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In-vitro Cytotoxicity Evaluation of DL-limonene on Vero E6 cell line

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Abstract

Studies have shown the beneficial effects of plant-derived small organic compounds, known as secondary metabolites, many of which have significant therapeutic potentials on cardiovascular disease, diabetes certain types of cancer. A wide variety of active phytochemicals have been found to influence cellular functions, membrane permeability, viral replication. Thus, naturally based pharmacotherapy such as DL-limonene may be a proper alternative for treating viral diseases such as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV- 2) infection. However, there is a need for proper documentation of the toxicity safety profile of these natural compounds. The aim of this study is to evaluate the cytotoxicity of DL-limonene on normal kidney cells of African Green Monkey (Vero E6 cell line),which is a non-cancerous cell line. In vitro cytotoxicity of DL-limonene on Vero E6 cells was evaluated by WST-8 assay. Afterwards, microscopic examination of the morphological alteration of the cells exposed to 5000.00 µM to 4.9 µM concentrations of DL-limonene was performed. The viability of cells treated with high concentrations (1250-5000 µM) of DL-limonene was reduced by more than 80% while increased cell growth proliferation were observed in cells treated with lower concentrations (625-4.9 µM). Morphological changes, such as shrinking, blebbing, cell rounding, were observed at varying concentrations (1250-5000 µM) of DL-limonene. In contrast, lower concentrations (625-4.9 µM) of DL-limonene showed no observable changes in the morphology of the cells. Therefore, DL-limonene is non-toxic to normal cells at low concentrations, supporting its safe utilisation for therapeutic applications.

Keywords: Cytotoxicity, Cell morphology, DL-limonene**,** Vero E6 cell line, WST-8 assay

INTRODUCTION

Phytochemicals are bioactive substances derived from plants that they manufacture to protect themselves. Whole grains, fruits, vegetables, nuts, herbs are just a few of the many sources from which they can be obtained. More than a thous phytochemicals have been discovered thus far (Sharma *et al*., 2018; Kumar, *et al*., 2023). Dietary fibre, certain polysaccharides, carotenoids, polyphenols, isoprenoids, phytosterols, saponins, terpenoids, steroids, flavonoids tannins are a few of the significant phytochemicals. These phytochemicals have strong antioxidant qualities as well as antibacterial, antiviral, antiallergic, antidiarrheal, anthelmintic effects are useful for treating curing human illnesses (Krishnaiah, *et al*., 2007; Kumar, *et al*., 2023).The greatest sources of health-promoting substances, including vitamins, β-carotene, minerals, flavonoids, phenolics, polyphenolics with notable bioactivities, are

fruits, vegetables, medicinal plants (Abdallah, *et al*., 2022). Citrus fruits are rich in a variety of phytochemicals, including flavonoids (like hesperidin naringin), limonoids (like limonin nobilin), carotenoids (such beta-carotene lutein), vitamin C. These phytochemicals impact the vibrant colours, unique Flavors, distinctive fragrances of citrus fruits (Shilpa, *et al*., 2023). Among the many natural compounds, limonene, due to its widespread presence in the essential oils of citrus fruit has a variety of pharmacological effects a high commercial value (Kwangjai *et al*., 2021). Limonene, the primary constituent of citrus essential oil, mainly includes d-limonene, l-limonene, dl-limonene, with d-limonene accounting for up to 90% of the citrus essential oil content (Negro *et al*., 2016).The limonene content of different citrus plants varied considerably, with blood oranges containing 63% limonene, sweet oranges 88%, lemons 78%, bergamot 72%, bitter oranges 48.85%, marin 69% (Tounsi *et al*., 2011, Moufida Marzouk, 2003).

