Mutagenicity and genotoxicity effects of *Verbena officinalis* leaves extract in Sprague-Dawley Rats

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**ABSTRACT**

**Ethnopharmacological relevance:** Traditionally, *Verbena officinalis* L. has been used for reproductive and gynaecological purposes. However, the mutagenicity and genotoxicity of *V. officinalis* have not been extensively investigated.

**Aim of the study:** To assess the *in vitro* mutagenicity and *in vivo* genotoxicity of aqueous extract of *V. officinalis* leaves using a modified Ames test and rat bone marrow micronucleus assay according to OECD guidelines.

**Materials and methods:** *In vitro* Ames test was carried out using different strains of *Salmonella* (TA97a, TA98, TA100, and TA1535) and *Escherichia coli* WP2 uvrA (pKM101) in the presence or absence of metabolic activation (S9 mixture). For micronucleus experiment, male and female Sprague-Dawley rats (n = 6/group) were received a single oral daily dose of 500, 1000, and 2000 mg/kg of *V. officinalis* extract for three days. Negative and positive control rats were received distilled water or a single intraperitoneal injection of 50 mg/kg of cyclophosphamide, respectively. Following dissection, femurs were collected and bone marrow cells were stained with May-Grünwald-Giemsa solution for micronucleus assessment.

**Results:** Ames test results demonstrated that 5, 2.5, 1.25 and 0.625 mg/ml of *V. officinalis* extract induced a significant mutagenic effect against TA100 and TA98 strains (with and without metabolic activation). Findings of the animal study showed there were no significant increase in the micronucleated polychromatophilic erythrocytes (MNPE) and no significant alterations in the polychromatophilic erythrocytes (PCE) to normochromatophilic erythrocytes (NCE) ratio of treated rats as compared with their negative control. Meanwhile, significantly increased in the MNPEs was seen in the cyclophosphamide-treated group only.

**Conclusion:** Aqueous extract of *V. officinalis* has mutagenic effect against TA98 and TA100 strains as demonstrated by Ames test, however, there is no *in vivo* clastogenic and myelotoxic effect on bone marrow micronucleus of rats indicating that the benefits of using *V. officinalis* in traditional practice should outweigh risks.

**Keywords:**
- Ames test
- Genotoxicity
- Micronucleus assay
- Mutagenicity
- *Verbena officinalis* L.

**1. Introduction**

Traditionally, *Verbena officinalis* L. (*V. officinalis*), or commonly referred as vervain (family: Verbenaceae) is a medicinaly-used herb native to the Mediterranean (Shamsarkani et al., 2010), where the majority of people still depend on the folk medicine, despite the great progress in allopathic medicines, particularly to alleviate anxiety, insomnia, depression (Kaur et al., 2014), amenorrhea and dysmenorrhea (Pharmacopoeia, 2010). *V. officinalis* has been used in traditional medicine for reproductive health-related issues and gynaecological problems (Akour et al., 2016), and also used during pregnancy in stimulating uterine smooth muscle (Liu et al., 2012), and as herbal galactagogues to induce milk secretion (Al-Qura’n, 2009). The leaves of *V. officinalis* water decoction are popularly consumed to obtain the desired effects or claimed medical benefits.

According to the literature, *V. officinalis* has antioxidant effects or claimed medical benefits.

**Abbreviations:** OECD, Organization for Economic Cooperation and Development; MNPE, micronucleated polychromatophilic erythrocytes; PCE, polychromatophilic erythrocytes; NCE, normochromatophilic erythrocytes; PCE/NCE, ratio of polychromatophilic erythrocytes to normochromatophilic erythrocytes; *V. officinalis*, *Verbena officinalis* L.; NC, negative control; PC, positive control; V500, 500 mg/kg aqueous extract of *V. officinalis* L.; V1000, 1000 mg/kg aqueous extract of *V. officinalis* L.; V2000, 2000 mg/kg aqueous extract of *V. officinalis* L.; AST, aspartate transaminase; ALT, alanine transaminase; LDH, lactate dehydrogenase

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(Schonbichler et al., 2013), anti-inflammatory and anti-fungal activities (Casanova et al., 2008; Pascaud et al., 2001). On the other hand, V. officinalis has been used in chronic prostatitis, hematuria and digestive problems such as spasm and stomach pain (Yang et al., 2013). The aforementioned activities could be due to the wide range of the phytochemical constituents of V. officinalis such as, essential oils (citral, geraniol, limonene and verbene), caffeic acid glycosides, β-sitosterol, ursolic acid, apigenin, luteolin and polyphenols (Depak and Handa, 2000).

