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Assessment of genotoxicity and cytotoxicity of standardized aqueous extract from leaves of *Erythroxylum cuneatum* in human HepG2 and WRL68 cells line

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ABSTRACT

Objective: To investigate the cytotoxicity and the genotoxicity of standardized aqueous of dry leaves of *Erythroxylum cuneatum* (*E. cuneatum*) in human HepG2 and WRL68 cells. **Methods:** The cytotoxicity of *E. cuneatum* extract was evaluated by both MTS and LDH assays. Genotoxicity study on *E. cuneatum* extract was assessed by the single cell gel electrophoresis (comet assay). The protective effect of *E. cuneatum* against menadione-induced cytotoxicity was also investigated. **Results:** Results from this study showed that *E. cuneatum* extract exhibited cytotoxic activities towards the cells with IC_{50} value of (125±12) and (125±14) μg/mL for HepG2 and WRL68 cells respectively, after 72 h incubation period as determined by MTS assay. LDH leakage was detected at (251±19) and (199.5±12.0) μg/mL for HepG2 and WRL68 respectively. Genotoxicity study results showed that treatment with *E. cuneatum* up to 1 mg/mL did not cause obvious DNA damage in WRL68 and HepG2 cells. Addition of *E. cunaetum* did not show significant protection towards menadione in WRL68 and HepG2 Cells. **Conclusions:** *E. cuneatum* standardized aqueous extract might be developed in order to establish new pharmacological possibilities for its application.

1. Introduction

Human have relied on plants as a source of medicinal agents for centuries to treat a wide range of health issues. The belief that natural medicines are much safer than synthetic drugs has gained popularity in recent years, leading to a tremendous growth of phytopharmaceutical usage. In recent years there has been a growing interest in identifying naturally constituents against the development of several diseases. There is a trend towards increasing the variety of plant products consumed by the population by introduction of herbal `remedies′ or health foods in Western societies[1]. The search for inhibitors of mutagenesis may be useful as a tool to discover anticarcinogenic agents. On the other hand most of the traditional medicinal plants have never been the subject of exhaustive toxicological tests such as is required for modern pharmaceutical compounds. Based on their traditional use for long periods of time they are often assumed to be safe. However, research has shown that a lot of plants which are used as food ingredients or in traditional medicine have *in vitro* mutagenic[2,3] or toxic and carcinogenic properties[4]. Within this context, it is also important to screen medicinal plants for their mutagenic properties. Plants exhibiting clear mutagenic properties should be considered as potentially unsafe and certainly require further testing before their continued use can be recommended. Plants with obvious antimutagenic potential can, on the other hand, be considered interesting for therapeutic use and merit further in depth investigations of their pharmacological properties *Erythroxylum cuneatum (E. cuneatum)* which belongs to Erythroxylaceae family is a tropical flowering plant from the genus of Erythroxylum. The Malaysian species is confined to the substage of the primary

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rain forest up to 1 600 m, obviously avoiding areas subject to dry season. *E. cuneatum* is an evergreen and sometimes deciduous, small to fairly large trees, reaching 40 m high, with fine-leafed crown; trunk brown, the bark closely and narrowly ridged and fissured. The twigs are green, flattened and marked with transverse lines (stipule-scars). The leaves are alternate, often distichous, simple, entire, often with two longitudinal lines on the upper surface; stipules triangular, clasping the twig, caduceus[5].

One of the 250 species in the genus is *Erythroxylum coca*, the source of the drug cocaine. Calystegines were identified in the genus *Erythroxylum* with the dry leaves containing 0.2% total calystegines. Simultaneous occurrence of calystegines, cocaine and other alkaloids of a 3 α -hydroxy- or 3 β -hydroxytropane structure together with nicotine showed that these alkaloids share common biosynthetic steps in *Erythroxylum*[6].

E. cuneatum has remained elusive in traditional medicine except for few reports saying that the leaves of this tree were used as a fish poison in the Philippines and might be used as tonics for miscarriage in Malaysia[5]. Data from our laboratory has shown that the aqueous extract of dry leaves showed antioxidant properties and reduction of glucose levels in animal experiment (data not published). However data on the cytotoxicity and genotoxicity of the aqueous extract of dry leaves of *E. cuneatum* is unavailable at present. Cytotoxicity and genotoxicity studies using cell lines provide some indication of a plant's safety profile. In order to establish new pharmacological possibilities for *E. cuneatum* application, the overall objective of the present work was to investigate the cytotoxicity and genotoxicity of the standardized aqueous extract of dry leaves of *E. cuneatum* on human HepG2 and WRL68 cells line.

