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In vitro cytotoxicity of ethanolic extract of the leaf of *Calotropis gigantea* from Ie Jue Geothermal Area, Aceh-Indonesia, and its mouthwash formulation against dental pulp cells

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ABSTRACT

The ethanolic *Calotropis gigantea* leaf extract (ECGLE) from Ie Jue geothermal area, Aceh-Indonesia, and ECGLE-based mouthwash formulation has been prepared. The formulation was prepared with various extract concentrations ranging from 0 to 25% of ECGLE. Both the extract and formulation were evaluated for antibacterial and *in vitro* cytotoxic activity in order to determine their potential medicinal value in the oral cavity. Antibacterial tests were carried out against Gram-negative bacteria (*Porphyromonas gingivalis*), Gram-positive bacteria (*Solobacterium moorei*), and a mix of both Gram-negative and Gram-positive bacteria (*P. gingivalis* + *S. moorei*). The cytotoxic activity was evaluated against human dental pulp primary cells (hDPPC) by calorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. All formulations passed the stability test with a pH of 5.35–5.92. Antibacterial activity testing revealed that the higher the ECGLE concentration, the more effective it is against bacteria. In comparison with other formulations, formulation-3 containing 3 gr of ECGLE demonstrated the highest activity. The minimum inhibitory concentration (MIC) value and % inhibition of formulation-3 against *P. gingivalis, S. moorei,* and a mix of both bacteria were 0.089, 0.075, and 0.083 µg/ml and 88.924%, 90.691%, and 89.72%, respectively. The cytotoxicity activities (IC_{50}) for both ECGLE and a formulation containing ECGLE were 6.44 and 0.27 gr/ml, respectively. The ability of cells to undergo apoptosis showed a strong correlation between cell viability and the ECGLE extract (R^2 = 0.973) as well as ECGLE-based mouthwash formulation (R^2 = 0.897). The greater the concentration of ECGLE extract or ECGLE-based mouthwash formulation, the lower the viability of hDPPCs, but the greater the antibacterial activity.

INTRODUCTION

Aceh has several geothermal sites which are contributed by volcanic activities from Jaboi (Idroes *et al*., 2021b), Seulawah Agam (Marwan *et al*., 2019a, 2019b), and Burni Geureudong (Dharma *et al*., 2021; Marwan *et al*., 2021; Putri *et al*., 2019). This condition has an effect on the biodiversity that exists in the area (Idroes *et al*., 2016, 2017). Ie Jue is one of the geothermal manifestations in Seulawah Agam volcano (Idroes *et al*., 2019a, 2019c; Marwan *et al*., 2021). This manifestation has higher acidity and temperature than the others (Ie Seu'eum and Ie Broek) (Idroes *et al*., 2018, 2019b). This situation contributes to plants' characteristics with thermal and dry resistances that grow massively in the Ie Jue area. The extreme environment forces the synthesis metabolism to produce more secondary metabolites (Nuraskin *et al*., 2019, 2020). As a result, the plants that grow there may have medicinal properties, such as antioxidants (Cane *et al*., 2020; Suhartono *et al*., 2019), antimicrobial (Estevam *et al*., 2015; Ningsih *et al*., 2019), antibacterial (Nuraskin *et al*.,

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2019, 2020; Rahmad *et al*., 2019; Tallei *et al*., 2020), antiviral (Khairan *et al*., 2021; Tallei *et al*., 2020, 2021a, 2021b; Tumilaar *et al*., 2021), antibiofilm (Pratiwi *et al*., 2015), and wound healing (Earlia *et al*., 2019a, 2019b).

One of the plants which are plenteous in the Ie Jue area is *Calotropis gigantea*. This plant is categorized as a weed that can thrive in unfertilized soil, geothermal area, near the coastal area, or places with direct sunlight exposure (Tezara *et al*., 2011). *Calotropis gigantea*, from the family Asclepiadaceae, is a unique plant from Southeast Asia. This plant is also known as giant milkweed/swallow-wort (English), *erukku* (Indian), *remiga* (Malaysian), and *widuri* or *biduri* (Indonesian), and in Aceh, it is known as *rubeek* (Sampath Kumar *et al*., 2015).

Calotropis gigantea is commonly used as a therapy for various diseases, such as antibacterial, anticancer, antitumor, antioxidant, and wound healing (Bairagi *et al*., 2018; Deshmukh *et al*., 2009; Idroes *et al*., 2021; Jacinto *et al*., 2011; Kar *et al*., 2018; Seniya *et al*., 2011; Singh *et al*., 2010). All parts of this plant can be used as herbal medicine, including its leaf (Bairagi *et al*., 2018). *Calotropis gigantea* leaf has been reported to contain several compounds, such as flavonoids, alkaloids, saponins, triterpenoids, tannins, and polyphenols. The use of this plant in traditional therapy tends to disregard the concentration and side effects which may be caused by the compound content in the leaves. Usually, the medicinal plant has biological and chemical activities that can influence the cell of an organism, such as cytotoxic activity.

Based on Nguyen *et al*. (2017) and Jacinto *et al*. (2011), the methanol and dichloromethane extracts of *C. gigantea* leaf have high cytotoxicity against human pancreatic cancer cell lines, colon carcinoma, lung non-small-cell adenocarcinoma, and liver hepatocarcinoma (Bairagi *et al*., 2018; Deshmukh *et al*., 2009; Idroes *et al*., 2021; Jacinto *et al*., 2011; Kar *et al*., 2018; Nguyen *et al*., 2017; Seniya *et al*., 2011; Singh *et al*., 2010). However, there is no investigation yet concerning the cytotoxic activities of this plant extract against the oral cells such as human dental pulp primary cells (hDPPCs). The exposure of *C. gigantea* leaves extract or its formulation has the potential to produce positive or negative effects against the hDPPCs, which can be investigated by *in vitro* cytotoxicity test.

