JEPIBIO
Journal of Epidemiology and Infection Biology

Research Article

Cytotoxic and Genotoxic Effects of *Eucalyptus globulus* on Vero Cell line

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Date of Receiving: 08/09/2024
Date of Acceptance 05/11 /2024
Date of Publishing 10/12/2024

ABSTRACT *Eucalyptus globulus* (*EG*) of the Myrtaceae family has been traditionally used in the treatment of various infectious disorders. Cytotoxic and Anti-proliferative studies of Eucalyptus extracts and oils have been undertaken, enlightening beneficial effects against numerous cancer cell lines. However, no study has been documented on the cyto-genotoxic effects of *EG* on Vero cell line. This study aims to assess the potential cytotoxic and genotoxic effects of *EG* in Vero cell lines. The MTT, Comet assay, Micronucleus (MN), and chromosomal aberrations were performed on Vero cell line after the exposure of *EG*. Furthermore, three concentrations i.e. LC50/2 (GB1), LC50 (GB2), 2*LC50/2 (GB3), were used to assess the concentration dependent association in DNA damage, micronuclei, and chromosomal aberrations assessment. *Eucalyptus globulus* showed cytotoxic effects and reduced cell viability at higher concentration after 24 h exposure. Similarly, the *EG* exhibited the DNA damage in concentration dependent manner. The highest damage was revealed by the GB3 concentration. Increased in the Micronuclei formation and chromosomal aberrations showed significant genotoxic effects by the *EG* on Vero cell line. Moreover, the findings of the present study show that *EG* has potential cyto-genotoxic effects on Vero cell line and this could be used a good candidate for the treatment of cancer. However further studies should be needed to authenticate and for practical implications.

KEYWORDS DNA Damage, Micronuclei, Cell Viability, Toxic Effects

Introduction

E. globulus is a rich source of phytochemical complexes as flavonoids, tannis, propanoids and alkaloids found in the leaf, root and stem of the plant (Pirbalouti *et al*, 2014). A few flammable chemical constituents such as globulol, ß-pinene, α-gurjunene, pipertone, α-,ß- and γ-terpinen-4-ol andallo-aromadendrene were identified in both shoots and leaves. Shoots contain eucalyptol, borneol, eudesmol, citral, fenchone, p-menthane, myrtenol, myrecene, α-terpineol, verbinone, cysteine, asparagine, glycine, glutamic acid, threonine, and ornithine were obtained from fruits (Shiezadeh *et al*, 2013), while forming acid, sucrose and dextrin were obtained from flowers (Surbhi *et al*, 2023). Despite the fact that about 18 compounds were detected in Eucalyptus, eucalyptol which is the main active constituent represents 79.85% of the total chemical composition determined.

Eucalyptus also revealed a high matter of oxygenated monoterpenes, which differ with every Eucalyptus species, and the possible difference in healing efficacy (Khedhri *et al*, 2023). The phenomena involving makeup pattern of essential oil affects by the seasons and geographical location and in return impacts biological behaviors (Freitas *et al*, 2022; dos Santos *et al*, 2023; Khedhri *et al*, 2023). Thus, Eucalyptus is widely used in many countries as India, China, South Africa, Portugal, Tasmania, and Brazil and for, cosmetics, perfumery, aromatherapy, for food and phytotherapy products and alcoholic beverages production. The phytoconstituents from this plant's tissues and several volatile ingredients in shoots are the prime and most vital component found in eucalyptus tree. Fresh Eucalyptus inner bark and leaves can be conventionally boiled in water and the resultant decoctions used to treat fever, flu and colds and a small quantity of the decoction can also be ingested for further therapy (Surbhi *et al*, 2023).

Cytotoxic and anti-proliferative studies of Eucalyptus extracts and oils have been undertaken previously. These studies were aimed to understand the beneficial effects of Eucalyptus extracts against numerous cancer cell lines. However, no study has been documented on the cyto-genotoxic effects of *EG* on Vero cell line. So, the current study has been planned

comprehensively to evaluate the cell viability and DNA damaging effects of *EG* on Vero cell line.