2. Materials and methods

2.1. Chemicals

Dulbecco's minimum essential medium (DMEM), RPMI, foetal bovine serum (FBS), penicillin, streptomycin, trypsin and phosphate buffered saline (PBS), were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Co., St. Louis, Missouri). (100 IU/mL penicillin and 100 mg/mL streptomycin) was purchased from PAA Laboratories (PAA Laboratories, Austria). tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) was purchased from Promega (Promega USA). Hydrogen peroxide (H_2O_2) was purchased from Bendosen Laboratory Chemicals (Bendosen Chemicals, Selangor, Malaysia).

2.2. Plant extract

The standardized aqueous extract of *E. cuneatum* was obtained from the Forest Research Institute of Malaysia (FRIM).

2.3. Cytotoxic effect of E. cuneatum on HepG2 and WRL68 cells (MTS assay)

Human liver cancer cells (HepG2 cells) and normal embryonic liver cell line (WRL 68) were purchased from the American Type Culture Collection (ATCC), USA. Cells were seeded in 96-well micro plates (2 x104 cells/well in 100 uL of complete DMEM for HepG2, WRL68 and in 100 μL of complete RPMI for 24 h. Cells were then, incubated with *E. cuneatum* (0.005-5 mg/mL) for 72 h. At the end of incubation, 20 μL of MTS was added to each well and incubation was allowed to continue for a further 2 h. Finally, the plates were read using a Microplate Reader (Palkin Elmer, USA), at a wavelength of 490 nm. The dose-response curve was plotted and the concentration which gave 50% of cell growth (IC_{50}) was calculated.

2.4. Determination of cytotoxicity using the lactate dehydrogenase assay (LDH)

Cytotoxicity was also determined by lactate dehydrogenase (LDH) leakage. Cells (2×10^4) were seeded into a 96-well plate and pre-cultured for 24 h. Then, cells were treated with different concentrations of *E. cuneatum* (0.005-5 mg/mL), H_2O_2 (10-100 μM), menadion (10-100 μM) and with medium containing 1% Triton X-100 as a high control for 24 h, and then incubated in an incubator (5%CO_2, 95%O_2, 37 ℃) for the appropriate time of treatment determined for the test substance, cells were centrifuged at 250 rpm for 10 min, 100 μL/well supernatant was transferred into corresponding wells of an optically clear 96-well plate, 100 μL of the reaction mixture was added to each well followed by incubation for up to 30 min at room temperature, with protection from light, the absorbance of all samples was measured at 490-520 nm using a Microplate Reader (Palkin Elmer, USA), with the reference wavelength greater than 520 nm. LDH activity is determined by a coupled enzymatic reaction. LDH oxidizes lactate to pyruvate which then reacts with tetrazolium salt to form formazan. The increase in the amount of formazan produced in culture supernatant directly correlates to the increase in the number of lysed cells. The formazan dye is water soluble and can be detected by spectrophotometer at 520 nm, (Bio Vision Research products).

2.5. Analysis of DNA damage (Comet assay)

Cells were plated on multiwell system at a density of 4 $\times10^5$ cells/mL culture medium. After 24 h of growth, cells were exposed to different concentrations of *E. cuneatum* (0.005 -1 mg/mL) and 50 μM of H_2O_2 as a positive control.

