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In vitro Antioxidant and Cytotoxic Evaluation of *Euphorbia royleana* Ethanol Lyophilized Latex towards Hepatic Cancer Cell Line

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Abstract *Euphorbia royleana*, is a member of the family Euphorbiacea, known for high contents of secondary metabolites with potential biological activities. In this work, antioxidant and anti-cancer potentials of lyophilized latex from *Euphorbia royleana* (*E. royleana)*, were investigated. For examination of antioxidative capacity, spectrophotometric methods were used. DPPH neutralization and NO scavenging ability were determined, and reduction potential was examined using the FRAP assay. IC_{50} value for $DPPH^{\cdot}$ neutralization is 96.3 µg/mL, and for $NO^{\cdot}$ scavenging 1.87 mg/mL. Reduction potential is 15.8 mg vitamin C equivalents per g of dry weight. High reduction potential and free radical scavenging ability makes the *E. royleana* lyophilized latex a good source of antioxidant agents. On the other hand, Anti-cancer potential of *E. royleana* lyophilized latex have been assessed in vitro towards human liver (HEGP2) cancer cell line in addition to murine fibroblast (BALB/3T3) as normal cell line using the MTT test method. The lyophilized latex cytotoxic activity has a medium toxic effect on liver (HEGP2) cancer cell line with an IC_{50} grade of 80.2 µg /mL. In comparison, normal BALB/3T3 has a feeble harmful impact with an IC_{50} rate of 352.1 µg /mL. It could have culminated that the *E. royleana* lyophilized latex has antiradical activity and has a moderate toxic effect on HEGP2 cancer cells but is weak against normal BALB/3T3 cells. It may be promote as a remedy for the management of liver cancer.

Keywords *Euphorbia royleana*, HEGP2 cancer cell line, normal BALB/3T3 cells, antioxidant

Introduction

Cancer is a large group of disease that involves abnormal growth of cells along with potential; for invading & spreading to other parts for body [1]. It contrasts along with benign tumor that does not spread [2]. Cancer compromised larger family for disease which involves abnormal cell growth along with potential; for invading other of the parts of body [3]. Many of treatment option is there for the prevention of cancer. The primary includes: chemotherapy, surgeries, hormonal therapies, targeted therapies, radiation therapies and much more [4]. For fewer side effects of chemotherapy treatment, many natural products derived from different plants are suggested to be used in curing of cancer diseases [5]. Natural products are the most important anti-cancer agents. Three quarters of anti-tumour compounds used in medicine are natural products or related to them. Of the 140 anti-cancer agents approved since 1940 and available for use, over 60% can be traced to a natural product. Of the 126 small molecules

among them, 67% are natural in origin [6]. In 2000, 57% of all drugs in clinical trials for cancer were either natural products or their derivatives [7]. From 1981 to 2002, natural products were the basis of 74% of all new chemical entities for cancer. Of the 225 natural product-based drugs in various stages of clinical testing in 2008 mentioned above [8] the therapeutic categories targeted included 86 for cancer.

Compounds with anti-tumor activity belong to several structural classes such as anthracyclines, enediynes, indolocarbazoles, isoprenoids, polyketide macrolides, non-ribosomal peptides including glycopeptides, and others. Most of the polyketides are produced by bacteria and fungi [9]. They include a number of anti-tumor drugs such as taxol, which is made by both plants and fungi. Halogenated anti-tumor candidates include salinosporamide A and rebeccamycin [10].

The anti-tumor compounds act by several mechanisms such as inducing apoptosis (programmed cell death) through DNA cleavage mediated by topoisomerase I or II inhibition, mitochondrial permeabilization, inhibition of key enzymes involved in signal transduction (e.g. proteases), or cellular metabolism, and by inhibiting tumor-induced angiogenesis (recruitment of new blood vessels).

