Original Research

Mutagenicity evaluation of *Anastatica hierochuntica* L. aqueous extract *in vitro* and *in vivo*

Siti Rosmani Md Zin¹, Zahurin Mohamed², Mohammed A Alshawsh², Won F Wong³ and Normadiah M Kassim¹

¹Department of Anatomy, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia; ²Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia; ³Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia

Corresponding author: Mohammed A Alshawsh. Email: alshaweshmam@um.edu.my

Impact statement

In this paper, we report on the mutagenicity evaluation of *Anastatica hierochuntica* aqueous extract. This is a significant research in view of the popularity of this herb consumption by the people across the globe despite of limited scientific evidence on its toxicity potential. This study is intended to encourage more extensive related research in order to provide sufficient evidence and guidance for determining its safe dosage.

Abstract

Anastatica hierochuntica L. (A. hierochuntica), a folk medicinal plant, was evaluated for mutagenic potential via in vitro and in vivo assays. The in vitro assay was conducted according to modified Ames test, while the in vivo study was performed according to Organisation for Economic Co-operation and Development guideline for mammalian erythrocyte micronucleus assay. Four groups (n= 5 males and 5 females per group) Sprague Dawley rats were randomly chosen as the negative control, positive control (received a single intramuscular injection of cyclophosphamide 50 mg/kg), 1000 and, 2000 mg/kg A. hierochuntica aqueous extracts. All groups except the positive control were treated orally

for three days. Findings of the *in vitro* assay showed mutagenic potential of AHAE at 0.04 and 0.2 mg/ml. However, no mutagenic effect was demonstrated in the *in vivo* study up to 2000 mg/kg. No significant reduction in the polychromatic and normochromatic erythrocytes ratio was noted in any of the groups. Meanwhile, high micronucleated polychromatic erythrocytes frequency was seen in cyclophosphamide-treated group only. These findings could perhaps be due to insufficient dosage of *A. hierochuntica* aqueous extracts to cause genetic damage on the bone marrow target cells. Further acute and chronic *in vivo* toxicity studies may be required to draw pertinent conclusion on the safety aspect of *A. hierochuntica* aqueous extracts consumption.

Keywords: *Anastatica hierochuntica*, mutagenicity, genotoxicity, Ames test, mammalian erythrocyte micronucleus assay, reverse mutation assay

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Introduction

Herbal products are gaining increasing popularity among consumers in developing and industrialized countries. This is shown by the rapid growth of the total global herbal market. According to the 2015 report by Global Industry Analyst (GIA) on herbal supplements and remedies, Europe represents the largest market worldwide. Meanwhile, Asia-Pacific has emerged as the fastest growing market with a compound annual growth rate (CAGR) of 9% over the analysis period. China is one of the main suppliers of herbal supplements to the United States and West European markets. The global herbal supplements

and remedies market are projected to be worth US\$115 billion by 2020.²

The increasing demand for herbal products is believed to be due to many factors. These include cultural and historical influences³ and the inherent perception that such products are safer and more efficient compared with modern medicine.^{4,5} Despite the claims of medical benefits of natural products, there have been many reports on various toxicity effects following prolonged consumption of herbal medicines.⁶ In addition, several studies have shown a relationship between herbal products consumption and the development of kidney problems.⁷

The World Health Organization (WHO) has expressed great concern regarding the safety of herbal products. This is shown by the efforts made by the WHO in developing the guidelines on the collection, preparation, and manufacture of herbal products. The WHO also supports a global regulatory network of the International Regulatory Cooperation on Herbal Medicines (IRCH) aimed at coordinating regulations on herbal medicines.3 The safety of herbal medicines is a major concern both to national health authorities and to the public.8 Therefore, in-depth studies on the safety and potential toxicity effects of herbal products would be helpful in this regard.

In this study, we investigated the mutagenicity of A. hierochuntica (Brassicaceae)9 - one of the commonly consumed herbs by people across the globe. 10-13 This medicinal plant is known by many names including Kaff Maryam, Rose of Jericho, Hand of Maria, Hand of Fatima, Sanggul Fatimah, Kembang Fatimah, and Akar Kayu Bunga Fatimah. 10,11,13-15 The literature regarding mutagenicity studies of A. hierochuntica is limited. Hence, further exploration of its mutagenicity potential is crucial. The plant used in this study was imported from the country of origin, Saudi Arabia. Other countries that grow A. hierochuntica include Egypt, Jordan, Oman, Libya, Iraq, the United Arab Emirates, Iran, Israel, Kuwait, and North Africa. 10,11 When dried, the plant is a tight, woody ball that will expand and straighten when soaked in water (Figure 1). Its water decoction is consumed to obtain the desired effects or claimed medical benefits. It is believed to treat reproductive system-related disorders such as menstrual cramping and uterine hemorrhage. Some women also use it to ease childbirth. 11,12,16,17 Other diseases purportedly treated using A. hierochuntica include diabetes mellitus, epilepsy, gastric disorders, arthritis, malaria,



Figure 1. Anastatica hierochuntica L. (A color version of this figure is available in the online journal.)

depression, mouth ulcers, and bronchial asthma. 11,12,17-19 Scientific studies have demonstrated some of the biological activities of A. hierochuntica including antioxidant, antimicrobial, antifungal, anti-melanogenic, anti-inflammatory, hepatoprotective, gastroprotective, hypoglycemic, and hypolipidemic activities.¹³

