Acute oral toxicity and LD_{50} values of standardized Melaleuca Alternifolia Concentrate (MAC) in the rats and rabbits

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ABSTRACT

Until recently most researches on Melaleuca oil have been focused on topical uses either in animals or humans, and are scarcely conducted systemically in the living systems to explore its efficacy and safety. It is important therefore to first-recognize the safety of the oil in animals before it could be introduced ultimately for human use.

In order to uncover the safety, we first study the acute toxicity and determine the LD_{50} values of the oil, namely *Melaleuca alternifolia* concentrate (MAC) which has been purified and standardized to contain 60% of biologically active compound terpinen-4-ol by a gas chromatographic method.

Non-pathogenic, male Wistar rats weighing 180-220 g, and domestic male rabbits weighing 1.5-2 kg were used in the studies. All the animals were acclimatized for one to two weeks in the laboratory with standard food and water *ad libitum* at 22° C and humidity of 50-60%. They were divided randomly into the treatment groups.

For the study in the rats, the animals were administered orally 10% MAC solution at the single dose of 0 (vehicle), 5, 10, 20, or 40 g/kg (each dosing group comprised 5 animals), whereas that in rabbits, each dosing group comprised 4 animals, received a single dose of 0 (vehicle), 13.3, 16.7, or 20 g/kg of 10% MAC solution. Toxic signs were observed for al least 24 hours and recorded in relation to dose and time. Animals dying during the observation period, as well as rats surviving to the end of the observation period were autopsied under ether anesthesia. A histopathological examination was conducted on any organ or tissue showing macroscopic changes at autopsy.

The results of the studies show that the calculated oral LD_{50} value of pure undiluted MAC was 2.1 g/kg of the rats, while that for rabbits was 1.56 g/kg, which are classified as moderately toxic. With 10% of MAC solution at the doses of 5 and 10 mg/kg the rats underwent wobbly gait, rather deep respiration, pale ears, but all animals were still alive. The weight of lungs, heart, stomach, spleen, liver, and kidney of the rats and rabbits, and also their histological profiles mostly appeared normal at all doses, although some

animals had inflammation, congestion, or necrosis in the stomach or liver at the doses near the LD_{50} value. Paralyses of the legs and ears were also observed in the rabbits after receiving MAC at the oral doses around and above its LD_{50} value. The information thus obtained in the present study may be useful for further studies in estimating the dose-effect and safety relationships of Melaleuca oil in other living systems including humans.

I. INTRODUCTION

Melaleuca Alternifolia Concentrate (MAC) is purified essential oil isolated from a native Australian plant, *Melaleuca alternifolia* (Webb, 2000). The preparation has been approved by the Australian TGA for topical use at 25% concentration as well as the FDA-USA as a herbal medicine. The oil has been reported to contain mainly terpinen-4-ol (56-58%) and to have an anti-inflammatory activity (Hart et al, 2000; Koh et al, 2002; Caldefie-Chezet et al, 2004 and 2006), and antimicrobial activities against various microbial species (Carson et al, 2006; Mondello et al, 2006; Papadopoulos et al, 2006). Witarto (2007) and Huang (2007) as well as Jiang (2007) have conducted a series of invitro studies and have found that MAC can inhibit the growth of Den 1, 2, 3 and 4 viruses, and ultimately kill the organisms.

However, the safety of Melaleuca oil has not been studied thoroughly in living animals before its possible use in humans. In order to look into the safety of MAC, therefore, we study the acute toxicity and determine the LD_{50} of MAC in rats and rabbits according to WHO Guidelines (1993).

II. METHODS OF THE STUDIES

Materials

Non-pathogenic male Wistar rats and domestic male rabbits obtained from the Faculty of Pharmacy, Gadjah Mada University, Indonesia. Both 10% MAC aqueous solution and vehicle solution (containing 1% w/w vitamin E) were supplied by NeuMedix Biotechnology Pty Ltd., Brisbane, Australia. Undiluted MAC contains 60% of terpinen-4-ol by a GC method (Wahyuni et al, 2009). Other reagents used were pro-analysis (Merck Darmstadt, Germany).