The lyophilized latex s of many plants has been used in alternative medicine [11]. *Euphorbia royleana*, commonly known as Sullu spurge and Royle's spurge, is a member of the genus *Euphorbia* in the family (Euphorbiacea). It is a succulent and almost cactus like in appearance although unrelated.

The present study aimed at evaluating the antioxidant and anticancer action of the lyophilized latex from *Euphorbia royleana* natural latex compared to traditional drugs on liver cancer (HEPG2) and normal murine fibroblast (BALB/3T3) cell lines in vitro.

Materials and Methods

Chemicals and Reagents

The materials used include aluminum foil, aqua dest (One Med), DMSO (Dimethyl Sulfoxide) (Merck, Germany), linoleic acid p.a (Aldrich), ethanol 96% (Merck, Germany), DMEM (eagle media modified by Dulbecco, Sigma Aldrich) media, FBS (Fetal Cow Serum, Sigma Aldrich), HEPES buffer media (Sigma Aldrich), chloroform (German brand), L-Glutamine, MTT (3- (4,5-dimethyl-thiazol-2-il-2,5- diphenyltetrazolium bromide, Gibco), n-Hexane (Merck, Germany), PBS (Phosphate Buffered Saline, Gibco®), Penicillin-streptomycin (Gibco), SDS (Sodium dodecyl sulfate) (Merck, Germany), trypsin EDTA 0.25% (Gibco), hydrogen peroxide (H_2O_2), HCl, *Angiopteris ferox* Copel Tuber, β-carotene (Japanese TCI), Tween 20 p.a (Merck, Germany)

Plant material:

Plants: *E. royleana* (Euphorbiaceae) are commonly in gardens as living fences and known as ornamental plants. Cultivation of *E. royleana* is simple and their multiplication is done by means of a vegetative process (asexual reproduction), which do not require frequent watering or application of pesticides or fertilizers [11-12].

Methods

Latex lyophilized latex ion and collection: The samples of the latex of *E. royleana* were collected through transversal sectioning, about 10 cm below the apical meristem of each branch. This collection process has the advantage of keeping the integrity of the plants, allowing them to remain continually productive. The raw latex that sprouted was collected in a close glass essay tube supplied with a screwball lid, so as to avoid coagulation, and transported to the laboratory. The natural latex samples were freeze for three days in a deep freezer and then transformed to a freeze-dryer for the lyophilization process.

Lyophilization of the latex: Lyophilization of the latex collected from *E. royleana* was done using a freeze-dryer (Dura-Top™ Digital Programmer Bulk Tray Dryer, FTS system, U.S.A.) for a period of three days till the complete drying of the latex.

General Experimental Procedures

Hydroxyl Radicals Assay

Testing the antioxidant activity of the lyophilized latex in reducing hydroxyl radicals was carried out based on the method of [12] with a slight modification. Hydroxyl radicals are initiate from the reaction of Fenton between $FeSO_4$ and H_2O_2. The sample performed the investigation by setting up a reaction fusion conceiving 1 mL of Ferri (II) Sulphate 1.5 mM, 0.5 mL Hydrogen Peroxide 6 mM, and sodium salicylate 20 mM. Furthermore, each concentration of the sample solution (10-1000 µg / mL) and quercetin as a positive control (10-100 µg/mL) was adding. The mixture kept the fusion for 30 minutes at 37ºC. After the incubation period, the absorbance was quantifying by UV-Visible spectrophotometry at 520 nm. Determination of antioxidant action by the following equation:

$$\text{Hydroxyl Radical Inhibition (\%)} = [1 - (A_1 - A_2)/A_0] \times 100.$$

A_0 shows the absorbance of the reagent only, A_1 shows the absorbance of the lyophilized latex or positive control, and A_2 shows the absorbance in the absence of reagents (sodium salicylate).

