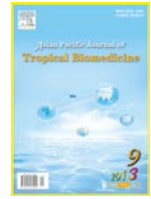




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## Cytotoxicity and genotoxicity assessment of *Euphorbia hirta* in MCF-7 cell line model using comet assay

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## PEER REVIEW

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## Comments

This is a good work. *E. hirta* L., known as “asthma weed” is widely used in traditional medicine for treatment of many ailments. However, there is little toxicological information available regarding the safety evaluation on this plant. The present study evaluated the potential cytotoxicity and genotoxicity of *E. hirta* which can apply in pharmaceutical products developments. Details on Page 696

## ABSTRACT

**Objective:** To evaluate the cytotoxicity and genotoxicity activity of *Euphorbia hirta* (*E. hirta*) in MCF-7 cell line model using comet assay.

**Methods:** The cytotoxicity of *E. hirta* extract was investigated by employing brine shrimp lethality assay and the genotoxicity of *E. hirta* was assessed by using Comet assay.

**Results:** Both toxicity tests exhibited significant toxicity result. In the comet assay, the *E. hirta* extract exhibited genotoxicity effects against MCF-7 DNA in a time-dependent manner by increasing mean percentage of DNA damage. The extract of *E. hirta* showed significant toxicity against brine shrimp with an LC<sub>50</sub> value of 620.382 µg/mL (24 h). Comparison with positive control potassium dichromate signifies that cytotoxicity exhibited by the methanol extract might have moderate activity.

**Conclusion:** The present work confirmed the cytotoxicity and genotoxicity of *E. hirta*. However, the observed toxicity of *E. hirta* extracts needs to be confirmed in additional studies.

## KEYWORDS

*Euphorbia hirta*, Cytotoxicity, Genotoxicity, Cell Line Model

### 1. Introduction

Medicinal plants have played important roles in protecting humans from various diseases throughout the world. Many of the modern drugs existing in clinical use today are of medicinal plant origin. Despite the profound therapeutic advantages possessed by some of the medicinal plants, some constituents of medicinal plants have been found to be potentially toxic, mutagenic, carcinogenic and

teratogenic[1]. However, the potential toxicity of herbs has not been recognized by the general public or by traditional healers[2]. This raises concern about the potential toxic effects resulting from the short-term and long-term use of such medicinal plants. Therefore, evaluating the toxicity effects of any medicinal plants extracts intended to be used in humans and animals is of greatest significance.

*Euphorbia hirta* L. (*E. hirta*) belongs to the family Euphorbiaceae and genus *Euphorbia*, which is commonly

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known as asthma weed. The herb is widely used in traditional medicine to treat a variety of diseased conditions including asthma, coughs, diarrhea and dysentery<sup>[1]</sup>. The sedative, anxiolytic, analgesic, antipyretic and anti-inflammatory properties of *E. hirta* have been reported in the literature<sup>[3]</sup>. Furthermore, studies revealed that *E. hirta* possesses galactogenic, anti-anaphylactic, antimicrobial, antioxidant, anticancer, antifeedant, anti-platelet aggregation, aflatoxin inhibition, antifertility, anthelmintic, antiplasmodial, antiamebic, antimalarial, and larvicidal activities<sup>[4]</sup>. Recent studies also reported various pharmacological activity of this plant extract namely standardization of the extract, anticandidal activity, and antioxidant activity<sup>[5–7]</sup>. Yuet Ping *et al.*<sup>[1]</sup> also tested the potential genotoxic effects of methanolic extracts of *E. hirta* by using *Allium cepa* assay. The extracts of 125, 250, 500 and 1000 µg/mL were tested on root meristems of *Allium cepa*. Ethylmethanesulfonate was used as positive control and distilled water was used as negative control. Result of this study confirmed that the methanol extracts of *E. hirta* exerted significant genotoxic and mitodepressive effects at 1000 µg/mL. Considering that there are few data on the biological effects of the extracts of *E. hirta*, this work aims to evaluate genotoxicity using the comet assay and cytotoxicity activity using brine shrimp.

## 2. Materials and methods

### 2.1. Sample collection and preparation of *Euphorbia hirta* methanol extract

Sample of *E. hirta* was collected from various areas in University Sains Malaysia, Penang in July 2012 and identified by Mr Shanmugam Vellosamy at the Herbarium of School of Biological Sciences, University Sains Malaysia, Pulau Pinang, Malaysia where the voucher specimen was deposited (Number: USM/HERBARIUM/11215). Whole part of *E. hirta* was thoroughly rinsed with tap water and distilled water before air-dried at room temperature. Then, the plant samples were ground to fine powder and soaked in absolute methanol for 4 d with frequent agitation. Extract was filtered with Whatman paper No. 1 and the residue was then re-soaked with a fresh portion of methanol twice. The filtrate was then concentrated by rotary evaporator under reduced pressure. The yield of the extract was 17.1% based on dry weight. The dried residue of plant extract was resuspended in dimethylsulfoxide (DMSO) (Sigma, USA) for further biological assays.

