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Cytotoxicity test of Bovine Gelatin- based hydrogel with *Hibiscus rosa-sinensis* L. leaf extract in topical drug development

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Abstract

Introduction: Herbal drugs have healing properties to prevent and treat various health conditions, including topical treatments, which contain therapeutic active ingredients like *Hibiscus rosa-sinensis* L. leaf extract combined with a carrier substance such as bovine gelatin hydrogel. *Hibiscus rosa-sinensis L.* extract also exhibits other pharmacological effects including antioxidant, antibacterial, antifungal, anti- inflammatory, antidiabetic, and anticancer properties. Bovine gelatin-based hydrogels have non-immunogenic properties derived from natural materials, with controlled drug release, as well as good biodegradability and biocompatibility, making them non-toxic. The combination of bovine gelatin hydrogel with *Hibiscus rosa-sinensis* L. leaf extract has been studied to develop a new topical drug. This research focuses on assessing the safety of this combination through cytotoxicity testing.

Materials and Methods: This *in vitro* experimental study, utilized a post-test only control group design. The control group utilizes a bovine gelatin-based hydrogel, while the treatment group uses the same hydrogel combined with *Hibiscus rosa-sinensis L.* leaf extract on BHK-21 fibroblast cell cultures. Preclinical testing using the MTT assay served as a cytotoxicity test to validate the safety of this topical drug. Comparative study using a T-test with a significance level of $p<0.05$. Data analysis was carried out using Statistical Product and Service Solution (SPSS) Software for Mac version 29.

Results: The cytotoxicity test results for bovine gelatin hydrogel and the combination with the extract of *Hibiscus rosa-sinensis* L. leaves were shown in the percentage of viable cells of 92.3% and 96.4%. The parametric T-test revealed no significant difference between the two groups ($p = 0.754$).

Conclusions: The combination of bovine gelatin hydrogel with the extract of *Hibiscus rosa-sinensis* L. leaves in the development of herbal drugs are non-toxic

Keywords: Herbal drugs; Drug carrier; Bovine gelatin; *Hibiscus rosa-sinensis* L.; Drug safety

1. Introduction

Herbal drugs involve the use of plants with medicinal properties for the prevention and treatment of conditions that can affect general health. Herbal drugs are an important and often underestimated part of health services. In some countries, herbal drugs have a long history of use in health maintenance, disease prevention, and treatment, particularly for certain diseases [1,2].

Recently, there has been an increased interest in combining conventional herbal therapy with contemporary synthetic medications. Herbal drugs serve as the primary source of healthcare for nearly 80% of people, particularly in developing

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countries. Oral healthcare is considered an important aspect of overall health. According to the World Health Organization (WHO), oral health significantly impacts overall health and quality of life. The use of herbal drugs for treating pathological oro-dental disorders is reasonable because they are readily available, affordable, and tend to have fewer adverse effects than pharmaceutical treatments. In recent years, herbal ingredients have been widely incorporated into dental care products alongside traditional treatment techniques. Herbal ingredients are included in the composition of drugs to enhance their therapeutic benefits [1].

The use of herbal drug is increasingly diverse and on the rise due to their perceived lack of side effects typically associated with chemical drugs [3]. One example is plant extracts such as *Hibiscus rosa sinensis* L., a plant native to East Asia. *Hibiscus rosa sinensis* leaves contain antibacterial compounds like flavonoids and tannins [4]. These compounds have the ability to accelerate fibroblast proliferation in the hemostasis process [5]. The active compounds in *Hibiscus rosa sinensis* L. leaves offer a promising potential for more effective wound care, readily available, and considered to have minimal significant side effects [6]. Additionally, *Hibiscus rosa sinensis* L. extract also exhibits other pharmacological effects including antioxidant, antibacterial, antifungal, anti-inflammatory, antidiabetic, and anticancer properties [7,8].

