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The *in vivo* rodent micronucleus assay of Kacip Fatimah (*Labisia pumila*) extract

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Abstract. *Kacip Fatimah* also known as *Labisia pumila* (Myrsinaceae), is a traditional herbal medicine with a long history in the Malay community. It has been used by many generations of Malay women to induce and facilitate childbirth as well as a post-partum medicine. We tested the genotoxic potential of *Kacip Fatimah* in bone marrow cells obtained from Sprague-Dawley rats using micronuclei formation as the toxicological endpoints. Five groups of five male rats each were administered orally for two consecutive days with doses of 100, 700 and 2000 mg/kg body weight of *Kacip Fatimah* extract dissolved in distilled water. Micronucleus preparation was obtained from bone marrow cells of the animals following standard protocols. No statistically significant increase in micronucleated polychromatic erythrocytes (MNPCEs) was observed at any dose level and sacrifice/harvest time point (24, 48 and 72h). However, a significant decrease in polychromatic erythrocytes/normochromatic erythrocytes (PCE:NCE) ratio was observed from the highest dose level (2000 mg/kg of body weight) at 48h harvest time point. In this study, we investigated the effect of *Kacip Fatimah* on mammalian bone marrow cells using micronuclei formation to assess the genotoxicity of the herb.

INTRODUCTION

A large proportion of medicines used by the locals in Malaysia are still derived from plants or their extracts and little is known about the safety and efficacy of such alternative treatments. *In vivo* and *in vitro* studies have shown that some natural constituents of plant parts (fruits, leaves, roots) play a modulating role in xenobiotic effects (Roncada *et al.*, 2004). While some herbs might be pharmacologically and clinically effective, they are not necessarily free of toxicity and side effects. Therefore, investigation into the traditionally used medicinal plants is valuable as a source for potential chemotherapeutic drugs and as a safety measure for the continued use of medicinal plants (Verschaeve *et al.*, 2004).

It was proposed by Schmid and Heddle that a simpler approach to assess chromosome damages *in vivo* was to measure the micronucleus (Fenech, 2000). Micronuclei (MN) are small chromatin bodies that appear in the cytoplasm by the condensation of acentric chromosome fragments or by whole chromosomes (Martinez *et al.*, 2005), often induced by clastogenic substances or spindle-poison in dividing cells such as bone marrow (Fenech, 2000). Micronuclei frequencies have been considered to be a reliable index for detecting chromosome breakages and loss (Lajmanovich *et al.*, 2005). For genetic toxicology studies, the formation of micronucleus in bone marrow and peripheral blood erythrocytes is recognised as one of the established methods for *in vivo* cytogenetic assays (Fenech, 2000). Bone marrow cytogenetics is also a useful short-term technique, for elucidating the mechanisms involved for the clastogenic

and anticlastogenic activity of substances (Shukla & Taneja, 2002). It has proven to be a sensitive screening procedure for the detection of freshly induced structural chromosomal damages in bone marrow cells (Muller *et al.*, 1995).

Kacip Fatimah (*Labisia pumila* (B1.) F. -Vi11) from the family of Myrinaceae, is a sub-herbaceous plant with creeping stems and found mainly in the lowland and hill forests of peninsular Malaysia at an altitude between 300 and 700 m. In the Malay tradition, a water decoction from the roots or whole plant of *L. pumila* is often given to pregnant woman between one to two months before delivery, as this is believed to induce and expedite labour. It was also reported that *Kacip Fatimah* can be used for post-partum medication to assist contraction of the birth channel, to delay fertility and to regain body strength; while some other folkloric uses include treatment of flatulence, dysentery, dysmenorrhoea, gonorrhoea and "sickness in the bones" (Jamia *et al.*, 2003).

However, the roots from both ethanol and water extracts of KF (*Labisia pumila*) were found to be cytotoxic and *in vivo* toxicology studies were recommended to asses the possibility of toxicity for human consumption (Jamia *et al.*, 2003). Thus, the aim of this preliminary study was to evaluate the incidence of micronuclei/chromosome damages induced by KF (*L. pumila*) when administered orally to rats at different doses and harvest periods.

MATERIALS AND METHODS

Plant material

Plant material used in this study was *Kacip Fatimah (L. pumila)* extract prepared by the Chemical Engineering Pilot Plant (CEPP) from Universiti Teknologi Malaysia (UTM), Skudai, Johor, Malaysia. The plant extract was mixed with distilled water at concentrations of 100, 700 and 2000 mg/kg of the rat body weight.

Chemicals and reagents

Mitomycin C (MMC CAS NO. 50-07-7, Batch No. 59524) was purchased from Calbiochem, EMP., Bioscience Inc. CA. Fetal calf serum (FCS) was obtained from GmbH. Giemsa and May-Grunwald stains were obtained from Gurr, BDH. Methanol was purchased from Fidin. All chemicals used in this study were of analytical grade purity and all test solutions were freshly prepared before each experiment.