Unfortunately, the herb is not sold according to properly approved standard guidelines regarding its safe dosage. Consequently, the amount ingested is based on advice from suppliers and other consumers. As far as we are aware of, there were only two studies on the toxicity effects of A. hierochuntica. 17,20 Furthermore, genetic toxicology studies (mutagenicity and genotoxicity) of the plant have never been done. Therefore, we conducted in vitro and in vivo assays called bacterial reverse mutation test and mammalian erythrocyte micronucleus test, respectively, to evaluate the potential mutagenic effects of A. hierochuntica aqueous extract (AHAE). Mutagenicity is a component of genotoxicity, which results in events that alter the DNA and/or chromosomal structure, that are passed to subsequent generations.²¹ The bacterial reverse mutation test uses Salmonella typhi (S. typhi) and Escherichia coli (E. coli) strains which contain identified mutations in amino acid biosynthesis gene that prevent their growth. Exposure to potential mutagen (test substance) may induce a second mutation (a reversion) that will restore the functional capability of the bacteria to synthesize the essential amino acid.²¹ Meanwhile, the *in vivo* mammalian erythrocyte micronucleus test was performed to detect presence of micronuclei in polychromatic erythrocytes (PCEs). Micronuclei formation is induced by mutagenic substance and may originate from acentric chromosomes, lagging chromosome fragments or whole chromosomes.²¹ The in vivo assay is required to develop weight of evidence in assessing results of in vitro assay.

We also assessed the toxicity effects of the AHAE on body weight (BW) gain, relative organ weight, some biochemical parameters and histological features of the kidneys and livers via the in vivo study.

Materials and methods

Preparation of A. hierochuntica aqueous extract

A. hierochuntica was obtained from Mahnaz Food, Shah Alam, Malaysia, which imported the dried plant from Saudi Arabia. The voucher sample is deposited in the herbarium of University of Malaya (KLU) with voucher number KLU49457 as determined by a botanist. The plant was ground to powder using a high-voltage grinder and mixed with sterile distilled water (ddH₂O) (1:10 w/v) in a glass Schott bottle. The mixture was subsequently left in a shaking water bath at 60°C for 4 h. The plant infusion was filtered twice using Whatman No. 1 filter paper, and was freeze dried into powder form. The percentage of the aqueous yield extract is 4.6%.

For the *in vitro* study, the AHAE powder was mixed thoroughly with a desired volume of ddH₂O to produce a series of concentrations, 5.0, 1.0, 0.2, and 0.04 mg/ml. For the in vivo assay, the AHAE powder was mixed with ddH₂O and doses of 1000 and 2000 mg/kg were used for treatment. The concentrations for *in vitro* and doses for *in vivo* assays were determined by the results of preliminary studies.

In vitro Ames test

Bacteria strains and mutagenicity assay

The *in vitro* Ames test was conducted according to Organization for Economic Co-operation and Development (OECD) guidelines no. 471.²² The study proposal was approved by the Institutional Biosafety and Biosecurity Committee (IBBC), University of Malaya. It involved four different strains of *S. typhi*, TA 100, TA 98, TA 97a and TA, 1535 and one strain of *E. coli* called WP2. All bacterial strains were purchased from Environmental Biodetection Product Inc. (EBPI), Canada. One day prior to the day of assay, one vial of growth media was thoroughly mixed with one vial of bacterial strain followed by incubation in 37°C incubator for 16 to 18 h.

Chemicals and reagents

The in vitro Ames test or so-called bacterial reverse mutation assay was performed using Muta-Chromo plate assay, purchased from Environmental Bio-Detection Products (EBPI, Canada) according to the manufacturer's protocol. The plate assay consists of five strains of bacteria, nutrient broth (growth media), S-9 components, and standard mutagens. Other reagents are components of 'reaction mixture' that consist of Davis Mingioli salts (A), D-glucose (B), bromocresol purple (C), D-biotin (D), and L-histidine (for S. typhi) or L-tryptophan (for E. coli) (E). They were mixed thoroughly with proper volume [A (43.24 ml) + B (9.50 ml) + C $(4.76 \, \text{ml}) + D \, (2.38 \, \text{ml}) + E \, (0.12 \, \text{ml})$] according to the supplier guideline in order to produce 60 ml of reaction mixture. The S-9 components are magnesium chloride (MgCl₂)+ potassium chloride (KCl), glucose-6-phosphate (S9B), nicotine amide di-nucleotide phosphate (NADP), phosphate buffer (pH 7.4) (S9D), ddH₂O (S9E), and rat-liver extract (S9F). Components S9A to S9F were mixed thoroughly $(0.40 \,\mathrm{ml}) + \mathrm{S9B}$ $(0.09 \, \text{ml}) + \text{S9C}$ $(0.81 \, \text{ml}) + \text{S9D}$ $(9.98 \,\mathrm{ml}) + \mathrm{S9E} \, (8.47 \,\mathrm{ml}) + \mathrm{S9F} \, (0.25 \,\mathrm{ml})$] according to the supplier guideline to produce 20 ml of S-9 mixture.

The standard mutagen used for all bacteria strains in the *in vitro* assays with metabolic activity was 2-Amino Anthracene (2 AA). For the assays without metabolic activity, sodium azide (SA) was the standard mutagen used for TA, 1535, and TA 100 bacteria strains. Meanwhile, Nitrofluorene (NF), 9- Amino Acridine (9 AA) and 4- nitroquinolone (4 NQ) were the standard mutagens for TA 98, TA 97a, and WP2 bacteria strains, respectively.

Reverse mutation assay (muta-chromo plate assay)

Muta-chromo plate assay without S-9 (without metabolic activation)

In the assay without metabolic activation, 2.5 ml of the "reaction mixture" were dispensed into each sterile tube.