The traditional herbs are wildly accepted by people for maintaining and improving general health, easily obtained and there is no restriction on their availability as traditional remedies in many countries. The lack of the minimum information needed for the proper use of herbal medicines and medicinal plants makes them easy targets for self-medication without responsibility (Costa et al., 2012). Moreover, the common misconception about the absence of side effects and toxicity of natural herbs may lead to uncontrolled and exaggerated consumption. Consequently, the amount of ingested dosage depends on advice from friends, suppliers and other consumers. Nevertheless, the plant materials like therapeutic agents, when overdosed or incorrectly used they also have the potential to induce adverse effects. Unfortunately, there is still a lack of information about human exposure and possible adverse health effects of many herbal and traditional medicines. The traditional plants and herbal products are usually not prepared according to the standard procedures of formulation and drug safety evaluation, hence the herbal toxicity has become an issue of concern due to its wide use (De Smet, 1995). Therefore, the efficacy and possible risks of herbal preparations should be carefully considered, to ensure that the benefits compensate for any risks and adverse effects produced. Regardless of the traditional or scientifically reported medicinal uses, the toxicity of most traditional herbal medicines has not been fully evaluated since plants that are frequently used in folk medicine might be potentially genotoxic (Ananthi et al., 2010; Shin et al., 2011). Furthermore, overutilization of natural materials or even synthetic compounds could act as a mutagen or a carcinogen (Roberts et al., 2014).

The identification of compounds or chemicals that have the potential to induce mutations is crucial in safety assessment since mutagenic compounds can potentially induce cancer (Sugimura, 2000). The most highly recommended tests to assess the safety of herbal medicines during pregnancy include in vitro mutagenicity, in vivo genotoxicity and teratogenicity assays (Chuang et al., 2006). Although V. officinalis is a plant which is widely used as aforementioned, it's mutagenic and genotoxic potentials have not yet been investigated. Therefore, in this study, both in vitro assay called bacterial reverse mutation assay (Ames test) and in vivo rat bone marrow micronucleus assay were carried out to evaluate the potential mutagenic and genotoxic effects of V. officinalis aqueous extract. The toxicity effects of V. officinalis aqueous extract on the liver and kidney was also investigated.

2. Materials and methods

2.1. Chemicals and reagents

Ames kit (AMES-MOD ISO™ 96 Well Format) was obtained from Environmental Bio-Detection Products Incorporation, EBPI, Ontario, Canada). For the in vivo study, methanol was purchased from Merck Chemicals, Germany. Foetal bovine serum (FBS) was obtained from Gibco, cyclophosphamide (CAS 6055-19-2) was purchased from Merck Millipore, US and May-Grünwald-Giemsa stain from Merck, Germany. Ketamine and xylazine were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Plant materials

The plant, Verbena officinalis, was purchased from a local market in Yemen and identified by a taxonomist at Pharmacognosy Laboratory, Faculty of Pharmacy, Sana’a University, Yemen. The plant was given a voucher specimen (VO/2015/72), which was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Sana’a University.

2.3. Preparation of an aqueous extract of V. officinalis

The leaves of the plant (1 kg) were dried, ground to a homogeneous powder and were soaked in distilled water at 70 °C for four hours in a water bath with continuous shaking. After that, the plant infusion was filtered through a cotton wool plug in the neck of filter funnel and then through a filter paper (Whatman No. 1). The solution was evaporated and concentrated at −80 °C using freeze dryer (Alpha 1–4 LSC; Martin Christ Gefriertrocknungsanlagen GmbH, Germany) (Kadhim et al., 2016). The percentage yield of extract was 11.77% w/w. The dried extracts were stored at −20 °C until used.

2.4. In vitro Ames test mutagenicity assay

Ames test (Salmonella typhimurium/microsome assay) was used to investigate the mutagenic effect of V. officinalis extract using a commercial kit (EBPI, S Modified Ames ISO kit, EBPI, Ontario, Canada). This test kit is based on the validated Ames bacterial reverse-mutation test (Ames et al., 1975; OECD, 1997), and this procedure is capable of identifying mutagenic substances.

Ames test was performed using the bacteria test strains Salmonella typhimurium (TA100, TA1535, TA98 and TA97a) and Escherichia coli (WP2uvrA) PKM101. The test was conducted under aseptic conditions techniques according to the method described by Hubbard et al. (1984).

The plate assay consists of nutrient broth (growth media), S-9 components, and standard mutagens. The purple colour wells were scored as negative, while yellow colours were scored as positive. For herbal extract to be mutagenic, the number of positive wells had to be significantly higher than that in the background microplate (spontaneous revertor mutation). The standard mutagens sodium azide (SA) was used with TA 100 and TA 1535 bacterial strains-S9, 2-nitrofluorene (2-NF) was used with TA 98 bacterial strains-S9, 9-aminooacridine (9-AA) was used with TA97a strain-S9, 4-nitroquinoline 1-oxide (4-NQO) was used with WP2 strain-S9, and 2-aminoanthracene (2-AA) was used for all strains with + S9 metabolic activation. The same solvent system as tested samples was used for negative control plate. The Modified Ames ISO kit test was performed according to the instructions of the manufacturer.

