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Cytotoxicity of 12.5% *Hibiscus rosa-sinensis Linn* leaf extract using 96% ethanol combined with sodium alginate excipient from brown algae for drug development

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Abstract

Topical drug products consist of active ingredients for therapeutic effect and excipients as a drug carrier. Hibiscus rosa-sinensis Linn leaf extract has therapeutic benefits such as antioxidants, antibacterials, and anti-fungi which are often applied in medicinal products. Sodium alginate is widely researched as a drug carrier because of its biocompatibility properties to stabilize the polymorphic form of active ingredients and improve their solubility and bioavailability. It also enhances the stability of the active ingredients in the final dosage form. Cytotoxicity tests are mandatory during the development of drugs to ensure their suitability for use. This study investigated the cytotoxicity of sodium alginate sponge and a combination of sodium alginate sponge as an excipient with 12,5% Hibiscus rosa-sinensis L. leaf extract as treatment group against BHK-21 fibroblast cells. This was an in vitro experimental laboratory research using a posttest only control group design involving two groups, the control group and the treatment group. Data were analyzed statistically using a T-test to compare the mean cell viability between the two research groups, with a significance level of $p<0.05$ considered significant. The results of the cytotoxicity test demonstrated that sodium alginate alone showed a certain level of cell viability. The combination of 12.5% Hibiscus rosa-sinensis L. leaf extract with sodium alginate as an excipient is not cytotoxic in drug development.

Keywords: Excipients; Sodium alginate; Hibiscus rosa-sinensis Linn; Cytotoxicity; Drug safety

1. Introduction

Drug development involves discovering and evaluating chemical compounds that can be effectively used to treat and cure diseases (Anusha et al., 2020). Herbal products play a significant role in this process due to their medicinal and phytochemical properties. According to the WHO, 70-95% of the global population relies on herbal products for traditional medicine (Carmona & Pereira, 2013; Moghadam *et al*, 2020; Sam, 2019). *Hibiscus rosa-sinensis L.* is a herbal product that can accelerate hemostasis and serves as a wound healing agent, exhibiting antibacterial and antifungal activity, and show antioxidant effects due to its metabolite activity, which includes tannins, flavonoids, saponins, calcium oxalate, terpenoids, peroxidase, and taraxeryl acetate (Anand & Sarkar, 2017; Gunawan *et al.*, 2016; Hermarana *et al*., 2014)

The selection of excipients or carrier materials and the form to be produced is very important. Sodium alginate is a suitable excipient due to its biocompatibility and capability of natural degradation. (Tønnesen & Karlsen, 2002; Zhang *et al*., 2021; Jadach *et al*., 2022; Guo *et al*., 2023). The interaction between excipients and active ingredients is expected to enhance the absorption of the active ingredients by altering the physical properties of the dosage form, thereby

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increasing the stability of the active ingredients (Reker *et al*., 2019). These interactions are generally divided into two types: physical interactions which often lead to changes in the dissolution rate or the properties of the active ingredients, and chemical interactions, which tend to be harmful as they can lead to the formation of unwanted degradation products. These degradation products have the potential to interact with the active ingredients, causing a decrease in the physicochemical stability and reducing the clinical efficacy of the active compounds. As a result, the quality and safety of the drug may be compromised if not properly controlled (Alfaridz & Musfiroh, 2020).

One of the necessary stages in drug development is testing for toxic contents in the product's excipient (Osterberg & See, 2003). In the initial phase of formulation development, it is crucial to conduct comprehensive cytotoxicity screening of all components used, not only the active pharmaceutical ingredient, but also each excipient must be thoroughly evaluated (Buckner et al., 2016). Cytotoxicity testing is a biological evaluation and screening test, using tissue cells in vitro to observe cell growth, reproduction, and morphological effects of a drug candidate. The MTT test is an enzymatic test using the reagent *3 - (4,5-dimethyl thiazol - 2 - yl) - 2,5 -diphenyltetrazolium bromide*, and is commonly used to measure cell viability in cytotoxic tests. Mammalian cell lines, such as baby hamster kidney (BHK-21) are widely used to detect the level of cellular toxicity in ethnopharmacological studies (Korkmaz *et al*., 2022). Fibroblasts were used as markers to test cytotoxicity by calculating the percentage of fibroblast cell viability, given their crucial in healing process, as inactive fibroblasts can lead to autolytic debridement and decrease fibrin clots, thereby inhibiting the healing process (Zhang *et al*., 2010; Bainbridge, 2013). The cytotoxicity level of a product is based on the ISO 10993-5 standard, which divides the cytotoxicity into four levels; a cell viability percentage above 80% is considered non-cytotoxicity; in the range 80%–60% is considered weak; in the range of 60%–40% is considered moderate; and below 40% is considered strong cytotoxicity (López-García *et al*., 2014).