In the development of herbal drug, the ideal excipient or carrier must possess the capability to target specific sites with properties that are biocompatible, safe, efficient, stable, nontoxic, non-immunogenic, and optimal bioavailability [9]. Bovine gelatin can be formulated in the form of hydrogel preparations as excipient agents [10]. Hydrogel preparations are formed by polymer networks that are cross-linked [11]. The crosslink structure of hydrogel can retain and protect active substances from negative external influences, while providing good and controlled swelling properties in the hydrogel [12]. Due to its high water content, porosity, and consistency similar to natural tissues, hydrogel is very similar to natural tissues [13]. Therefore, bovine gelatin-based hydrogels have non-immunogenic properties, with controlled drug release, as well as good biodegradability and biocompatibility, making them non-toxic [14–16].

According to research analyzing clinical trial data from 2010 to 2017, difficult-to-manage toxicity is one of the causes of 90% of drug development clinical failures [17]. Preclinical trials, including pharmacological and toxicological tests both *in vitro* and *in vivo,* play a vital role in providing information about the safety and efficacy of a drug candidate before clinical testing [18,19]. Cytotoxicity testing is one of the main indicators of *in vitro* testing useful for determining the potential toxicity of test substances, including those derived from plant extracts, by observing cell growth, reproduction, and morphological effects [20]. In laboratory research, fibroblast cells are commonly used because they are readily available and easily cultured [21]. BHK-21 (baby hamster kidney) fibroblast cells are widely used in bio-pharmaceutical research and industry [22]. BHK-21 cell lines are a good cell culture if processed correctly and subcultured regularly, consistently yielding replicable results [23].

This study aims to analyze the cytotoxicity of the combination of gelatin-based hydrogel and *Hibiscus rosa-sinensis* L. leaf extract, using a magnetic mixer to blend the extract, gelatin, and crosslinker [10]. This research aims to contribute to the achievement of Sustainable Development Goal (SDG) 3, "Good Health and Well-Being," with a primary focus on ensuring access to safe, effective, high-quality, and affordable essential medicines and vaccines. To achieve this, cytotoxicity tests will be conducted on bovine gelatin-based hydrogel combined with *Hibiscus rosa-sinensis* L. leaf extract and on bovine gelatin-based hydrogel without the extract. The tests will be performed *in vitro* on BHK-21 (baby hamster kidney) fibroblast cells using the MTT assay method, followed by a comparison of the results from both formulations.

2. Material and methods

This study was conducted in accordance with the protocol approved by the Ethical Clearance of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, under number 864/HRECC.FODM/VII/2023. The type of research used is laboratory experimental *in vitro*, employing a post-test only control group design. This study compares the cytotoxicity of bovine gelatin-based hydrogel as the control group with a combination of bovine gelatin-based hydrogel and *Hibiscus rosa sinensis* L. leaf extract as the treatment group. This study was calculated using the Federer formula which resulted in a minimum number of replications carried out of 16 times for each group. The cytotoxicity test was carried out by calculating the percentage of living cells in the form of cell viability using the MTT Assay on fibroblast cell cultures from Baby Hamster Kidney (BHK-21) in a 96-well plate.

2.1. *Hibiscus rosa-sinensis* L. leaf extraction

The extract of *Hibiscus rosa sinensis* L. leaves is obtained through an extraction process using the maceration method with water as the solvent. The leaves of *Hibiscus rosa sinensis* L. are dried, then crushed using a hand grinder until it becomes a fine powder. In the extraction process, 15g of *Hibiscus rosa sinensis* L. leaf powder is mixed with 150 mL of

distilled water and heated for 2 days at a temperature of 50°C. Afterward, the extract is filtered using Whatman No. 1 filter paper, then dried at 50°C and cooled for further use [10].