Limonene (1-methyl-4-isopropenylcyclohex-1-ene), also known as dipentene, is a natural monocyclic monoterpene that is a colorless liquid with two optical isomers: dextrorotatory D or (+) limonene levorotatory L or (-) limonene a racemic mixture (DL-limonene), which have different bioactivities, which may be attributed to the fact that different spatial structures result in different affinities for the active site (Vieira *et al*., 2018, Di *et al*., 2023). Limonene in natural plant essential oils is mainly dominated by D-limonene, which is widely found in the peels of citrus other fruits as a plant biomarker volatile organic compound with a content of up to 80% or more (Chen *et al*., 2020), whereas L-limonene is found in *Cymbopogannardus Cymbopogan citratus*, with a content of about 3% (Kvittingen *et al*., 2021). D-limonene L-limonene have been extensively studied compared to DL-limonene; however studies on the safety profile of DL-limonene on normal mammalian cells have not been documented. Kidney cells of the African Green Monkey (Vero E6 cell line) has been widely used for toxicology, virology pharmacology research, as well as, on the production of vaccines diagnostic reagents (Matskevich *et al*., 2009). In particular, these cells have been used as model for assays to evaluate the toxicity of compounds of different nature, either chemical or microbial toxins. Some chemical substances tested include carbamazepine (Jos *et al*., 2003); triclosan (Jirasripongpun *et al*., 2008); lead nitrate (Romero *et al*., 2004); pentachlorophenol rotenone (Freire *et al*., 2009), where the Vero cell line revealed to be one of the most sensitive model used in these studies (Menezes *et al*., 2013). Establishing the cytotoxic effects of DL-limonene on normal cells is crucial for elucidating its pharmacological profile assessing its suitability for therapeutic use. This present study is aimed at evaluating the cytotoxicity of DL-limonene on normal kidney cells of African Green Monkey (Vero E6 cell line).

MATERIALS METHODS

DL-Limonene Preparation

DL-limonene (mixture of D- L-form ~1:1) for synthesis was purchased from Sigma-Aldrich (St. Louis, MO, USA), with a 95% purity. It was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) Phosphate Buffer Saline (PBS; Elabscience) to a reach the required final concentration.

Vero E6 Cell Culture

Vero E6 cell line was obained from the Centre for Human Zoonotic Virology (CHAZVY), Central Research Laboratory, University of Lagos Teaching Hospital (LUTH). The cells were propagated maintained in complete growth medium (CGM) composed of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% Fetal bovine serum (FBS) 1% penicillin-streptomycin in a tissue culture flask incubated in a humidified 5% CO_2 incubator at 37°C. Every two to three days, sub-culturing was carried out.

Cytotoxicity assay

The cytotoxicity of DL-limonene on Vero E6 cells was determined using a Cell Counting Kit (CCK-8) solution according to the manufacturer's protocol. Briefly, Vero E6 cells were seeded in 96-well plates (7.5×10^4 cells/mL) allowed to attach for 24 hours. Thereafter, the cells were treated with a 2-fold serial dilution of DL-limonene (5000.00 µM - 4.9 µM) for 24 hours. After the incubation period, 10 µL of CCK-8 solution was added to each well incubated for $3^1/_2$ hours, after which absorbance was read at 450 nm in a microplate reader. Per cent viability percent cytotoxicity were calculated with the following equations:

$$\% \text{ Viability} = \frac{\text{Test - blank}}{\text{Control - blank}} \times 100\%$$

% Cytotoxicity = 100 - % Viability

Microscopic Assessment of Morphology

The microscopic examination of cellular morphology was performed according to Khazaei *et al*. (2017), with certain adjustments. Vero E6 cells were seeded in 24 well-plates at 1 x 10^5 cells/well, (one plate for each test drug) incubated overnight. After 24 hours incubation, the medium was replaced with 2-fold serial dilution of DL-limonene (5000.00µM - 4.9µM) from well 1 - well 22. Well 23 24 were taken as negative containing cells complete growth medium. Thereafter all plates were incubated for 72 h. Dose time-dependent morphological effects of the treatments were assessed photomicrographs were taken. The morphological changes in treated cells were compared with those of the untreated cells that served as the negative control.

Data analysis

Cell viability data were processed on Microsoft Excel Spreadsheet then subjected to one-way analysis of variance (ANOVA) using GraphPad Prism (Version 10.5.0 774). Mean ± Stard Error of Mean (SEM) of two or three independent determinations. Values of $p < 0.05$ were considered to indicate a statistically significant difference.

Results

Cytotoxicity effect

The cytotoxic effect of DL-limonene was studied against Vero E6 cell line using WST-8 assay. The cytotoxic effect of varying concentrations of DL-limonene after 24 h treatment was expressed as percentage cell viability in Vero E6 cells as shown in figure 1. The cytotoxicity of DL-linomene was dose-dependent showing the highest cell viability at 9.5µM the lowest cell viability at 5000µM (Fig 1).