β– Carotene Degradation Assay

The antioxidant activity of the lyophilized latex of *E. royleana* in reducing lipid peroxidation radicals was carried out by the β-Carotene Degradation (BCD) assay. Emulsion of beta carotene contains 5 mg of beta carotene pollen and dissolving with chloroform. Four mL of polysorbate 20 as surfactant and 0.5 mL of linoleic acid as peroxide radical originator (chloroform in the mixture was evaporating), the volume was adequately upset into distilled water to 250 mL and swing until a transparent emulsion of beta carotene was obtaining. A series of ethyl acetate fractions (10-1000 µg / mL) made in the flask and 2 ml of the β-Carotene emulsion was appending. Furthermore, the mixtures' volume was added with distilled water to 5 mL in a volumetric flask and incubated in an oven for 20 minutes at 50ºC. After the incubation period, the absorbance quantifying by a spectrophotometer (461 nm). The mixtures monitored measurements for 0-120 minutes at 30 minutes intervals. The antioxidant effect was deliberated based on the dissimilarity in the reduction rate of the sample and control (β-carotene emulsion only). The samples calculated the percentage (%) of inhibition of β-carotene reduction rate based on the following formula [13].

$$\text{Inhibition of Degradation Rate (\%)} = [(\text{Ln } (a/b) \; x \; 1/t \,] \; x \; 100$$

Oxidative Stress-induced H_2O_2

The procedure of antioxidant activity using of Human Dermal Fibroblast adult (HDFA) cell-induced by hydrogen peroxide (H_2O_2) was carried out based on [14] with modification. The lyophilized latex solution was made by 10 mg of the fraction and diffused with 100 µL of DMSO, and the capacity was sufficient into 1 mL with DMEM (10000 µg/mL). It made the concentration series of the sample from 500 to 31.25 µg/mL. A total of 100 µL of hydrogen peroxide (H_2O_2 1000 µM) was used as an oxidative stress-inducing agent. An amount of 0.1 mL (2 x 10^4 cells/well) of the cell was dispensed into 96-well plates then keep for 24 hours at CO_2 incubator at 37º C so as the cells stick fast to the wells and added diver's concentration series of samples to the medium of culture. The well plates were kept for 24 hours in a CO_2 (5%) incubator at 37ºC, thrown out the culture medium after an incubation interval. The well plate cleansed cells with 0.1 mL of phosphate-buffered saline. A sum of 0.1 mL of H_2O_2 (1000 µM) was attached to each well, then kept in a 5% CO_2 incubator for 2 hours, at 37ºC. Afterward, thrown away the medium and rinsed the cells with 100 µL of media. Add to every well with MTT reagent (0.1 mL) in PBS and kept into the CO_2 incubator at 37ºC for four h. The well plate was added with SDS 10% and then held one night till the reaction stops. The absorbance was scan with a microplate reader at 595 nm.

Cytotoxic assay against HEGP2 and BALB/3T3 cell lines

Isolation and Cell Harvest

Cell lines: human liver (HEPG2) cancer cell line was obtained from American Type Culture Collection (Rockville, Maryland, USA) and are being maintained in the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy (Wrocław, Poland). Murine fibroblast normal cell line (BALB/3T3) was cultured in DMEM (Gibco, UK) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (GE Healthcare, Logan, UT, USA).

Cell lines growth was observed, and every two days replaced the media with entire DMEM media. After cell confluent surface about> 80%, harvest the cells. Cells are attached to the plate and washing with DMEM media. Furthermore, rinsed the layer of cells attached to the dish using the trypsin-EDTA solution to remove the cells from the bottom of the plate and incubated at 37ºC for 5 minutes, observed until the cells no longer stick to the dish and expand on the media. Added 5 mL of complete media to activate trypsin, and then the suspension was transferred to the centrifugation tube and centrifuged for 10 minutes at a speed of 1000 rpm. Then rejected The supernatant from the tube and the suspended residue (cells) in 1 mL of whole media. A total of 10 µL of cells were collected and transferred into a haemocytometer, and the cells were sum up using a microscope. Several cells must be conveyed into sterile tubes and added with the culture medium based on the hoped-for concentration.