Niles *et al*. (2009) stated that the *in vitro* cytotoxicity simplified the threshold test of drug safety and validated the target for the basis of drug modification. Furthermore, cytotoxicity studies play a role in safeguarding the safety, reducing the production cost, observing the reaction of the cellular membrane, and penetration ability of the drug to the body, including the oral cavity (Bácskay *et al*., 2018; Niles *et al*., 2009). Investigation of cytotoxic activities of *C. gigantea* leaves, utilized for oral cavity, is still limited, especially in the form of herbal mouthwash formulation. Hence, this research aimed to identify the antibacterial and cytotoxic activities of the ethanolic *C. gigantea* leaf extract (ECGLE) and its formulation in the form of mouthwash against the hDPPCs.

MATERIALS AND METHODS

Materials and apparatus

This research was a laboratory experiment with a posttest-only control group design. It was conducted in the Laboratory of Oral Biology, Faculty of Dentistry, Universitas Indonesia. *Calotropis gigantea* leaves were collected from Ie Jue geothermal area, Aceh Besar Regency (location coordinate: 5°30′24″N 95°37′46″E), Aceh-Indonesia. Leaf samples were extracted with ethanol and their cytotoxicity against hDPPCs was determined. The pulp cell was the sixth generation maintained by Oral Biology Laboratory, Faculty of Dentistry, Universitas Indonesia.

Preparation of ECGLE

The leaves of *C. gigantea* were washed and dried for 14 days at room temperature. The dry leaves were cut and ground using a crusher to produce a simplicia. One part of dried simplicia was added to a macerator, followed by the addition of 10 parts of ethanol 96%, rinsed for 6 hours, and allowed to rest for 18 hours. The resulting macerate was precipitated and filtered. The filtrate was evaporated using a rotary evaporator to obtain a concentrated ECGLE.

Preparation of the ECGLE-contained formulation

The procedure for preparing the mouthwash formulation was modified from a previously published study (de Paula *et al*., 2014). The composition of this formulation can be seen in Table 1. All materials were dissolved in distilled water up to 100 ml. Each formulation is distinguished by the amount of ECGLE added to the mouthwash formulation.

Quality control tests of the ECGLE-based mouthwash formulation

The stability test of the ECGLE-based mouthwash formulation was carried out using the shock thermal method by combining high and low temperatures during storage. The formulated mouthwash was put into a heat-resistant container and then exposed to high temperature (60°C) for 1 day. Furthermore, the mouthwash was exposed to low temperatures for 1 day (−20°C). This process was carried out continuously for 6 days (3× cycles) and then transferred to room temperature. During these processes, physical conditions were observed, especially changes in consistency, color, smell, and appearance (Ahmad *et al*., 2018).

Bacterial strains and culture conditions

Porhyromonas gingivalis ATCC 33277 and *Solobacterium moorei* ATTC 22971 strains were used in this research. Bacteria were spread separately on an agar brain heart infusion medium (BHI-Himedia Laboratories, India) and incubated for 24 hours under a microaerophilic atmosphere (10% H_2 : 10% CO_2 : 80% N_2).

Antibacterial activity test

Antibacterial tests were carried out using the microdilution method. 100 µl of ECGLE extracts (25%, 12.5%, 6.25%, 3.13%, and 1.56%) and ECGLE-based mouthwash formulation (F0, F1, F2, and F3) were added to the culture plate 96 well (microplate). Then 100 µl of bacteria (*P. gingivalis*, *S. moorei*, and a mix of both *P. gingivalis* and *S. moorei* bacteria, which have been diluted previously) was added. The microplate was closed and put in an anaerobic jar at 37°C for 24 hours (10% H_2:10% CO_2 :80% N_2). After incubation, the microplates were removed and read using a microplate reader (M965+, Metertech Inc., Taipei, Taiwan) at 600 nm. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that completely inhibits the growth of the microorganism or no visible microbial growth observed within

Table 1. Formulation composition with a variation of ECGLE concentration.

	Formulation (gr)			
Composition	**Formulation 0**	**Formulation 1**	**Formulation 2**	**Formulation 3**
C. gigantea extract	-	0.1	0.2	0.3
Sodium benzoate	0.1	0.1	0.1	0.1
Saccharin	0.1	0.1	0.1	0.1
Propylene	15	15	15	15
Mint aroma	0.5	0.5	0.5	0.5
Sodium fluoride	0.05	0.05	0.05	0.05
Distilled water	100 ml	100 ml	100 ml	100 ml

the plate. The percentage of inhibition of the extract/formulation against the tested bacteria was calculated by the following formula (Saquib *et al*., 2019):

$$\% \text{ inhibition} = \frac{\text{Abs. Control} - \text{Abs. sample}}{\text{Abs. Control}} \times 100$$

Cell culture

Preparation of alpha minimum essential medium (MEM)

Alpha MEM was prepared in a sterile condition by dissolving fetal bovine serum 10% and AA 1% into a 50 ml volumetric flask. The medium solution was filtered using a 0.2 µm syringe filter (Laredo-Naranjo *et al*., 2016).