Methods

The studies were performed according to the WHO Guidelines (1993). All the animals were acclimatized for one or two weeks in the laboratory with standard food and water *ad libitum* at 22° C, humidity of 50-60%, and about equal day-night cycles. They were divided randomly into the control and treatment groups according to a completely randomized block design.

Two species were used, *ie.* five Wistar rats (rodents) weighing 180-220 g per dose group, and four rabbits (non-rodents) weighing 1.5-2 kg per dose group. The rats received four single dose levels, whereas the rabbits received three single dose levels of 10% MAC solution orally to find the LD_{50} values. All studies included a vehicle control group of the test animals. The MAC solution was administered in one dosing during a 24 hour period.

Toxic signs were observed for at least 24 hours and recorded in relation to dose and time. Animals dying during the observation period, as well as those surviving to the end of the observation period were autopsied under ether anesthesia. A histopathological examination was conducted microscopically on any organs or tissues showing macrospic changes at autopsy. Non-parametric statistics Kruskal-Wallis and Mann-Whitney test were used to evaluate differences among and between treatments at the probability of 0.05.

III. RESULTS OF THE STUDIES

Acute toxicity study in the rats

Since the knowledge of the toxicity of MAC was precisely unknown, it was necessary to select appropriate doses of the oil that may cause toxic to some animals. The preliminary doses were first predicted from human experiencing toxicity of tea tree oil which was then converted to animal doses based on the body surface area method (Gad & Chengelis, 1998; Shargel et al, 2005). The results are presented in Table 1.

Table 1. A Preliminary dose ranging study of 10% MAC solution in the rats

Single oral dose (g/kg)	n	Clinical signs
Vehicle	2	No death of animals.
5	2	No death of animals but underwent pale ears, wobbly gait, and respiratory depression
12.5	1	No dead animals, but with wobbly gait
25	4	One rat died after 20 min administration, 2 rats dying, one who survived underwent pale ears, wobbly gait & respiratory depression. One rat died during 24 hrs period.
30	2	Two rats died 24 hours later with respiratory depression
35	2	One rat died; one was dying due to respiratory depression, but finally survived after 24 hours

The rats were all survived after receiving vehicle solution, 5 and 12.5 g/kg of 10% MAC solution, but already showed intoxication with these two doses of the oil. Animal death was seen from the doses of 25 to 35 g/kg, but no consistent results occurred with the oral doses of 30 and 35 g/kg. Based on these preliminary findings, the oral doses of 10% of MAC solution were rearranged for the determination of LD_{50} value, as seen in Table 2.

Table 2. Dose levels of 10% MAC solution for determination of LD_{50} in the rats

Single oral dose (g/kg)	n	Clinical signs
Vehicle	5	No animals death, all clinical signs and behaviour of the animals were normal
5	5	Wobbly gait, rather deep respiration, pale ears, but all animals were alive
10	5	Wobbly gait, rather deep respiration, pale ears, all underwent urination, but all animals were alive
20	5	Two animals died about 10 hours after oral administration. Three animals were alive after dying or a deep sleep with deep respiration
40	5	All animals died within 1 hour to 20 hours after oral administration

Based on the data in Table 2, the calculated oral LD_{50} value of undiluted MAC solution was 2.1 g/kg of the rats which is classified as moderately toxic (Lu, 1991).

Investigation continued with the oral administration of doses around the LD_{50} value of MAC in separate goups of the rats to look into its effect on vital organs weight. Changes in organs weight may be regarded as early toxic signs occurred in animals (Lu, 1991).

Table 3. Organs weight of rats after a single oral dose of 10% MAC

Dose (g/kg)	Organs weight in grams (Mean ± SD, n = 5)					
	Lungs	Heart	Stomach	Spleen	Liver	Kidney
Vehicle	0.94 ± 0.07	0.65 ± 0.07	1.45 ± 0.11	0.48 ± 0.03	5.52 ± 0.25	1.28 ± 0.06
5	1.03 ± 0.08	0.66 ± 0.08	1.49 ± 0.08	0.41 ± 0.07	6.35 ± 0.81	1.51 ± 0.23
10	1.01 ± 0.04	0.62 ± 0.04	1.50 ± 0.16	0.39 ± 0.07	7.07 ± 0.95	1.36 ± 0.14
20	0.97 ± 0.18	0.56 ± 0.11	1.42 ± 0.11	0.30 ± 0.05	8.39 ± 0.94	1.57 ± 0.21
40	1.23 ± 0.26	0.58 ± 0.05	1.41 ± 0.28	0.46 ± 0.22	6.24 ± 0.79	1.51 ± 0.19