Then, a proportion of AHAE with concentration of (5.0, 1.0, 0.2, and 0.04 mg/ml), standard mutagen and sterile water were added to a total volume of 17.5 ml into each respective tube. No S-9 mixture is required for this assay.

Muta-chromo plate assay with S-9 (with metabolic activation)

For the assay with S-9 activation, 2.5 ml of the "reaction mixture" were dispensed into each sterile tube followed by 2.0 ml of S-9 mixture. After that, AHAE with concentration of (5.0, 1.0, 0.2, and 0.04 mg/ml), standard mutagen and sterile water were added to a total volume of 15.5 ml into each respective tube. The total volume of the final solution then became 17.5 ml. The mixture was mixed thoroughly. Then, 5 μl of grown bacterial strains were added and mixed with the prepared solution in each sterile tube except blank tubes of both assays, with and without S-9. Subsequently, 200 μl final solutions were added into each well of a 96-well microtiter plate using a multichannel pipette. All procedures were conducted under a biosafety cabinet to assure aseptic conditions. Finally, the plates were incubated in a 37°C incubator for five days.

Each well of the 96-well plate is considered as a colony. If a reverse mutation has occurred, the *S. typhi* and *E. coli* in the colony regained the ability to synthesize histidine and tryptophan, respectively, to grow. The continuous growth of the bacteria will turn the color in the well from purple to yellow. All yellow, partially yellow or turbid wells were scored as positive and all purple wells were scored as negative (Figure 2).

In vivo rat micronucleus assay

Animals

The experiment was conducted according to proper ethical use of laboratory animal guidelines. The proposal of the study was approved by Institutional Animal Care and Use Committee (IACUC), University of Malaya (UM) with ethical number 2014–03-05/ANAT/R/SRMZ. Female and male Sprague Dawley (SD) rats aged seven to nine weeks old were purchased from the animal breeding house, University Putra Malaysia (UPM), Malaysia. They were housed in the satellite facility of Animal Experimental Unit (AEU), UM at 12-h light/dark cycle. Two to four rats were placed per cage bedded with dried corn. They were given rat chow and water ad libitum.

Micronucleus assay

The assay protocol was based on OECD guideline 474 for the mammalian erythrocyte micronucleus test. The SD rats were randomly divided into four groups (n=5 males and 5 females per group): (1) treated with ddH₂O (negative control), (2) cyclophosphamide (CPA) (positive control), (3) 1000 mg/kg AHAE, and (4) 2000 mg/kg AHAE. All animals except the positive control group were treated accordingly using oral gavage tube for three consecutive days. These animals were euthanized not later than 24 h after the last dose. The positive control group that was

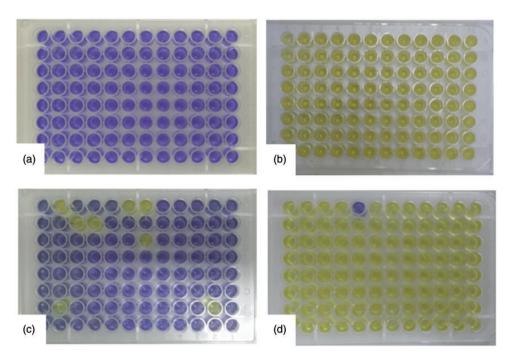


Figure 2. Photographs of 96-well plates used in the Muta-Chromo Plate assay showing all purple wells of the blank sterility plate (a), a positive control plate with 96 colonies of revertant bacteria (b), a negative control plate with 8 yellow wells containing revertant bacteria (c) and a treated plate with 95 colonies of revertant bacteria (d). Purple color represents no mutation, yellow color represents reverse mutation occurred. (A color version of this figure is available in the online journal.)

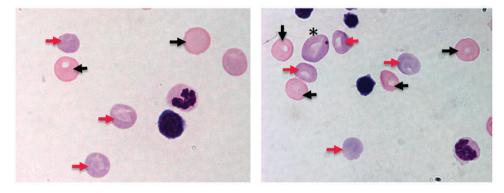


Figure 3. Photomicrographs of May-Grunwald and Giemsa-stained bone marrow (100× magnification) showing polychromatic erythrocyte (red arrow), normochromatic erythrocyte (black arrow), and micronucleated polychromatic erythrocyte (asterisk). (A color version of this figure is available in the online journal.)

treated with intraperitoneal (IP) injection of 50 mg/kg CPA (CAS 6055-19-2, Merck Millipore, US), these animals were euthanized 24 h after the injection, ²³ using carbon dioxide (CO₂) chamber.

After euthanization and blood collection, the skin over the rat thigh was incised to expose the femur. The femur was taken out and cut at the epiphyses level. Following which, the bone marrow (BM) was syringed out from both femurs of each rat using 23-gauge needle attached to a syringe filled with sterile fetal bovine serum (FBS). The collected BM was then placed in 2 ml micro centrifuge tube and subjected to centrifugation at 1000 r/min for 5 min at temperature of 20°C. The supernatant was discarded and the pellet was spread onto glass slides, dried, fixed with absolute methanol and stained with May-Grunwald and Giemsa stains (Figure 3).²⁴

The stained BM was viewed under 100× magnification using Nikon Eclipse 80i upright light microscope equipped with a digital color camera controller (DS-5Mc-U2) with **NIS-Elements** software (NIS-Elements Advanced Research, Nikon, Japan) for further analysis. PCE and normochromatic erythrocytes (NCE) were identified to determine the PCE to NCE (PCE/NCE) ratio from a total of 200 erythrocytes. The PCEs were further observed and calculated manually for the frequency of micronucleated PCE (MNPCE).