Materials and Methods

Frozen vero cell line was thawed rapidly in water-bath, maintained at 37°C to further sub culture the cell line for experimental purposes. Then cells were pipetted out quickly into a flask and added the right amount of medium in 25 cm^2 flask and kept in CO_2 Incubator. Logarithmically growing cells were observed until grown to up to 80% confluence then trypsinized and plated on 75 cm^2 flask. Further, MTT assay was performed as described by the Ali and Cigerci (2019). Briefly, series of concentrations (1, 1.5, 2.5, 5, 10, 20, 40, 80, 100 ppm) of aqueous extracts of *EG* were employed on Vero cells (1 X 10^5 cells in each well) in 96 well plate for 24 h. Reading of optical density was taken from spectrophotometer at 540 nm and cell viability was calculated to find LC50.

Then further three concentrations LC50/2 (GB1), LC50 (GB2), 2*LC50/2 (GB3), were used to perform DNA damage, micronuclei, and chromosomal aberrations assessment. The DNA damage was assessed using the alkaline comet assay. To each flask (25 cm^2), 1 X 10^8 cells were cultured and the flasks were incubated for 24 h for stabilization. Vero cells were treated with EG extract using GB1, GB2, GB3 concentrations. After 24 h exposure, samples cells were washed with PBS and 10 µl cells were mixed with low melting point (LMP) agarose (100µl). The normal melting point (NMP) agarose coated slides were prepared then mixed with a mixture of cells and LMP agarose. Then slides were kept at the cold slabs for 2-3 min and covered with long coverslips. Then coverslips were removed and the slides were placed in an alkaline lysis solution for one hour. Subsequently the slides were kept in electrophoresis buffer for 20 min and followed by electrophoresis for 20 min using 25V/300mA. Immediately the slides were washed with cold distill water followed by staining with ethidium bromide at a concentration of 200 µg/ml. The DNA damage score was determined as explained by the Liman *et al* (2021). In brief, there was scoring from 0-4 for the tail length, where 0 represented no damage and 4 represented the highest damage. The data of the comet assay were analyzed for variance analysis (ANOVA), ($p < 0.05$).

To find the MN and chromosomal aberrations, the *EG* treatment was administered to the cells similarly, as it was done in comet assay. The cells were centrifuged for 5 min at 2000 rpm and at 4˚C. The supernatant was carefully poured off and the cells were resuspended in 1 mL of PBS. After that, centrifugation was performed again at 1200 rpm for 5 min and at 4˚C. The debris and supernatant were removed and cells were resuspended in PBS. Fixative II of 0.5 mL was added on the cell pellet and spun at 4˚C for 5 minutes. After this the fixative I was placed on the cell pellet and centrifuged for 5 mins after which the supernatant was discarded. To the cell pellet 0.1% KCL was then added. Isolated cells were covered with a drop of water on a cold slide and the slide was left open to dry at the room temperature. After that, Giemsa stain was added on the slide for 20 mins. Microscopic examination for micronuclei and chromosomal aberrations was conducted on compound microscope at 40x.

Results

Determination of cell viability

Concentration dependent decrease in cell viability was observed by the EG on Vero cells in MTT assay (Fig. 1). Highest cell viability (100%) was noticed at the lowest concentrations. LC50 was found as 46 ppm.

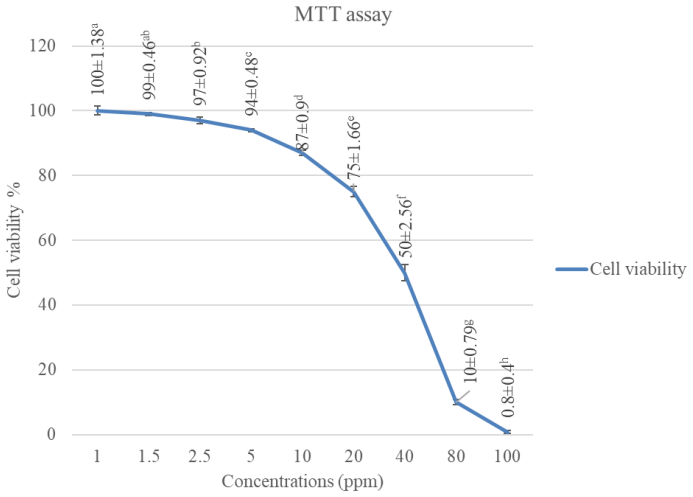

Fig. 1: Graph showing the cell viability by *Eucalyptus globulus* aqueous extract at the different series of concentrations.

