The Effect of Maklua (Diospyros mollis) in Mice
(พิษของมะเกลือในหนู)

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Abstract

The effect induced by various forms and doses of Maklua were conducted in 1,000 mice. The doses being used in this experiment were 1, 10, 20, 25 and 30 times the human therapeutic doses. Fresh Maklua juice with calcium hydroxide solution, oxidized form and fresh Maklua juice with distilled water were used for A, B and C forms respectively. The results indicated that the LD_{50} values of A and C forms were approximately 22 and 22. 25 times the human therapeutic doses. Most of the mice died within 24 hours and had a sign of tremor at extremities before died. There were no significant difference in the BUN, SGPT and SGOT levels from controls. No evidence of pathological alternations have been observed in eye ball and optic nerve. However, There was brown pigment which is suggestive as Maklua particle was observed in the collecting tubules of kidney, cytoplasm of liver cells and lamina propria of small and large intestines. In Thirty and twenty – five times the human therapeutic doses, brown pigment deposited mostly in small intestine and kidney, a few in liver and large intestine. In the twenty times the human therapeutic dose, small intestine was mostly deposited

with brown pigment, a few in kidney and large intestine but none in the liver cell. In ten times of human therapeutic dose, only A – form that found brown pigment deposited in kidney and small intestine. Non of brown pigment was found in the dose that was equal to the human therapeutic dose either any form.

Introduction

In Thailand, Maklua has been extensively used as an anthelmintic for hundreds of years. Several clinical trials have been carried out with good results. Following the administration of Maklua there have been some slight contraindications reported such as nausea, vomitting, abdominal pain, mild drowsiness, vertigo and blurred vision (Sadum and Vajarasthira, 1954). Recently Limpaphayom *et al*, (1977) reported that a case of bilateral retrobulbar neuritis with subsequent optic atrophy occurred 17 hours after taking Maklua. The toxic substance was suspected to be the oxidized form of tetra – hydroxy di – methyl binaphthalene of Maklua. Thereafter Unhanand (1979) reported cases of children aged 6–7 years suffering blindness after taking Maklua from the local health officer. The possible cause of the blindness in these cases was assumed to be the same as Limpaphayom *et al*, (1977) described. The studies conducted by Grant (1962) and Zagora (1962) indicated that naphthalene possessed this toxic effect to the eye of experimental rabbits.

As previously mentioned, it seems that the contraindication of the eye injury in man is probably associated with consuming the oxidized form of Maklua preparation. However, the reason for this phenomenon has not been elucidated particularly on the point of the mechanism by which this herbal medicine induces the toxic effect, and toward what organs of the host. Therefore, the purpose of this study is to try to clarify whether or not Maklua produces the toxic effect to the host by using mice as the experimental model. The study primarily focuses on the pathological changes induced by various forms and dosages of Maklua prepartion.

Materials and Methods

Animals

Animals used in the experiment were adult male CRC mice weighing 25 – 35 gm. Five mice were kept in one cage and fed with commercial mouse pellets, and tab water *ad libitum*. The temperature of the laboratory room was 22 – 35°C and humidity at approximately 50 – 60 %. All animals were fasted overnight before weighing and dosing but given free access to drinking water.

Maklua *(Diospyros mollis)*

Maklua berries were collected from Lopburi, Saraburi and Rajburi provinces. The weight of each berry was about 6 – 8 gm. The black spotted or streaked berries were discarded. They were stored in the refrigerator at 4°C. Maklua being used in the experiment was not kept in the refrigerator longer than 10 days.

Maklua preparations

Seventy grams of fresh *D. mollis* berries were selected, avoiding all those that were black or streaked with black spots. The calyxes of the berries were removed before weighing. The berries were washed, crushed in a juicer blender (National MJ – 28ON) and thoroughly mixed with 15 ml. of calcium hydroxide solution. The mixture was yellowish (A – form) in color.

The oxidized form of the *D. mollis* was prepared by exposing the fresh berry juice to the air for 48 hours and the color of the juice turned from yellowish to black. Distilled water was then added in order to obtain the original volume (B – form).

The third form was prepared similarly to the first one, but mixing the juice with distilled water instead of calcium hydroxide solution (C – form).

Experiment

To determine LD_{50} of Maklua in mice with various forms and doses. Fifty mice were used for each dose. The doses were varied, using 1, 10, 20, 25 and 30 times the dosage being used for humans. They were given single doses of A, B and C forms of Maklua mixtures by oral administration using an intubation needle (4 inches, 17 gauze) blunted tip attached to a syringe, which was passed down the esophagus to the stomach. All mice were observed periodically up to seven days after a given dose of A, B and C forms.