BW gain and relative organ weight

BW was determined daily starting from a day before commencement of the treatment until the day of sacrifice. Daily and total BW gain was then calculated and analyzed. The abdomen of rats was incised and the main organs including

Table 1. Reverse mutation assay results for A. hierochuntica aqueous extract in four strains of S. typhi and one strain of E. coli.

| | Colony of revertants per plate | | | | | | | | | |
|------------------------------|--------------------------------|------|---------------------|------|-------------------|------|--------------------|------|----------------|--------------|
| | S. typhi TA 100 | | S. typhi TA 1535 | | S. typhi TA 98 | | S. typhi TA 97a | | E. coli WP2 | |
| Groups | - S 9 | +S9 | -S9 | +S9 | -S9 | +\$9 | - S 9 | +\$9 | -S9 | + S 9 |
| Blank ^a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| -ve control ^b | 15 | 75 | 2 | 8 | 6 | 22 | 7 | 80 | 59 | 52 |
| AHAE ^c 5.0 mg/ml | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| AHAE ^c 1.0 mg/ml | 12 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 |
| AHAE ^c 0.2 mg/ml | 18 | 95** | 0 | 41** | 10 | 96** | 0 | 96** | 74* | 70** |
| AHAE ^c 0.04 mg/ml | 5 | 81 | 1 | 75** | 5 | 27 | 1 | 88* | 66 | 66* |
| +ve controls ^d | 37** | 90** | 94** | 45** | 91** | 96** | 96** | 84 | 95** | 82** |

AHAE: A. hierochuntica aqueous extract.

the liver, kidney, spleen, heart, and lung were harvested and weighed. The organ weight data were expressed relative to the animal's BW.

Serum biochemical analysis

After euthanization, blood sample was collected and sent to Gribble Pathology laboratory, Petaling Jaya, Malaysia, for serum renal profile (RP) and protein level.

Histological analysis

Liver and kidney of rats were fixed with 10% formalin. After that, the tissue samples were processed with series of alcohol (Thermo Fisher Scientific, USA) and xylene (BDH, England) and then embedded in paraffin wax (Paraplast, USA). Paraffin blocks were sectioned at 5 μm thickness placed on glass slides, incubated and stained with hematoxylin and eosin (H&E). The slides were viewed under $100\times$ magnification using Nikon Eclipse 80i upright light microscope (Nikon, Japan), equipped with a digital color camera controller (DS-5Mc-U2) and NIS-Elements software for histological analysis.

Statistical analysis

The muta-chromo plate kit was used, and it utilized the same principle of an Ames test which compares the rate of spontaneous reverse mutation in the background group to the rate of reverse mutation within a sample assay. The statistical significance of the data was determined using the table for analysis of fluctuation tests (Table S1) provided by the manufacturer (EBPI, Canada).²⁵

The BW gain, serum biochemical parameters, percentage of MNPCE frequency, and statistical comparisons of PCE/NCE ratio were analyzed using univariate analysis of variance (SPSS, version 22.0). The results were considered as statistically significant when the difference has P value of less than 0.05 (P <0.05).

Results

In vitro reverse mutation assay

A significant increase in the number of revertants was observed in the presence of two AA as the positive reference (standard mutagen) in all bacteria strains except for TA 97a with S-9 (with metabolic activation). There was also a significant increase in the number of revertants in all the *S. typhi* strains tested with 0.2 mg/ml AHAE with metabolic activation. As for the WP2 *E. coli* tested with the same AHAE concentration, the number of revertants was noted to be increased in well plates with and without S-9. Exposure to the lowest concentration, 0.04 mg/ml resulted in increased revertants in WP2 *E. coli* and only two *S. typhi* strains namely TA, 1535, and TA 97a with presence of metabolic activation. Absence of revertant bacteria was observed in most of the well plates containing higher concentrations (1.0 and 5.0 mg/ml) of AHAE (Table 1).

In vivo micronucleus assay

Polychromatic and normochromatic erythrocytes ratio

The PCE/NCE ratio of female and male SD rats treated with 2000 mg/kg AHAE showed significant increase (P < 0.05) compared with the control group. In contrast, decreased PCE/NCE ratio was observed in positive control group in both genders. Although the result was not statistically significant (Tables 2 and 3), the trend suggests CPA as an effective positive control as shown by lowest PCE/NCE ratio in CPA-treated group compared with the other groups.

Percentage of MNPCE frequency in 2000 PCE

In the male rats, the percentage of MNPCE frequency in 2000 PCE in the positive control group was significantly increased compared with the negative control group and other groups. The same trend was also observed in the CPA-treated female rats but the results were not

^aBlank: Sterility check.

^bNegative control: Sterile distilled water.

^cAnastatica hierochuntica aqueous extract (mg/ml).

⁻S9: without metabolic activator rat liver extract. +S9: with metabolic activator rat liver extract.

^dPositive controls: 2- Amino anthracene (all bacterial strains; +S9); Sodium azide (TA 100 and TA 1535; -S9); Nitrofluorene (TA 98; -S9); 9- Amino acridine (TA 97a;

⁻S9) and 4-Nitroquinolone (WP2; -S9). **P < 0.001, *P < 0.05.

statistically significant. On the other hand, there was no significant difference of MNPCE frequency between the AHAE-treated animals and the negative control group (Tables 2 and 3).