After 24 h, cells were washed with PBS, and added with 300 μL of Trypsin, then incubated for 3 minutes and simultaneously added with 1 mL of MEM+10%FMS media. Cells were detached by pipetting. The cells suspension were placed in eppendorf tubes and centrifuged for 3 min at 1000 rpm. The supernatant was removed and the cells were resuspended in 100 μL of PBS (kept on ice). DNA damage was estimated using single cell gel electrophoresis (SCGE or comet assay). Fully frosted slides were covered with 0.6% of NMA as the first layer, a mixture of cell suspension and 0.6% of LMA as the second layer, and finally with 0.6% of LMA (without cell) as the third layer. After solidification at 4℃, all slides were immersed in the lysing buffer containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris and 1% Triton-X (pH 10) at 4℃ for 1 hour, and the slides were then placed in a horizontal electrophoresis tank. The tank was filled with freshly prepared electrophoresis solution consisting of 300 mM NaOH and 1 mM Na_2EDTA (pH 13), and then the slides were left in the solution for 20 minutes to allow DNA unwinding and expression of alkali labile damage before electrophoresis. Electrophoresis was then conducted at 4℃ for 20 minutes using 25 V and 0.3 A. After electrophoresis, the slides were neutralized in neutralization buffer, stained with ethidium bromide, and kept in a humidified airtight container and examined using a fluorescence microscope. The degree of DNA damage was graded visually into 5 categories according to the amounts of DNA in the tail[7]. Grade 0, no damage, 5%; Grade 1, low level damage, 5%-20 %; Grade 2, medium level damage, 20%-40 %; Grade 3, high level damage, 40%-95 %; Grade 4, total damage, 95 %.

2.6. Protective effect of E. cuneatum against cell lines treated with menadione

To determine any protective effect of *E. cuneatum* in menadione-treated cells HepG2 and WRL68 cells were precultured in 96-well microplates (2×10^4 cells / well in 100 μL of MEM+10%FBS) for 24 h. Menadione-induced toxicity was estimated by incubating cells with different concentrations of menadione (10-100 uM) for 24 h. The effect of *E. cuneatum* extract on menadione-induced cytotoxicity was determined by treating cells with different concentrations of *E. cuneatum* (5-50 μg/mL) and (12-30 μM) of menadione in 100 μL of MEM+10%FBS for 24 h. MTS (20 μL) was added to each well and incubation was allowed to continue for a further 2 h. Finally, the plate was read using a microplate reader at a wavelength of 490 nm using a Microplate Reader (Palkin Elmer, USA).

2.7. Statistical analysis

Each experiment was repeated at least thee times and the data are reported as the mean± SD. One-way ANOVA was used to compare the results from different treatments and control cells. The statistical significance was indicated by *P*-values < 0.05. Data were analyzed using the SPSS window program version 14.0.

3. Results

3.1. The cytotoxicity of E. cuneatum in HepG2 cells and WRL68 cells (MTS assay)

As shown in Figure 1 and 2, the growth of the HepG2 and WRL68 cells in the presence of various concentrations of *E. cuneatum* ranging between 0.005 and 5 mg/mL was examined. Under the experimental conditions, *E. cuneatum* extract exhibited growth inhibitory effects on both HepG2 and WRL68 cells over a 72 h period. The IC_{50} values were (125±12) and (125±14) μg/mL for HepG2 and WRL68 cells respectively.

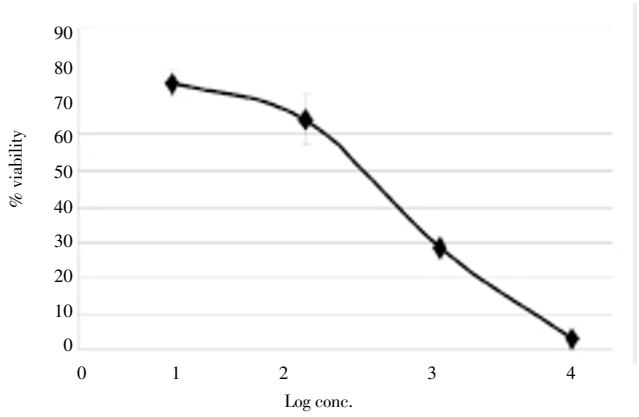

Figure 1. Cytotoxic effect of *E. cuneatum* against HepG2 cells. HepG2 cells were seeded into 96-well plate and incubated overnight, then cells were exposed to medium containing different concentrations (0.005-5 mg/mL) of *E. cuneatum* for 72 h. The IC_{50} value was (125±12) μg/mL as detected by the MTS method. Each value presents the average of 3 replicates± SD.