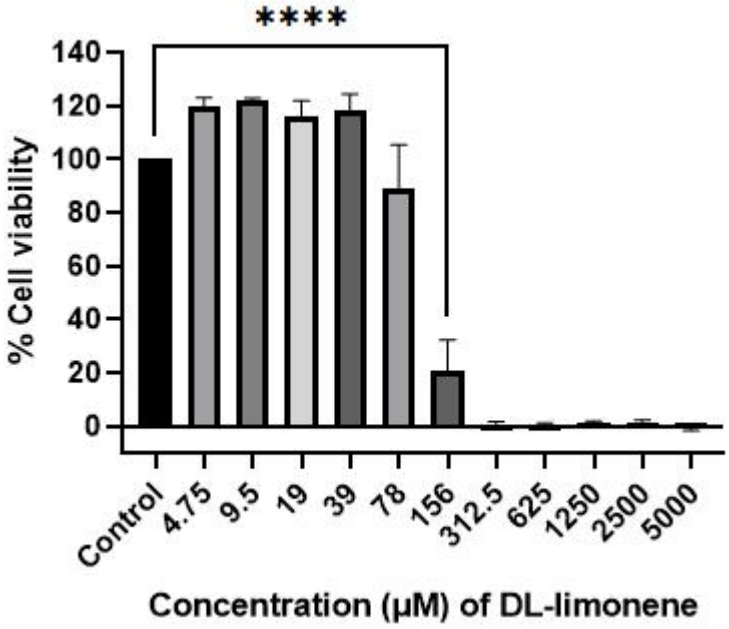

Figure 1: Cytotoxic effects of DL-limonene on Vero E6 cells after 24 hours of exposure.Values expressed as mean ± stard deviation.

Morphological alteration of Vero E6 cells upon treatment with varying concentrations of DL-limonene was evaluated for 24 h, 48 h 72 h using an inverted light microscope as shown in figures 2-12. These results depicts the observed morphological changes increasing in sequential order with treatment duration for Vero E6 cells treated with 5000, 2500 1250µM of DL-limonene.

Compared to the untreated group (Figures 2A, 3A 4A), Figures. 2B, 3B 4B showed cell shrinkage as a result of loss of cellular fluid into the extracellular matrix, membrane blebbing which is characterized by small protrusions of the membrance cell rounding which is detachment from other cells the floor of the flask utilmately leading to death as treatment duration increased. High concentration of DL-limonene caused the cells to lose their normal shape, with some of the cells detaching after 24 h the shape of the cells changing more more as time progressed towards 72 h. The cells treated with concentrations below 1250µM of DL-limonene (Figures 5B-12B) continued to exhibit their original morphological characteristics, such as epithelial cell morphology, while also increasing in number despite the duration. The result showed that cells treated with high concentration of DL-limonene showed cell characteristics such as shrinkage, blebbing, lifting from the walls of the plate.

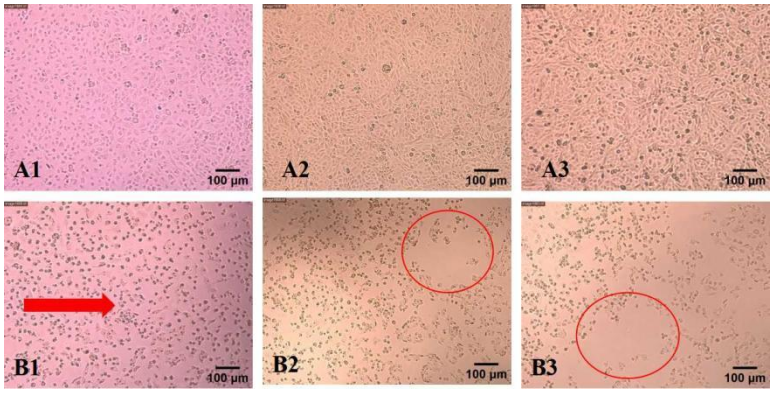

Figure 2: Morphological changes observed in Vero E6 cells after treatment with 5000µM DL-limonene (B1, B2 B3) shows cells are rounded dettached from other cells (Arrow) the floor of the flask (Circle) control,normal Vero E6 cells (A1, A2 A3) for 24hours,48hours 72hours respectively.