Cell inoculation/growing

Alpha MEM, which had previously been prepared, was taken up to 2 ml, diluted with hDPPCs, and added to a 15 ml Falcon tube. The solution was centrifuged for 5 minutes at 2,000 rpm. The supernatant was removed before adding 1 ml new MEM. It was stirred and removed into a 25 cm^2 flask, followed by the addition of another 4 ml MEM. The medium containing the cells was incubated at 37°C with 5% CO_2. The medium was replaced every 2 days until it reached 70%–80% confluence (Naz *et al*., 2019).

Cell harvesting, calculation, and seeding

The MEM was removed from the flask after the incubation. Three ml of PBS was added to the flask, then discharged, and added with 3 ml 0.25% Trypsin ethylenediaminetetraacetic solution, followed by 5 minutes incubation. An inverted microscope (Carl Zeiss type Axiovert 40 CFL) was used to ensure that all cells were detached from the flask surface. The cell suspension was poured into 15 ml Falcon tube and added with MEM medium to reach 3 ml. The procedure was followed by a 5-minutes centrifugation at 2,000 rpm at room temperature. Afterward, the supernatant was discharged and 1 ml of new MEM medium was added to the tube. The cell suspension was then diluted by a factor of 10. A total of 10 µl of suspension was taken, homogenized, and dropped dropwise onto a hemocytometer for cell calculation using the following equation:

$$\text{Total cell} = \frac{\text{number of cell in hemocytometer}}{\text{number of cell counted}} \times \text{dilution factor} \times 10^4 \text{ (coef)}$$

After that, the cell suspension was dripped into a 96-well plate based on the calculation. The plate was reincubated at 37°C with 5% CO_2 for 72 hours (Naz *et al*., 2019).

Exposure of the sample to cells

Ethanolic extract of *C. gigantea* leaves (30 µl) was added to a 96-well plate containing cells after the medium had been replaced with 70 µl new MEM. The microplate was incubated for 24 hours at 37°C with 5% CO_2. The microscope observation was carried out after 24 hours incubation with 10× magnification. The same procedure was applied to study the mouthwash formulation (Naz *et al*., 2019).

Determination of cytotoxicity using MTT

After the medium was renewed with 100 µl MEM, the microplate was added with 50 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and incubated for 3 hours at 37°C with 5% CO_2. Acidified isopropanol (100 µl) was added and the microplate was incubated on the shaker at room temperature for 1 hour. The absorbance was measured using Elisa Reader (Metertech type Accu Reader +) at 600 nm, where the cell viability was calculated based on the following formulation (Tabari *et al*., 2017):

$$\% \text{ viability cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100\%$$

Morphological analysis of hDPPC

Cell morphology was analyzed following ECGLE exposure to determine the changes in these cells. These include cell shrinkage, membrane decay, swelling, chromatin condensation, and the formation of apoptotic bodies. The observation was intended to predict the apoptosis mechanism. Meanwhile, vacuolation of the cytoplasm and the formation of double vesicle membranes containing organelles were observed in order to ascertain autophagic cell death (Bustillo *et al*., 2009).

Results of the analysis

The data were analyzed using Microsoft® Excel (Office SP, 2007). Data analysis included average, standard deviation

of absorbance, linearity, correlation, IC_{50} of the ECGLE, and ECGLE-based mouthwash formulation on hDPPC viability.

RESULTS

The stability test revealed that all mouthwash formulations had the same organoleptic, viscosity, and temperature parameters at 0 and 6 days (3× cycles). A slightly different result was shown by the formulation acidity. The increase in pH occurred with the increase in ECGLE concentration. The increase in pH also occurred between before the cycle (0 days) and after passing through three cycles (6 days). The pH before the cycle was lower compared to that after three cycles. An increase in pH (more alkaline) occurred at F3 (Table 2). However, the pH value of all formulations was still in the neutral range (5.35–5.92).

Antibacterial activities

The MIC activity from both ECGLE- and ECGLE-based mouthwash formulation showed that the smaller the concentration used, the lower the MIC resulted (Fig. 1A). The lowest MIC value was shown at a concentration of 1.56%, where *P. gingivalis* had a lower MIC value of 0.059 mg/ml compared to other bacteria (0.079 mg/ml for *S. moorei* and 0.055 mg/ml for a mix of both bacteria).

However, when compared to the ECGLE-based mouthwash formulation, the MICs were significantly lower (Fig. 1B) than ECGLE MIC (Fig. 1B). Overall, the resulting MIC value ranges were 0.089–0.094 mg/ml for *P. gingivalis*, 0.075–0.800 mg/ml for *S. moorei*, and 0.083–0.096 mg/ml for a mix of both bacteria (dual-species). ECGLE-based mouthwash formulation 3 had the lowest overall MIC values for each bacterium.

The small MIC value generated was inversely proportional to the percentage of inhibition produced. The smaller the MIC, the greater the percentage of inhibition. In ECGLE, the percentage of inhibition was much smaller than mouthwash ECGLE-based mouthwash formulation. The highest percentage of inhibition produced by ECGLE was 63.361% for *P. gingivalis,* 90.400 ± 0.498 for *S. moorei,* and 93.327 ± 1.199 for dual-species. Meanwhile, the percentage of mouthwash ECGLE-based mouthwash formulation inhibitors was 88.924 ± 2.531 (*P. gingivalis*), 90.691 ± 2.342 (*S. moorei*), and 89.72 ± 2.417 (dual-species). The results of statistical tests showed that there were significant differences in MIC and the percentage of inhibition produced in this study between ECGLE concentrations (Saquib *et al*., 2019).