2.5. In vivo genotoxicity bone marrow micronucleus assay

2.5.1. Animal ethics

The animal study was performed in accordance with the approval issued by the Faculty of Medicine Institutional Animal Care and Use Committee (FOM-IACUC) with an ethic number (2016–190105/PHAR/R/AMH). The maintenance of animals followed the Guide for the Care and Use of Laboratory Animals published by the Institute for Laboratory Animal Research (2010) and the EEC Directive (1986). Healthy specific-pathogen-free female and male Sprague-Dawley rats aged 9–11 weeks were obtained from the Animal Experiment Unit (AEU), Faculty of Medicine, University of Malaya. The animals were housed in polypropylene cages (three rats per cage). Environmental controls were set to maintain conditions of 19–23 °C and 40–70% relative humidity, with a 12-h light/dark cycle, and animals were received normal rodent diet and tap water ad libitum.

2.5.2. Study design

A total of 60 animals were divided into two groups, males (n = 30) and females (n = 30) according to OECD guideline 474 for the mammalian erythrocyte micronucleus test (OECD, 2016). Rats were
randomly arranged in cages (3 rats/cage) and allowed to acclimatize for 1 week prior to the experiment. Then each group was subdivided into 5 subgroups (n = 6) designed as negative control (NC), low dose 500 mg/kg *V. officinalis* (V500), medium dose 1000 mg/kg *V. officinalis* (V1000), high dose 2000 mg/kg *V. officinalis* (V2000) and positive control (PC). The subgroups were treated orally with a single daily dose of 5 ml/kg B.W of vehicle, 500, 1000 or 2000 mg/kg of *V. officinalis* extract in distilled water via oral gavage for three days, respectively. The 5th group (PC) was treated with a single I.P. injection of 50 mg/kg cyclophosphamide (CPA) 24 h prior to euthanasia. The plant extracts were always freshly prepared before use. Rats were monitored for any changes in body weight (BW), behaviour and health status. The clinical signs of toxicity and body weight after treatment were observed daily. After the last treatment, animals were anaesthetized with ketamine and xylazine (80:10 mg/kg I.P) to minimize pain distress and suffering. Then blood samples were collected via heart puncture. Finally, rats were euthanized by an overdose of ketamine and xylazine (150:15 mg/kg I.P) to harvest bone marrow, liver and kidney.

### 2.6. Statistical analysis

Within 3 days (Jenssen and Ramel, 1978). PCEs appear in PCEs not earlier than 10 h after injection of the animal with from chromosomal fragments produced during the preceding cell cycle stained reddish to yellow. The treatment-induced micronuclei derived slightly larger than the NCEs. Normochromatic erythrocytes (NCEs) of the high content of ribonucleic acid in the cytoplasm, and also aminated under the light microscope using oil immersion 100 X magnification was carried out to visualize any visible lesions on the surface fixed in 10% buffered formalin, processed, dehydrated, and embedded in paraffin wax. After that, 5 μm sections in thickness were stained with haematoxylin-eosin stain and observed microscopically for any abnormalities.

#### 2.5.5. Preparation of bone marrow smears and scoring of micronuclei

Bone marrow micronuclear assay was carried out as recommended by Schmidt (1976) and OECD guideline No 474 (OECD, 2016). Briefly, after dissections the femurs were immediately removed and bone marrow was flushed by 2 ml of sterile foetal bovine serum (FBS) into centrifuge tubes to obtain bone marrow cells. The cells suspension was centrifuged at 1000 rpm for 10 min the supernatant was discarded, and the pellet was re-suspended in a drop of FBS and smears were prepared on clean coded slides. The slides were air-dried for 24 h and stained with May–Grünwald and Giemsa solution. For the analysis of micronucleated cells, 2000 polychromatic erythrocytes (PCE) per animal were scored for determination of the mutagenic property of *V. officinalis* extract. To evaluate genotoxicity, 200 PCE per animal were scored to determine the PCE: NCE ratio (Salmani et al., 2015). Slides were examined under the light microscope using oil immersion 100 X magnification. Under the microscope, PCEs stained bluish to purple because of the high content of ribonucleic acid in the cytoplasm, and also slightly larger than the NCEs. Normochromatic erythrocytes (NCEs) stained reddish to yellow. The treatment-induced micronuclei derived from chromosomal fragments produced during the preceding cell cycle appear in PCEs not earlier than 10 h after injection of the animal with the test chemical. In fact, micronuclei appear much later than this within 3 days (Jenssen and Ramel, 1978).

#### 2.6. Statistical analysis

In vivo data were explored to meet assumptions of one-way ANOVA, where possible followed by post hoc Tukey test (if homogeneity was assumed) or post hoc Games-Howell test (if homogeneity was not assumed) (Freundenberg et al., 2016). Differences in means were expressed as the mean ± SD. P value ≤ 0.05 represented a significant difference, and P ≤ 0.01 represented a moderately significant difference, and

#### 3. Results

##### 3.1. Mutagenicity Ames test

Mutagenicity test of aqueous extract of *V. officinalis* was carried out using *Salmonella* (TA97a, TA98, TA100, and TA1535 strains) and *Escherichia coli* WP2uvrA strain. *V. officinalis* showed a moderately significant (P < 0.01) mutagenic effect against TA98 strain (with and without metabolic activation S9) at 5, and 2.5 mg/ml and a slight significant effect (P < 0.05) at 1.25 and 0.625 mg/ml. Ames test showed that *V. officinalis* demonstrated a moderately significant (P < 0.01) mutagenic effect against TA100 strain without metabolic activation (S9) at 5, 2.5 and 1.25 mg/ml and a less significant (P < 0.05) at dose 0.625 mg/ml, however, *V. officinalis* exerted a significant (P < 0.05) mutagenicity against TA100 with S9 at 5, 2.5,1,25 and 0.625 mg/ml. In addition, *V. officinalis* has no mutagenic effect against TA97a, TA1535 and WP2uvrA strains (Table 1). Meanwhile, a significant increase in the number of revertants, (P ≤ 0.001) was observed in all bacteria strains with or without S9 activation treated with standard reference mutagens.