To study the histopathological changes in the tissues of some visceral organs and the eyes of mice after treatment with various forms and doses of Maklua.

A group of ten mice were given a single oral administration of A, B and C forms of Maklua at the doses of 1, 10, 20, 25 and 30 times the usual human therapeutic dose (1.7 ml/kg BW). Placebo and control mice received equal volume of calcium hydroxide solution (D – form) and distilled water (E – form) respectively. They were sacrificed on 1, 3, 7, 15, 30 days after treatment (Table 1). Visceral organs and eye balls with optic nerve were excised rapidly

and fixed in 10% formalin solution. The process of the section being performed step by step as described in Manual of Histologic and Special Staining Technics. Sections 5 micron thick were cut and stained with hematoxylin and eosin.

Table 1. Doses and number of mice used in various forms of Maklua preparations

Dose (Times the human therapuetic dose)	No. of mice used in various forms of Maklua preparations					Total mice used
	A*	B*	C*	D*	E*	
1	50	50	50	25	25	200
10	50	50	50	25	25	200
20	50	50	50	25	25	200
25	50	50	50	25	25	200
30	50	50	50	25	25	200
Total miee used	250	250	250	125	125	1,000

A* = Maklua juice + calcium hydroxide solution

B* = Oxidized form of A

C* = Maklua juice + distilled water

D* = Calcium hydroxide solution

E* = Distilled water

Each group of mice (10 mice) were sacrificed on 1, 3, 7, 15, 30 days after treatment.

To study the relationship between the enzyme activity (SGOT, SGPT) and the liver cell changes in mice by feeding various forms and doses of Maklua. Mice were anaesthetised with ether and exsanguinated directly from the heart with needle and syringe. Serum was pooled from each group of mice after centrifugation. Measurement was carried out accordingly to the description given in Sigma Technical Bulletin No. 505 (Sigma Chemical Co. St., Louis, U.S.A.). Each assay was performed in duplicate and the mean was calculated. To study the relationship between the blood urea nitrogen (BUN) and kidney cell changes induced in mice after administration of Maklua. The blood was drawn from the mouse heart by disposable heparinized plastic syringe and transferred into a centrifuge tube. The determinations of blood urea nitrogen were followed according to Techniques of Chemical Chemistry (Naletson, 1971).

Results

Following the administration of A, B, C, D and E forms of Maklua, the mice were sequentially observed for contraindication and death. The mortality rate of mice was strikingly high in the group to which the high doses of A and C forms were administered, as illustrated in Table 2. Thirty times the human therapeutic dose of A and C forms showed a 95.83 and 84.78 percent mortality rate, which B form showed 40 percent mortality. Similar results were also obtained in the group of mice which were given 25 times the human therapeutic doses of A and C forms (87.5 and 95 % mortality rate respectivcly). When the doses were decreased to 20 and 10, the mortality rate of mice gradually declined, and none of them died following feeding with a dose equal to that given to humans. For all doses of D and E forms, there were no deaths of mice.

Table 2. Comparison of mortality rate in group of mice treated orally with various doses and forms of Maklua

Doses*	Forms	Mortality rate (%)	Time in which death resulted after dosage administration
1	A	0	–
	B	0	–
	C	0	–
10	A	4	1 hr – 1 d
	B	0	–
	C	4.44	35 min – 1 hr 45 min
20	A	20	35 min – 1 hr 45 min
	B	0	–
	C	16	20 min – 2 d
25	A	87.5	45 min – 5 d
	B	39.47	1 hr 15 min – 12 hr
	C	95	20 min – 2 hr 30 min
30	A	95.83	10 min – 2 hr
	B	40	10 min – 4 d
	C	84.78	20 min – 2 d

Doses* = times the human therapeutic dose.
d = day

After feeding with Maklua, the obvious contraindications such as inactivity, drowsiness, high respiration rate and diarrhea were observed, particularly among the group of mice given high doses. The mice showed convulsive seizures and tremor of the extremities before death. Most of them died between 10 mins–24 hrs after feeding in Maklua. Those mice which survived after the treatment would usually recover within 24 hours. For the placebo (D–form) and control (E–form) groups, the mice remained alert and active throughout the experiment. The LD_{50} was determined from the mortatity rate for A and C forms, which were approximately 22 and 22.25 times the human therapeutic dose respectively (Fig. 1)

Considering the result of BUN, SGPT and SGOT levels obtained from mice after feeding Maklua, no marked difference was observed as compared to those in the control group ($P > 0.05$).