Table 2. Parameters of bone marrow micronucleus test in the female SD rats

| | Polychromatic/ normochromatic erythrocytes ratio | | Micronucleated polychromatic erythrocytes for 2000 PCE | | |
|--|---|-------------------------|---|-------------------------|--|
| Groups | Mean ± SEM | P | Mean ± SEM | P | |
| -ve control ^a +ve control ^b AHAE ^c 1000 mg/kg AHAE ^c 2000 mg/kg | 0.54 ± 0.14 0.15 ± 0.14 0.66 ± 0.14 $0.99 \pm 0.14^*$ | 0.056 0.565 0.033 | $\begin{array}{c} 1.12 \pm 0.66 \\ 3.00 \pm 0.66 \\ 1.75 \pm 0.66 \\ 0.92 \pm 0.66 \end{array}$ | 0.054 0.507 0.833 | |

AHAE: A. hierochuntica aqueous extract.

Note: Data are expressed as mean \pm standard error. *P <0.05.

Table 3. Parameters of bone marrow micronucleus test in the male SD rats.

| | Polychromatic normochroma erythrocytes r | tic | Micronucleated polychromatic erythrocytes for 2000 PCE | | |
|--|---|-------------------------|--|-------------------------|--|
| Groups | Mean ± SEM | P | Mean ± SEM | P | |
| -ve control ^a +ve control ^b AHAE ^c 1000 mg/kg AHAE ^c 2000 mg/kg | 0.50 ± 0.14 0.16 ± 0.14 0.70 ± 0.14 $1.29 \pm 0.14^*$ | 0.098 0.321 0.000 | 0.97 ± 0.66 $3.38 \pm 0.66^*$ 1.08 ± 0.66 0.89 ± 0.66 | 0.015 0.907 0.933 | |

AHAE: A. hierochuntica aqueous extract.

Note: Data are expressed as mean \pm standard error. *P <0.05.

BW gain

Male SD rats orally treated with 1000 mg/kg and 2000 mg/ kg AHAE showed significant reduction in BW gain after 24 h of treatment as compared with the negative control group. However, the BW gain seemed to normalize on subsequent days. Meanwhile, significant reduction of BW gain was observed in both genders treated with CPA 24 h before sacrifice (Table 4).

Weight of relative organs

The kidney weights of female rats treated with 2000 mg/kg AHAE were significantly higher than those of the negative control group. Meanwhile, the spleen weights of the positive control group in both genders were significantly lower compared with the negative control. In addition, the heart weights were significantly reduced in female CPA-treated group than those of the negative control group (Table 5).

Table 5. Relative organs weight (g/100 g BW) of female and male SD

| | Groups | | | | | | | |
|------------|-----------------------------------|---|-----------------------------------|-----------------------------------|--|--|--|--|
| Parameters | -ve control ^a | +ve control ^b | AHAE ^c 1000 mg/kg | AHAE ^c 2000 mg/kg | | | | |
| Female | | | | | | | | |
| Liver | $\boldsymbol{4.76 \pm 0.27}$ | $\boldsymbol{4.97 \pm 0.27}$ | $\boldsymbol{4.96 \pm 0.27}$ | 4.85 ± 0.27 | | | | |
| Kidneys | $\boldsymbol{0.97 \pm 0.05}$ | $\textbf{0.94} \pm \textbf{0.05}$ | $\boldsymbol{1.06 \pm 0.05}$ | $1.10\pm0.05^{\ast}$ | | | | |
| Spleen | $\boldsymbol{0.27 \pm 0.01}$ | $0.20\pm0.01^{\star}$ | $\textbf{0.25} \pm \textbf{0.01}$ | $\boldsymbol{0.27 \pm 0.01}$ | | | | |
| Heart | $\boldsymbol{0.50 \pm 0.03}$ | $\textbf{0.42} \pm \textbf{0.03}^{\star}$ | $\boldsymbol{0.47 \pm 0.03}$ | $\textbf{0.48} \pm \textbf{0.03}$ | | | | |
| Lungs | $\boldsymbol{0.73 \pm 0.05}$ | $\boldsymbol{0.73 \pm 0.05}$ | $\textbf{0.81} \pm \textbf{0.05}$ | $\boldsymbol{0.76 \pm 0.05}$ | | | | |
| Male | | | | | | | | |
| Liver | $\boldsymbol{5.60 \pm 0.27}$ | $\boldsymbol{5.09 \pm 0.27}$ | $\boldsymbol{5.34 \pm 0.27}$ | $\boldsymbol{5.19 \pm 0.27}$ | | | | |
| Kidneys | $\boldsymbol{1.02 \pm 0.05}$ | $\textbf{1.01} \pm \textbf{0.05}$ | $\boldsymbol{1.04\pm0.05}$ | $\boldsymbol{1.03\pm0.05}$ | | | | |
| Spleen | $\textbf{0.26} \pm \textbf{0.01}$ | $\textbf{0.20} \pm \textbf{0.01*}$ | $\boldsymbol{0.29 \pm 0.01}$ | $\boldsymbol{0.27 \pm 0.01}$ | | | | |
| Heart | $\textbf{0.52} \pm \textbf{0.03}$ | $\boldsymbol{0.45 \pm 0.03}$ | $\boldsymbol{0.46 \pm 0.03}$ | $\textbf{0.45} \pm \textbf{0.03}$ | | | | |
| Lungs | $\boldsymbol{0.70\pm0.05}$ | $\textbf{0.68} \pm \textbf{0.05}$ | $\textbf{0.71} \pm \textbf{0.05}$ | $\boldsymbol{0.70\pm0.05}$ | | | | |

AHAE: A. hierochuntica aqueous extract.

Note: Data are expressed as mean \pm standard error. *P < 0.05.