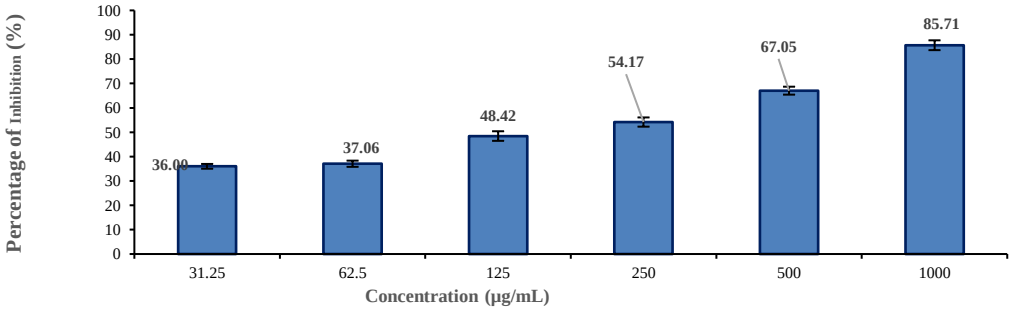
Cytotoxic test by MTT

The harvested cells (2 x 10^4 cells/well) were distributed to the wells according to the number of samples tested [15]. Then the mixture was kept in an incubator CO_2 at 37ºC for twenty-four hours to adjust and stick to wells until cells were ripe to be managed. In the following sitting period, took the well plate containing the cell from the incubator, and then the media was discarded. Insert each 100 µL concentration series of the sample solution into well-containing HEGP2 cells, then kept the plate for twenty four hours into the CO_2 incubator at 37ºC. In the wake of incubation season, thrown out the cell media and a total of 0.1 mL, the MTT reagent was distributed into the well plate. The well plate was kept for four h. Then the well plate was added with a stopper reagent (100µL of SDS) and was kept overnight then, the absorption was scanned at 595 nm with a plate-reader.

Results and Discussion

Hydroxyl radical testing

The principle of reducing hydroxyl radicals is the Fenton reaction mechanism, namely the reaction between Fe^{2+} and H_2O_2 to produce hydroxyl radicals. The hydroxyl radicals formed react with salicylates become 2,5-dihydroxy-benzoic-acid and 2,3-dihydroxy-benzoic-acid was measured by spectrophotometer.



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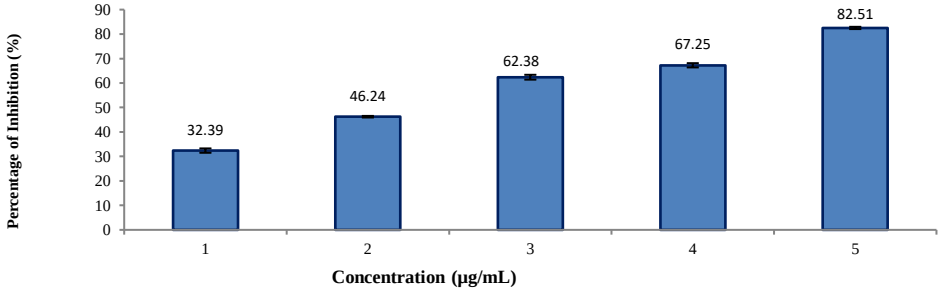