##### 3.2. In vivo bone marrow micronucleus assay

**3.2.1. Body weight of rats**

Fig. 1 shows that the body weight of treated female and male rats was not significantly different (P > 0.05) as compared to untreated rats group (negative control).

**3.2.2. Frequencies of micronucleus and the ratio of polychromatic to normochromatic erythrocytes**

Table 2 shows that the frequencies of micronucleus (MN) of female and male rats treated with various treatments. All treated female and male rats with different doses of *V. officinalis* demonstrated no significant increase in micronucleated polychromatic erythrocytes (MNPE) and no significant alteration in the ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE: NCE) as compared to their negative controls, however, cyclophosphamide (positive control) showed significantly increased MNPEs and significantly reduced PCE: NCE ratio (Figs. 2 and 3).

**3.2.3. Biochemistry markers**

The results of biochemistry (Tables 3, 4) show that there were no significant effects on the evaluated biomarkers in rats. However, serum AST levels of female and male rats treated with V500, V1000, and V2000 mg/kg *V. officinalis* were significantly different (P < 0.001) as compared to negative control. Female and male rats treated with positive control (cyclophosphamide) showed a significant increase in AST, ALT and LDH as compared to negative control. The ALT enzyme was increased in both male and female treated rats but the increase was not significant when compared with the negative control group.

**3.2.4. Histopathology evaluation**

The liver histological sections of NC, V500, V1000, and V2000 of both female and male rats showed a normal architecture of hepatocytes...
### Table 1

**Mutagenicity Ames test of aqueous extract of *V. officinalis***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colony of revertants per plate (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salmonella typhimurium</strong> TA97a</td>
<td>SC 0 ± 0.00, BG 5.0 ± 0.00, PC 48.0 ± 0.00, V500 4.3 ± 0.58, V2000 4.7 ± 0.58, V2000 4.7 ± 0.58</td>
</tr>
<tr>
<td><strong>Salmonella typhimurium</strong> TA98</td>
<td>SC 0 ± 0.00, BG 3.7 ± 0.58, PC 41.0 ± 1.41, V500 3.7 ± 0.58, V2000 4.3 ± 1.53</td>
</tr>
<tr>
<td><strong>Salmonella typhimurium</strong> TA100</td>
<td>SC 0 ± 0.00, BG 3.3 ± 0.58, PC 48.0 ± 0.00, V500 3.3 ± 0.58, V2000 3.3 ± 0.58</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong> WP2uvrA</td>
<td>SC 0 ± 0.00, BG 3.3 ± 0.58, PC 48.0 ± 0.00, V500 3.3 ± 0.58, V2000 3.3 ± 0.58</td>
</tr>
</tbody>
</table>

The values of outcome variables were expressed as mean ± standard deviation of triple microplates.

*P* ≤ 0.05, **P** ≤ 0.01, ***P*** ≤ 0.001 vs. background control. SC: Sterility check, BG: background, PC: positive control, V: *V. officinalis* leaves, -/+S9: without or with metabolic activator rat liver. V5, V2.5, V1.25, V0.625, V0.312, V0.156 and V0.078 denoted different serial dilutions of *V. officinalis* extract in mg/ml. For each tester strain, a specific positive control was used as follows; 9-aminoacridine (9-AA) for TA97a, 2-nitrofluorene (2-NF) for TA 98, 4-nitroquinoline 1-oxide (4-NQO) for TA 100 and *E. coli* WP2, sodium azide (SA) for TA 1535, and 2-aminoanthracene (2-AA) was used as positive control for all strains with S9 metabolic activator. No contaminant colonies were observed in the sterility microplates.

### 4. Discussion

#### 4.1. *In vitro* mutagenicity (*Ames test*)

The present study was focused on the evaluation of the *in vitro* and *in vivo* mutagenicity of aqueous crude extract of *V. officinalis* leaves. Several studies have reported that some chemicals are capable of causing mutation on genetic materials (DNA) of bacteria or human (Phillips, 1983). *In vitro* Ames test mutagenicity assay plays an important role due to its high sensitivity and rapidity (MacGregor, 1986). The Ames test is one of the most important non-clinical safety studies which enables the detection of mutagenic effects of dietary, plant extract, and it shows a high predictive value results (Lewis et al., 1993). It can be carried out using various types of *Salmonella typhimurium* and *Escherichia coli* tester strains with and without S9 metabolic activation. The Ames test in absence of S9 metabolic activation can only detect direct mutagens, while in the presence of S9 metabolic activation allows the detection of indirect mutagens (Hong and Lyu, 2013). Direct mutagen interacts directly with DNA, whereas indirect mutagen acts by inducing a DNA damage by its metabolic products (i.e. activation of promutagens) or its reactive metabolites (Goldman and Shields, 2003).