Table 4. Daily body weight gain (g) of female and male SD rats.

| | Day 1 | | Day 2 | | Day 3 | |
|--|---|---|--|--|--|--|
| Groups | Female | Male | Female | Male | Female | Male |
| -ve control ^a +ve control ^b AHAE ^c 1000 mg/kg AHAE ^c 2000 mg/kg | 2.80 ± 1.51 5.40 ± 1.51 2.80 ± 1.51 5.06 ± 1.51 | 10.20 ± 1.51 12.20 ± 1.51 $4.80 \pm 1.51^*$ $5.80 \pm 1.51^*$ | $\begin{array}{c} 1.20\pm1.43\\ 4.00\pm1.43\\ 5.00\pm1.43\\ 3.00\pm1.43\\ \end{array}$ | $6.00 \pm 1.43 \\ 8.60 \pm 1.43 \\ 5.00 \pm 1.43 \\ 5.80 \pm 1.43$ | 8.40 ± 1.78 $1.20 \pm 1.78^*$ 5.00 ± 1.78 5.00 ± 1.78 | $12.40 \pm 1.78 \\ 0.40 \pm 1.78^* \\ 10.20 \pm 1.78 \\ 9.40 \pm 1.78$ |

AHAE: A. hierochuntica aqueous extract.

Note: Data are expressed as mean \pm standard error. * P <0.05.

^aNegative control: The rats were administered distilled water for three consecutive days.

^bPositive control: Single intramuscular injection of cyclophosphamide (50 mg/kg) 24 h before sacrifice.

^cThe rats were administered Anastatica hierochuntica aqueous extract (mg/kg) for three consecutive days.

^aNegative control: The rats were administered distilled water for three consec-

^bPositive control: Single intramuscular injection of cyclophosphamide (50 mg/ kg) 24 h before sacrifice.

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^cThe rats were administered *Anastatica hierochuntica* aqueous extract (mg/kg) for three consecutive days.

^aNegative control: The rats were administered distilled water for three consecutive days.

^bPositive control: Single intraperitoneal injection of cyclophosphamide (50 mg/kg) 24 h before sacrifice.

^cThe rats were administered Anastatica hierochuntica aqueous extract (mg/kg) for three consecutive days.

Histological analysis

The kidneys of AHAE-treated female SD rats (Figure 4) exhibited a few small cysts and dilated renal tubules. These findings were present in two out of five rats treated with 1000 mg/kg AHAE and in four out of five rats treated with 2000 mg/kg AHAE. Meanwhile, kidneys of CPA-treated female rats exhibited dilated renal tubules in two out of five rats. The kidneys of female negative control showed normal histology.

In kidneys of male SD rats (Figure 5), renal cysts were observed in three out of five rats in both groups treated with AHAE (1000 and 2000 mg/kg). Dilated renal tubules were also seen in one out of five rats in both groups treated with AHAE (1000 and 2000 mg/kg). In the negative control group, only a small cyst was seen in two out of five rats with presence of dilated renal tubule in one of the animals. Meanwhile, histological examination of kidney of CPA-treated rats showed dilated renal tubules in three out of five rats and renal cyst in one of them.

These abnormal histological findings are consistent with the higher kidney weights in AHAE-treated groups. Meanwhile, histological analysis of the livers showed normal histology and no obvious differences between negative control, positive control, and AHAE-treated groups.

Serum biochemical analysis

Oral administration of AHAE for three consecutive days did not significantly affect serum RP. However, significant decrease in albumin level was seen in female groups treated with CPA and AHAE (1000 and 2000 mg/kg) compared to the control group (Table 6).

Discussion

A. hierochuntica is a natural product which is widely consumed by people in many countries, yet there is limited scientific evidence for its potential toxicity effects. In order to address this, we conducted this study to investigate the mutagenicity and other toxicity potentials of this herb.

The mutagenicity evaluation of *A. hierochuntica* was carried out using *in vitro* and *in vivo* assays. This is the first mutagenicity study ever conducted on AHAE. Therefore, the definitive assay was carried out following optimization in a preliminary assay. AHAE had significant mutagenic potential at 0.2 and 0.04 mg/ml, which demonstrated two types of gene mutation, namely base-pair substitution and frameshift mutation. However, the AHAE bioactive constituents that caused the mutations in the tested bacterial strains (mostly those with S9 mix) are still unknown.

Moreover, we obtained negative results (absence of revertant bacteria) in well plates containing higher concentrations of AHAE (5.0 and 1.0 mg/ml). This could have been due to the antibacterial activity of *A. hierochuntica*, which inhibited bacterial growth when used at high concentrations. Therefore, only concentrations <1 mg/ml can be interpreted to explore its mutagenicity effect. Nevertheless, we included the data of 1.0 and 5.0 mg/ml in this report as it could be an important reference point for future mutagenicity studies of AHAE.

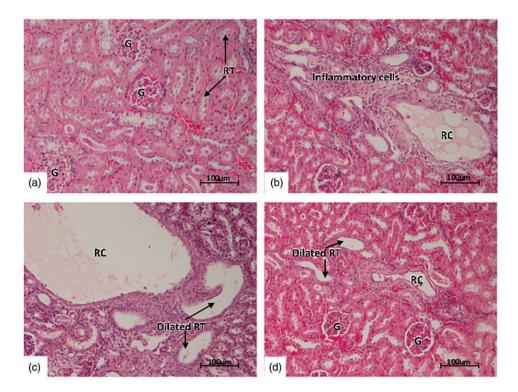


Figure 4. Photomicrographs of H&E-stained kidneys of female rats (20× magnification) showing normal glomerulus (G) and renal tubules (RT) of negative control (a), renal cyst (RC) and inflammatory cells infiltration in 1000 mg/kg group (b), presence of renal cyst (RC) and dilated renal tubules (RT) in 2000 mg/kg group (c) and CPA-treated group (d). (A color version of this figure is available in the online journal.)