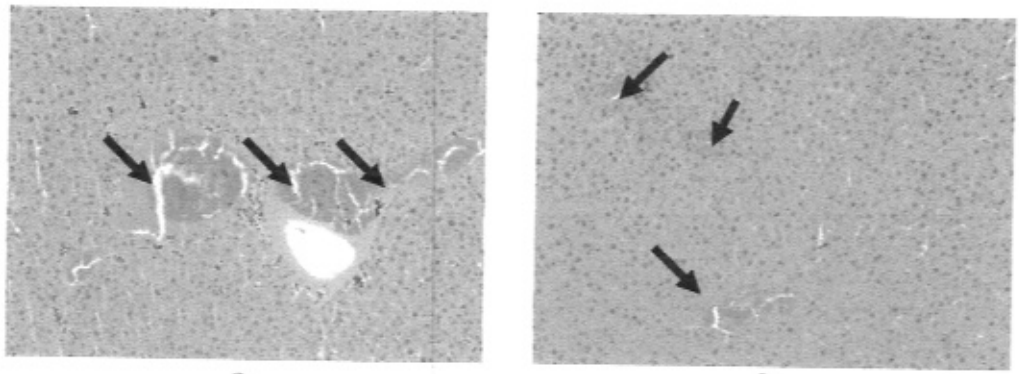
Data presented in Table 3 shows that all organs weight after a single oral dose of 10% MAC were similar to those after the control vehicle ($p>0.05$), except for the liver which caused organ weight increases after high doses of 10 and 20 g/kg (these doses are 48 and 95% of its oral LD_{50}, respectively). However, at the higher dose (40 g/kg), although there was a slight increase in the liver weight, the change was statistically insignificant ($p>0.05$). The same is true for the kidney weight, although there was a slight increase in the organ weight, the changes were statistically insignificant ($p>0.05$). These findings were in accordance with those presented in Table 4 where histological profiles of the vital organs were mostly normal.

Table 4. Histology of rat organs after a single oral dose of 10% MAC solution

Dose (g/kg)	Organs (n = 5)					
	Lungs	Heart	Stomach	Spleen	Liver	Kidney
Vehicle	N	N	N	N	N	N
5	N	N	N	N	C = 2**	N
10	N	N	G = 1*	N	N	N
20	N	N	N	N	N	N
40	N	N	N	N	N	N

N = normal, G = gastritis, C = congestion, * G=1 means that gastritis occurred in one animal, ** C=2 means that congestion occurred in two animals.

Table 4 shows that after a single oral dose of 10% MAC solution of various doses, the microscopic profiles of organs appeared normal compared with control animals, except that after 5 g/kg has caused liver congestion in two rats (out of 5 rats) and after 10 g/kg has caused swelling of the stomach (Figures 1 and 2). However, these clinical phenomena did not happen even at higher doses (20 and 40 g/kg).



a. b.

Figure 1. Histopathology of rat liver undergoing (a) congestion near blood vessel, and (b) congestion of hepatocytes after a single oral dose (5 g/kg) of 10% MAC solution

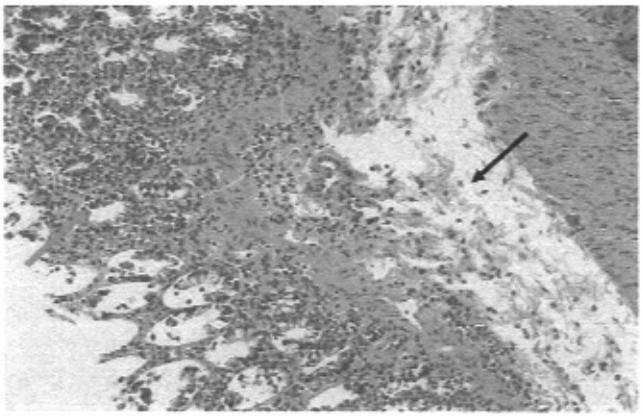


Figure 2. Histopathology of rat stomach swollen after a single oral dose (10 g/kg) of 10% MAC solution

Acute toxicity test in the rabbits

Dose determination in rabbits was performed by extrapolation based on the body surface area method from the oral LD_{50} value of MAC found in the rats according to Gad & Chengelis (1998) and Shargel et al (2005), and the results are presented in Table 5.