### 2.2. Chemicals

Dulbecco's modified eagle's medium, fetal bovine serum, trypsin, penicillin and streptomycin were purchased from Gibco (Invitrogen, USA). OxiSelect™ Comet Assay Kit was purchased from Cell Biolabs, Inc. Doxorubicin hydrochloride

(Sigma) was the standard drugs used as positive control in this study.

### 2.3. Brine shrimp lethality assay

#### 2.3.1. Hatching shrimp

Brine shrimp eggs, *artemia salina*, were hatched in artificial seawater prepared by dissolving 38 g of sea salt in 1 L of distilled water. After a 24-h incubation period at room temperature (22–29 °C), the larvae were attracted to one side of the vessel with a light source and collected with a pipette. The larvae were separated from the eggs by aliquoting them three times in small beakers containing seawater.

#### 2.3.2. Brine shrimp assay

The cytotoxicity of the *E. hirta* extract was monitored by the brine shrimp lethality test<sup>[8]</sup>. Samples were dissolved in DMSO and diluted with artificial seawater. Two milliliters of seawater was placed in all the bijoux bottles. A twofold dilution was carried out to obtain the concentration from 5000 to 19.531 µg/mL. Potassium dichromate served as positive control and was prepared by dissolving in artificial seawater to obtain the concentration from 500 to 1.953 µg/mL (Colegate & Molyneux, 1993). The last bottle was filled with sea salt water and DMSO only, serving as a drug-free control or negative control. A suspension of larvae (0.1 mL) containing about 10–15 larvae was added into each bottle and incubated for 24 h. The bottles were then examined, and the number of dead larvae in each bottle was counted after 24 h. The total number of shrimp in each bottle was counted and recorded. The mean percentage mortality was plotted against the logarithm of concentrations, and the concentration that could kill 50% of the larvae (LC<sub>50</sub>) was determined from the graph.

#### 2.3.3. Calculations and statistics

Lethal concentration (LC<sub>50</sub>) for *A. salina* with 95% confidence level was determined by Probit analysis on a Finney computer program (BioStats 2009)<sup>[9]</sup>. Percentage mortalities were corrected for the natural mortality observed in the negative controls using Abbott's formula,  $p = (p_i - C) / (1 - C)$ , where  $p_i$  denotes the observed mortality rate and C means the natural mortality. Extracts giving LC<sub>50</sub> values greater than 1.0 mg/mL were considered to be nontoxic<sup>[10]</sup>.

### 2.4. Comet assay

#### 2.4.1. Cell culture

MCF-7 (breast adenocarcinoma cells) was obtained from the American Type Culture Collection (ATCC, USA). All cells were cultured in Dulbecco's modified eagle's medium (Gibco) supplemented with 10% fetal bovine serum, penicillin-streptomycin 1% (v/v) (Gibco, USA). The cells were cultured in 5% CO<sub>2</sub> incubator at 37 °C in a humidified atmosphere.

### 2.4.2. Evaluation of genotoxicity

The comet assay was performed under alkaline conditions according to Singth *et al.*[11]. Cells were seeded in 6-well tissue-culture plates and incubate for 24 h for cell attachment. Subsequently, cells were treated with increasing concentration of *E. hirta* and doxorubicin hydrochloride (positive control) for 24 h. Cells were harvested by trypsinisation, washed with PBS and resuspended in ice-cold PBS. About 7.5  $\mu$ L of the resuspended cells was mixed with 75  $\mu$ L of low melting point agarose at 37 °C and spread the suspension over the well with the pipette tip. The slides were placed at 4 °C in the dark until gelling occurred and then immersed in pre-chilled lysis buffer at 4 °C. After 60 min incubation, the buffer was aspirated and replace with pre-chilled alkaline solution for 30 min at 4 °C. After lysis and unwinding, the slides were placed in a horizontal electrophoresis tank filled with freshly prepared alkaline electrophoresis buffer. The electrophoresis was run for 20 min at 35V and 300 mA. After electrophoresis, the slides were transferred to pre-chilled distilled water and immerse for 2 min, aspirate and repeat twice. The final water rinse was aspirated and replace with cold 70% ethanol for 5 min. Thereafter, the slides were allowed to air dry and 100  $\mu$ L/well of diluted Vista Green DNA dye was added to each slide for 15 min in the dark at room temperature for DNA staining. DNA migration was observed using fluorescence microscope at a magnification of 10X (Carl Zeiss Apo Tome, Germany). For each concentration, 100 randomly selected cells (50 cells from each of the two replicate slides) were analysed. The tail DNA % and tail moment were analyzed using TriTek CometScore TM software.