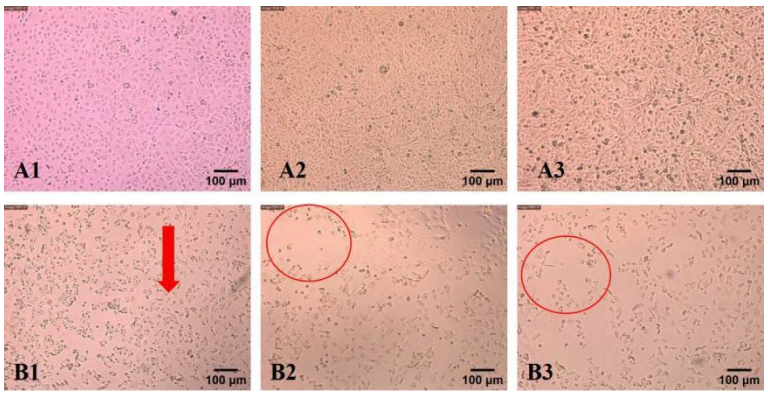

Figure 3: Morphological changes observed in Vero E6 cells after treatment with 2500µM DL-limonene (B1,B2 B3) shows cell shrinkage resulting from lossof cellular fluid, loss in morphology enlongation of cells (Arrow) leading to cell death, clearing from the wall of the flask (Circle) control,normal Vero E6 cells (A1, A2 A3) for 24hours, 48hours 72hours respectively

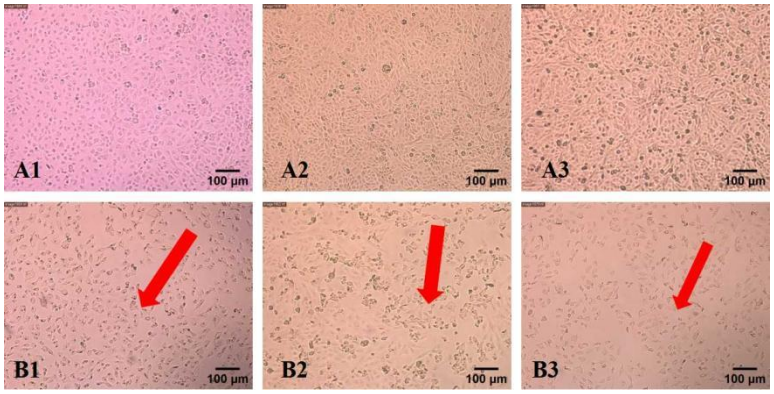

Figure 4: Morphological changes observed in Vero E6 cells after treatment with 1250µM DL-limonene (B1,B2 B3) shows shrinkage blebbing which is small protrusions of the membrane (Arrow) control, normal Vero E6 cells (A1, A2 A3) for 24hours, 48hours 72hours respectively

The result showed that treatment with decreasing concentration of the compound had no effect on the morphology of the cells as these concentrations increased cell proliferation growth when compared with the control as shown in Figures 5-12.

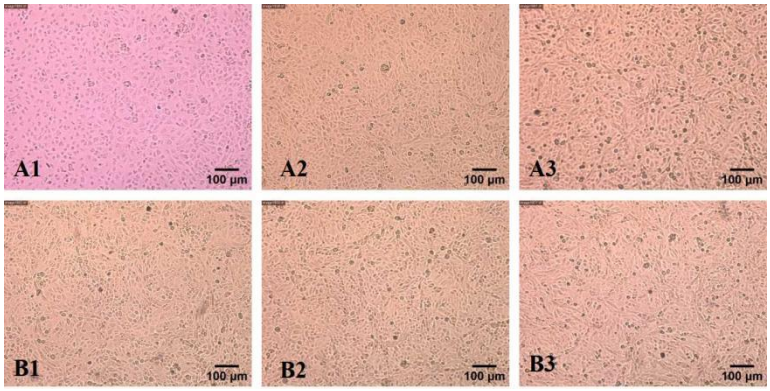

Figure 5: Morphological changes observed in Vero E6 cells after treatment with 625µM DL-limonene (B1,B2 B3) shows no change in morphology as compared with control (A1, A2 A3) for 24hours, 48hours 72hours respectively