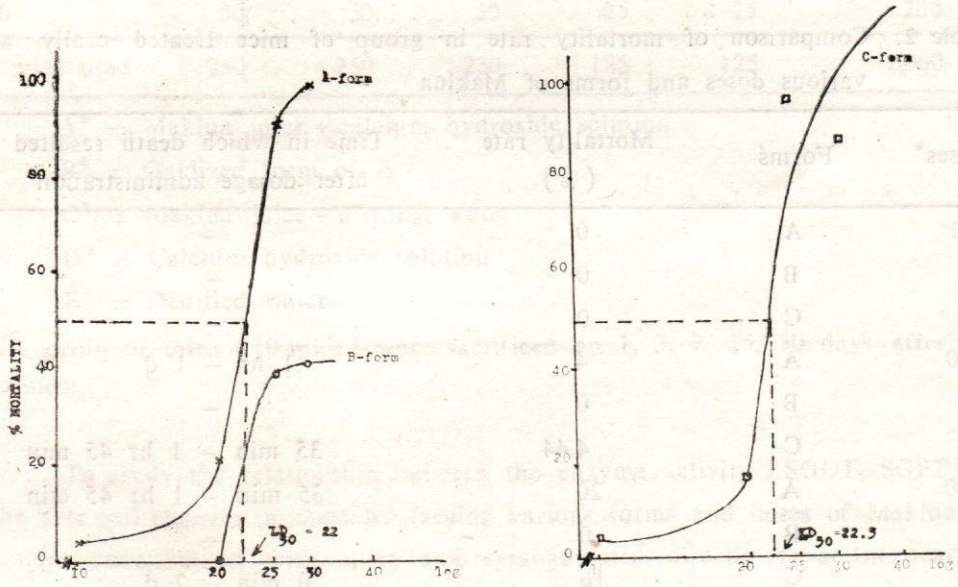

Fig 1. Mortality rate of mice treated orally with various doses of A, B, and C forms of Maklua.

The morphological changes observed in the liver, kidney, intestines and eye ball with optic nerve of mice treated with various doses and forms of Maklua indicated no marked histologic alteration except there was some deposition of brown pigment which may be particles of Maklua in some of previously

mentioned organs. The structure of the mucosa, submucosa, muscularis external and serosa of the intestinal wall of small and large intestines were histologically normal. But in the lamina propria particularly in the region adjacent to the tip of villi, there was the deposition of brown pigment in mice treated with Maklua as shown in (Figure 2.)

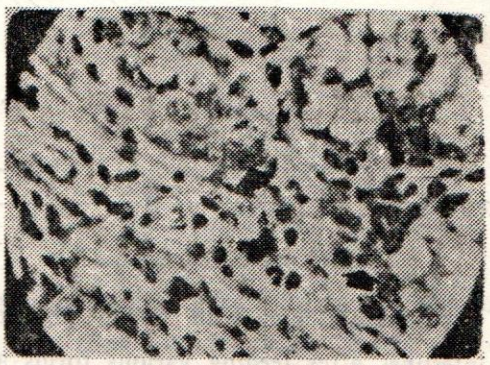

Figure 2. Cross section of the large intestine of a mouse, sacrificed on day 30 following feeding with B – form of Maklua (25 times the human therapeutic dose). Arrow indicates the deposition of brown pigment in the lamina propia. H & E stain $\left(\frac{10 \times 10}{2}\right)$.

The pigment was first observed in the lamina propria of the small intestine in mice treated with 10 times the human dose (A – fom) and appeared more obviously in group of mice treated with the higher doses. The percent of mice with brown pigment increased responding to the increment of doses as high as 75 in mice treated with A form (30 times of the human dose) and similar results were also obtained in mice treated with B and C forms. The pigment was found in the lamina propria of large intestine of mice treated with B form at the dose of 20, 25 and 30 times of human dose. It was also observed from day 1 to day 30 after treatment (Fig 3). The liver of mice treated with 25 times the human dose in A and B forms indicated the appearance of brown pigment in the cytoplasm of hepatocytes. It was not observed in the other groups of mice and in the control.