Value of IC_{50} using MTT assay

The results indicated that the higher concentration of ECGLE used, the higher the absorbance obtained. It also revealed that the absorbance of ECGLE-based mouthwash formulation was higher than ECGLE (Table 1).

Table 1 exhibits that the higher concentration of ECGLE, the higher the number of living hDPPCs, or in other words, the cell viability increased with R^2 = 0.973 (Fig. 1). The value of R^2 is close to 1, which indicated a significant positive correlation.

On the other hand, the determination of linear correlation obtained from that of ECGLE-based mouthwash formulation gave a value of R^2 = 0.8968 (Fig. 3). This value showed a strong correlation between the concentrations of each formulation with the cell viability percentage. The correlation was negative, in which if the formulation concentration is high, then the percentage of cell viability is low, or vice versa.

The linear equation indicated that the ECGLE had a greater IC_{50} (6.44 gr/ml) in comparison with that of the ECGLE-based mouthwash formulation (0.27 gr/ml). From LINEST, the R^2 value generated from ECGLE or mouthwash formulation was close to 1, allowing an assumption that the experimental values of x and y will not be significantly different from that of the theoretical values. Moreover, the slope reaches 0.8145 with an intersection on the y-axis at 1.2112. Meanwhile, the formulation had a slope of R^2 = −58.668 with an intersection on the y-axis at 17.055 (Table 4).

Morphology of hDPPCs

The changes on hDPPCs after the exposure with the ECGLE- and ECGLE-based mouthwash formulation were observed using an inverted microscope with 10× magnification (Figs. 4 and 5). Morphological observation of hDPPCs was as much important as investigating the cytotoxicity effect of ECGLE.

Cell morphology analysis revealed that ECGLE exposure resulted in visible bulges. This was due to the blebbing of the cells, indicating an initial phase of cell apoptosis mechanism (programmed cell death). It was marked by the cell image with bulges as indicated by → and the living cells indicated by →. Additionally, the images suggested the presence of cell shrinkage,

Table 2. The stability test of mouthwash formulation contained ECGLE.

Formulation test	F0 (day)		F1 (day)		F2 (day)		F3 (day)	
	0	6	0	6	0	6	0	6
1. Organoleptic								
a. Color	Transparent, green	Transparent, green	Transparent, green	Transparent, green	Transparent, green	Transparent, green	Transparent, green	Transparent, green
b. Taste	Sweet	Sweet	Sweet	Sweet	Sweet	Sweet	Sweet	Sweet
c. Smell	Unique	Unique	Unique	Unique	Unique	Unique	Unique	Unique
2. pH	5.35	5.52	5,48	5,56	5,56	5,59	5.78	5.92
3. Viscosity	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
4. Temperature	25.8	25.8	25.8	25.8	25.8	25.8	25.8	25.8

Nd: not detected.