2.2. Bovine Gelatin Hydrogel Preparation

Bovine gelatin-based hydrogel is a semi-solid formulation formed through cross- linking. In this study, we utilized bovine gelatin-based hydrogel without *Hibiscus rosa sinensis* L. leaf extract for the control group, and bovine gelatin-based hydrogel with a mixture of *Hibiscus rosa sinensis* L. leaf extract for the treatment group. Bovine gelatin 8% (1.6g) was dissolved in 20 ml of distilled water for the control group and in 20 ml of *Hibiscus rosa sinensis* L. leaf extract for the hydrogel with extract. Glutaraldehyde was added as a cross-linker at a concentration of 1% (0.2g). The solution was stirred using a magnetic stirrer at 70°C with a speed of 250 rpm until completely dissolved. The hydrogel is then stored at 4°C until further use. When ready for use, the hydrogel stored at 4°C is washed with distilled water and intermittently agitated to remove the toxic effects of glutaraldehyde (10).

2.3. BHK-21 Fibroblast Cell Culture

The fibroblast cell culture used in this study originated from Baby Hamster Kidney fibroblast cells (BHK-21) obtained from the Research Center, Faculty of Dentistry, Universitas Airlangga, Surabaya. The BHK-21 cell culture was seeded onto 5 cm diameter plates and incubated for 72 hours at 37°C with α-MEM media. The culture was then transferred to a 96-well microplate with 5 x 10^3 cells per well. Once the BHK-21 cell culture was ready, the Dulbecco's Modified Eagle's Medium (DMEM) culture media was removed using a micropipette, and the cells were washed and rinsed with Phosphate Buffered Saline (PBS). Trypsin-EDTA was added to PBS at a concentration of 1-2 ml to release clustered cells. The cells in the flask were then incubated in the incubator at 37°C until they detached. Subsequently, the cell suspension was centrifuged at 2000 rpm for 10 minutes. The resulting cell suspension was added to fresh media and transferred to a new flask. Subculture was then performed before the cells reached confluency (ISO 2009).

2.4. Hydrogel Medium Incubation Procedure

The hydrogel samples need to be pre-incubated in culture medium to ensure that the BHK-21 cells are not covered by the hydrogel during the test. The procedure involves cutting both sample groups using a surgical blade and scalpel. Samples from the bovine gelatin hydrogel group have dimensions of 5x5x5 mm with a weight of ±3 grams, showing a clear yellowish color, and having a solid elastic consistency. Samples from the bovine gelatin hydrogel with *Hibiscus rosa sinensis* L. leaf extract have a greenish-yellow color and solid elastic consistency, also measuring 5x5x5 mm with a weight of ±3 grams. Each of the two hydrogel groups is then added in an amount of 3 grams into each well (n=3) and incubated for 3 hours at 37°C using a 12-well cell culture plate. Next, MEM medium is added in an amount of 3 ml into the wells containing the hydrogel, with a medium-to-sample ratio of 1:1. Incubation is continued for 24 hours at 37°C. Afterward, the medium is filtered using Millex. Then, the culture medium is replaced with hydrogel medium.

2.5. Cytotoxicity Test MTT Assay

The MTT assay procedure for evaluating the cytotoxicity of a substance is conducted as follows (Kamiloglu et al., 2020): The pellet obtained from harvested cell centrifugation is given 3 ml of MEM medium. The pellet is then resuspended until fully dissolved, and 10 µl is taken and dropped onto a parafilm. Take 10 µl of trypan blue and drop it onto the parafilm. Mix the trypan blue with the cells until homogen. Perform cell counting using a scepter instrument at 5 x 10^3 cells per well, then add 100 µl of MEM medium to each well and the cells were incubated at 37°C for 72 hours until reaching confluence. After reaching confluence, pipette out the medium and replace it with hydrogel medium. Then, the plate is divided into 4 sections with each section having n=16 replicates:

- Section I: Contains BHK-21 cells added with bovine gelatin-based hydrogel without *Hibiscus rosa-sinensis* leaf extract.
- Section II: Contains bovine gelatin-based hydrogel with a combination of *Hibiscus rosa-sinensis* leaf extract.
- Section III: Control group with only culture media.
- Section IV: Control cell group with only culture media and BHK-21 cells without any specific treatment.