Morphological changes in the kidney of mice treated with various forms of Maklua was not evident. The cortex and medulla appeared normal. However, there was the deposition of brown pigment in the collecting tubules in

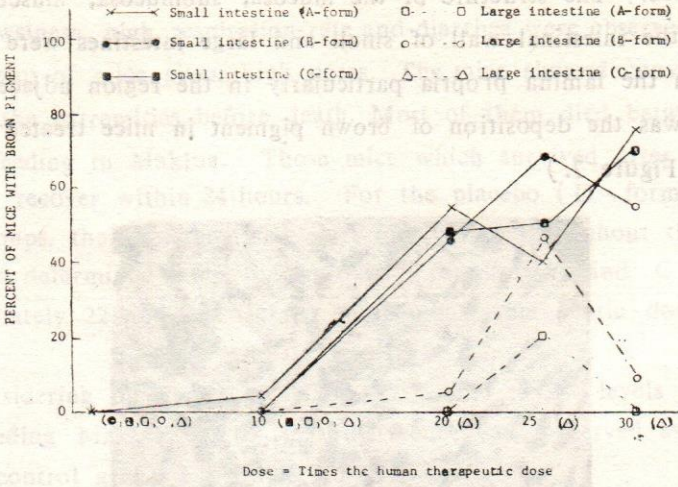

Figure 3. Percent of experimental mice with brown pigment deposited in small and large intestines after feeding various forms and doses of Maklua

the medulla area of mice treated 10, 20, 25 and 30 times the human dose as shown in Figure 4 but not found in the glomeruli. The percent of mice with brown pigment in the collecting tubule was found to be as high 50 and 57.14 in group of mice treated with 25 and 30 times and human dose (C–form) as illustrated in Fig 5. The pigment in the collecting tubules was found in mice sacrificed from day 1 to day 30.

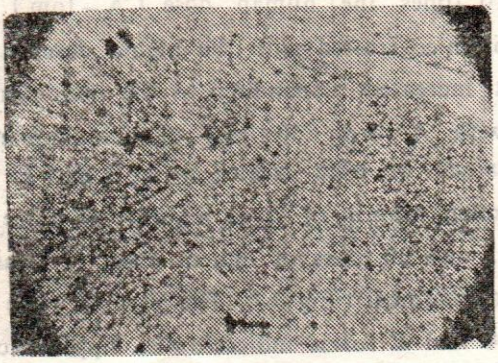

Figure 4. Cross section of the kidney of a mouse, sacrificed on day 3 following feeding with B–form of Maklua (30 times the human therapeutic dose). Arrow shows the deposition of brown pigment in the collecting tubule. H & E stain $\left(\frac{10 \times 4}{2}\right)$

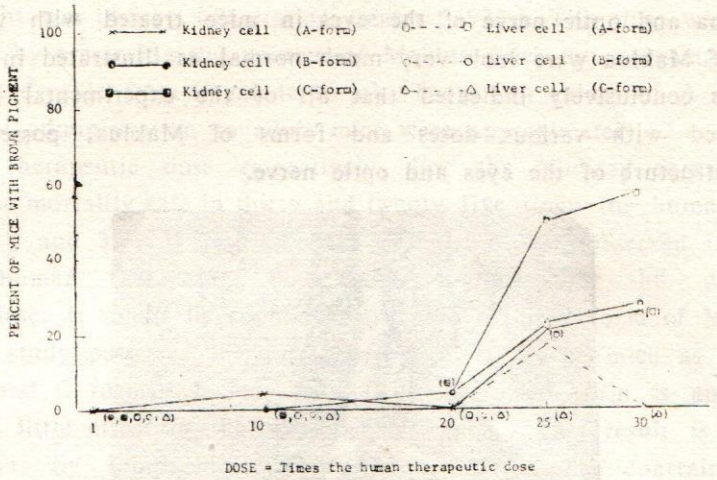

Figure 5. Percent of experimental mice with brown pigment deposited in kidney and liver cells after feeding various forms and doses of Maklua

The structure of the eyes of all mice were serially observed. Histological details of the structure are depicted in Figure 6, 7 and 8. The eye of mouse (Figure 6) treated with 20 times the human dose of Maklua A – form indicated the anterior surface of the cornea which is covered with non – papillated, stratified squamous epithelim and the layer of columnar cells rests on a typical membrane. The corneal cells which are the modified fibroblasts appeared normal. Figure 6. illustrated the normal iris and lens obtained from the same mouse. The sclera,

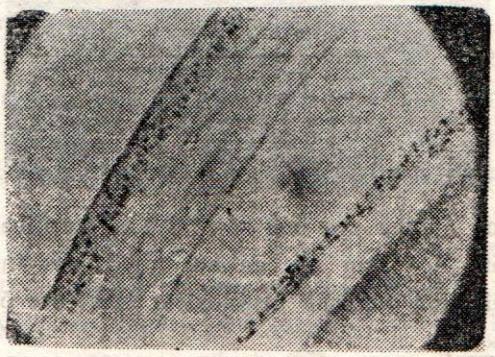

Figure 6. Cross section of the eye ball of a mouse, sacrificed on day 3 following feeding with A – form of Maklua (20 times the human therapeutic dose), indicating normal histological structure of cornea. H & E stain $\left(\frac{10 \times 10}{2}\right)$

choroid, retina and optic nerve of the eyes in mice treated with various doses and forms of Maklua were look very much normal as illustrated in Figure 6, 7 and 8. It is conclusively indicated that all of the experimental mice in the groups treated with various doses and forms of Maklua, possessed normal histological structure of the eyes and optic nerve.