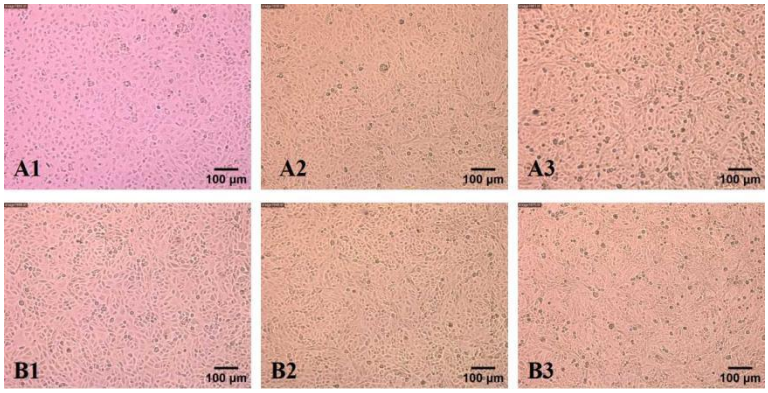

Figure 6: Morphological changes observed in Vero E6 cells after treatment with 312.5µM DL-limonene (B1, B2 B3) shows no change in morphology as compared with control (A1, A2 A3) for 24hours ,48hours 72hours respectively

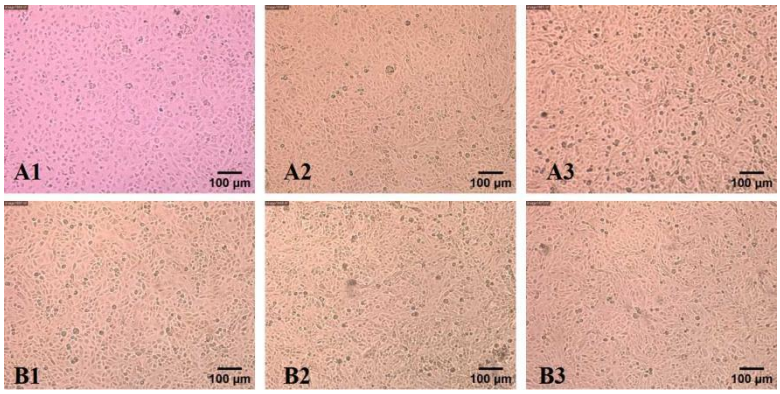

Figure 7: Morphological changes observed in Vero E6 cells after treatment with 156.3µM DL-limonene (B1, B2 B3) shows no change in morphology as compared with control (A1, A2 A3) for 24hours, 48hours 72hours respectively

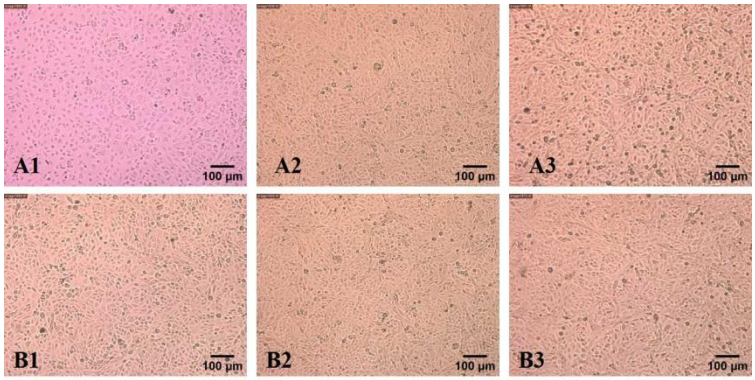

Figure 8: Morphological changes observed in Vero E6 cells after treatment with 78.1µM DL-limonene (B1,B2 B3) shows no change in morphology as compared with control (A1, A2 A3) for 24hours, 48hours 72 hours respectively

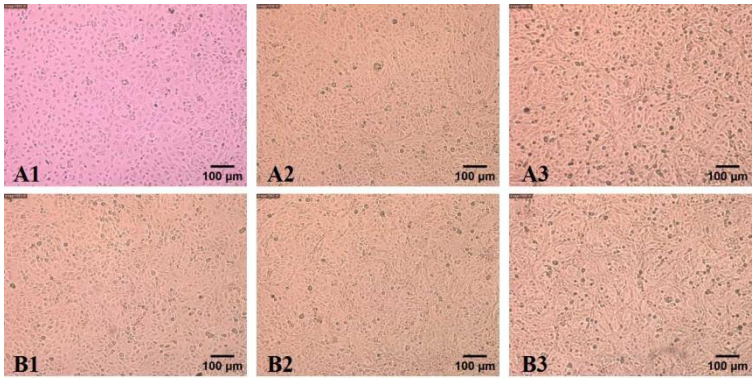

Figure 9: Morphological changes observed in Vero E6 cells after treatment with 39.1µM DL-limonene (B1, B2 B3) shows no change in morphology as compared with control (A1, A2 A3) for 24hours, 48hours 72hours respectively