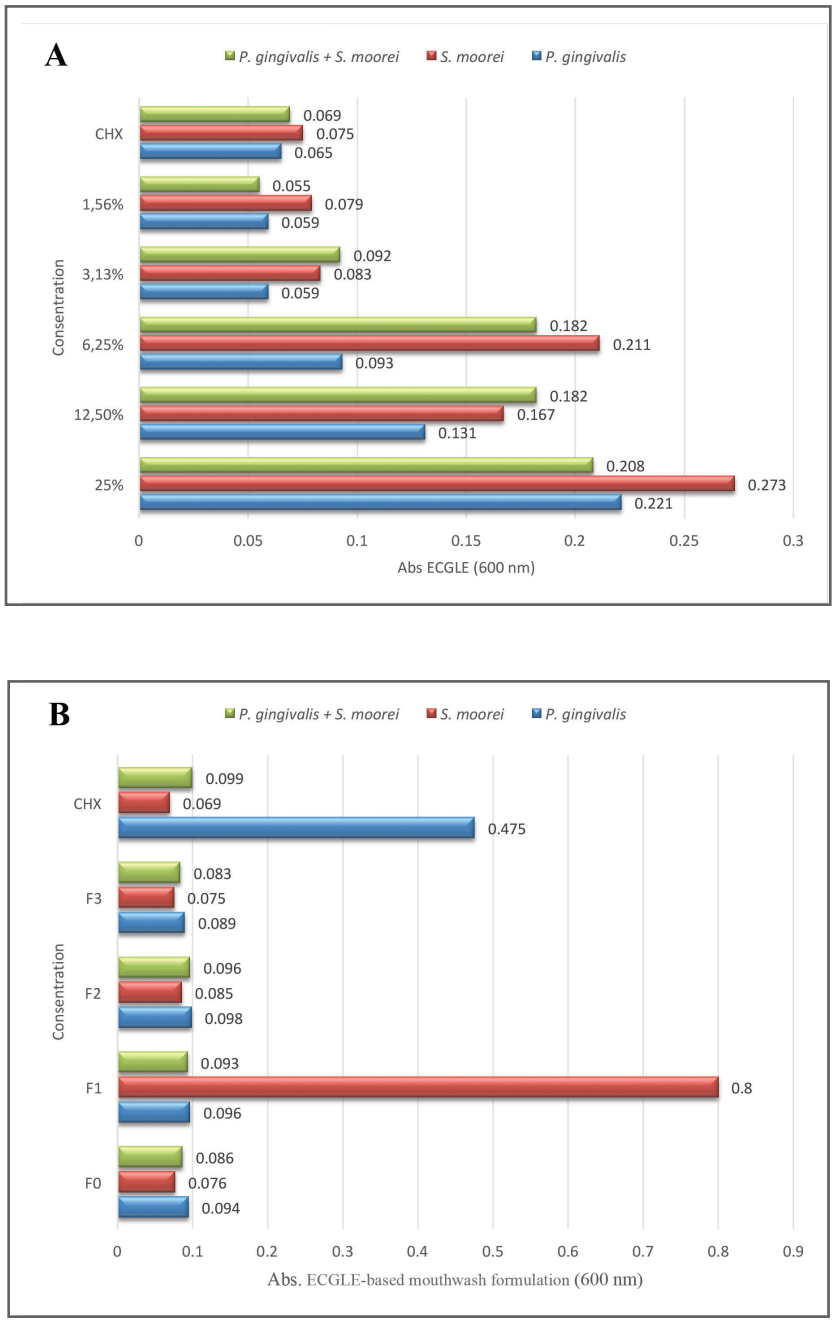

Figure 1. MIC activity at various concentrations: (A) EGCLE- and (B) ECGLE-based mouthwash formulation.

as indicated by a change in cell morphology from a basil to a spherical shape.

DISCUSSION

Stability tests conducted on the mouthwash formulation using organoleptic, temperature, viscosity, and pH parameters (Table 2) revealed that formulation 3 (F3) performed significantly better than the other formulations. However, overall the entire formulation was very non erosive to the teeth (Table 1). All formulations showed a pH in the range 5.35–5.92. This highly meets the recommended mouthwash pH standards according to ISO 16408-2015, that is, 3.0–10.5 (ISO, 2015), and the standards according to Collares, that is 4.11–7.0 (Collares *et al*., 2014). Based on this fact, it was ascertained that mouthwash ECGLE-based mouthwash formulation is safe to use (pH 5.35–5.92).

Based on *in vitro* analysis, both ECGLE- and ECGLE-based mouthwash formulation showed excellent antibacterial activity. The lowest MIC to inhibit the growth of all tested bacteria occurred at the lowest ECGLE concentration (1.56%) and ECGLE-based mouthwash formulation. It was assumed that the active substance produced was much greater so that the higher the extract concentration, the greater the ability to inhibit bacterial growth. Both extract and ECGLE-based mouthwash formulation were shown to be more able to inhibit Gram-positive

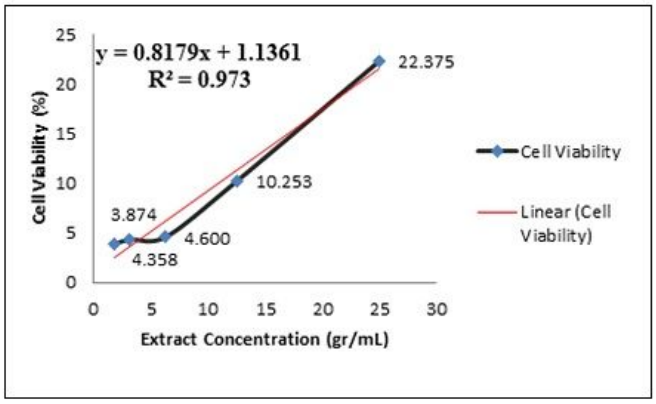

Figure 2. Cell viability and linearity of ethanolic extract of *C. gigantea* leaves.

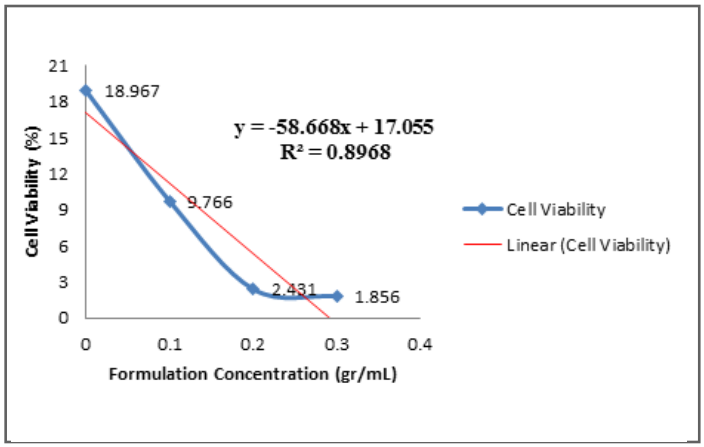

Figure 3. Cell viability and linearity of the ECGLE-based mouthwash formulation.

Table 3. The relationship between MIC and the % inhibition occurred in the absorbances of ECGLE and formulation containing ECGLE.

Concentration/ formulation	*P. gingivalis*		*S. moorei*		*P. gingivalis* + *S. moorei*	
	MIC (mg/ml)	% inhibition	MIC (mg/ml)	% inhibition	MIC (mg/ml)	% inhibition
			ECGLE			
25%	0.221 ± 0.110	73.041 ± 13.190	0.273 ± 0.031	66.625 ± 3.431	0.208 ± 0.113	74.477 ± 14.126
12,50%	0.131 ± 0.050	84.039 ± 0.426	0.167 ± 0.035	79.546 ± 4.555	0.182 ± 0.297	77.756 ± 3.383
6,25%	0.093 ± 0.016	88.634 ± 1.775	0.211 ± 0.026	74.231 ± 3.490	0.182 ± 0.021	77.721 ± 2.845
3,13%	0.059 ± 0.005	92.847 ± 0.525	0.083 ± 0.003	89.848 ± 0.232	0.092 ± 0.008	88.811 ± 0826.
1.56%	0.059 ± 0.019	92.830 ± 2.416	0.079 ± 0.005	90.400 ± 0.498	0.055 ± 0.009	93.327 ± 1.199
CHX	0.065 ± 0.011	92.056 ± 1.295	0.075 ± 0.008	90.831 ± 0.935	0.069 ± 0.019	91.573 ± 2.327
			ECGLE-based mouthwash formulation			
F0	0.094 ± 0.002	88.304 ± 2.687	0.076 ± 0.004	90.684 ± 1.919	0.086 ± 0.002	89.231 ± 2.965
F1	0.096 ± 0.001	88.062 ± 3.173	0.8 ± 0.000	90.07 ± 2.498	0.093 ± 0.002	88.552 ± 2.625
F2	0.098 ± 0.001	87.909 ± 2.957	0.085 ± 0.002	89.479 ± 2.902	0.096 ± 0.001	88.107 ± 2.822
F3	0.089 ± 0.002	88.924 ± 2.531	0.075 ± 0.000	90.691 ± 2.342	0.083 ± 0.001	89.72 ± 2.417
CHX	0.475 ± 0.007	94.093 ± 1.570	0.069 ± 0.028	91.928 ± 1.284	0.099 ± 0.067	88.823 ± 5.262