A metabolic activation system with S9 fraction (a microsomal fraction or a post-mitochondrial supernatant) is usually prepared from liver homogenate of rats through treating the rats with a potent inducer of drug-metabolizing enzymes before being killed (De Méo et al., 1996). Sodium azide is the standard mutagen that is used in the absence of S9 metabolic activation to detect the direct mutation in TA 1535, and TA 100 bacteria strains. Meanwhile, nitrofluorene, 9-amino acridine and 4-nitroquinoline are the standard mutagens that are used with TA 98, TA 97a, and *E. coli* WP2 bacteria strains, respectively in the absence of S9.
fraction. On the other hand, 2-amino anthracene is the standard that is used with S9 metabolic activation for the detection of indirect mutagens (Hong and Lyu, 2013; Md Zin et al., 2018). The principle of Ames test depends on the fact that *Salmonella* strains contain reverse mutations in the histidine operon, which makes them unable to synthesize the amino acid histidine, and therefore called histidine auxotrophs (h-). Similarly, *Escherichia coli* strains cannot synthesize the amino acid tryptophan (trp-). Exposure to test substance (potential mutagen) may induce a second mutation (a reversion) that will restore the functional capability of the bacteria to synthesize the essential amino acid.

The most frequently used *Salmonella* strains namely; TA97a, TA98, TA100 and TA1535, and *E. coli* WP2 pKM101 were exposed to different

### Table 2: Frequencies of micronucleus and the ratio of polychromatic to normochromatic erythrocytes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MNPE/2000</th>
<th>PCE/NCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>NC</td>
<td>2.83 ± 0.75</td>
<td>3.33 ± 0.82</td>
</tr>
<tr>
<td>PC</td>
<td>39.17 ± 7.36***</td>
<td>37.50 ± 5.36***</td>
</tr>
<tr>
<td>V500</td>
<td>3.00 ± 0.89</td>
<td>3.00 ± 0.89</td>
</tr>
<tr>
<td>V1000</td>
<td>3.67 ± 1.03</td>
<td>3.00 ± 0.89</td>
</tr>
<tr>
<td>V2000</td>
<td>3.33 ± 1.03</td>
<td>3.83 ± 1.17</td>
</tr>
</tbody>
</table>

The values of outcome variables were expressed as mean ± standard deviation. *P* < 0.05, **P** < 0.01, ***P** < 0.001 vs. normal control. MNPE: micronucleated polychromatic erythrocytes, PCE/NCE: polychromatic erythrocytes to normochromatic erythrocytes ratio, NC: negative control group, PC: positive control group, V500: 500 mg/kg *V. officinalis*-treated group, V1000: 1000 mg/kg *V. officinalis*-treated group, V2000: 2000 mg/kg *V. officinalis*-treated group.

Fig. 2. Micronucleus cells of bone marrow of female rats. The bone marrow slides were stained with May-Grünwald and Giemsa stain. A: negative control group, B: positive control group, C: 500 mg/kg *V. officinalis*-treated group, D: 1000 mg/kg *V. officinalis*-treated group, E: 2000 mg/kg *V. officinalis*-treated group. Black arrows point to polychromatic erythrocyte (PCE), red arrows point to normochromic erythrocyte (NCE), and yellow arrows point to micronucleus polychromatic erythrocyte (MNPE). Power oil immersion 100x. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).
dilutions of *V. officinalis* with and without metabolic activation. To achieve a valid test, a number of concentrations were tested and positive controls (well-known mutagenic agents) were also included along with negative controls that lack only the test compound. Moreover, the entire test was conducted in triplicate as recommended by manufacturer’s kit. In addition, no contaminant colonies were observed in sterility microplate wells which used for sterility assessment of the test.

According to Modified Ames ISO kit, at least five serial dilutions of the test sample should be carried out at different plates (Rao and

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**Table 3**

Biochemistry parameters of female rats.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>PC</th>
<th>V500</th>
<th>V1000</th>
<th>V2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>49.50 ± 4.84</td>
<td>166.18 ± 24.33***</td>
<td>59.50 ± 5.46</td>
<td>69.80 ± 13.72</td>
<td>70.60 ± 13.09</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>79.45 ± 5.93</td>
<td>192.85 ± 19.66***</td>
<td>136.80 ± 25.81***</td>
<td>161.35 ± 25.97***</td>
<td>187.08 ± 8.93***</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>467.50 ± 45.27</td>
<td>797.25 ± 103.92***</td>
<td>371.00 ± 48.42</td>
<td>364.00 ± 15.66</td>
<td>366.75 ± 30.70</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>87.25 ± 12.63</td>
<td>82.75 ± 7.72</td>
<td>60.25 ± 3.95</td>
<td>55.50 ± 3.11</td>
<td>61.25 ± 4.99</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>7.80 ± 1.14</td>
<td>8.85 ± 1.71</td>
<td>6.35 ± 1.06</td>
<td>5.80 ± 0.48</td>
<td>6.60 ± 0.94</td>
</tr>
</tbody>
</table>