This study aims to investigate the effect of sodium alginate excipient from brown algae as an excipient for *Hibiscus rosa-sinensis L.* leaf extract. The study was conducted by performing in vitro cytotoxicity tests on BHK-21 fibroblast cells using the MTT assay method.

2. Materials and Methods

The research was an experimental *in vitro* model conducted through cytotoxicity testing on two study groups: sodium alginate sponge as the control group and combination of sodium alginate sponge as an excipient with 12.5% extract of *Hibiscus rosa-sinensis L.* leaf as the treatment group. The medium used in this study was baby hamster kidney (BHK-21) fibroblast cell cultures, characterised by flat and elongated shapes with oval-shaped nuclei. The BHK-21 fibroblast cells were prepared using frozen stock available at the Dental Research Center, University of Airlangga which had undergone a thawing process for culture purposes in this study.

The MTT assay procedure began with the addition of 1 ml of medium to the pellet resulting from cell harvesting centrifugation. Then, the pellet was resuspended until dissolved, and 10 µl of the mixture was pipetted into parafilm. Then, 10 µl of trypan blue was added and mixed with the cells for the staining process. Cell counting was performed using a sceptre with a total of 5 x 10^3 cells for each well, then 100 µl of medium was added to all wells, and the cells were incubated at 37 °C for 72 hours until confluence. The microwell plate was then divided into four sections according to the treatment groups: Part I, contains BHK-21 fibroblast cells with sodium alginate sponge medium (n=16); Part II, contains BHK-21 fibroblast cells with a combination of sodium alginate sponge medium and *Hibiscus rosa-sinensis L.* leaf extract (n=16); Part III, medium control group containing only culture medium (n=16); Part IV, cell control culture containing only culture medium and BHK-21 fibroblast cells without any treatment (n=16).

All cells were incubated for 24 hours in a 5% CO_2 incubator at 37 ℃, afterward the culture medium was removed, the cells were washed with PBS, and 10 µl of MTT solution was added and incubated again for 4 hours in a CO_2 incubator at 5% CO_2 and 37°C in the dark. Observed under the microscope whether the formazan crystals had formed. If they had formed, 50 µl of DMSO was added, and then it was incubated for 10 minutes in the incubator or at room temperature. The results of the cell staining by formazan were blue and were read using an ELISA reader with a wavelength of 540 nm, and then converted into a percentage.

The data generated from cell cytotoxicity testing using the MTT assay are in the form of absorbance values, which are then converted into cell viability. The percentage of cell viability is calculated using the following formula (Kamiloglu *et al*., 2020): $\% cv = \frac{TA - MA}{CA - MA} \times 100\%$, where *% cv* represents the percentage of the number of cells that are alive after treatment, *TA* represents the Optical Density (OD) value of formazan for each sample after treatment, *CA* represents the OD value of formazan in the average control cells, and *MA* represents the OD value of formazan in the average of each medium control. Cell viability was measured using an inverted microscope.