The cells are then incubated for 24 hours in a 5% CO2 incubator at 37°C. Subsequently, the culture media is removed, and the cells are washed with PBS. After that, 10 µl of MTT solution is added to each well and incubated again for 4 hours in a 5% CO2 incubator at 37°C in the dark room. Next, the wells are observed under a microscope to determine if formazan crystals have formed. If they have formed, 50 µl of DMSO can be added and incubated for 10 minutes in the incubator or at room temperature. The staining results by formazan are evaluated based on the optical density or absorbance read by an ELISA reader at a wavelength of 540 nm.

2.6. Data Collection Procedure

The data generated from the cell proliferation test using the MTT assay is in the form of absorbance values which are then converted into cell viability. The percentage of cells is converted to a nominal scale using the following formula:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treatment} - \text{Absorbance of media}}{\text{Absorbance of cells} - \text{Absorbance of media}} \text{ x } 100\%$$

% cell viability represents the Percentage of living cells after testing, absorbance of treatment represents Optical Density (OD) value of formazan for each sample treatment after testing, absorbance of media represents OD value of formazan on average for each media control, absorbance of cell represents OD value of formazan on average cell control. Based on ISO 10993-5, if the percentage of cell viability is above 80% it is considered non-cytotoxic.

2.7. Processing and Statistical Analysis

The obtained research data consist of descriptive data, which are then statistically tested using the Shapiro-Wilk normality test to determine whether the data come from a normal population, and the Levene test for variance homogeneity. Subsequently, the data undergo significance testing using the T-test. In this study, the significance value is determined using a p-value < 0.05 to observe significant differences in BHK-21 cell viability between treatment groups. Data analysis is performed using Statistical Product and Service Solution (SPSS) software for Mac version 29.

3. Results

Microscopic images of BHK-21 fibroblast cells from two groups before and after the addition of the MTT solution (Figure 1). The BHK-21 fibroblast cells from both samples after treatment but without the MTT solution (Figure 1A and Figure 1B) exhibit live cells with characteristics of transparent cytoplasm and nuclei. Meanwhile, the BHK-21 fibroblast cells exposed to the MTT solution (Figure 1C and Figure 1D) appear bluish-purple, indicating active cell metabolism through mitochondria that absorb formazan. The group treated with bovine gelatin hydrogel with *Hibiscus rosa sinensis* L. leaf extract (Figure 1D) shows denser cell images, indicating higher cell viability.