Table 5. A Preliminary dose ranging study of 10% MAC solution in rabbits

Single oral dose (g/kg)	n	Clinical signs
Vehicle	4	All animals were alive and normal
8	1	Signs of leg and ear paralyses, smaller irish
13.3	1	Signs of leg and ear paralyses, smaller irish
16.7	1	Signs of leg and ear paralyses, smaller irish
20	1	Animal died about 10 hours after oral ingestion

From the preliminary data, the oral LD_{50} value of MAC in rabbits was determined using the single oral doses of 13.3, 16.7, and 20 g/kg, each dosing group comprised of 4 animals, as seen in Table 6.

Table 6. Dose levels of 10% MAC solution for determination of LD_{50} in the rabbits

Single oral dose	n	Clinical signs

(g/kg)		
Vehicle only	4	All animals were alive and normal
13.3	4	No animal died, but all animals underwent leg and ear paralyses, slower respiration rate, and smaller irish.
16.7	4	Two animals died. Two animals were alive with leg and ear paralyses, slower respiration rate, and smaller irish.
20	4	All animals died.

Using the probit analysis, the oral LD_{50} value of MAC solution in the rabbits was found to be 1.56 g/kg which is classified as moderately toxic (Lu, 1991). It is clear from these findings that rabbits were more sensitive to MAC than rats in which all four animals died after the same dose of 20 g/kg (Table 6). The slower respiration rate and paralyses produced by MAC at the doses near their LD_{50} values were smilar in both species which might indicate the depression of central nervous system and somatomotoric disturbance.

Table 7. Organs weight of rabbits after a single oral dose of 10% MAC solution

Dose (g/kg)	Organs weight (in grams)* , Mean ± SD, n = 4					
	Lungs	Heart	Stomach	Spleen	Liver	Kidney
Vehicle	6.75 ± 0.47	3.02 ± 0.90	22.84 ± 1.52	44.45 ± 1.58	0.52 ± 0.15	8.32 ± 0.84
13.3	8.39 ± 3.46	4.16 ± 0.92	25.10 ± 4.68	54.68 ± 6.26	0.65 ± 0.15	9.47 ± 1.46
16.7	7.31 ± 3.14	2.90 ± 0.46	23.36 ± 4.14	47.51 ± 6.01	0.56 ± 0.28	8.80 ± 0.75
20	6.47 ± 0.59	3.95 ± 0.84	22.69 ± 7.47	51.19 ± 3.62	0.70 ± 0.17	9.29 ± 0.97

* Each organ weight was normalized to animal body weight.

It appears in Table 7 that MAC at the doses around and above its LD_{50} value, all of the organs weight differs to some extent to that of control group ($p>0.05$), and also the histological profiles were mostly unaltered. The results of statisical analyses have shown that all the doses of MAC did not affect significantly the organs weight of rabbits following single oral dosing of the herbal medicine ($p>0.05$). These findings were in accordance with those presented in Table 8 where histological profiles of the vital organs were mostly normal, except at a high dose (13.3 g/kg) showing liver necrosis and kidney congestion in one rabbit (out of four animals), as seen in Figures 3 and 4. However, at the higher doses (16.7 and 20 g/kg) these clinical outcomes were unseen microscopically.

Table 8. Histology rabbit organs after a single oral dose of 10% MAC solution

Dose (g/kg)	Organs, n = 4					
	Lungs	Heart	Stomach	Spleen	Liver	Kidney
Vehicle	N	N	N	N	N	N

13.3	N	N	N	N	NEC = 1	C = 1
16.7	N	N	N	N	N	N
20	N	N	N	N	N	N

N = normal, NEC = 1 means liver necrosis in 1 animal, C = 1 means kidney congestion in 1 animal.