The measurement was carried out in triplicate.

Table 4. Absorbance of ECGLE- and ECGLE-based mouthwash formulation.

ECGLE (%)	A_{600} nm ± SD	Mouthwash formulation	A_{600} nm ± SD
Control negative (medium)	0.088 ± 0.004	Control negative (medium)	0.088 ± 0.004
Control positive (cell)	0.501 ± 0.053	Control positive (cell)	0.501 ± 0.053
25	0.095 ± 0.035	F0	0.078± 0.022
12.5	0.042 ± 0.022	F1	0.040± 0.029
6.25	0.019 ± 0.017	F2	0.010± 0.002
3.13	0.018 ± 0.004	F3	0.008 ± 0.005
1.56	0.016 ± 0.008		

The measurement was carried out in triplicate.

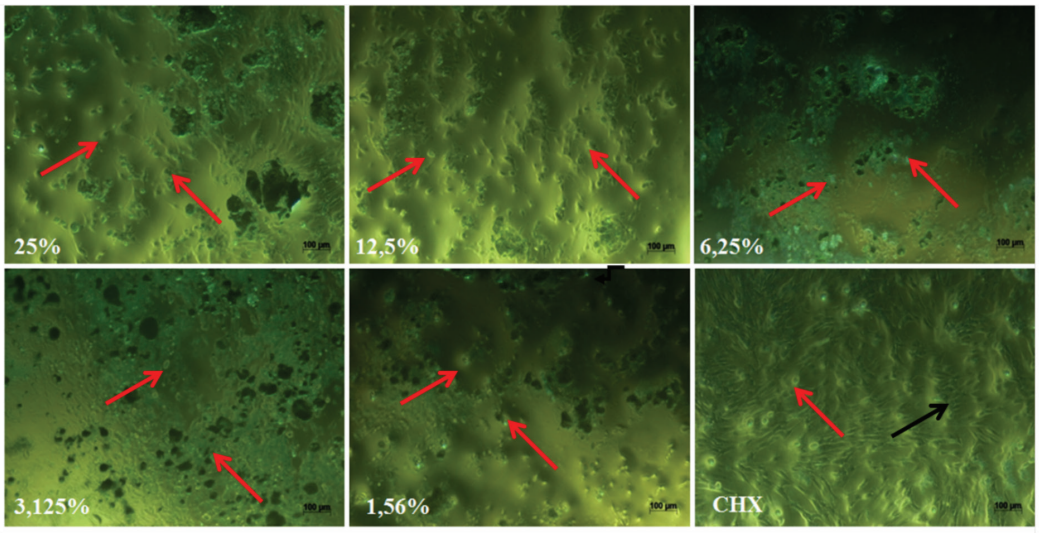

Figure 4. The effect of ECGLE addition on the morphology of hDPPCs. Living cells were indicated by the black arrow (→) and dead cells were indicated by the red arrow (→). The cells were observed using an inverted microscope with 10x magnification.

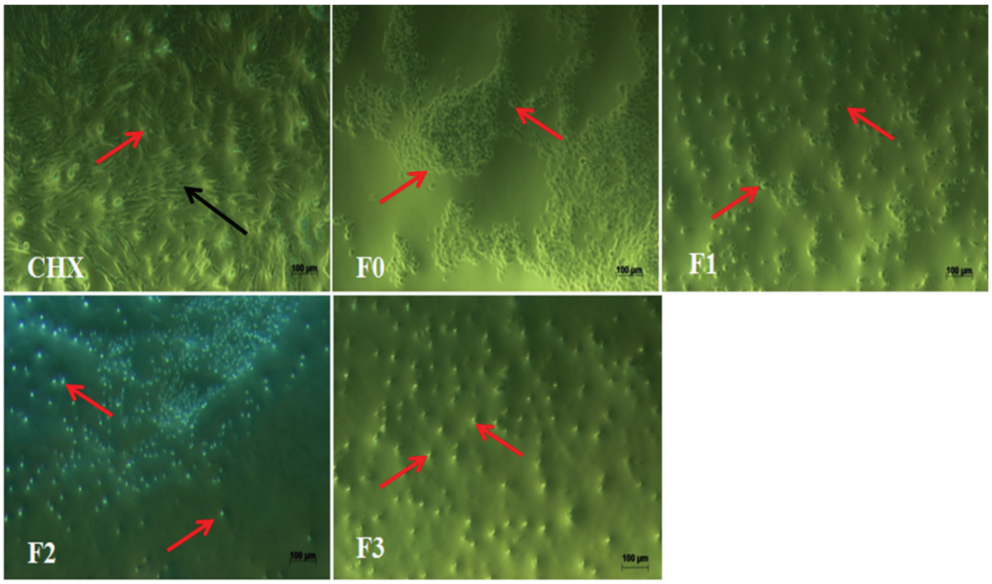

Figure 5. The effect of ECGLE-based mouthwash formulation addition on the morphology of hDPPCs. Living cells were indicated by the black arrow (→) and dead cells were indicated by the red arrow (→). The cells were observed using an inverted microscope with 10x magnification.