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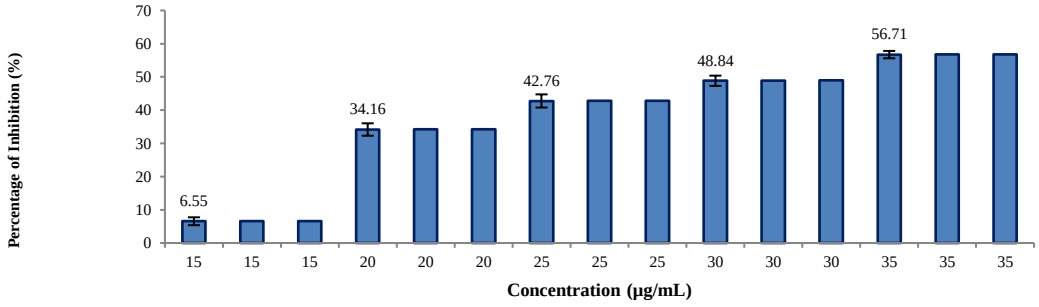
Figure 1: Graph the relationship between sample concentration and percent inhibition of lyophilized latex (1) and standard quercetin (2). The data with triplicate (n=3)

The research results in reducing hydroxyl radical are show that the supreme level in the ethyl acetate fraction, the greater the strength of inhibition (**Figure 1).** Significant effects occur at the highest concentration, 1000 µg/ml, with a percent inhibition value of 85.70%. Inhibiting hydroxyl radicals up to 50% (IC_{50}) required a concentration of 126.62 µg/ml (100-150 µg/ml), a moderate level of antioxidant power, and the IC_{50} value of quercetin as a comparison was 2.32 µg/ml (<50 µg/ml) extreme antioxidant levels [16].

β- Carotene Degradation Assay

In the antioxidant activity test by the beta-carotene method of linoleic acid form free radicals from hydroperoxides produced by linoleic acid. Free radicals form from linoleic acid oxidation due to the reduction of the hydrogen atom from one diallyl methylene group, which strike out the double bond in beta carotene, causing beta carotene oxidation which causes the loss of the chromophore group, which gives the orange color [17].



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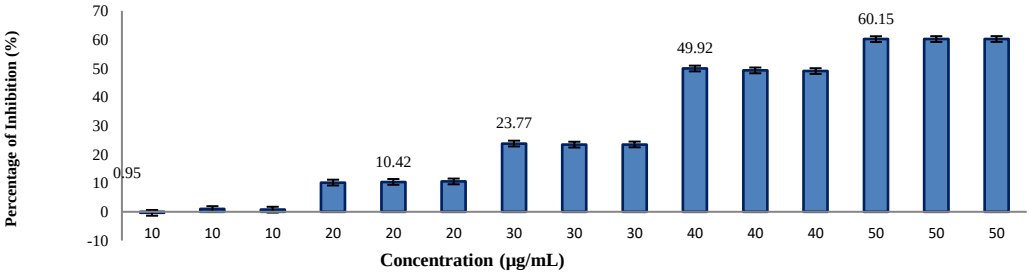

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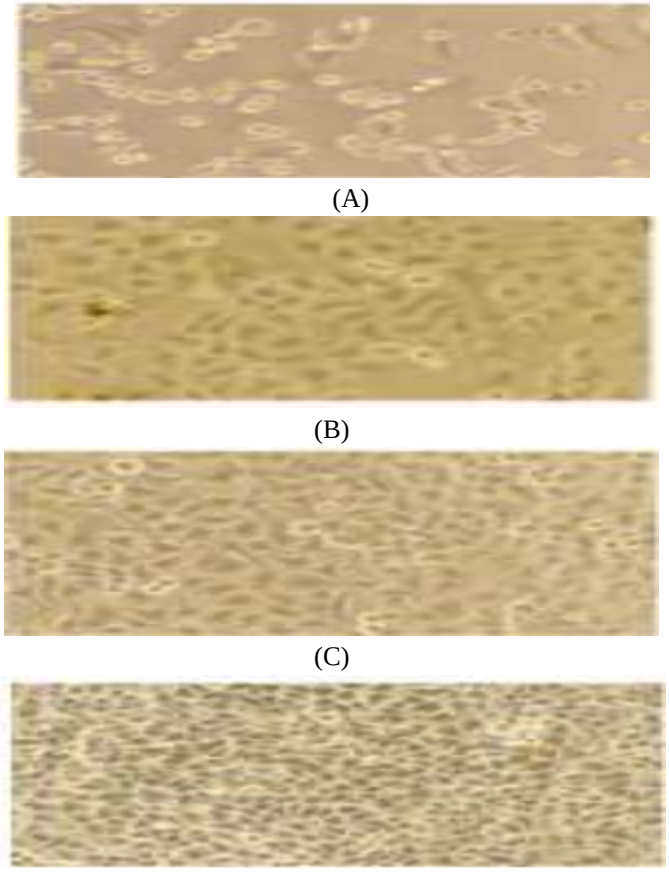
Figure 2: Graph of inhibition percentage and sample concentration of lyophilized latex (1) and positive control of quercetin (2). The data with triplicate (n=3)