Concentration dependent increase in DNA damage was by the *EG* (Fig. 2). Highest DNA damage was observed by the highest dose (GB3) of *EG*. Similarly, higher level of MN was noticed in Vero cells by the exposure of EG at highest concentrations. More number of Binuclei were observed in Vero cells (Fig. 3).

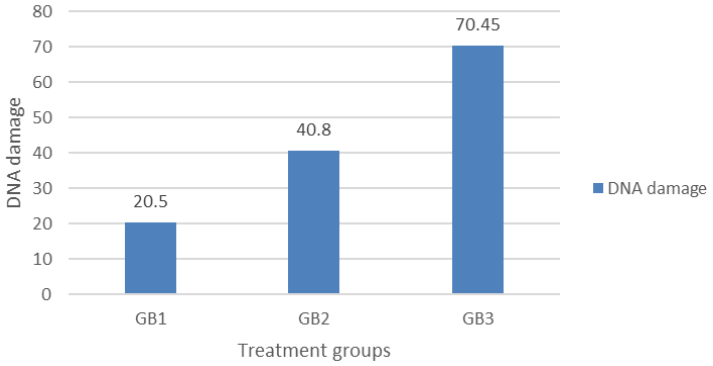

Fig. 2: DNA Damage assessment after exposure of *Eucalyptus globulus* extract on Vero cell line.

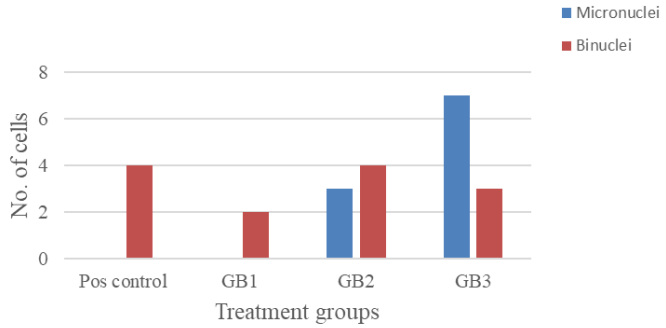

Fig. 3: Findings of Micronuclei and Binuclei after exposure of *Eucalyptus globulus* concentrations.

Various chromosomal aberrations were noticed, i.e., breaks, gaps, aneuploidy etc. But these were very few and non-significant. However, chromosomal aberrations were higher at all concentrations compared to the control group (Fig. 4).

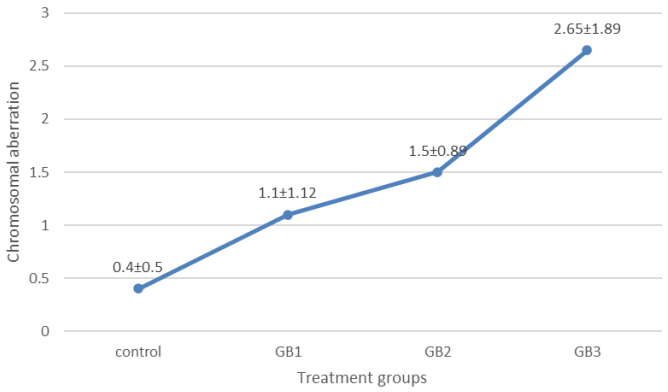

Fig. 4: Findings of chromosomal aberrations after exposure of *Eucalyptus globulus* concentrations.