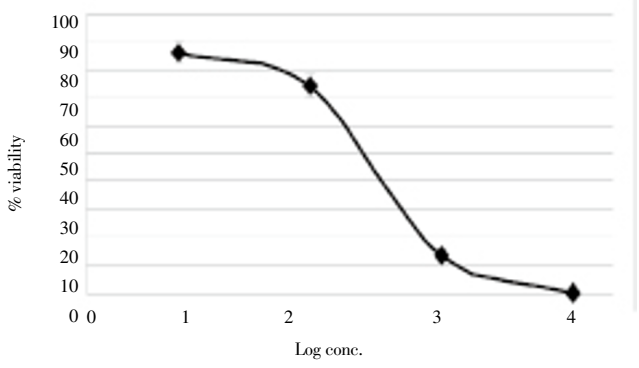

Figure 2. Cytotoxic effect of *E. cuneatum* against WRL68 cells. Each value presents the average of 3 replicates± SD. WRL68 cells were seeded into 96-well plate and incubated overnight, then cells were exposed to medium containing different concentrations (0.005-5 mg/mL) of *E. cuneatum* for 72 h. The IC_{50} value was (125±14) μg/mL as detected by the MTS method. Each value presents the average of 3 replicates± SD.

3.2. The cytotoxicity of E. cuneatum in HepG2 and WRL68 (LDH assay)

As shown in Figure 3 and 4, LDH leakage were detected at (251±19) and (199.5±12.0) μg/mL for HepG2 and WRL68 cells respectively which indicates that *E. cuneatum* at indicated concentrations kill the cells via necrosis due to loss of membrane integrity and LDH leakage.

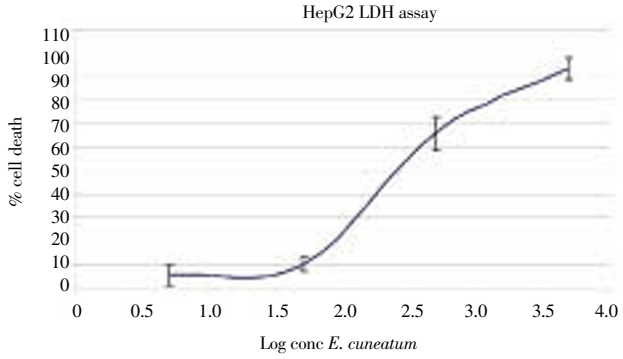

Figure 3. Analysis of LDH leakage for HepG2 cells.
HepG2 cells were cultured in 96 well plate and incubated with *E. cuneatum* (0.005-5 mg/mL) for 24 h. Data are presented as means± SD (*n*=3). LDH leakage level was detected at (251±19) μg/mL.

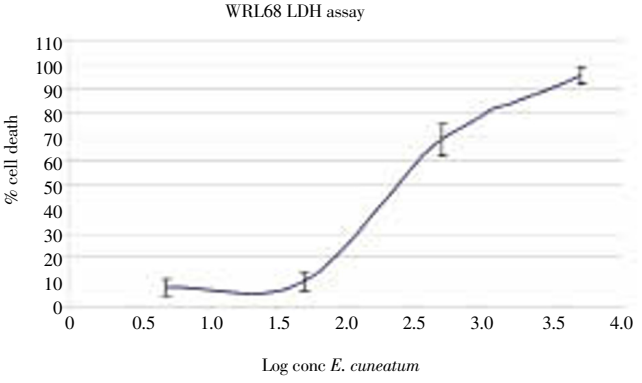

Figure 4. Analysis of LDH leakage for WRL68 cells.
WRL68 cells were cultured in 96 well plate and incubated with *E. cuneatum* (0.005-5 mg/mL) for 24 hr. Data are presented as means± SD (*n*=3). LDH leakage were detected at (199.5±12.0) μg/mL.

3.3. Effect of E.cuneatum on DNA damage

The effect of *E. cuneatum* on DNA damage is presented in Figure 5 and 6, it was found that WRL68 and HepG2 cells treated with 50 μM H_2O_2 resulted in serious DNA damage. The damage was mainly composed of grades 3 and 4 damage. The results also indicated that treatment with E.cuneatum up to 1 mg/mL did not cause obvious DNA damage in WRL68 and HepG2 cells.