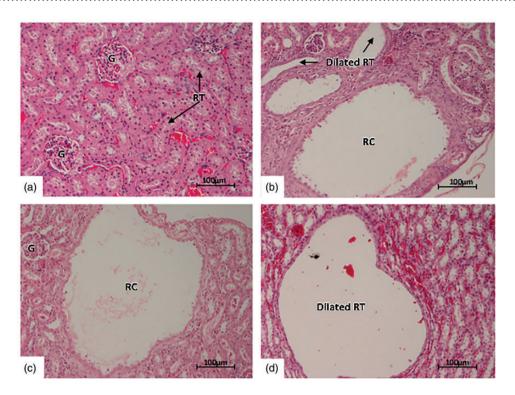


Figure 5. Photomicrographs of H&E-stained kidneys of male rats (20× magnification) showing normal glomerulus (G) and renal tubules (RT) of negative control (a), renal cyst (RC) and dilated renal tubule (RT) in 1000 mg/kg group (b), presence of renal cyst (RC) in 2000 mg/kg group (c) and dilated renal tubule (RT) in CPA-treated group (d). (A color version of this figure is available in the online journal.)

Table 6. Serum biochemical parameters of female and male SD rats treated with AHAE for three days.

| | | Groups | | | | |
|---------------------|------------------------------------|------------------------------------|-----------------------------------|------------------------------|--|--|
| Parameters | −ve control ^a | +ve control ^b | AHAE ^c 1000 mg/kg | AHAE° 2000 mg/kg | | |
| Females | | | | | | |
| Urea (mmol/L) | $\textbf{7.18} \pm \textbf{0.49}$ | $\textbf{7.16} \pm \textbf{0.49}$ | $\textbf{5.83} \pm \textbf{0.49}$ | $\boldsymbol{6.76 \pm 0.49}$ | | |
| Creatinine (μmol/L) | 36.20 ± 3.72 | 25.80 ± 3.72 | 30.00 ± 3.72 | 32.80 ± 3.72 | | |
| Total protein (g/L) | $\textbf{70.20} \pm \textbf{1.71}$ | $\textbf{63.80} \pm \textbf{1.71}$ | 62.00 ± 1.71 | 66.80 ± 1.71 | | |
| Albumin (g/L) | $\textbf{45.40} \pm \textbf{1.04}$ | $39.60 \pm 1.04^*$ | $39.50 \pm 1.04^{\ast}$ | $42.00 \pm 1.04^{*}$ | | |
| Males | | | | | | |
| Urea (mmol/L) | 7.26 ± 0.49 | $\textbf{6.64} \pm \textbf{0.49}$ | $\textbf{6.50} \pm \textbf{0.49}$ | $\boldsymbol{6.28 \pm 0.49}$ | | |
| Creatinine (µmol/L) | $\textbf{30.80} \pm \textbf{3.72}$ | 24.80 ± 3.72 | 30.60 ± 3.72 | 28.40 ± 3.72 | | |
| Total protein (g/L) | 64.40 ± 1.71 | $\textbf{63.00} \pm \textbf{1.71}$ | 65.40 ± 1.71 | 64.60 ± 1.71 | | |
| Albumin (g/L) | 40.60 ± 1.04 | 38.40 ± 1.04 | 39.40 ± 1.04 | 39.80 ± 1.04 | | |

AHAE: A. hierochuntica aqueous extract.

Note: Data are expressed as mean \pm standard error. *P <0.05.

The Muta-Chromo Plate kit is based on the most widely used and validated bacterial reverse mutation test, i.e. the Ames.²⁶ The Ames test is used worldwide as an initial screen for determining the mutagenic potential of new chemicals and drugs. The assay has a high predictive value for rodent carcinogenicity.²⁷ Testing the mutagenic potential of A. hierochuntica is a worthwhile effort, as the plant is widely used as a traditional medicine despite the lack of studies evaluating its toxicity potential. A positive result we obtained in the in vitro study does not by itself indicate that AHAE can cause cancer.²⁸ Nevertheless, it suggests that AHAE metabolites can produce mutations and serves as a guide for more extensive testing, such as gene expression profiling, micronucleus test, and acute, subacute, and chronic toxicity testing. 27,28 These assays are required to support the safety profiling of AHAE.

The in vivo micronucleus assay determined that AHAE is non-mutagenic in SD rats, as seen in the PCE/NCE ratio and the MNPCE frequency results. The former is an indicator of toxicity affecting cell formation in BM. Reduction of the numerator (PCE) suggests the mutagenic potential of the tested compound or plant. This was seen in the PCE/

aNegative control: The rats were administered distilled water for three consecutive days.

^bPositive control: Single intramuscular injection of cyclophosphamide (50 mg/kg) 24 h before sacrifice .

^cThe rats were administered Anastatica hierochuntica aqueous extract (mg/kg) for three consecutive days.

NCE ratio of the CPA-treated group, which was much lower PCE/NCE ratio compared to the negative control and all AHAE-treated groups. No ratio reduction was observed in the AHAE-treated groups.