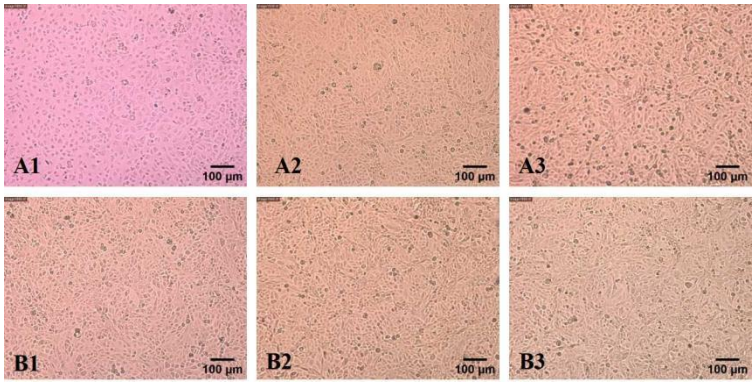

Figure 10: Morphological changes observed in Vero E6 cells after treatment with 19.5µM DL-limonene (B1,B2 B3) shows no change in morphology as compared with control (A1, A2 A3) for 24hours, 48hours 72hours respectively

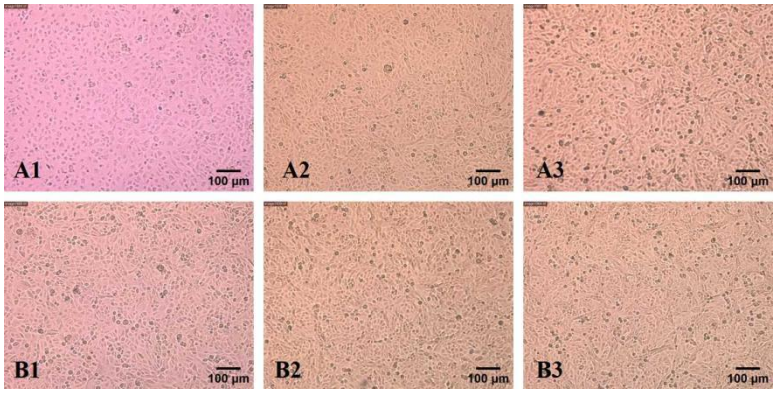

Figure 11: Morphological changes observed in Vero E6 cells after treatment with 9.8µM DL-limonene (B1, B2 B3) shows no change in morphology as compared with control (A1, A2 A3) for 24hours, 48hours 72 hours respectively.

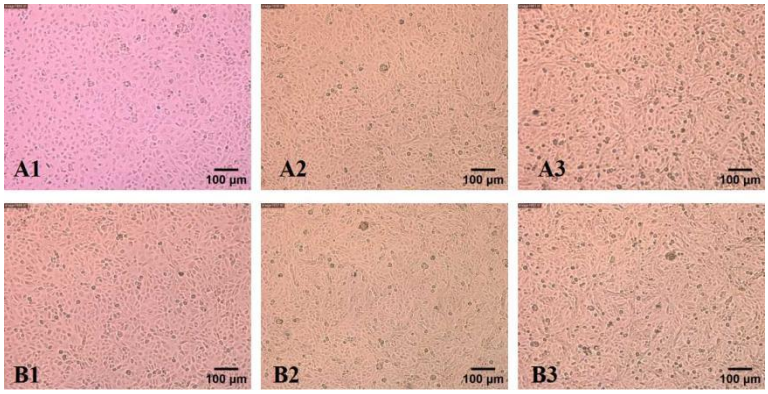

Figure 12: Morphological changes observed in Vero E6 cells after treatment with 4.9µM DL-limonene (B1, B2 B3) shows no change in morphology as compared with control (A1, A2 A3) for 24hours, 48hours 72 hours respectively.