3.4. Effect of E. cuneatum extract in menadione-induced cytotoxicity in HepG2 and WRL68

Menadione (30 μM, equivalent to its IC_{50} in these cells) was incubated with HepG2 cells under standard conditions. To determine any cytoprotective effects of the extract, cells were incubated with menadione (30 μM) and with the extract (5 and 50 μg/mL) (Figure 7, 8). Menadione (30 μM) produced about 50 % cell death while the extract of *E. cuneatum* (5 and 50 μg/mL) was non cytotoxic. Co-incubation of cells with menadione (30 μM) and extract (5 or 50 μg/mL) did not lead to any lessening of menadione's cytotoxicity (Figure 7).

Preliminary studies showed that the IC_{50} of menadione in WRL68 cells was about 25uM. To delineate any cytoprotictive effects in WRL68, cells were incubated with menadione 25 μM alone or together with *E. cuneatum* (5 or 50 μg/mL) (Figure 8). The results showed when WRL68 cells incubated with menadione (25uM) and *E. cuneatum* extract (5 or 50 μg/mL) had the same percentage of viability as the cells that were exposed to menadione only (Figure 8).

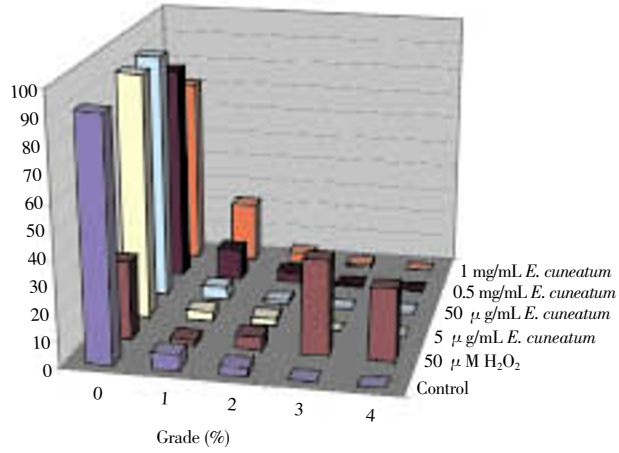

Figure 5. Effect of *E. cuneatum* on DNA damage in HepG2 cells estimated with the comet assay.
Data are presented as percentage of grade (*n* = 3).

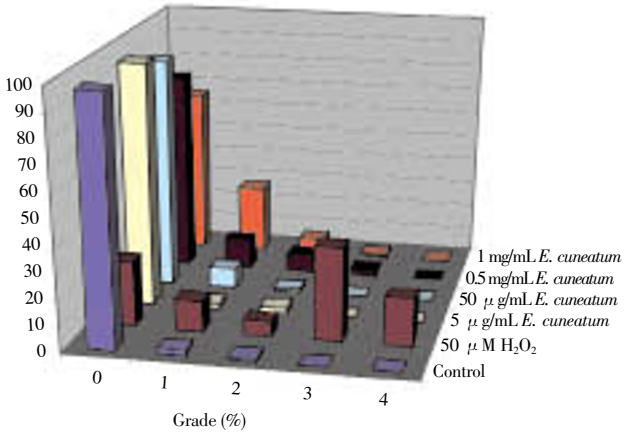

Figure 6. Effect of *E. cuneatum* on DNA damage in WRL68 cells estimated with the comet assay. Data are presented as percentage of grade (*n* = 3).

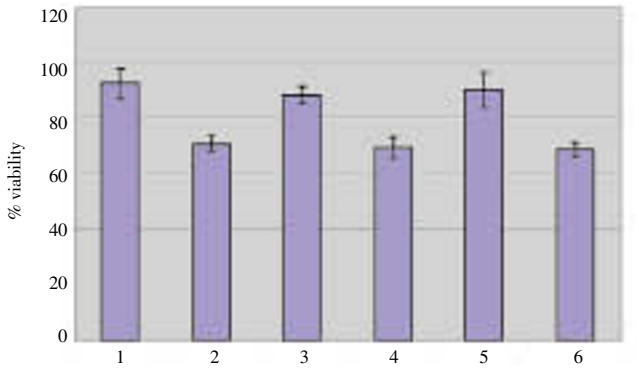

Figure 7. Effect of *E. cuneatum* extract in menadione-induced cytotoxicity in HepG2 cells, data are presented as the mean± S.D (n=3), (1) control, (2) 15 uM menadione, (3) 50 ug/mL *E. cuneatum*, (4) 50 ug/mL *E. cuneatum* + 15 uM menadione, (5) 5 ug/mL *E. cuneatum*, (6) 5 ug/mL *E. cuneatum* + 15 uM menadione.