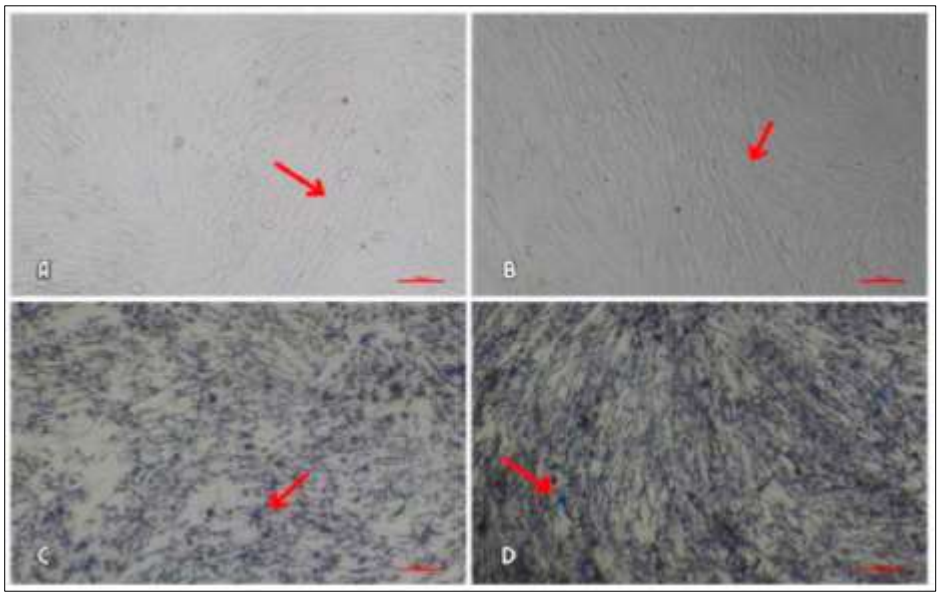

Figure 1 A microscopic image of BHK-21 cells from two groups after the MTT assay. This image was taken by using an inverted microscope at 100x magnification and a 100 µm scale. The red arrow indicates the nucleus of the BHK-21 cells. (A) Microscopic image of BHK-21 cells without extract before MTT Assay testing, (B) Microscopic image of BHK-21 cells with extract before MTT Assay testing, (C) Microscopic image of BHK- 21 cells without extract after MTT Assay testing, (D) Microscopic image of BHK-21 cells with extract after MTT Assay testing

The results of viable cells are presented in the form of descriptive and statistical data analysis, as shown in Table 1. The control group showed an average cell viability of about 0.968 ± 0.334, equivalent to 92.3%, indicating that this group is non-toxic. Meanwhile, in the group treated with bovine gelatin hydrogel with *Hibiscus rosa-sinensis* L. leaf extract, the average cell viability of BHK-21 cells was approximately 1.01 ± 0.395 equivalent to 96.4% which also indicating non-

cytotoxicity of the sample. The normality test using Shapiro-wilk test for both sample groups yielded a value of p = 0.161, which is greater than 0.05, indicating that the data in both groups are normally distributed. The homogeneity test using Levene test for both sample groups yielded a value of p = 0.252, which is also greater than 0.05, indicating homogeneity of data in both sample groups. Subsequently, a parametric difference test, namely the T-test, was conducted to evaluate the significance of the difference between the two data groups. The T-test results for both sample groups yielded a value of p = 0.754, which is greater than 0.05. Therefore, it can be concluded that there is no significant difference between the data in the two sample groups.

Table 1 Statistical Analysis

Group	**Descriptive Data**			**Statistical Data**		
	Number of Sample	**Average OD ± SD**	**Viable Count (%)**	**Shapiro-Wilk Test**	**Levene Test**	**T-Test Test**
Bovine Gelatin Hydrogel (control)	16	0.968 OD ± 0.334	92.3%	0.161*	0.252*	0.754#
Bovine Gelatin Hydrogel with *Hibiscus rosa-sinensis* L. Leaf Extract (Treatment)	16	1.01 OD ± 0.395	96.4%			
Control Medium	0.056		0%			
Control Cell	1.044		100%			

Note: (*) = Data is normally distributed if p > 0.05; (#) = H_0 is rejected if p > 0.05

4. Discussion

The extract of *Hibiscus rosa sinensis* L. as an active substance contains tannins and flavonoids that can stimulate fibroblast proliferation, trigger blood vessel vasoconstriction, and possess astringent properties, thus accelerating the wound healing process. Additionally, it can also function as an antioxidant and antibacterial agent [5,24–26]. Bovine gelatin hydrogel derived from cows serves as a promising candidate as a carrier material (vehicle) because it can preserve and protect these compounds from external influences. Moreover, this hydrogel has been recognized as safe by the United States Food and Drug Administration (FDA), exhibiting absorbent, biocompatible, low immunogenicity properties, and wide usage [27–29]. Preclinical tests such as cytotoxicity assays with MTT Assay can be used to evaluate toxic effects on various biological systems during drug development stages [30].

In this study, the extraction of *Hibiscus rosa sinensis* L. leaves is performed using a water solvent known as aqueous extract. Water has the potential to support microbial growth, so the extract needs to be stored in a frozen state to prevent spoilage. Phytochemical analysis of the aqueous extract of *Hibiscus rosa sinensis* L. leaves reveals significant levels of several secondary compounds and biochemical components such as phenols, flavonoids, sugars, and tannins, with high concentrations (± 1mg/g), particularly in tannins and flavonoids, compared to extracts obtained using ethanol solvent [31,32]. Water is considered the safest and most non-toxic universal solvent, economical, and readily available compared to other organic solvents [33] Using a 1:10 ratio with the maceration method ensures the concentration and preservation of active compounds in the extract, making it more effective for various applications such as antimicrobial, antioxidant, and anticancer properties, as well as being a suitable mixture to achieve wound dressing characteristics [10,34].

