Final Report

ACUTE AND SUBACUTE TOXICITY STUDIES OF *Melaleuca Alternifolia* Concentrate (MAC) IN THE LABORATORY ANIMALS

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ABSTRACT

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 or 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 acute toxicity 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/kgbw (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/kgbw 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. A histopathological examination was conducted on any organ or tissue showing macrospic changes at autopsy (WHO, 1993).

For the subacute toxicity study in the rats, the animals were administered orally 10% MAC solution at 0 (vehicle), 1.6, 3.15, or 6.3 g/kgbw (each dosing group comprised 10 animals) for 28 consecutive days, and 5 animals per dosing group were kept alive without the administration of MAC solution for 7 days. The animals were then sacrificed for biochemical and histopathological analyses according to WHO Guidelines (1993).

The results of the studies show that :

1. The calculated LD_{50} value is 21 g/kg bw of Wistar rats, while that for rabbits is 15.6 kg/kgbw, which are classified as practically non-toxic (Lu, 1991).
2. All organs examined (spleen, heart, lungs, kidney, stomach, intestine and liver) appear normal at the daily dose of 1.6 g/kg/day for 28 days.
3. Intestine and liver injuries occur dose-dependently starting from 3.15 g/kg/day.
4. Kidney function appears normal as seen from normality of serum creatinine and ureum, and from free-protein urine, and liver function appears normal as seen from normality of serum bilirubin concentration, after oral daily doses of 1.6, 3.15, and 6.3 g/kg/day for 28 days.
5. Increase levels of SGPT and SGOT are seen in MAC and vehicle groups.
6. All the blood constituents (white blood cells, red blood cells, hemoglobin, hematocrite, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, blood platelets counts) are normal (Mitruka et al.,1977), except that the white blood cells counts tend to increase to some extent and it appears 7 days after the withdrawal of 10% MAC solution.

I. INTRODUCTION

Melaleuca Alternifolia Concentrate (MAC) is an essential oil isolated from a native Australian plant, *Melaleuca alternifolia* (Webb, 2000). The preparation has been approved by the Australian TGA for human use at 25% concentration as well as by the FDA-USA as a herbal medicine.

However, the safety of MAC has not been studied thoroughly in laboratory animals. In order to look into the safety of MAC, therefore, the acute and subacute toxicity studies of MAC were performed in rats and rabbits. The subacute study is considered sufficient because MAC will be used in humans for one to two weeks periods.

II. METHODS

Materials

Non-pathogenic male Wistar rats weighing 180-220 g, and domestic male rabbits weighing 1.5-2 kg were used in the studies. Both 10% MAC aqueous solution and vehicle solution (containing vitamin E) were supplied by NeuMedix Biotechnology Pty Ltd., Australia.

Procedures

The studies were performed according to the WHO Guidelines (1993) : *Research guidelines for evaluating the safety and efficacy of herbal medicines*, Manila.

All the animals were acclimatized for one or 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.

1. Acute toxicity test

a. Two species were used, *ie.* 5 males per dose group of Wistar rats (rodents), and 4 males per dose group of rabbits (non-rodents).
b. 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 include a vehicle control group of test animals.
c. The MAC solution was administered in one dosing during a 24 hour period.

Observation :

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 rodents surviving to the end of the observation period were autopsied. A histopathological examination was conducted on any organ or tissue showing macrospic changes at autopsy (WHO, 1993).

2. Subacute toxicity test

a. One species was used, ie. 10 males per dose group of rats.
b. Three dose levels of 10% MAC solution were given orally everyday for 28 days. After completion of 28 days treatment, the animals were splitted into two groups of five animals per dose. The first groups were sacrificed on the day-29 for autopsy, whereas the second groups were kept alive for 7 days (recovery study). These later goups were then sacrificed and autopsied.
c. All studies included a vehicle control group of test animals.
d. The organs observed were the stomach, intestine, spleen, heart, lungs, kidney and liver.

Observations and examinations were performed according to WHO (1993).