Antioxidant activity testing using the BCD method can be determined quantitatively based on the IC_{50} value. The IC_{50} value is using to see how much concentration a sample needs to inhibit 50% of radicals. Antioxidant capacity

shows the smaller of IC_{50} value, the greater the potential sample in reducing free radicals. The results obtained from this study can be seen in Figure 2. The lyophilized latex can reduce the colour degradation of β-Carotene with an IC_{50} grade of 30.29µg/mL, contrasted to the quercetin with an IC_{50} rate of 43.38µg/mL. From the data, the IC_{50} value of the sample has almost the same potential as a positive control of quercetin. It is due to many flavonoids and phenolic contents in samples studied [18].

Antioxidant Activity by H_2O_2-induced Oxidative Stress Method

Changes in cell morphology are a sign of cytotoxic activity resulting from a compound after treatment of cells compared to control cells. Antioxidant activity against H_2O_2-induced cells from samples using normal HDFa cells can also observe microscopically to see the morphology of control HDFa cells, as shown in Figure 3A. HDFa cell morphology after H_2O_2 treatment (3B) Observation results of HDFa cells showed differences in cell morphology before and after the MTT reagent.



(A)

(B)

(C)

(D)

Figure 3: *Morphology of cells after treatment with MTT reagent (A) Control Cells, (B) negative control (H_2O_2 exposure without sample), (C) Cells after sample treatment (high concentration of 500 µg/mL) and H_2O_2 exposure (D) Cells after sample treatment (low concentration of 31.25 µg/mL) and H_2O_2 exposure*

In Figure 3C, more formazan crystals formed, while in Figure 3D, very few formazan crystals formed. These express that the higher the test sample level, the more formazan crystals created, meaning more cells were alive. On the other hand, exposure to H_2O_2 (1000 µM) causes cell death up to above 50%, characterized by less formazan formation. It occurs because H_2O_2 is a source of oxidative stress from an increase in Reactive Oxidative Species (ROS), which causes cell toxicity [19-20]. Therefore, the Lyophilized latex of a sample can prevent cells from

experiencing oxidative stress after exposure to H_2O_2 so that cells are still viable. Living cells have the mitochondrial reductase enzyme, which can react with the MTT reagent to indicate a purplish-blue formazan salt formation.

Table 1: Cell viability (%) of Lyophilized latex of *E. royleana by* H_2O_2-induced Oxidative Stress Method

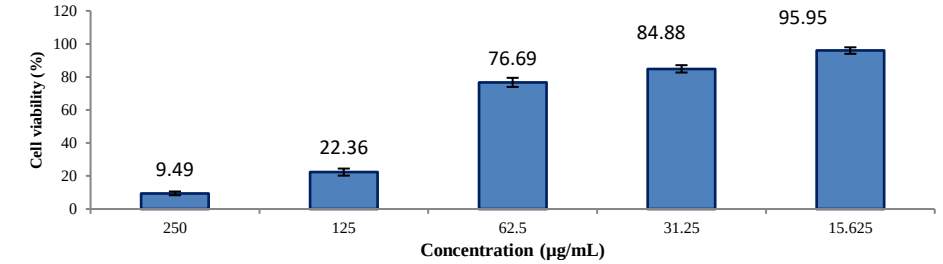
Sample	Viability of cells (%) with level (µg/mL)					IC_{50}
	31.25	**62.5**	**125**	**250**	**500**	
Control cell	100					
Negative Control (H_2O_2)	40.45±2.25					
Lyophilized latex	30.18±0.67	30.57±1.42	38.11±1.58	65.49±3.89	89.05±1.42	121.20
Positive Control (quercetin)	69.31+4.23	68.41±3.00	81.23±2.95	115.51±1.3	112.76±5.67	<30