The use of baby hamster kidney fibroblast cells (BHK-21) in this study is based on the crucial role of fibroblasts in the hemostasis process, and their presence is an essential part of the pulp, periodontal ligament, and gingiva [35,36].

The research findings indicate a viability rate of 92.3% for BHK-21 fibroblast cells in the bovine gelatin hydrogel group. This group, being a natural material with high amino acid content, exhibits non-cytotoxic properties [14]. The results obtained from the bovine gelatin hydrogel with *Hibiscus rosa sinensis* L. leaf extract show a cell viability percentage of 96.4%. This success is attributed to the non-toxic nature of the hydrogel base derived from bovine gelatin and the *Hibiscus rosa sinensis* L. leaf extract. It aligns with literature indicating that various parts of this plant, including its leaves, show no signs of toxicity [8]. The *Hibiscus rosa sinensis* L. leaf extract contains active compounds such as tannins and flavonoids, which possess antimicrobial properties, stimulate fibroblast proliferation, and help maintain the viability of BHK-21 cells [5,26,37].

The increase in cell viability percentage occurred in both groups after the extract was added to the bovine gelatin. When the active ingredient is added to the extract, phytochemical compounds also act as antioxidants to protect cells from damage caused by oxidative stress due to free radicals, thereby maintaining tissue cell viability [38]. Hydrogel is a semi-solid hydrophilic polymer formulation in the form of a three-dimensional (3D) network that swells in fluid and retains a large amount of water due to the cross-linking structure of individual polymer chains [39]. The ability of hydrogel to swell plays a crucial role in the controlled release of active substances and interaction with cells. Bovine gelatin hydrogel has been the subject of much research due to its controlled release properties and interaction with cells. The cross-link structure of hydrogel provides the ability to store and protect active substances from external influences while also providing good and controlled swelling properties [12]. The cross-link characteristics in gelatin hydrogel with active substances allow the active substances to be preserved and released regularly, thus the addition of the extract has no cytotoxic impact [10].

The decrease in cell viability percentage in both groups may be due to exposure to exogenous toxic substances, such as chemicals, environmental pollutants, and natural plant extracts. These exogenous substances can induce an increase in nitric oxide, reactive oxygen species (ROS), and oxidative stress [9]. The use of the crosslinker glutaraldehyde, which has toxic properties, can also affect cell viability results. Residual products that emerge after crosslinking with glutaraldehyde can have toxic effects depending on the concentration, duration, and form of exposure. However, the use of glutaraldehyde at concentrations below 8% is considered safe. Washing with aquades can remove residual glutaraldehyde products [10].

The explanation regarding bovine gelatin-based hydrogel aligns with the findings from both the bovine gelatin hydrogel group and the group with the addition of *Hibiscus rosa sinensis* L. leaf extract, showing non-toxic properties with a higher percentage of cell viability in the group with the extract. This success is linked to the active compound content in the *Hibiscus rosa sinensis* L. leaf extract and the characteristics of bovine gelatin hydrogel made from natural materials, making it non-toxic and effective in protecting active compounds.

This study has a limitation regarding the *Hibiscus rosa sinensis* L. leaf extract prepared with water as the solvent, making it an aqueous extract. The exact final concentration of the aqueous extract of *Hibiscus rosa sinensis* L. leaves used in this study is not known.

5. Conclusion

The study results indicate that both bovine gelatin hydrogel and bovine gelatin hydrogel combined with *Hibiscus rosa sinensis* L. leaf extract are non-toxic. Furthermore, the group treated with a combination of *Hibiscus rosa sinensis* L. extract exhibited a greater percentage of viability among BHK-21 fibroblast cells.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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