.05)

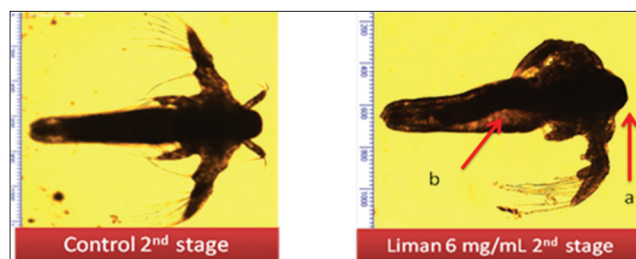


Fig. 2: Morphological abnormality of nauplii at the second stage. (a) Antenna did not grow, (b) arch at the thoracopod body part

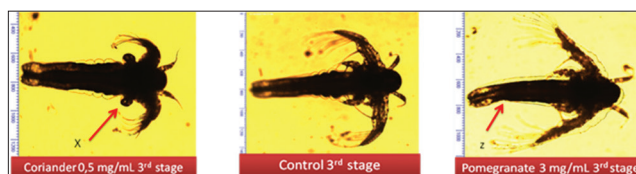


Fig. 3: Morphological abnormality of nauplii at the third stage. (x) Shorter *gnatobasten*, (z) arch at lateral of the tail

Table 3: Body length of nauplii in extracts

Sample	Body length (µm)		
	Second stage	Third stage	Fourth stage
Seawater	896.70±90.86	1074.00±70.27	1505.64±90.06
Methotrexate 15 µg/ml	872.76±78.73	1019.84±38.15	-
Fennel 1 mg/ml	887.96±60.10	964.87±235.76	-
Fennel 0.5 mg/ml	890.31±59.53	1002.99±104.95	-
Fennel 0.25 mg/ml	943.94±97.47	1060.92±85.70	-
Coriander 1 mg/ml	860.70±96.46	1035.59±24.46	2030.00±0.00
Coriander 0.5 mg/ml	876.86±68.75	1047.50±81.99	1735.97±494.99
Coriander 0.25 mg/ml	902.70±65.89	1097.28±43.24	1431.85±13.56
Pomegranate 6 mg/ml	851.54±159.93	1232.03±0.00	2179.04±0.00
Pomegranate 3 mg/ml	868.41±129.41	1008.99±108.23	-
Pomegranate 1.5 mg/ml	868.57±116.63	1104.40±159.38	1344.02±0.00
Kaempferia 6 mg/ml	-	-	-
Kaempferia 3 mg/ml	-	-	-
Kaempferia 1.5 mg/ml	-	-	-
Liman 6 mg/ml	789.27±99.87	-	-
Liman 3 mg/ml	896.95±114.21	1183.84±0.00	1353.65±0.00
Liman 1.5 mg/ml	917.19±127.49	1123.00±227.70	-

Replication is performed 5 times, n=50

therefore, the loss of antenna can cause disruption of reproductive system and sensors on adult *Artemia*.

In the present study, it could be seen the affect of extract to development of nauplii although it was not statistically significant. The retarded development that be found were retarded development of nauplii which should be in advance stage, but it still in below stage; however, it was not linear with concentration. The higher concentration did not always give increasing in retarded development. The previous research [12] showed that developmental delay disorder can be caused also by differences in time in hatch of cysts.

Although it was not significant different body length between nauplii in seawater and nauplii in extract, the higher concentration of extract gave shorter body length of nauplii, and the higher concentration showed more different body length compared to control. Some previous studies [12-14] stated that the differences in body length of nauplii indicated growth disturbance on *Artemia*.

Based on the brine shrimp teratogenic screening test which used five parameters and tested five plants extracts, it could be seen that liman leaves extract and coriander seeds extract complied almost all of parameters. Fennel seeds extract and pomegranate pericarp extract complied a few parameters, whereas kaempferia rhizomes extract just stop at one parameter only. Therefore, liman leaves extract and coriander seeds extract the most likely to have teratogenic effect.

CONCLUSION

Liman leaves extract and coriander seeds extract potentially had teratogenic properties. Fennel seeds extract and pomegranate pericarp extract had no teratogenic effect, while kaempferia rhizomes extract acts as larvicide its analog with embryo lethal in mammal.

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