

Physical Signs of Illness, Liver Functions and Reproductive Parameters of Female Rats Supplemented with *Melaleuca cajuputi* Methanolic Extract

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ABSTRACT

Melaleuca cajuputi is the main source of cajuput essential oil, which is widely used in the folk medicine in southeastern Asia. The study aimed to determine the effect of *M. cajuputi* methanolic extract (MCME) on liver functions and fertility of female Sprague Dawley rats. Twenty four female rats were divided into four groups and supplemented with 2 ml/kg bwt distilled water or several doses of MCME (50, 100, and 200 mg/kg bwt) for 30 days. Physical signs of illness were observed on a daily basis. Blood was collected at the end of the experimental period for liver functions and estrogen assay. All females also subjected to fertility test. No signs of illness and mortality were observed. Results also demonstrated that liver functions, estrogen level and fertility in female Sprague Dawley rats were not affected by MCME. These findings suggested that MCME at doses in between 50-200 mg/kg bwt were not toxic to female Sprague Dawley rats.

INTRODUCTION

The *Melaleuca* genus is an aromatic and medicinal plant genus, best known for the production of essential oils (Barbosa *et al.*, 2013). This genus grows as a shrub or tree with single flexible trunk ranging from a small tree to tree with 25 meters in height. *Melaleuca cajuputi* is the most widespread *Melaleuca* species, extending from northern Australia, Papua New Guinea, Indonesia, Malaysia, Thailand, Cambodia, and Vietnam (Craven, 1999). This species is well adapted to seasonally flooded soils, even salt-water flooding, and infertile soils. *M. cajuputi* contains eucalyptol, which is a monoterpene with medicinal purposes (Boland *et al.*, 1991). Traditionally, *M. cajuputi* is used as a medicine to treat cholera, diarrhea, muscular pain, scabies and intestinal worms (Al-Abd *et al.*, 2016). Pre-clinical data demonstrated that *M. cajuputi* possess anti-inflammatory, anti-bacterial, anti-microbial, anti-dengue, anti-oxidant, anti-cancer and anticonvulsant activities (Daud

et al., 2015). Widiana *et al.* (2014) reported that solid residue of *M. cajuputi* leaves rich with volatile fatty acids, dry matter digestibility, and organic matter digestibility, thus, has a potential to be used as a livestock feed.

However, very few studies have addressed the toxicity effect of *M. cajuputi* on reproductive as well as the non-reproductive system. Roszaini *et al.* (2013) documented that *M. cajuputi* essential oil less toxic against subterranean termite, *Coptotermes curvignathus*. Acute toxicity studies demonstrated that the lethal dose of cajuput oil derived from *M. cajuputi* was 5 g/kg in mice and 3870 mg/kg in rats (Russell, 1999). Previous authors reported that methanolic extract of *M. cajuputi* leaves at 200 mg/kg was not toxic to the male reproductive system of juvenile Sprague Dawley rats (Daud *et al.*, 2015). As far as our literature survey could ascertain, no attempts have been made to investigate the effect of this plant on the female reproductive system. Reproductive toxicology has recently become a rapidly extending area of research and testing. Since herbal and plant-based medicine have recently become a widespread form of therapy, reproductive toxicology should be considered as part of

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the safety evaluation process. Hence, the present study has been directed towards investigating the action of the *M. cajuputi* leaves methanolic extract on the female reproductive system in rats. In addition, the effect of this crude extract on the non-reproductive system (particularly on the liver functions) also has been investigated.

MATERIALS AND METHODS

Plant materials collection and methanolic extraction

Melaleuca cajuputi fresh leaves were collected in May-July 2012 from their natural habitats in Selangor, Malaysia. The plant was botanically identified by plant specialist from Herbarium, Universiti Kebangsaan Malaysia with voucher specimen number NLRA 3/A/2012. The leaves were washed thoroughly, air-dried and subjected to size reduction using an electrical blender (Pensonic PB-3203L, Malaysia). Then, the leaves were soaked in 80% methanol (HmbG Chemicals, Germany) for three days. The filtrate was collected and evaporated under reduced pressure using rotary evaporator (Buchi Rotavapor R-210, Switzerland). The *M. cajuputi* methanolic extract (MCME) obtained was stored in a container and was freshly reconstituted with distilled water immediately before administration to the female rats.