Discussion

A safety profile is exemplified, among other factors considerations, by cytotoxicity levels, although *in-vitro* results may not always simulate *in –vivo* conditions. However, *in-vitro* cytotoxicity determinations could serve as one of the adjuncts in profiling justifications for clinical trials (Morobe, *et al*., 2012). It is crucial to evaluate cytotoxicity in order to ascertain the hazardous concentrations of investigated substances (Abulila Sabry, 2025). This is important because substances that have therapeutic potential may also cause adverse effects (Sulong *et al*., 2025). In this study, the cytotoxic effects of various concentrations of DL-limonene were evaluated using Vero E6 cell line which is a normal cell line. Cytotoxicity of the natural compound was assessed by CCK-8 (Cell Counting Kit-8) assay which measures cell viability proliferation by using the water-soluble tetrazolium salt WST-8, which is reduced by cellular dehydrogenase enzymes in viable cells to produce a yellow-colored formazan dye. According to the findings in this study, the low viability observed in cells treated with high concentrations of test compound suggests cytotoxicity on the cells, while treatment with lower concentrations did not affect the viability of the cells indicating non-toxicity on the cells. Cell viability is defined as the proportion of living, healthy cells within a given population (Adan, *et al*., 2016; Madorran *et al*., 2025) As shown in Fig. 1, the

percentage viability for cells treated with 5000µM - 312.5µM was 0% while viability increased with decreasing (156µM - 4.8µM) concentration of test compound. The higher the concentration of the compound, the lower the viability of the cells the lower the concentrations the higher the viability of the cells. This observation is in agreement with Sulong et al. (2025) who observed similar results on Vero cell line after treatment with Taxol aqueous extract from *Asam Gelugur*. Their results showed that minimal cytotoxicity was observed with low concentration (0.0 µg/mL) of aqueous extract from *Asam Gelugur* as concentration increased, a fluctuation in cell viability was observed while Taxol at the lowest concentration (0.0 µg/mL), displayed no cytotoxicity, maintaining cell viability at 100%. However, as the concentration increased, a notable decrease in cell viability was observed. At concentrations of 0.00001 µg/mL 0.0001 µg/mL, the mean viability percentage dropped to 91.64% 88.70%, respectively, indicating the onset of cytotoxic effects.The decreasing trend continued with concentrations of 0.001 µg/mL, 0.01 µg/mL, 0.1 µg/mL, reaching a mean viability percentage of 85.91%, 86.44%, 70.21%, respectively. These concentrations demonstrate a dose-dependent cytotoxic response of Taxol on Vero cells. Results from this study also followed a dose-dependent cytotoxic response on Vero E6 cells. Zulkipli, et al. (2024) also reported that the ethanol, methanol aqueous extracts of *Prismatomeris glabra* (*P. glabra*) had a dose-dependent cytotoxic effect on human breast adenocarcinoma (MCF-7) cells with the highest cell viability at 100µg/ml the lowest cell viability at 500µg/mL for both ethanol methanol extract.

This study was not only conducted to determine the cytotoxicity of DL-limonene but also to examine the ability of the compound to induce changes on Vero E6 cells by analysing the morphology. Treatment with high concentrations of the test compound which showed loss in morphology as cells became enlongated, shrinking of cells as a result of loss of cellular fluid into the extracellular matrix, cell rounding which is membrane detachment from other cells the floor of the flask (clear circled area in Fig 2 3) ultimately leading to cell death suggests toxicity on the cells within the optimum time being 72h. These morphological changes were not observed in the cells treated with lower concentrations of the test compound as cell growth increased for the test period suggesting low toxicity. This result is in agreement with Vijayarathna Sasidharan (2012) who observed similar morphological alteration of MCF-7 cells upon exposure to *Elaeis guineensis* extract. Their results showed that the number of dead cells increased correspondingly with concentration increment of the extract treatment, 40%-50% of the cells showed membrane blebbing (demonstrated with small protrusions of the membrane) at highest concentration cells became rounder, shrunken showed signs of detachment from the surface of the wells denoting cell death. Zulkipli, et al. (2024) also reported that morphological changes characterised by blebbing, cell shrinkage, floating, as well as the appearance of apoptotic cells was observed in MCF-7 cells treated with varying concentrations of *P. glabra* in a dose time- dependent manner indicating toxicity on the cells at high concentrations.

DL-limonene is non-toxic to normal cells at low concentrations supporting its safe utilization for therapuetic applications paving the way for future investigations into its specific therauetic potential. Cytotoxicity assays are vital in the basic research of drug discovery development, therefore this finding is crucial for virology researchers evaluating bioactive compounds as potential antivirals.

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