Activity testing using the oxidative stress method was carried out colorimetrically by observing the formation of formazan and measuring its absorption at 595 nm. The potential of EA-fractions compared with negative control and control cells. The resulting absorbance and the intensity of the colour formed are comparable to the number of viability cells. The darker the purple colour produced and the greater the absorbance value, the more cells are alive, but more cells are dead if the colour is yellow. Table 1 show that the lyophilized latex of *E. royleana* gives a higher percentage of cell viability with increasing concentration. At the highest concentration (500 µg/mL) was able to maintain cell viability up to 89.05±1.42 %. The test results show that the IC_{50} value of the lyophilized latex was 121.20 µg/mL. The result indicates that a 121.2 µg/mL level of lyophilized latex could inhibit 50% of cell mortality after exposure to H_2O_2. It shows that the lyophilized latex can be in the moderate category (100-150 µg/ml) to prevent cell death due to oxidative stress.

Cytotoxic activity test with MTT

The cytotoxic evaluation of the lyophilized latex of *E. royleana* on liver cancer (HEGP2) cells to observe the potential of the sample to exert a toxic effect on cells with various series of sample concentrations. In addition, cytotoxic testing of the fraction also carrying out on normal BALB/3T3 cells aims to evaluate the safety of the sample so that it has a selective effect on normal cells. It hopes that the *E. royleana* lyophilized latex has a toxic outturn on HEGP2 cancer cells despite not BALB/3T3 cells developing as a supporting agent for cancer therapy. In a quantitative cytotoxicity test, an IC_{50} value was necessary, which indicates the concentration required to prevent the accretion of HEGP2 liver cancer cells by 50% of the total population. According to [17] an IC_{50} grade <50 µg/mL is classified as potent effect, if a grade of 50 µg/mL - <20µg/mL is classified as average, and a grade of >200 - <1000µg/mL is classified as poor effect. And the IC_{50} value >1000 µg/mL had no cytotoxic effect. It studied the determination of the IC50 grade according to the relationship between concentration and absorbance of the sample.

Figure 4 showed the lyophilized latex of *E. royleana* cytotoxic test results against HEGP2 liver cancer cells and BALB/3T3 normal cells. The graph in Figure 4 shows a trend that the higher the concentration, the lower the viability of cells. It indicates that the fraction has a toxic effect on cells. The lyophilized latex of *E. royleana* has a poisonous impact on HEGP2 liver cancer cells with a medium category (IC50 grade of 78.96 µg/mL). In contrast, BALB/3T3 normal cells had a weak category toxic effect (IC_{50} grade of 742.5 µg/mL. In this work, doxorubicin (Figure 4C), as an anticancer agent, is exerted to heal numerous kinds of cancer as though breast cancer is acute leukemia, bone cancer, liver cancer, and ovarian cancer. The analysis results show that doxorubicin has an IC_{50} value of 3.275 with a potent category. Based on the outcomes of this research, it showed that the lyophilized latex of *E. royleana* had moderate cytotoxic activity and was slightly selective against normal cells because it had a weak toxic effect. These results are similar to the study of Aisyah et al. [21] which stated that the lyophilized latex had average action against T47D cell line.