Animal maintenance

Young adult male, Sprague-Dawley rats weighing between 200-250 g (7 to 8 weeks old) were used in the present study. The animals were obtained from the Laboratory Animal Resource Unit (LARU), Institute for Medical Research (IMR), Malaysia. The use of the laboratory animals was approved by the Animal Care and Use Committee (ACUC), Ministry of Health (MOH), Malaysia, ACUC No: ACUC/KKM/01/2005. The research was conducted in accordance with the Principles and Guide to Ethical Use of Laboratory Animals, MOH. The rats were housed in polypropylene cages (two to three rats per cage) with steel wire tops and sawdust bedding. They were given commercial food pellets and water was made available at all times. They were maintained in a controlled atmosphere of 12 h dark/light cycle, 25 ± 2 °C temperature with 50-70% humidity.

Experimental design

The animals were divided into five groups each consisting five rats respectively. The first group was given distilled water and served as negative/vehicle control. The second, third and fourth test group received different concentrations of *Kacip Fatimah* (KF) extract at 100, 700 and 2000mg/kg of body weight respectively and the fifth group received Mitomycin C (0.75 mg/kg of body weight) to determine the reproducibility and applicability of the test which served as positive control. The KF extracts for the test and distilled water for the negative control groups were administered orally where as, Mitomycin C was administered intraperitoneally to the positive control rats (Shukla & Taneja, 2002; Singh *et al.*, 2003). We have selected intraperitoneal injection as

the rank of administration since Mitomycin C will maximise the chemical exposure to the bone marrow (Patlolla & Tchounwou, 2005). The KF extracts were administered daily for two consecutive days and subsequently the animals were sacrificed by cervical dislocation at 24, 48 and 72 h after the second medication. The dosing schemes are summarised in Table 1.

Bone marrow micronucleus assay

Animals were sacrificed at 24, 48 and 72 hours after the second administration of the KF extract. Immediately after the animals were sacrificed, both femurs of the rat were removed through the pelvic bone and the femur bone was freed from the extra muscles. The epiphyses were cut and the bone marrow was flushed out by gentle flushing and aspiration with fetal calf serum (Valette *et al.*, 2002). The cell suspension was centrifuged at 1000 rpm for 10 min and the supernatant was discarded. A small drop of the resuspended cell pellet was spread on to clean glass slides and air-dried. The bone marrow smears were made in five replicates and fixed in absolute methanol for 10 minutes and stained with May-Grunwald/ Giemsa at pH 6.8 (D'Souza *et al.*, 2002). According to Valette *et al.* (2002) the staining method will distinguish between the polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs). This study was conducted in compliance with OECD guideline 474 (Genetic Toxicity: Mammalian Erythrocyte Micronucleus Test, adopted 21 July 1997).

Scoring

Micronuclei and the PCE to NCE cell ratio (PCE:NCE) were scored from the smeared bone marrow slides. The micronucleus frequencies (expressed as percent micronucleated cells) were determined by analysing the number of micronucleated PCEs (MNPCEs) from at least 2000 PCEs per animal. The PCE:NCE ratio was calculated to evaluate the cytotoxic effect of KF by scoring the number of PCEs and NCEs in at least 1000 erythrocytes per animal (Ouanes *et al.*, 2003).

Statistical analysis

A compound is considered mutagenic in this test system if at any of the preparation intervals, a statistically significant increase in the number of micronucleated PCEs is found in comparison to the negative controls. Data were analyzed using ANOVA to observe any significant differences amongst the dosage sets and harvest periods to asses the genotoxicity effects at $p<0.05$ level of significance. All the statistical analysis were performed using SPSS 11.5 statistical programme.

RESULTS

The purpose for administration of *Kacip Fatimah* during proliferation of haematopoietic cells was to observe whether the test compound can cause chromosome damages or inhibition of mitotic apparatus. The chromosome fragments or whole

Table 1. Dosing scheme for the micronucleus assay with *Kacip Fatimah*, distilled water (vehicle/negative control) and Mitomycin C (positive control)

Groups	Treatment	No. of rat	Period of harvest (h)
I	Distilled water	15	24
II	KF (100mg/kg BW)	15	24, 48, 72*
III	KF (700mg/kg BW)	15	24, 48, 72*
IV	KF (2000mg/kg BW)	15	24, 48, 72*
V	MMC (0.75mg/kg BW)	15	24

KF: Kacip Fatimah; BW: body weight; MMC: Mitomycin C
* Period of harvest after second dose of treatment

chromosomes may lag behind during cell division and form micronuclei as seen in positive control Mitomycin-treated rats. The results of the *in vivo* micronucleus tests are presented in Table 2. The micronuclei induced after administration of KF at three different harvest times (24, 48 and 72 h) in PCE and PCE:NCE cells ratio were statistically analyzed. The values were comparable with the negative/vehicle control values. A compound is considered to be mutagenic in this test system if at any of the preparation dose (100, 700 and 2000 mg/kg of body weight) and harvest periods (24, 48 and 72 h), has increase significantly in the number of MNPCEs when compared to the negative controls (distilled water).