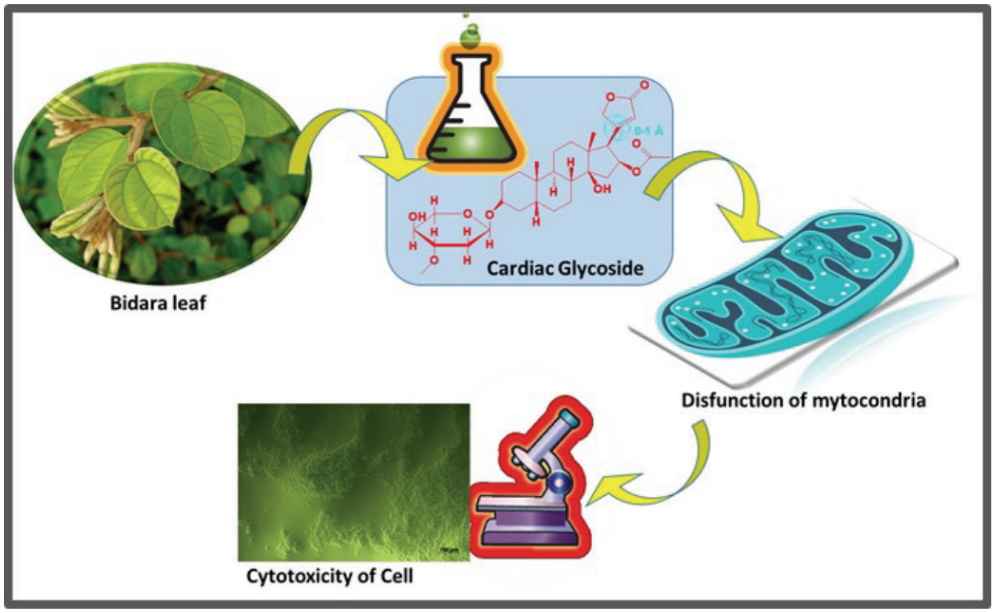

Figure 6. The graphic illustration of cardiac glycoside influence against hDPPCs cell.

than Gram-negative bacteria. This was thought to be caused by the lipopolysaccharide of Gram-positive bacteria being much thinner than that of Gram-negative bacteria. This finding was in line with Varposhti's research which stated that the thickness and composition of the bacterial membrane affected the bacteria inhibition ability (Varposhti *et al*., 2014).

The results of this study produced different cytotoxicity absorbance values, with ECGLE collected from the Ie Jue geothermal area yielding higher absorbance than the ECGLE-based mouthwash formulation (Table 4). It was based on the concentration difference between ECGLE- and ECGLE-containing formulation, with ECGLE having a higher concentration than ECGLE-based mouthwash formulation. The concentration/color intensity changed the produced absorbance from the real absorbance. It was in agreement with Lambert–Beer's law, which stated that there is a correlation between absorbance and sample concentration. Furthermore, the MTT method used in this research had a weakness. It was difficult to remove the formazan solution attached to the cell mitochondria, which affected the calculation results. This condition was in line with the report by Aslanturk (2018), where undissolved formazan used in the MTT method formed purple threads within the cells, which are difficult to be removed (Aslantürk, 2018). Research by Wang *et al*. (2010) suggested thorough evaluation on the method to study the *in vitro* cell proliferation which relied on the chemical properties of the studied plant supplemented (Wang *et al*., 2010).

These results on absorbance explained that the higher the sample concentration, the higher the absorbance (Table 4). Nonetheless, the contrary results were shown by the mouthwash formulation with a high ECGLE concentration. It was ascribed to the effect of interaction between the additives ingredients of the formulation. The addition of ingredients other than the ECGLE weakened or removed the ability of the mouthwash, affecting the absorbance from the mouthwash formulation.

In this research, the cytotoxicity activities of ECGLE- and the ECGLE-based mouthwash formulation against hDPPCs were observable (Figs. 2 and 3). It was in line with Nguyen *et al*. (2017) and Jacinto *et al*. (2011) reports that *C. gigantea* leaf possessed high cytotoxicity (Bairagi *et al*., 2018; Deshmukh *et al*., 2009; Idroes *et al*., 2021a; Jacinto *et al*., 2011; Kar *et al*., 2018; Nguyen *et al*., 2017; Seniya *et al*., 2011; Singh *et al*., 2010). The high cytotoxicity was attributed to the presence of secondary metabolite contents of *C. gigantea*, such as cardiac glycoside. This compound was similar to calotropone, possessing cytotoxic activities against human chronic myelogenous leukemia cells and gastric cancer, based on the *in vitro* studies using the MTT method with IC_{50} of 9.7 and 6.7 µg/ml, respectively (Wang *et al*., 2008).