III. RESULTS

A. Acute toxicity tests in rats and rabbits

1. Acute toxicity test in the rats

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

Single oral dose (g/kg bw)	n	Clinical signs
Vehicle	2	No animals death
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 with pale ears and respiratory depression, but finally survived after 24 hours

Animal death was seen from 25 to 35 g/kgbw, but no consistent results occurred with the oral doses of 30 and 35 g/kgbw. Based on these findings, the oral doses of 10% of MAC solution were rearranged as seen Table 2.

Table 2. Acute toxicity and oral LD_{50} value of 10% MAC solution in the rats

Single oral dose (g/kg bw)	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 10 hours after per oral administration. Three animals were alive after dying or a deep sleep with deep respiration
40	5	All animals died within 1-20 hours after per oral administration

Conclusion :

Based on the above data, the calculated LD_{50} value of oral 10% MAC solution was 21 g/kg bw of male Wistar rats which is classified as practically non-toxic (Lu, 1991).

2. Acute toxicity test in the rabbits

Dose determination in rabbits was performed by extrapolation based on the body surface area method from the LD_{50} value found in the rats.

Table 3. A Preliminary dose ranging study of 10% MAC solution in rabbits

Single oral dose (g/kg bw)	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 10 hours after oral ingestion

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

Table 4. Acute toxicity and oral LD_{50} value of 10% MAC solution in the rabbits

Single oral dose (g/kg bw)	N	Clinical signs
Vehicle only	4	All animals were alive and normal
13.3	4	No animal died, but all animals underwent leg and ear paralyses, and smaller irish.
16.7	4	Two animals died. Two animals were alive with leg and ear paralyses, and smaller irish.
20	4	All animals died.

Conclusion :

The oral LD_{50} value of 10% MAC solution to the rabbits was found to be 15.63 g/kg bw which is classified as practically non-toxic (Lu, 1991).

B. Sub-acute toxicity test in the rats

B.1. The results on the pathology of organs.

Table 5. Histopathological profiles of organs after male Wistar rats received 10% MAC solution at various doses for 28 consecutive days, and after withdrawal for 7 days (for recovery study).

Histopathological profiles of organs					
28 days treatment			7 days recovery (no treatment)		
Stomach	Intestine	Liver	Stomach	Intestine	Liver
Vehicle (n = 5)			(n = 4)		
Necrosis and Inflamm : 1	Necrosis and Inflamm : 5	Congestion : 4	Necrosis and Inflamm : 0	Necrosis and Inflamm : 1	Congestion: 2 Necrosis and Inflamm : 1
Oral dose : 1.6 g/kg/day (n = 5)			(n = 5)		
No macroscopic changes of the organs, then histopathological test is unnecessary.			-		
Oral dose : 3.15 g/kg/day (n = 5)			(n = 5)		
Normal	Necrosis and Inflamm : 1	Congestion : 1 Necrosis and Inflamm : 1	Normal	Necrosis and Inflamm : 1	Congestion: 5
Oral dose : 6.3 g/kg/day (n = 5)			(n =5)		
Normal	Necrosis and Inflamm : 4	Congestion : 3	Normal	Necrosis and Inflamm : 3	Congestion: 3
Oral dose : 12.6 g/kg/day (n = 5)			(n = 1) ; Four animals died during treatment		
Necrosis and Inflamm : 1	Necrosis and Inflamm : 5	Congestion : 4 Hydropic de-generation: 1	Normal	Normal	Normal

Conclusion :

1. Spleen, heart, lungs, and kidney appear normal (not shown in Table 5).
2. Stomach is slightly affected by the doses of MAC, but recovered after 7 days.
3. The intestine and liver injuries appear dose-dependent, and the recovery varies between animals (dose-independent).

B.2. The results of urinalysis and blood chemistry

1. Urine status

The colour, pH and specific gravity of urine are normal following the oral administration of three doses (1.6, 3.15 and 6.3 g/kg) of MAC to the rats for 28 days as well as after cessation of MAC for 7 days compared with those of vehicle group. The colour is yellow with the pH value of 8.5 and specific gravity of 1.000.

2. Urinary protein

Protein concentrations found in urine range from 0 to 3+ in the vehicle and in all three dosing groups of MAC as well as after cessation of MAC for 7 days.