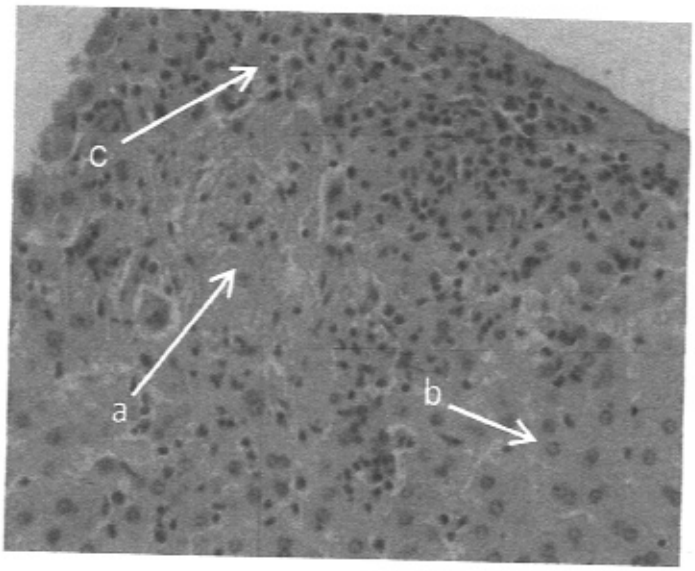


Figure 3. Histopathology of rabbit liver showing (a) necrosis and (c) swelling of hepatocytes after a single oral dose (13.3 g/kg) of 10% MAC solution in comparison with (b) normal hepatocytes.

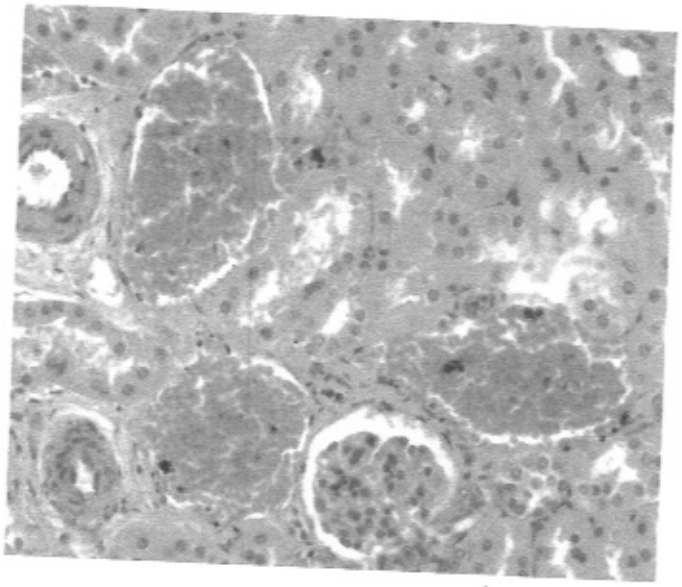


Figure 4. Histopathology of rabbit kidney showing congestion (red colour) after a single oral dose (13.3 g/kg bw) of 10% MAC solution

Based on these findings, it is now possible to perform further researches to explore the efficacy and safety of Melaleuca oil after the long-term use in the living animals, and also to estimate the dose-effect and safety relationships of Melaleuca oil in other biological systems including humans

CONCLUSION

The results of the studies have shown that the calculated LD_{50} value of MAC was 2.1 g/kg of male Wistar rats, while that for male rabbits was 1.56 g/kg, which are classified as moderately toxic.

With 10% of MAC solution at the doses of 5 and 10 g/kg, all of the rats were alive but underwent wobbly gait, rather deep respiration, pale ears. The weight of lungs, heart, stomach, spleen, liver, and kidney of the rats and rabbits, and also their histological profiles mostly appeared normal at all doses, although some animals had inflammation, congestion, or necrosis in the stomach or liver at the doses near the LD_{50} value. Legs and ears paralyses were also observed in the rabbits after receiving MAC at the oral doses around and above its LD_{50} value.

The information thus obtained in the present study may be useful to predict the safety of MAC in other animals and humans which corresponds to its therapeutic doses.

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