Cardiac glycosides were known to be capable of inducing apoptosis by disrupting the homeostasis and inducing the mitochondrial pathway (Fig. 6) (Muti *et al*., 2016). This ability was also in line with the increasing concentration percentage, leading to the lower survival rate of the cell. The presence of the lysis process was suspected of causing necrosis on cells due to its cytotoxicity. The necrosis leads to cell swelling and cellular structure damage, stopping the metabolism, and then releasing out its components. This activity caused the cells to stop dividing and growing. *In vitro* cells with necrosis did not have sufficient time or energy to activate apoptosis and did not release apoptosis marker leading to the disruption in intracellular communication (Muti *et al*., 2016).

Low IC_{50} given by the formulation, in comparison with the crude extract, was associated with the different ingredients of each sample (Table 5). In the extract, the ingredients only consisted of the ECGLE with concentration variation and distilled water as the solvent. Meanwhile, in the formulation, the ingredients consisted of other ingredients, including sodium benzoate, saccharin, propylene glycol, disodium EDTA, sodium fluoride, and flavoring and coloring agents (Table 1). The additional ingredients were suspected to contribute to these higher cytotoxic activities, indicated by the low surviving cell viability. It was corroborated by Garland *et al*.'s report (1989) that saccharin reduced cell viability. Jeng *et al*. (1998) reported that sodium fluoride was toxic against the fibroblast cells from oral mucosa and commonly used to prevent caries (Jeng *et al*., 1998). Park

Table 5. Calculation of IC_{50} from ECGLE- and ECGLE-based mouthwash formulation using linear regression.

Sample		Value		
ECGLE	IC_{50}	6.44 gr/ml		
	Slope	0.814544921	1.211190355	Intercept
	Sdslope	0.080912533	1.044061238	SD Intercept
	R-square	0.971180289	1.542274121	Sdreg
Formulation	IC_{50}	0.27 gr/ml		
	Slope	−58.668	17.0552	Intercept
	Sdslope	14.07108056	2.632458125	SD Intercept
	R-square	0.896821686	3.146389264	Sdreg

et al. (2011) agreed that sodium benzoate possessed cytotoxic activity in a mammalian cell by increasing the intracellular Ca^{2+} concentration and mitochondrial transmembrane potential in a dose-dependent manner.

By observing the viability value of <50%, *C. gigantea* was concluded to possess high cytotoxicity. However, these results should be compared with other cytotoxicity tests such as LDH, BrdU, RT-CES, and agar overlay. Yet, Bácskay *et al*. (2018) suggested not to compare the cytotoxicity test using MTT assay with XTT test owing to the use of tetrazolium-based material, which is predicted to have a similar limitation with MTT test (Bácskay *et al*., 2018). Furthermore, plants with high cytotoxicity levels cannot always be used in the body, in which the exposed cell is not merely one cell but many other cells forming a colony. It then contributes to the higher cell resistance to be protected from the extract. ECGLE concentration used in further research should be lower to be capable of reducing the toxic effect of the extract.

The cytotoxicity effect was observed further in Figures 3 and 4, where the higher the extract concentration administered, the higher the cell death occurred. The presence of blebbing also indicates one of the cell death patterns (autophagia). Blebbing condition was more intense on the formulation exposure in comparison with that on the extract. Thus, it was concluded that the formulation has higher activities than the extract. These high activities were associated with the presence of other ingredients added in the mouthwash formulation. The formulation ingredient could also be capable of causing cell shrinkage, and the swells were observable on the surface, as seen in Figures 4 and 5. Ghabanci *et al*. (2013), in similar research, reported the comparison of three mouthwash having the cytotoxic effect on the cultured cell. The commercial formulation was also revealed to have high cytotoxicity (Ghabanchi *et al*., 2013). Research by Muller *et al*. (2017) suggested that chlorhexidine 0.05% had moderate cytotoxic activities, where chlorhexidine and cocamidopropyl betaine 0.2 % showed strong cytotoxic and antibacterial activities (Sun *et al*., 2016).

The ECGLE- and ECGLE-based formulation were used as a mouthwash. The oral cavity contains many cells, not only single cells such as hDPPCs, but also other cells, which interact with each other to form cell layers. Logically, single cells tend to react on their own to fight external conditions so that it is thought to be easier to die than if several cells were combined together. This is in accordance with Muller's (2017) report, which described the ideal conditions that exist in the oral cavity (which contains several cells such as oral fibroblasts, epithelial cells, and immune system cells), which are thought to affect the ability of cells to survive external stimulation. Nevertheless, if we want to reduce the activity, it is suggested to use the extract in a lower concentration than that reported in this research. The most intriguing aspect of this research was the discovery that ECGLE extract/ECGLE-based mouthwash formulation with high cytotoxicity activity also has good antibacterial properties. This is believed to be because of the high antioxidant content, which inhibits antibacterial growth but is toxic to cells. The use of high antioxidants tends to lead to other cancer/degenerative treatments.

CONCLUSION

The ECGLE and ECGLE-based mouthwash formulation produced in this research had high cytotoxicity. As a result, additional research is required to establish a safety standard for its use on humans. The results revealed that the extract concentration of *C. gigantea* leaves determined the level of cytotoxicity on the dental pulp cell. In this research, it was observed that the ECGLE-based mouthwash formulation with 0.3 g/ml extract of *C. gigantea* and 25% extract was far more cytotoxic against the hDPPC. The use of a lower concentration of active compounds of the *C. gigantea* extract was recommended to reduce the cytotoxicity effect on the hDPPC. ECGLE extract and ECGLE-based mouthwash formulation have shown *in vitro* growth inhibition of hDPPC. This was due to the induction of cell cycle arrest and apoptosis. However, future study is needed to understand the mechanisms of cytotoxicity of this plant extract.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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