3. Urobilinogen and sediments in urine

Urobilinogen and sediments found in urine is also normal in the vehicle and in all three dosing groups of MAC as well as after cessation of MAC for 7 days.
Sediments usually found in all urine samples are tripple phosphate, uric acid and Ca oxalate.

4. Blood glucose

No	Oral Dose (g/kg)	Blood glucose (mg/dL); Mean ± SD		
		Day-0	Day-28	7 Days Recovery
1	Vehicle	65.7 ± 16.9	67.6 ± 17.0	104.9 ± 13.5
2	1.6	123.0 ± 24.1	89.9 ± 20.2	71.2 ± 6.1
3	3.15	48.1 ± 12.6	78.6 ± 21.9	75.9 ± 12.6
4	6.3	47.3 ± 18.5	117.9 ± 20.1	82.4 ± 9.3
n	-	10	10	5

5. Serum creatinine

No	Oral Dose (g/kg)	Serum creatinine (mg/dL); Mean ± SD		
		Day-0	Day-28	7 Days Recovery
1	Vehicle	0.21 ± 0.06	0.61 ± 0.29	0.63 ± 0.52
2	1.6	0.23 ± 0.08	0.45 ± 0.15	0.44 ± 0.05
3	3.15	0.31 ± 0.16	0.63 ± 0.21	0.24 ± 0.09
4	6.3	0.25 ± 0.08	0.62 ± 0.18	0.50 ± 0.14
n	-	10	10	5

6. Blood ureum

No	Oral Dose (g/kg)	Blood ureum (mg/dL); Mean ± SD		
		Day-0	Day-28	7 Days Recovery
1	Vehicle	26.32 ± 5.49	31.06 ± 5.79	28.15 ± 2.99
2	1.6	32.93 ± 4.61	30.45 ± 5.50	28.70 ± 2.98
3	3.15	31.65 ± 6.93	26.79 ± 2.35	35.56 ± 9.24
4	6.3	29.67 ± 3.90	37.03 ± 6.29	31.84 ± 2.14
n	-	10	10	5

7. Bilirubin in serum

No	Oral Dose (g/kg)	Serum bilirubin (mg/dL); Mean ± SD		
		Day-0	Day-28	7 Days Recovery
1	Vehicle	0.50 ± 0.18	0.37 ± 0.08	0.95 ± 0.29
2	1.6	0.36 ± 0.11	0.37 ± 0.14	0.38 ± 0.11
3	3.15	0.48 ± 0.19	0.34 ± 0.07	0.34 ± 0.09
4	6.3	0.64 ± 0.21	0.41 ± 0.18	0.64 ± 0.25
n	-	10	10	5

8. SGPT

No	Oral Dose (g/kg)	SGPT (mg/dL); Mean ± SD		
		Day-0	Day-28	7 Days Recovery
1	Vehicle	33.34 ± 10.59	77.62 ± 17.21	62.75 ± 16.11
2	1.6	66.85 ± 9.72	52.48 ± 10.71	43.78 ± 6.11
3	3.15	38.25 ± 16.02	78.68 ± 12.87	63.28 ± 8.21
4	6.3	33.83 ± 11.06	64.47 ± 15.49	63.34 ± 18.03
n	-	10	10	5

9. SGOT

No	Oral Dose (g/kg)	SGOT (mg/dL); Mean ± SD		
		Day-0	Day-28	7 Days Recovery
1	Vehicle	95.18 ± 13.89	146.89 ± 50.52	126.15 ± 24.13
2	1.6	94.87 ± 16.77	87.32 ± 11.04	172.40 ± 38.43
3	3.15	69.27 ± 27.38	143.00 ± 45.84	140.16 ± 22.44
4	6.3	93.88 ± 13.90	118.21 ± 24.01	144.50 ± 30.07
n	-	10	10	5

Conclusions :

1. Kidney function appears normal as seen from normality of serum creatinine and ureum, and from free-protein urine.
2. Liver function is normal as seen from serum bilirubin concentration.
3. SGPT and SGOT levels increase in MAC as well as in vehicle groups.