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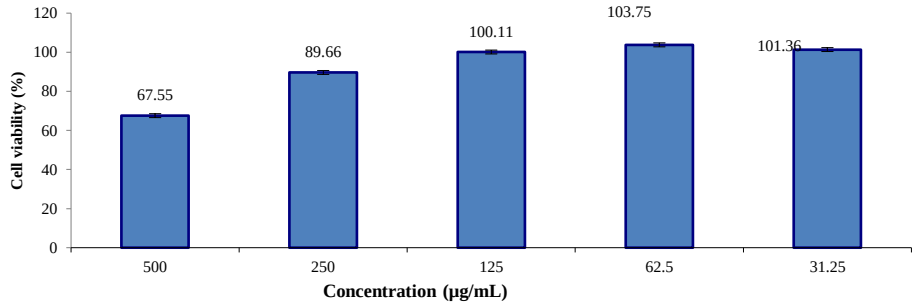

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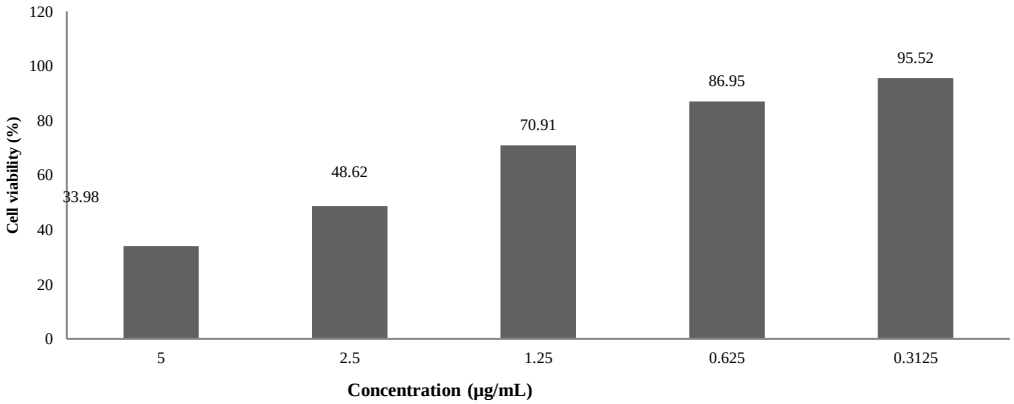

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Figure 4: The graph of the cytotoxic effect of the E. royleana lyophilized latex on cells. (1) HEGP2 liver cancer cells, (2) BALB/3T3 normal cells, and (3) cytotoxic effects of doxorubicin as a positive control against HEGP2 liver cancer

The presence of phenolic or flavonoid compounds in the sample may influence cytotoxic activity with the moderate category of Lyophilized latex against HEGP2 liver cancer cells [13]. Several studies have reported on compounds with anticancer properties such as phenolic or flavonoids and saponins. Natural phenolic has been declared to lead cell cycle inhibition at distinct cell stages: G1, G2, S, and S-G2 by immediately adjusting cyclins-dependent-kinases (CDKs) or implicitly inducing gene expressions p21, p27, and p53. Also, several studies have shown that natural phenolic indicate different effects on cancer cells than normal cells [22]. Flavonoids play a role in cancer cells by inhibiting MAPK in the receptor signaling pathway like tyrosine kinase. Flavonoid also has a role in inactivating proteins that present a bit part in transduction signal and culminate in the blockade of growth factor receptors. Flavonols take part a role in targeting cell surfaces of enzyme transduction signals, like a protein tyrosine kinase, adhesion focal kinase (AFK), and angiogenesis processes [23].

Conclusions

The bioactivity test of the *E. royleana* lyophilized latex on its antioxidant power and toxicity to liver and normal cell line has been done. The results show intercourse in the middle of antioxidant activity and its toxicity effect on liver cancer cells. The moderate impact on liver cancer cells and the weak effect on BALB/3T3 normal cells allowed compounds in the *E. royleana* lyophilized latex as supportive therapy for liver cancer.

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