In the present study, there was no clear dose-effect relationship in the frequency of MNPCEs. No significant increase in the mean frequencies of MNPCEs was observed when KF was administered at different concentrations (100, 700 and 2000 mg/kg of body weight) and at different periods of harvest (24, 48 and 72h) as compared to the vehicle control. Nevertheless, the mean values of PCE:NCE ratio decrease significantly ($p<0.05$) when treated with 2000 mg/kg dose level of KF extract at 48 h (0.45±0.02). A significant increase ($p<0.01$) in the frequency of micronuclei in polychromatic erythrocytes (MNPCEs) was associated with a significant decrease ($p < 0.05$) of the PCE:NCE ratio in the group treated with Mitomycin C (positive control) as compared to the vehicle control, with mean and standard error of 1.22 ± 0.02% and 0.41 ± 0.02, respectively.

DISCUSSION

The evaluation of micronucleus frequencies *in vivo* is one of the primary genotoxicity tests recommended internationally by regulatory agencies for product safety assessment (Krishna & Hayashi, 2000). An increase in the frequency (percentage) of micronucleated polychromatic erythrocytes (MNPCEs) in treated rats is an indication of

Table 2. Bone marrow micronucleus assay in *Kacip Fatimah*-treated rats

Treatment	Group	Dose (mg/kg)	Harvest time (h)	% MNPCEs of 2000 PCEs (mean ± S.D)	PCE:NCE (mean ± S.D)
Distilled water (negative)	I	0	24	0.04 ± 0.04	0.55 ± 0.01
Kacip Fatimah	II	100	24	0.02 ± 0.02	0.58 ± 0.02
			48	0.08 ± 0.05	0.56 ± 0.02
			72	0.04 ± 0.02	0.61 ± 0.02
	III	700	24	0.04 ± 0.03	0.56 ± 0.02
			48	0.03 ± 0.01	0.55 ± 0.14
			72	0.06 ± 0.01	0.57 ± 0.02
	IV	2000	24	0.07 ± 0.02	0.59 ± 0.02
			48	0.06 ± 0.02	0.45 ± 0.02*
			72	0.04 ± 0.02	0.55 ± 0.02
MMC (positive)	V	0.75	24	1.22 ± 0.02**	0.41 ± 0.02*

Each treatment and control group consisted of five rats.
PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes; MMC: Mitomycin C.
*$p < 0.05$ significantly than the vehicle/negative control
**$p < 0.01$ significantly than the vehicle/negative control

induced chromosome damages (EPA, 1996; Krishna & Hayashi, 2000; Santos-Mello *et al.*, 2001).

Genotoxicity activity is indicated by statistically significant dose-related incidence of MNPCEs in the treatment group (KF). Inhibition of cell proliferation in the bone marrow illustrates the cytotoxicity of KF. Bone marrow cell toxicity (or depression) is normally indicated by a dose-related decrease in the proportion of immature erythrocytes (Krishna & Hayashi, 2000). Young adult rats were used in this study because of their high proliferative activity and low fat content which favour good quality preparations (EHC, 2004). With regard to the frequency of MNPCEs obtained (no significant increase or decrease), there was no indication that KF induced clastogenic effects and therefore KF was evaluated as negative at any level of dose administered and period of harvest. However, a significant decrease in the PCE:NCE ratio was noted only at the highest dose level of KF (2000 mg/kg) at 48 h. This reduction could be due to either direct cytotoxicity or micronuclei formation and heavy DNA damages leading to cell death or apoptosis (Ouanes *et al.*, 2003). Nevertheless, the mean number of MNPCEs frequencies in the positive control receiving Mitomycin C showed significant increase value at 24 h administration. These findings demonstrate the validity of the experiment and the sensitivity of the animal strains to a clastogenic agent as observed by Krishna & Hayashi (2000) and Valette *et al.* (2002).

In this study, there was no indication of dose-effect relationship. The lack in dose-related effect could be attributed by the cell depression in the dividing bone marrow cells, which will interfere with the expression of the genotoxic potential (Nesslany *et al.*, 2004). This could also be due to pharmacokinetic/pharmacodynamic factors that need further investigation in *Kacip Fatimah.* Majer *et al.* (2001) reported the limitation of the bone marrow micronucleus test which was governed by the short lifespan of the DNA-reactive metabolites found in the organ, remote from the target cells, and had failed to induce genetic damage.

The exact mechanism of action of KF is still unclear. No attempt has been made to identify the chemicals present in KF extract to explain the mechanisms responsible for dose-related effects. This raises the issue with regard to the safety for long term usage of KF. It is also important to know whether micronuclei induced by this extract are the results of chromosome breakages or chromosome loss because the latter may be interesting since many anticancer drugs are mitotic spindle disruptors (Krishna & Hayashi, 2000).

KF extracts should be investigated further for active compound(s), mutagenic and/or antimutagenic properties, if there is any, using cell lines. In conclusion, *Kacip Fatimah* extracts do not have any genotoxicity effect or mutagenic potency as demonstrated from the micronucleus assays.

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