B.3. The results on haematological profiles

Dose (g/kg)	Blood Constituents	Units	Mean ± SD		
			D-0 (n=10)	D-28 (n=10)	7-D Recovery
Control (Vehicle)	WBC	10^3/uL	6.19 ± 2.44	8.89 ± 3.06	8.95 ± 2.34
	RBC	10^6/uL	7.83 ± 0.83	6.53 ± 1.44	7.18 ± 0.19
	HGB	g/dL	14.36 ± 1.61	13.64 ± 3.03	12.39 ± 3.51
	HCT	%	43.13 ± 4.92	37.77 ± 8.41	40.93 ± 2.41
	MCV	fL	55.08 ± 1.57	57.81 ± 2.07	57.03 ± 2.99
	MCH	pg	18.35 ± 0.72	20.96 ± 1.54	19.40 ± 0.65
	MCHC	g/dL	33.28 ± 0.54	36.22 ± 1.97	34.00 ± 0.83
	PLT	10^3/uL	843.1 ± 334.2	724.0 ± 180.1	1251.8 ± 242.5
1.6	WBC	10^3/uL	6.67 ± 2.18	10.14 ± 3.11	12.54 ± 3.79
	RBC	10^6/uL	7.95 ± 0.30	7.23 ± 0.59	7.41 ± 0.59
	HGB	g/dL	14.57 ± 0.72	14.11 ± 0.59	13.88 ± 0.86
	HCT	%	43.66 ± 2.89	41.90 ± 2.06	41.08 ± 2.64
	MCV	fL	55.26 ± 1.91	58.16 ± 2.80	55.50 ± 1.57
	MCH	pg	18.48 ± 1.12	19.61 ± 1.27	18.76 ± 0.78
	MCHC	g/dL	33.49 ± 2.50	33.71 ± 0.88	33.82 ± 0.86
	PLT	10^3/uL	1204.6 ± 203.8	915.7 ± 151.6	1268.6 ± 265.5
	WBC	10^3/uL	8.47 ± 3.83	7.34 ± 2.19	10.24 ± 3.32
	RBC	10^6/uL	8.78 ± 0.55	6.95 ± 0.99	6.96 ± 0.39
	HGB	g/dL	16.14 ± 1.06	14.25 ± 1.89	14.28 ± 0.99

3.15	HCT	%	48.68 ± 2.93	41.48 ± 5.37	40.74 ± 2.42
	MCV	fL	55.46 ± 1.18	59.84 ± 1.59	58.58 ± 1.76
	MCH	pg	18.41 ± 0.70	20.56 ± 0.76	20.52 ± 0.87
	MCHC	g/dL	33.15 ± 0.91	34.35 ± 1.22	35.02 ± 0.55
	PLT	10^3/uL	923.8 ± 142.1	920.1 ± 332.3	1029.8 ± 301.4
6,3	WBC	10^3/uL	7.29 ± 2.92	7.19 ± 3.09	10.16 ± 3.93
	RBC	10^6/uL	8.00 ± 0.93	7.19 ± 1.10	6.92 ± 0.19
	HGB	g/dL	14.97 ± 1.87	14.08 ± 2.15	13.56 ± 1.08
	HCT	%	44.68 ± 5.74	42.61 ± 6.33	40.06 ± 1.88
	MCV	fL	55.79 ± 1.37	59.68 ± 1.82	57.9 ± 2.84
	MCH	pg	18.71 ± 0.52	19.71 ± 0.73	19.60 ± 1.67
	MCHC	g/dL	33.54 ± 0.68	33.07 ± 0.98	33.68 ± 2.55
	PLT	10^3/uL	797.4 ± 309.2	980.8 ± 282.6	1164.4 ± 180.2

WBC = white blood cells, RBC = red blood cells, HGB = hemoglobin, HCT = hematocrite, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, PLT = blood platelets counts.

Conclusion :

All the blood constituents (white blood cells, red blood cells, hemoglobin, hematocrite, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, blood platelets counts) are normal (Mitruka et al.,1977), except that the white blood cells counts tend to increase to some extent after leaving the animals for 7 days without MAC solution.

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