Many previous studies have also reported inconsistent findings between *in vivo* and *in vitro* assays.^{29–32} The authors of those studies have postulated that the negative *in vivo* micronucleus assay findings may have been due to insufficient doses of the plant extract or its mutagenic metabolite(s), which could have enabled them to reach the BM target cells to produce deoxyribonucleic acid (DNA) damage.^{29–32} The AHAE effects *in vivo* could also been influenced by absorption, distribution, metabolism, and excretion, which are not factors in *in vitro* assay.³³ It might also be possible that the genotoxic metabolites of AHAE could have reached the BM but predominantly induced point mutations rather than chromosome aberrations. This might explain the non-significant findings in the AHAE-treated SD rats in the micronucleus assay.

Nevertheless, we noted that high-dose AHAE had a stimulatory effect on PCE numbers. Compared with the negative control, the PCE/NCE ratio was significantly increased in both female and male SD rats treated with 2000 mg/kg AHAE. This finding indicates that there were more reticulocytes than mature erythrocytes, i.e. the BM of rats exposed to 2000 mg/kg AHAE produced more red blood cells (RBCs). Similar results trends have been reported previously. Those researchers believed that it could have been due to the induction of erythropoietin, a growth factor hormone necessary for erythropoiesis.

The ability of *A. hierochuntica* to promote RBC production is consistent with the folk belief that *A. hierochuntica* is beneficial for treating menorrhagia, ^{11,12,16,17} a condition that requires RBC replacement. Further studies should be conducted to determine whether AHAE has medicinal benefits in hematopoietic dysfunction such as leukopenia and thrombocytopenia resulting from the administration of immunosuppressive drugs such as CPA.

Another biomarker of a mutagenic effect in the micronucleus assay is the MNPCE frequency. MN or Howell-Jolly bodies were originally identified and described in erythrocytes by William Howell and Justin Jolly. Howell-Jolly bodies mainly originate from acentric chromosome fragments, acentric chromatid fragments or whole chromosomes that fail to be included in the daughter nuclei at telophase completion during mitosis. This occurs due to improper attachment to the spindle during segregation in anaphase. The displaced chromosome or chromosome fragments are eventually enclosed by a nuclear membrane. They are morphologically similar to the nucleus after conventional nuclear staining but are smaller. The secretary of the microscopic fragments are eventually enclosed by a nuclear membrane.

The molecular mechanisms of MN formation described above could have been caused by deficiencies in vitamins, such as folate and vitamin B12, and exposure to ionizing radiation or genotoxic chemicals.³⁸ In this study, we investigated the possible mutagenic effects of AHAE in the BM of SD rats following three-day oral administration of 1000 and 2000 mg/kg AHAE. We found no significant increase in the percentage of MN frequency among 2000 PCE compared to the negative control. However, as expected, there

was a marked increase in this parameter in the positive control group (CPA-treated) in rats of both sexes, with a significant difference among the male rats.

In addition to the mutagenicity parameters, we also analyzed the effect of AHAE on BW gain, relative organs weight, and biochemical parameters. The BW gain of male rats treated with 1000 and 2000 mg/kg AHAE was significantly reduced 24 h after extract administration as compared to the negative control group. However, there was fair recovery of BW gain on subsequent days. Conversely, the extract did not interfere with BW gain in female rats, while CPA significantly decreased BW gain in both female and male rats at 24 h after injection.

The BW reduction in the CPA-treated rats was consistent with the decreased spleen weight in the same group. Reduced spleen weight in mice following IP injection of CPA has been reported previously. This was also observed in our study, where a single IP injection of 50 mg/kg CPA significantly reduced spleen weight in both male and female rats. The spleen is an important component of the immune system in addition to the thymus and lymphatic system. The depletion of a major population of spleen cells, consisting largely of B lymphocytes, would contribute to the reduction in spleen weight. On the other hand, the spleen weight of the AHAE-treated rats was not significantly different from that of the negative control group.

Analysis of other organs weight revealed significantly increased kidney weight in female rats treated with 2000 mg/kg AHAE. This is consistent with the presence of cysts in some of the rats. Renal cysts are a feature of nephropathy following long-term herbs consumption. In our study, only small numbers of cysts were noted in the affected rats, which could have been due to the short period (three days) of AHAE exposure. A longer duration of exposure could enhance the effect and is expected to result in the development of more advanced renal cysts and other nephrotoxic features. In addition, several studies have reported that acquired cystic kidney disease is associated with the development of renal cell carcinoma.

Another analyzed parameter, i.e. serum albumin levels, was significantly lower in female rats treated with CPA and AHAE compared to the negative control group. Despite the significant difference, the albumin levels of all animals were within the normal range, and thus the rats were not hypoalbuminemic. The normal albumin levels and liver histological features indicate the absence of hepatotoxicity in the SD rats following three-day exposure to AHAE.

Conclusion

The *in vitro* bacterial reverse mutation assay demonstrates that AHAE is mutagenic, either through base-pair substitution or frameshift mutation in the bacteria. However, further evaluation of the mutagenic potential through *in vivo* mammalian erythrocyte micronucleus testing demonstrated that up to 2000 mg/kg, AHAE does not induce significant mutagenicity in SD rats. Although the *in vivo* results were negative, it does not represent a definite absence of mutagenic potential of AHAE *in vivo* at higher doses.

Further work is necessary to identify the genotoxic risk of AHAE in humans. It may also be worthwhile to perform acute and chronic toxicity studies to draw firm conclusions regarding the safety of AHAE consumption.

Authors' contributions: MAA, ZM and SRMZ designed the study. SRMZ conducted the experiments, analyzed data and drafted the manuscript. MAA analyzed data and wrote parts of the manuscript. ZM, WFW and NMK wrote parts of the manuscript. The manuscript was reviewed by all authors.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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