Figure 7. Cross section of the eye ball of a mouse, sacrificed on day 7 following feeding with B – form of Maklua (30 times the human therapeutic dose), indicating normal histological structure of retina. H & E stain $\left(\frac{10 \times 10}{2}\right)$

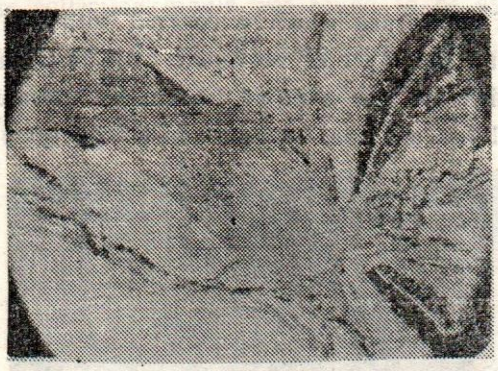

Figure 8. Cross section of the eye ball of a mouse, sacrificed on day 3 following feeding with A – form of Maklua (30 times the human therapeutic dose), indicating normal histological structure of optic nerve. H & E stain $\left(\frac{10 \times 4}{2}\right)$

Discussion

The dosage of Maklua preparation given to experimental mice was approximately the same dosage being used in man (A form). That was 70 ml. of Maklua preparation II for the treament of 5–9 years old Thai children whose

average weight was 14.9 kg. (Sanitation Center I 1973). This dose was applied for using in mice by preserving the volume of Maklua juice but reducing the volume of calcium hydroxide from 60 ml. to 15 ml. The LD_{50} values of A and C forms following oral administration to adult mice were approximately 22 and 22.25 times the human therapeutic dose respectively. For the oxidized form of Maklua (B form), the mortality rate in thirty and twenty five times the human therapeutic dose was 40% and 39.47% respectively. No death was observed in 20 and 10 times the human therapeutic dose, and similarly for the usual human therapeutic dose. It might be concluded that the oxidized form of Maklua being used in this study possessed less effect to the mortality of mice as compared to those in A and C forms. It may be that the oxidized form is an inert form which causes little effect to the experimental mice. This result is contrary to the study done by Limpaphayom and et al. (1977). The contraindications of Maklua treatment in mice were diarrhea and drowsiness but not vomiting. The cause of death in the experimental animals was not apparent and further investigation is needed to clarify this issue.

With the dosages and forms of Maklua used, no evidence of pathological alterations has been observed in the liver and kidney cell and no significant difference in the SGPT, SGOT and BUN levels as compared to those in the control, was seen either before operation or until the end of the experimental period.

The chemical constituent of Maklua was tetrahydroxy di-methyl bi-naphthalene, composed of two naphthalenes. Naphthalene as the cause of catarat in human and animal has been reported by many investigators (Adams, 1930; Grant, 1962; Zagora, 1962). Verdi (1958) found the area of necrosis to be in all strata of the retina, with a moderate number in the choroid also involved. But from the results of this experiment, no evidence of histopathological changes has been observed in the cornea, lens, sclera, choroid, retina or optic nerve. Although the chemical structure of Maklua contains two naphthalenes which produced many of histopathological changes observed in man and animal eyes, but the methyl and hydroxil groups that attached at the naphthol rings may affect the properties of naphthalenes in producing the toxicity. On the other hand, the animal used in studies reported in the literature were rabbits. Possibly, eyes of the mice may be resistent to the toxic effects of this substance because there have not been any reports in which mice were used as the experimental animal.

On this histopathological study, numerous brown pigments were found deposited in the visceral organs of the experimental mice, but were not found in the placebo and control groups. It may be concluded that these brown pigments found in the collecting tubules of the kidney cell were Maklua. In the small

and large intestines it was found in the lamina propia at the tip of villi and in the hepatocytes of the liver. But these pigments were not found in the eyes. From these findings ti may be assumed that Maklua can be absorbed in the small intestine and then eliminated through the kidney. However, these brown pigments will be the residual or particle of Maklua need to be further confirmed by histochemical analysis or specific stain. The deposit of brown pigment presence in the small intestine in greater qualities than in the large intestine may be due to the fact that in the large intestine, there are many goblet cells which prevent the absorption of all substance except water, and in the small intestine there were more villi which provided more area for absorption.

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