Experimental animals and experimental design

The handling of the laboratory animals was performed according to the national and international regulations and the prior ethical approval was taken before starting the experiment (AREC 3/669/07). All animals were maintained in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) under uniform lighting and were given commercial rat chow pellets (Gold Coin Feedmills Sdn Bhd, Malaysia) and water *ad-libitum*. Twenty-four female Sprague Dawley rats were divided into four groups with six animals each. Group 1 ($n = 6$) was served as a control and orally supplemented with 2 ml/kg bwt of distilled water. Group 2-4 ($n = 6$ for each group) were supplemented with 50, 100, and 200 mg/kg bwt of *M. cajuputi* leaves methanolic extract (MCME), respectively. All the animals were supplemented with respective doses of MCME for 30 days. Physical signs of illness and toxicity (piloerection, lethargy, hair loss, dark urine, diarrhoea, paleness of skin, locomotor activity and mortality) were monitored, twice daily at 7 am and 5 pm as previously described (Basir *et al.*, 2012). After 30 days of the experimental period, all animals were subjected to several tests including liver functions test, estrogen assay, and fertility test.

Table 1: Physical signs of illness among female Sprague Dawley rats supplemented with distilled water (dH_2O) or different doses of *M. cajuputi* methanolic extracts (MCME).

Physical signs	Control (2 ml/kg dH_2O)	MCME (50 mg/kg bwt)	MCME (100 mg/kg bwt)	MCME (200 mg/kg bwt)
Piloerection	-	-	-	-
Lethargy	-	-	-	-
Hair loss	-	-	-	-
Dark urine	-	-	-	-
Diarrhoea	-	-	-	-
Paleness of skin	-	-	-	-
Locomotors	Active	Active	Active	Active
Mortality	-	-	-	-

Note: + (presence of the sign of illness), - (absence of the sign of illness).

Blood collection, liver functions, and estrogen assay

The blood was collected in the morning, via retro-orbital bleeding into K_2EDTA tube. Blood serum was harvested and subjected to liver functions test (ALT, AST, ALP, and TP) and estrogen assay. Analysis of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was performed by Reitman-Frankel colorimetric method (Reitman and Frankel, 1957). Serum alkaline phosphatase (ALP) was estimated as previously described by Kind-King method (Kind and King, 1954). Total protein (TP) was determined by biuret method (Doumas, 1975). The estrogen level was measured by quantitative enzyme immunoassay using Enzyme-linked Immunosorbent Assay Kit for Estrogen (Cloud-Clone Corp, USA) according to manufacturer's manual.

Fertility test

Fertility test was conducted as previously described (Al-Hamood *et al.*, 1998). Female rats were mated with proven fertile males and the presence of copulation plug or sperm in the

vaginal smear in the following morning was regarded as a day 1 of pregnancy. All females were sacrificed on the day 20 of pregnancy under diethyl ether anesthesia. During the autopsy, the number of a foetus along the uterine horns were recorded.

Statistical analysis

The results were expressed as mean \pm SEM. All data were analyzed by analysis of variance (ANOVA) and p values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Physical signs of illness and liver functions test

Daily supplementation of *M. cajuputi* methanolic extract (MCME) for 30 days was well tolerated and no physical signs of illness were observed in female rats (Table 1). The findings also demonstrated that alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total

protein (TP) did not differ significantly ($p > 0.05$) among female Sprague Dawley rats supplemented with distilled water (control) or with different doses of MCME (Table 2). No significant variations in behaviors, health conditions, zero mortality and liver functions parameters (ALT, AST, ALP, and TP) were basic evidence

indicating the non-toxic nature of MCME. Liver functions can be detrimentally altered by toxicants. The liver is the first organ to encounter drugs and environmental toxicants that enter the hepatic portal vein from the digestive system (Shyamal *et al.*, 2010).

Table 2: The effects of different doses of *M. cajuputi* methanolic extracts (MCME) on liver functions of female Sprague Dawley rats. Values are expressed as mean \pm SEM (n = 6).