### 2.4.3 Statistical analysis of data

Data were expressed as mean $\pm$ standard error of mean (SD) from at least two independent experiments. Statistical analysis was performed by using Student's t-test or one-way analysis of variance (ANOVA). A value of  $P < 0.05$  was considered to be statistically significant. SPSS Version 16.0 was used.

## 3. Results

### 3.1. Brine shrimp lethality assay

Results of the toxicity evaluation against brine shrimp of the *E. hirta* extract are shown in Figures 1, 2, and 3. The extract of *E. hirta* showed significant toxicity against brine shrimp with an  $LC_{50}$  value of 620.382  $\mu$ g/mL (24 h) Figure 1. Figure 3 shows a light microscope micrograph of the death of *A. salina* after treatment with the methanol extract of *E. hirta*. However, the positive control, potassium dichromate was exhibited significant toxicity ( $LC_{50}$  value < 1.0 mg/mL) against the brine shrimp (Figure 2).

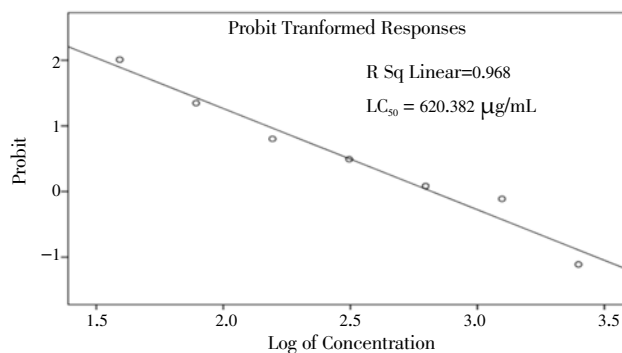


Figure 1. Toxic effects of the *E. hirta* extract after 24 h using brine shrimp lethality assay.

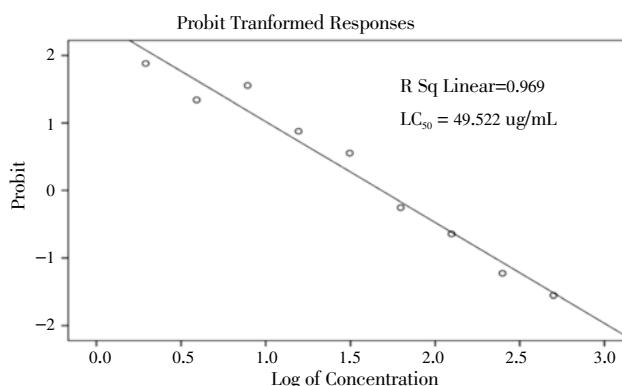


Figure 2. Toxic effects of potassium dichromate after 24 h using brine shrimp lethality assay.



Figure 3. Light microscope micrograph of the *E. hirta* extract-treated *Artemia salina*.

### 3.2. Comet assay

The results of mean percentage of DNA damage and photomicrographs of DNA damage are shown in Table 1 and Figure 4, respectively. Methanol extract of *E. hirta* showed genotoxicity activity in an incubation time-dependent manner. The exhibited genotoxicity effects were also comparable to those of the positive control doxorubicin hydrochloride tested in this study. In addition, photomicrographs of different DNA migration profiles were obtained (Figure 4). In the group

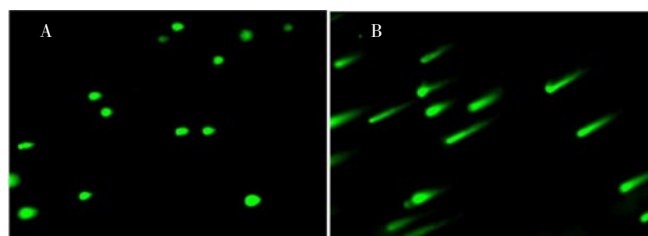
treated only with *E. hirta*, the DNA was completely damaged and the amounts of tail DNA were significantly increased over times of incubation (Table 1).

**Table 1**

Mean percentage of DNA damage by the comet assay in MCF-7 cells treated with different concentrations of *E. hirta* and doxorubicin hydrochloride.

Treatments	%DNA in tail Mean±SD	Tail Movement Mean±SD
Control	16.3518±4.7091	3.6292±2.8380
25µg/ml – 24h	42.4681±9.3168*	42.2773±16.4798*
25µg/ml – 48h	45.1525±10.4713*	45.7786±19.9437*
25µg/ml – 72h	48.1694±9.5570*	55.3530±20.2604*
Doxorubicin hydrochloride (0.3993µg/mL)	44.223±9.3756**	40.3232±15.3359*

A total of 100 nucleoids were analyzed for each group to obtain % DNA in tail. Significant differences compared between control and treated cells (\* $P < 0.05$ ).