Discussion

Majority of cancer chemotherapeutics are alkaloids derived from plants which are toxic to various cancer cells (Mathur *et al*, 2009; El-Menshawi *et al*, 2010; Shiezadeh *et al*, 2013). Essential oils of *EG* have some pharmacological properties such as anti-inflammatory, antimicrobial and also antioxidant healing characteristics (Ghaffar *et al*, 2015; Hafsa *et al*, 2016). In current study, cytotoxic effects have been observed by the *EG* due to concentration dependent decrease cell viability. The cytotoxicity of the extracted eucalyptus oil on the A549 lung cancer cell line has already been investigated, and it was concluded that the extracted eucalyptus oil had lower toxicity compared to Fluorouracil 5-FU (Adnan, 2019).

In other studies, on plant extracts, concentration, and time-effect relationships, decreased cell viability was observed; additionally, the cytotoxicity increased with time (Hafsa *et al*, 2016; Ali and Ciğerci, 2017). The resulting from the present study findings illustrates that *EG* cytotoxicity tends to rise with the increase in concentration of the administered dose.

In another study, just Eucalyptus alcoholic extract at the concentration of 20 µg/ml slowed the growth rate and promoted death of the lung cancer cells (A549) compared to doxorubicin as a chemotherapeutic agent (Sharifi *et al*, 2018). It is stated that at higher concentrations, the eucalyptus essential oil (0.5-5%) imposes a toxicity on the normal cells including the fibroblast cells. One study has found that during low concentrations (about 0.1%) did not have a toxic effect at peripheral blood mononuclear cells, but according to this research on cancer cell lines and fibroblast cells above concentration is not harmless.

Similarly, it has been demonstrated that the genotoxic components derived from plant origin can act as a chemotherapeutic agent. It is well known that podophyllotoxin, catharanthine and scopolamine possess toxicity and these are used in therapy (Pirbalouti *et al*, 2014).

Condensation of nuclear chromatin due to endonucleolytic cleavage of nuclear DNA (nucDNA) is one of the hallmarks of cell death. In our study, DNA damage was measured by comet assay and belongs to method used to analyze effects caused by a diverse variety of adverse environmental factors such as chemicals, mutagens, plant extracts etc. (Zhiqun *et al*, 2017; Ali *et al*, 2024). It has been described as a relatively selective approach to assess inter-nucleosomal breakage. It has been reported as a sensitive method to detect inter-nucleosomal damage which is specific for programmed cell death. Because of the the most useful parameters of the comet assay among other genotoxic assays for evaluating DNA damage, extent of DNA damage was measured in this study by this assay. Frequency of DNA damage in cell line increased as the concentration of *EG* increased in the current study. Other researchers found that during the mutagenicity assessment of plant aqueous extracts, frequency of comets was increased with the increasing doses of various extracts (Frescura *et al*, 2013; Ali and Cigerci, 2019).Thus, internucleosomal fragmentation of genomic DNA points out to an active process of cell death that may have been elicited by *EG* (van Doorn, 2011).

It is therefore imperative to determine the degree of cytotoxicity required for the functioning of the *EG* for various therapeutic purposes. Even though *EG* has been used in folk medicinal systems extensively. Toxic nature of *EG* on both colon cancer cell lines and normal human fibroblast cells have been observed. The essential oil killed SW48 cancer cells and that the non-toxic concentration is 0.05% with no effects on normal fibroblast cells. It is accordingly assumed to be a candidate for colon cancer treatment. Comparing the toxicity of 5-FU to eucalyptol on fibroblast cells and carcinogenic SW48 cells indicated that eucalyptus alone has caused a significant decrease in the viability of fibroblast cells (Hafsa *et al*, 2016; Sharifi *et al*, 2018).

In current study, increase in MN and chromosomal aberrations were observed. Micronuclei and Binuclei are recognized as an obligate consequence of exposure to genotoxic agents. It exhibits chromosomal breakage or malfunction in mitosis due to presence of compounds in Eucalyptus globulus extracts (Luzhna *et al*, 2013; Fenech *et al*, 2020). Chromosomal changes represent the primary indicators of genotoxicity. In this case the results indicate *Eucalyptus globulus* may cause disruption of chromosome stability possibly through oxidative stress or interaction with replication machinery.

Declaration of Competing Interest

The authors declare that they have no competing or conflict of interests.

Author Contributions

ZA: Conceptualization, Methodology, formal analysis, Writing—original draft preparation. **MHS:** Formal analysis, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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