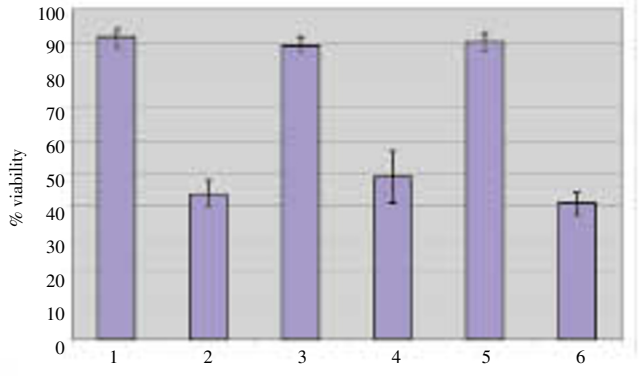

Figure 8. Effect of *E. cuneatum* extract in menadione-induced cytotoxicity in WRL68, data are presented as the mean± S.D (*n*=3), (1) control, (2) 25 μM menadione, (3) 50 μg/mL *E. cuneatum*, (4) 50 μg/mL *E. cuneatum* + 25 μM menadione, (5) 5 μg/mL *E. cuneatum*, (6) 5 μg/mL *E. cuneatum* + 25 μM menadione.

4. Discussion

This study was performed in order to investigate the cytotoxicity and the genotoxicity of standardized aqueous of dry leaves of *E. cuneatum* in HepG2 liver cancer cells and WRL68 normal liver cells. The HepG2 cells, have been shown to be very promising for assessing the genotoxicity[8,9]. The cell proliferation assays were performed in order to demonstrate the cytotoxic effects of aqueous of dry leaves of *E. cuneatum* on HepG2 and WRL6 cells growth. Results indicate a significant decrease (50%) in proliferation of the cells at of (125±12) and (125±14) μg/mL for HepG2 and WRL68 cells respectively, after 72 hours incubation period. *E. cuneatum* showed a concentration-dependent cytotoxicity in HepG2 and WRL68K562 (Figure 1, 2). According to scale by Abbas *et al.*,[10]. E.cuneatum showed to be moderately cytotoxic effect against HepG2 and WRL6 cells growth.

Another study done in our labaroratoy (Data not published) showed that the IC_{50} value of alcoholic *E. cuneatum* extract was (64±4) μg/mL, indicate that the aqueous *E. cuneatum* extract less cytotoxic than alcoholic *E. cuneatum* extract. The differences in the IC_{50} values obtained from this study and the previous study in regard to the HepG2 cells growth may be due to different extracts of *E. cuneatum* being used.

In metabolic active cells, the mitochondrial dehydrogenase enzyme breaks down MTS to purple blue formazan particles. This assay uses MTS and the electron coupling reagent, phenazine methosulfate (PMS). MTS is chemically reduced by cells into formazan, which is soluble in tissue culture medium[11]. PMS is an electron acceptor and carrier in enzyme systems. The oxidized form is yellow and the reduced form is colorless. Since the reduced PMS is easily oxidized by oxygen, it is used in assays as an electron carrier between enzymes and oxygen. Since the production of formazan is proportional to the number of living cells, the intensity of the produced color is a good indication of the viability of the cells.

This study showed that LDH leakage were detected at (199.5±12.0) and (251±19) μg/mL for WRL68 and HepG2 respectively which indicates that *E. cuneatum* at indicated concentrations kill the cells via necrosis due to loss of membrane integrity and LDH leakage. There are two main mechanisms by which cell death occurs. They are apoptosis and necrosis. Apoptosis or cell death which occurs under normal physiological conditions. It is energy dependent cell death. Morphological features of apoptosis involve membrane blebbing (but no loss of membrane integrity), shrinking cytoplasm[12]. Compared with apoptosis, necrosis is energy independent death due to unexpected and accidental cell damage. A number of toxic chemicals or physical events can cause necrosis such as toxins, radiation, heat and trauma. The morphological features of necrosis involve loss of membrane integrity, selling of organelles and complete cell lysis.