**Figure 4.** Photomicrographs of stained DNA of MCF-7 cells for alkaline comet assay. (A) Untreated showing no DNA damage and (B) Treated with *E. hirta* (25 µg/mL) for 24 h showing DNA damage in comet tail.

#### 4. Discussion

During the past decade, traditional systems of medicine have become increasingly important in view of their safety. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs[12]. Isolation of bioactive compounds from natural sources requires toxicity information on the constituent of interest. It should be emphasized that toxic effects of the antimicrobial agent on the host cells must be considered, as a substance may exhibit an apparent antimicrobial activity by virtue of its toxic effect on the cells[13]. In this report, results of screening of methanolic extract of *E. hirta* used in the traditional medicine for genotoxicity and lethality towards brine shrimps larvae are presented.

The brine shrimp lethality bioassay was used in this study to determine the cytotoxicity of the *E. hirta* extract. The brine shrimp lethality bio assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and antitumor properties. The assay is considered to be a very useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, toxicity of plant extracts, heavy metals, and cytotoxicity testing of dental materials[14]. Since LC<sub>50</sub>

value less than 1000 µg/mL are consider significant for crude extract[15], the lethality of the extract of *E. hirta* increased with concentration suggesting the presence toxic compound/s in *E. hirta* crude extract which deserved further detail study on phytochemistry of this plants. Comparison with positive control Potassium dichromate signifies that cytotoxicity exhibited by the methanol extract might have moderate activity.

The balance between the therapeutic and toxicological effects of a compound is a very important measure of the usefulness of a pharmacological drug. Therefore, the determination of the potential mutagenic effect of *E. hirta* extract which exhibited cytotoxic activity is mandatory[16]. Consequently, comet assay was used in this study for further evaluation on the potential mutagenic effect of *E. hirta* extract. In the comet assay, it was possible to quantify and to distinguish cells with different rates of DNA damage, thus the analysis of the average values of the scores for each treatment group was very important[17]. The comet assay has been established as a simple, rapid, cheap, flexible and, most importantly, sensitive method to detect DNA damage, which is also able to detect DNA damage in individual cells. In this assay, cells are embedded in agarose, lysed in an alkaline buffer, and subjected to an electric current. Relaxed and broken DNA fragments stream further from the nucleus than intact DNA, so the extent of DNA damage can be measured by the length of the stream[18]. In the present study, the treatment of 25 µg/mL of *E. hirta* extract for 72 h on MCF-7 cells caused an increase in DNA damage by approximately 48.16% compared to the unchallenged control. These data confirm genotoxic effect of *E. hirta* extract. Therefore, there are genotoxic compounds in this plant that must be evaluated in future studies. Our previous results are in comparison with findings of this study, which showed that the methanol extracts of *E. hirta* exerted significant genotoxic and mitodepressive effects at 1000 µg/mL[1] in *A. cepa* assay.

The present study documents that *E. hirta* extract display cytotoxicity activity against brine shrimp and marked genotoxicity in the comet assay. However, the observed toxicity of *E. hirta* extracts needs to be confirmed in additional studies. After detailed *in vivo* and *in vitro* evaluation and thorough toxicologic studies, *E. hirta* methanol extract may find use as a pharmacological agent in known dosages, especially in rural communities, where conventional drugs are unaffordable or unavailable and health facilities are inaccessible.

#### Conflict of interest statement

The authors declare no conflict of interest.

#### Acknowledgements

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## Comments

### Background

The objective of this study was to evaluate the cytotoxicity and genotoxicity assessment of *E. hirta* in MCF-7 cell line model using Comet assay and *A. salina*.

### Research frontiers

The author evaluated the genotoxicity of *E. hirta* by using cellular model.

### Related reports

Ping et al., 2012 reported the genotoxic effects of *Euphorbia hirta* by using using *Allium cepa* assay. The result showed that mitotic index decreased as the concentrations of *E. hirta* extract increased. A dose-dependent increase of chromosome aberrations was also observed. Abnormalities scored were stickiness, c-mitosis, bridges and vagrant chromosomes. Micronucleated cells were also observed at interphase. Result of this study confirmed that the methanol extracts of *E. hirta* exerted significant genotoxic and mitodepressive effects at 1000 µg/mL.

### Innovations and breakthroughs

This study has showed that the *E. hirta* extract display cytotoxicity activity against brine shrimp and marked genotoxicity in the comet assay.

### Applications

*E. hirta* extract commonly used in the traditional medicine. Therefore extra precautions will need to be followed in setting the safe concentration of this plant during the development of pharmaceutical products based on the reported data.

### Peer review

This is a good work. *E. hirta* L., known as “asthma weed” is widely used in traditional medicine for treatment of many ailments. However, there is little toxicological information available regarding the safety evaluation on this plant. The present study evaluated the potential cytotoxicity and genotoxicity of *E. hirta* which can apply in pharmaceutical products developments.

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