Parameters	Control (2 ml/kg dH ₂ O)	MCME (50 mg/kg bwt)	MCME (100 mg/kg bwt)	MCME (200 mg/kg bwt)
ALT (U/l)	49.48 \pm 3.18	53.79 \pm 9.21	51.13 \pm 6.83	52.43 \pm 2.95
AST (U/l)	73.35 \pm 2.64	76.35 \pm 4.29	72.99 \pm 1.09	75.61 \pm 3.27
ALP (U/l)	153.60 \pm 7.81	146.83 \pm 8.42	149.11 \pm 5.39	150.98 \pm 4.15
TP (g/l)	75.37 \pm 4.37	72.73 \pm 6.11	70.15 \pm 2.95	74.44 \pm 2.07

ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, TP: total protein. Note: No differences occurred ($p > 0.05$) in between treatments.

Table 3: The effects of different doses of *M. cajuputi* methanolic extracts (MCME) on estrogen level and fertility of female Sprague Dawley rats. Values are expressed as mean \pm SEM (n = 6).

Parameters	Control (2 ml/kg dH ₂ O)	MCME (50 mg/kg bwt)	MCME (100 mg/kg bwt)	MCME (200 mg/kg bwt)
Estrogen (ng/ml)	8.37 \pm 0.15	8.23 \pm 1.09	7.98 \pm 0.47	8.13 \pm 0.29
Pregnancy (%)	100	100	100	100
Foetus (n)	8.6 \pm 1.7	8.3 \pm 1.8	8.8 \pm 1.2	8.5 \pm 1.4

Note: No differences occurred ($p > 0.05$) in between treatments.

Previously, Hammer and co-authors (2006) indicated that *Melaleuca* species has the potential to be developmentally toxic if ingested at higher doses, especially in the form of essential oil with a high concentration of terpinen-4-ol. Moderate increases in ALT and AST were reported in cats exposed dermally to concentrated *Melaleuca* species essential oil (Bischoff and Guale, 1998). Our results contradict others, where no signs of toxicity were detected. Perhaps, this can be explained by the different doses and extracts used. In the current study, crude methanolic extract of *M. cajuputi* was tested on female Sprague Dawley rats at a relatively low dose (50-200 mg/kg). Meanwhile, other researchers focused their investigations on the toxicity effects of concentrated essential oil harvested from several *Melaleuca* species, namely *M. cajuputi* and *M. alternifolia*.

Estrogen level and fertility test

Estrogen assay revealed that circulating estrogen in female rats was not significantly ($p > 0.05$) affected by MCME supplementation (Table 3). The percentage of pregnant females and the number of the foetus also did not differ significantly ($p > 0.05$) in between female Sprague Dawley rats received distilled water (control) compared to female Sprague Dawley rats supplemented with different doses of MCME (Table 3).

The pituitary-gonadal axis is important for the maintenance of the reproductive system, any distortion to this axis can be deleterious (Adewale *et al.*, 2014). Female reproductive hormones are crucial for ovulation, preparing the uterus for embryo implantation and also milk production. To date, there is no report on the reproductive toxicity of this plant, particularly in the female. Our findings suggest that MCME at doses in between

50-200 mg/kg bwt were not harmful to the reproductive system of female Sprague Dawley rats. However, according to the previously published work, eucalyptol is a major component of *M. cajuputi* leaves (Barbosa *et al.*, 2013; Boland *et al.*, 1991; Russell, 1999) and eucalyptol has been demonstrated to possess insecticidal activities (Wu *et al.*, 2015). Exposure to high concentration of insecticide during pregnancy proves to have a negative impact on embryo development. Therefore, it is rational to assume MCME at very high doses may involve in abortifacient and anti-fertility activities. Further MCME mechanism of action in the female reproductive system should be explored in the future experiments.

CONCLUSION

From the evidence presented in this study, 50-200 mg/kg bwt of *M. cajuputi* methanolic extract (MCME) has apparently demonstrated non-toxic effects on the non-reproductive system (particularly on liver functions) and on the reproductive system in female Sprague Dawley rats. However, the toxicology studies on MCME are still lacking at this stage. Thus, further investigation on acute and sub-chronic toxicity of MCME are required.

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CONFLICT OF INTEREST AND AUTHOR'S CONTRIBUTION

The authors declare that there is no conflict of interests regarding the publication of this paper. All authors were involved

in the writing, revision and final approval of the paper.

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