The values of outcome variables were expressed as mean ± standard deviation. *P* < 0.05, **P** < 0.01, ***P*** < 0.001 vs. normal control. ALT: alanine aminotransferase, AST: aspartate aminotransferase, LDH: lactate dehydrogenase, NC: negative control group, PC: positive control group, V500: 500 mg/kg *V. officinalis*-treated group, V1000: 1000 mg/kg *V. officinalis*-treated group, V2000: 2000 mg/kg *V. officinalis*-treated group.
For our experiment, seven serial dilutions (5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 mg/ml) of *V. officinalis* extract were tested. The highest concentration for this assay was set at 5 mg/ml based on the preliminary study, which showed that higher concentrations had the ability to inhibit the growth of bacterial strains and exert toxic effect on the bacteria, therefore higher concentrations above 10 mg/ml were excluded. These findings are in accordance with the reported antimicrobial activity of *V. officinalis* extract (Ahmed et al., 2017). Experimentally, our in vitro findings of the Ames test showed that *V. officinalis* demonstrated mutagenic effect against TA100 strain without metabolic activation (S9) at 5, 2.5 and 1.25 mg/ml of *V. officinalis* with a less effect at dose of 0.625 mg/ml. However, *V. officinalis* showed a significant mutagenic effect at 5, 2.5, 1.25 and 0.625 mg/ml against TA100 strain with S9. In addition, *V. officinalis* also showed a

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>PC</th>
<th>V500</th>
<th>V1000</th>
<th>V2000</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALT (U/L)</strong></td>
<td>54.45 ± 10.16</td>
<td>151.33 ± 21.17 **</td>
<td>81.32 ± 13.08</td>
<td>78.20 ± 13.84</td>
<td>87.48 ± 30.33</td>
</tr>
<tr>
<td><strong>AST (U/L)</strong></td>
<td>74.40 ± 7.01</td>
<td>288.75 ± 33.84 **</td>
<td>123.23 ± 10.56 **</td>
<td>172.83 ± 19.97 **</td>
<td>174.20 ± 17.08 **</td>
</tr>
<tr>
<td><strong>LDH (U/L)</strong></td>
<td>424.75 ± 138.56</td>
<td>752.00 ± 61.8 8 **</td>
<td>405.00 ± 121.39</td>
<td>398.75 ± 58.70</td>
<td>446.25 ± 49.18</td>
</tr>
<tr>
<td><strong>Urea (mmol/L)</strong></td>
<td>75.00 ± 9.42</td>
<td>71.50 ± 12.87</td>
<td>74.50 ± 17.31</td>
<td>70.25 ± 12.45</td>
<td>52.25 ± 2.36</td>
</tr>
<tr>
<td><strong>Creatinine (mmol/L)</strong></td>
<td>7.80 ± 0.62</td>
<td>6.65 ± 1.24</td>
<td>7.25 ± 2.65</td>
<td>7.53 ± 1.79</td>
<td>6.08 ± 0.13</td>
</tr>
</tbody>
</table>

The values of outcome variables were expressed as mean ± standard deviation. *P* < 0.05, **P** < 0.01, ***P** < 0.001 vs. negative control. ALT: alanine aminotransferase, AST: aspartate aminotransferase, LDH: lactate dehydrogenase, NC: negative control group, PC: positive control group, V500: 500 mg/kg *V. officinalis*-treated group, V1000: 1000 mg/kg *V. officinalis*-treated group, V2000: 2000 mg/kg *V. officinalis*-treated group.
moderately significant mutagenic effect against TA98 strain with and without S9 at 5, and 2.5 mg/ml and a significant effect at 1.25 and 0.625 mg/ml, whereas *V. officinalis* has no mutagenic effects against TA97a, TA1535 and WP2uvrA strains. These results of TA98 and TA100 showed an increased pattern in the number of positive wells of histidine revertants by increasing the concentration of *V. officinalis*, which indicates that their mutagenicity was concentration-dependent. On the other hand, standard mutagens as positive controls including 9-aminacridine (9-AA), 2-nitrofluorene (2-NF), 4-nitroquinoline 1-oxide (4-NQO), sodium azide (SA), and 2-aminoanthracene (2-AA) with and without S9 metabolic activator significantly induced a positive response. The above-mentioned findings suggested the existence of some compounds in *V. officinalis*, which might be responsible for the frame-shift mutations in TA98 and base pair substitutions in TA100 with S9 metabolic activator (Guan et al., 2017). However, a positive result was obtained in the *in vitro* study does not by itself indicates that *V. officinalis* can cause cancer (Wu et al., 2011). Nevertheless, it proposes that *V. officinalis* metabolites might produce mutations.