Lactate dehydrogenase (LDH), an enzyme that catalyzes the conversion of lactate to pyruvate. This is an important step in energy production in cells. Many different types of cells in the body contain this enzyme. Some of the organs relatively rich in LDH are the heart, kidney, liver, and muscle. LDH requires NAD+ (Nicotinamide adenine dinucleotide) as a hydrogen acceptor[13]. Cytotoxicity measurement is based on the lactate dehydrogenase (LDH) released from damage cells. LDH activity is determined by a coupled enzymatic reaction. LDH oxidizes lactate to pyruvate which then reacts with tetrazolium salt to form formazan. The increase in the amount of formazan produced in culture supenatant directly correlates to the increase in the number of lysed cells. The formazan dye is water soluble and can be detected by sectrophotometer at 520 nm, (Bio Vision Research products). Genotoxicity studies on *E. cuneatum* extract in both cancer and normal cell liens were assessed by the single cell gel electrophoresis (comet assay). The comet assay, also known as the single-cell gel-electrophoresis (SCGE) assay, is a very sensitive test for the quantification of DNA damage and provides direct determination of DNA single- and doublestrand breaks in individual cells. Many authors have used the comet assay to evaluate the *in vitro* and/or *in vivo* genotoxicity/ antigenotoxicity of several chemicals with various cell lines[14]. Cells were treated

with hydrogen peroxide (50 μM) as a positive control and different concentrations of *E. cuneatum* (0.005-1 mg/mL), results showed that there was low level DNA damage at concentration 1 mg/mL. It may be due to the difference of cell physiology like the cell cycle status when the experiments occurred. In fact, chromatin structure changes depending on the cell cycle phase. This chromatin structure can affect the role of the DNA during the comet formation[15]. Another source of heterogeneity in cellular response to H_2O_2 is the interindividual variability[16]. On the another hand, no DNA damage at other concentrations. Previous studies done by[17]. On Zuccagnia punctata ethanolic extract which is native shrub, known under the common names of jarilla pispito, puspus and jarilla macho, is used in folk medicine as foot antiseptic and against bacterial and fungal infections, asthma, arthritis and rheumatism[18,19]. Different concentrations of Zuccagnia punctata were tested with HepG2 cells to check the genotoxicity profile of this extract. Results showed absence of genotoxic response by Zuccagnia extract against HepG2 cells and the extract developed to be traditional medicine and it considered to be safe at indicated concentrations. Compare to our studies E.cuneatum extract even at 500 μg/mL did no show any genotoxic effects, it safer than *Zuccagnia punctata* at indicate concentration and it conceder a positive step forward in determining the safe use of *E. cuneatum* in traditional medicine.

Menadione undergoes one and two electron reduction resulting in the formation of the semi-quinone and hydroquinone, respectively. Semi-quinone radicals react with molecular oxygen at diffusion limited rates to first produce the superoxide anions radical which undergo further reactions including the fenton reaction to produce the highly reactive hydroxyl radical. These reactive oxygen species can directly damage macromolecules including DNA, proteins, and lipid membranes[20]. HepG2 and WRL68 cells were treated with different concentrations of menadione (10-100 μM), maximum cell death was observed with the highest concentration (100 μM). The inhibitory concentration IC_{50} values for menadione in HepG2 and WRL68 cells were (30.0 ±2.2) and (25±2) μM, respectively. E.cuneatum extract at different doses of 5 and 50 μg/mL) was not protective to when cells were incubated with menadione (IC_{50}) indicating that the extract was not able to rescue cells from the cytotoxicity effects of menadione. That may be was due to the used dose of the extract was not the active dose.

These results indicate that *E. cuneatum* is not potent against menadione at the used dose and more experiments need to be carried out to determine whether *E. cuneatum* is cytoprotective via its antioxidant effect or via different mechanisms. Further studies should be designed to isolate, identify, and characterize the active constituents of *E. cuneatum* standardized aqueous extract. also there is a need to perform an animal study provide a greater understanding of the safety of the *E. cuneatum* extract.

Conflict of interest statement

We declare that we have no conflict of interest.

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