In general, the aqueous extracts from herbal medicine are complex mixtures that have different biological potential in their cells such as antimutagenic, mutagenic and toxic activities. When acting together, the synergism, additivism and antagonism of these substances can lead to a different pattern of response for each of the mixtures (Schimmer et al., 1988). The pharmacological properties of *V. officinalis* were attributed to the presence of several biologically-active constituents, such as iridoid glycosides, flavonoids, ursolic acid and apigenin (Chen et al., 2006; Deepak and Handa, 2000; Tian et al., 2005). In addition, various solvent extracts of *V. officinalis* were reported to exert antioxidant and anti-fungal properties (Casanova et al., 2008). The safe use of herbal medicine is commonly presumed in traditional medicine. This safety is based on their belief that herbal medicines are natural and safe, therefore are free from the harmful effects of drugs as compared to synthetic drugs. Additionally, the long-term use of the herbal medicines for the treatment of various diseases according to knowledge which has been accumulated over centuries had led to the perception that these drugs must be safe (Della Torre et al., 2011). However, recent scientific

![Fig. 5. Histological sections of liver of male rats. The liver sections were stained with Hematoxylin and eosin stain. A: negative control group, B: positive control group treated with 50 mg/kg cyclophosphamide, C: 500 mg/kg *V. officinalis*-treated group, D: 1000 mg/kg *V. officinalis*-treated group, E: 2000 mg/kg *V. officinalis*-treated group. Arrows point to mild cells infiltration. Otherwise, the liver sections showed normal hepatocytes. Magnification power 50 x.](image-url)
data have demonstrated that many natural plants used as food ingredients or in traditional medicine could be potentially toxic, mutagenic or carcinogenic (Fennell et al., 2004). Moreover, the examination of faecal samples of animals and humans indicated that a number of plants contained mutagenic substances that exist in plants as an inactive conjugated form such as glycosides (Maron and Ames, 1983). Furthermore, the mutagenicity of some flavonoids and tannins have been reported (Brown and Dietrich, 1979; De Serres and Hollaender, 2012; Macgregor and Jurd, 1978; Sugimura and Nagao, 1980). One of those flavonoids is apigenin, which was isolated previously from the leaves of *V. officinalis* (Deepak and Handa, 2000), has been reported to be mutagenic particularly in TA100 and TA98 strains with S9 activator (Nagao et al., 1981). Perhaps our above-mentioned *in vitro* findings of mutagenic activity of *V. officinalis* could be attributed to the presence of apigenin. In addition, luteolin, a flavonoid compound, also showed signs of mutagenicity in TA102 strain using Ames assay (Resende et al., 2012).

4.2. *In vivo* bone marrow micronucleus assay

*In vivo* bone marrow micronucleus assay (MN test) is a valuable technique for assessing genetic damage, which has been utilized for a long time for examination of genotoxicity of several herbal medicines, particularly those of pharmaceutical interest (Junior et al., 2015). The *in vivo* bone marrow micronucleus protocol was based on OECD guideline number 474 for the mammalian erythrocyte micronucleus test. The data of the mammalian erythrocyte micronucleus test demonstrated relevant differences between females and males, particularly in metabolism, systemic toxicity, bioavailability and bone marrow toxicity (in the range-finding study), which would encourage the use of both male and female (OECD, 2016). The mutagenic potential of mutagen is estimated by measurement of micronucleated polychromatic erythrocytes (MNPCE) in bone marrow of rodents, therefore an increase in the percentage of (MNPCE) in treated rats is indicating of induced chromosomal damage (Thybaud et al., 2007). While the alteration in
the ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE: NCE) as compared to their negative control animals provides a good cytotoxicity index (Krishna and Hayashi, 2000).

OECD guidelines 474 (OECD, 2016) has suggested three dose levels to cover a range from the maximum to little or no toxicity. According to OECD guideline, if genotoxicity would not be obtained, then the maximum dose of 2000 mg/kg/day can be considered. For our experiment, three dose levels (500, 1000 and 2000 mg/kg) of *V. officinalis* extract were tested and findings of animal study showed neither signs of toxicity nor mortality or morbidity were observed in female or male rats during the treatment period. In addition, no significant changes were observed in body weight of females or males even in those treated with 2000 mg/kg of *V. officinalis*.

Our findings demonstrated that exposure to different doses of *V. officinalis* could not provoke a significant increase in MNPE frequency as compared to negative control indicating no genotoxic effects in PCE of bone marrow. To detect genotoxicity, the number of immature to mature erythrocytes (PCE/NCE ratio) was calculated (Salmani et al., 2015). When bone marrow cells proliferation is affected by a toxic substance, a decrease in PCE/NCE ratio occurred reflecting bone marrow toxicity and cell depression (Kim et al., 2011), which was similar to the findings in the positive control (CPA). Our findings showed no significant reduction in PCE/NCE ratio at any dose of *V. officinalis* as compared to negative control. Therefore, these results indicated that *V. officinalis* has no genotoxic effect, which might be due to the antioxidant activity of phenolic compounds of *V. officinalis* that provide a protection against genotoxicity (Casanova et al., 2008).

Several studies have also reported inconsistent findings between in *vitro* and *in vivo* assays (Dearfield et al., 2002). For mutagenicity evaluation, the Ames test (bacterial mutation assays) is used as an initial screening test because it produces reliable results (Mortelmans and Zeiger, 2000). Reports from several laboratories have shown that many non-carcinogenic agents produce misleading positive results in one or other regulatory genotoxicity assays (Walmsley and Billinton, 2011).

Fig. 7. Histological sections of kidney of male rats. The kidney sections were stained with Hematoxylin and eosin stain. A: negative control group, B: positive control group treated with 50 mg/kg cyclophosphamide, C: 500 mg/kg *V. officinalis*-treated group, D: 1000 mg/kg *V. officinalis*-treated group, E: 2000 mg/kg *V. officinalis*-treated group. All kidneys sections showed normal histology. Magnification power 50 x.
However, it was also noted that some detected mutagens by in vivo micronucleus test were normal. Furthermore, it was recognized that not all animal's carcinogens are bacterial mutagens (McCann et al., 1975). This was thought to reflect the differences between animals and bacteria, however, these different findings may be due to different environments and variation between the in vivo and in vitro models particularly that human or animals have detoxifying systems which biotransform foreign compounds metabolically to inactive compounds (Kebano et al., 2015). Furthermore, the organism contains vital substances (antigenotoxins) like vitamins that can reduce the activity of genotoxins (Sylianco, 1991). Other studies have postulated that the negative in vivo micronucleus assay findings may have been due to insufficient doses of the herbal extract or its mutagenic metabolite(s), which could not be delivered to the target cells of the bone marrow to produce deoxyribonucleic acid (DNA) damage (Dearfield et al., 2002; Mayer et al., 1998). Although bacterial mutation assays have a high predictive value for carcinogenicity, in most validation studies at least 10% of compounds give results that contradicted with the animal cancer data. Hence, some authorities such as the Organization for Economic Cooperation and Development (OECD), the European Economic Community (EEC) and the US Environmental Protection Agency (EPA) require specific tests to be carried out on certain types of chemicals. Finally, it was recognized that many carcinogens do not actually have a genotoxic mode of action (Shaw and Jones, 1994).

Some of these are potentially useful pharmaceuticals, however, evidence has indicated that the in vitro negative results are usually considered sufficient to indicate lack of mutagenicity, whereas an in vitro positive result is not considered sufficient to indicate that the chemical represents a mutagenic hazard (i.e. it could be a false positive) (Benigni et al., 2012). Moreover, various research studies have been carried out in independent laboratories, to try and find the correlations between mutagenic activity in bacteria and carcinogenicity in animals (Simmon, 1979). The results from all these studies clearly indicated that a chemical found to be mutagenic in any living system should be suspected of possessing a carcinogenic effect. However, it is impossible to provide a single number to express the degree of confidence with which a mutagen can be considered to be a carcinogen or with which a non-mutagen can be regarded as one with no carcinogenic effect (Council, 1982).

The hepatotoxicity and nephrotoxicity potential of V. officinalis is not well established. Accordingly, this study tried to measure vital biomarkers of liver and kidney functions to explore the toxicity of the plant using an animal model (Stickel and Shouval, 2015). The increased level of both ALT and AST are generally associated with hepatotoxicity or toxicity of other organs (Lalibette and Villeneuve, 1996). In addition, urea and creatinine reflect the function of kidneys (Alsalahli et al., 2012). However, lactate dehydrogenase (LDH) levels may also rise in hepatic disease, myocardial and pulmonary infarction. Our findings indicated that the activity of liver enzymes was not altered, however, AST levels of female and male rats were increased after receiving V500, V1000 and V2000 treatment. The activity of liver enzymes may increase with herbal remedies not only with overdose but also with the recommended doses (Danan and Teschke, 2015; Teschke et al., 2013).

On the other hand, the findings of kidney functions demonstrated that creatinine was not affected which could indicate that kidney functions remained normal. Nonetheless, the histopathology sections of liver and kidney of male and female rats showed normal findings, which indicated that V. officinalis is devoid of hepato- or nephrotoxic effects.

5. Conclusion

This study indicated that aqueous extract of V. officinalis has a mutagenic effect, either through base-pair substitution or frameshift mutation in the bacteria as demonstrated in vitro by the Ames test. However, there appears to be no in vivo clastogenic and myelotoxic effect in bone marrow micronucleus of rats. Furthermore, there was a moderate alteration in liver AST enzyme activity of male and female rats, however, histopathology of liver sections of male and female rats indicated normal histology. The presence of tannins and flavonoids in the aqueous extracts may have contributed to positive mutagenic activity, however further studies need to be carried out with a careful analytical approach to unravel the exact identity and quantity of the compounds from the complex mixture which is responsible for the mutagenic effect.

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Author contributions

Abdulmannan H. Fateh performed the experiments and drafted the manuscript; Zahurin Mohamed involved in study design, supervision and reviewed the manuscript; Zamri Chik involved in supervision and reviewed the manuscript; Siti Rosmani Md Zin helped in animal experiment and data curation; Abdul Samad Alsalahi contributed in data analysis and writing the manuscript; Mohammed A. Alshawah involved in study design, supervision and reviewed the manuscript. All authors approved the final manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jeph.2019.02.007.

References