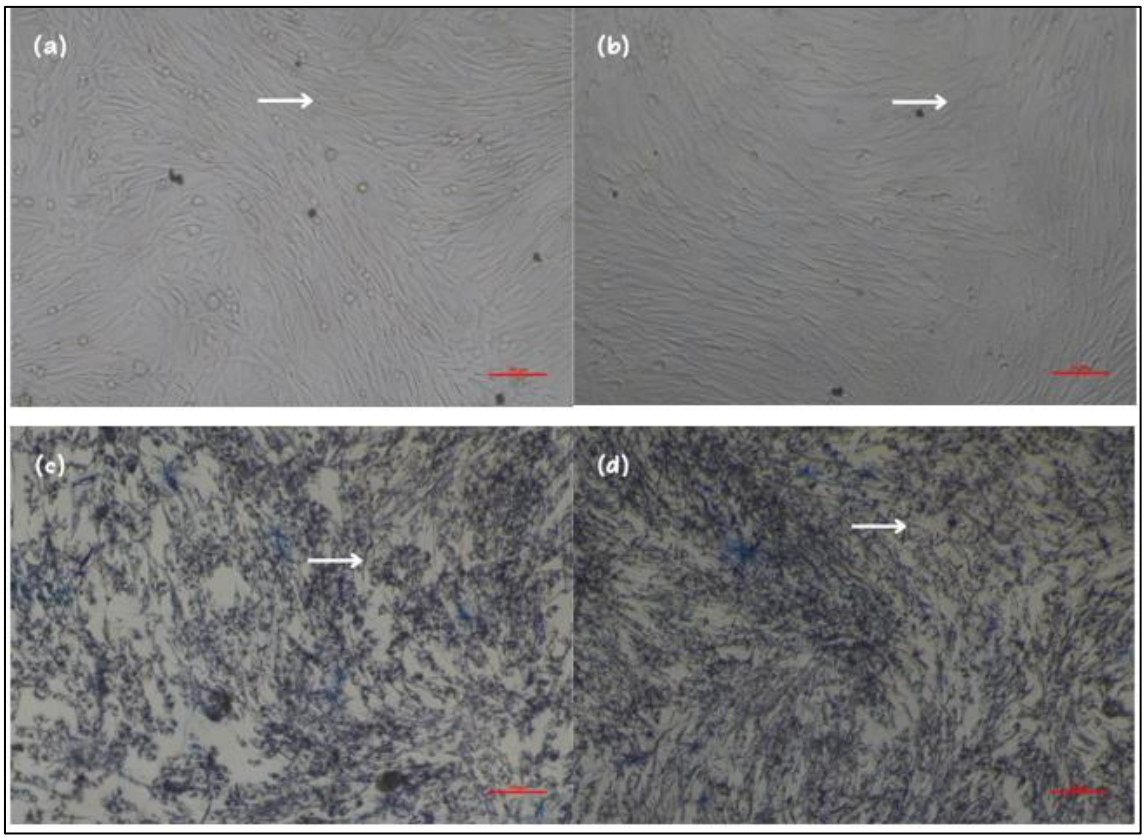
3. Results

Data was analysed with a descriptive test and presented in Table 1. The Shapiro-Wilk test was used as the normality test. The both groups have $p < 0.05$ and thus the data is non-normally distributed. On the other hand, the data have homogeneous variations as the Levene test obtained was $p > 0.05$. As the data were non-normally distributed, a Mann-Whitney test was performed and there were no significant differences, $p = 0.940$ ($p > 0.05$) between the control and treatment groups. The control group showed an average fibroblast cell viability of 0.765 OD ± 0.237, equivalent to 90.13%, and in the treatment group, it was 0.811 OD ± 0.292, equivalent to 95.87% (Table 1).

Tests using an inverted microscope showed that BHK-21 fibroblast cells appeared denser in the treatment group than in the control group (Figure 1a, 1b). Cytotoxicity test by administering MTT solution to both the control and treatment groups showed that the microscopic image of BHK-21 fibroblast cells appeared blue, indicating the formation of formazan crystals (Figure 1c, 1d).

Table 1 Mean and standard deviation (SD) for the optical density of group sample

Group	Mean	SD
Sodium alginate	0.765	0.237
Sodium alginate + Hibiscus	0.811	0.292



Note : Microscopic images of BHK-21 fibroblast cells using an inverted microscope with 100x magnification on a 100µm scale, with cells indicated by white arrows: (a) control group before MTT solution application, (b) treatment group before MTT solution application, (c) control group after MTT solution application, showed the formation of blue formazan crystals, indicating the presence of fibroblast cells, and (d) treatment group after MTT solution application showed more concentrated formazan crystals than the control group.

Figure 1 Microscopic images of BHK-21 fibroblast

4. Discussion

The results showed that the percentage of BHK-21 fibroblast cell viability was quite high in both groups. Based on ISO 10993-5, the sodium alginate sponge group is classified as non-toxic because it shows a percentage of 90.13%. This is due to the natural properties possessed by sodium alginate as a polysaccharide derived from brown algae extract, which has a unique structure such as human extracellular matrix, broad biological spectrum, high biocompatibility, good biodegradability, the ability to absorb and retain good fluids, and is colloidal (Jayasinghe *et al*., 2022). In the sodium alginate combination sponge group with 12.5% extract of *Hibiscus rosa-sinensis L.* leaves showed a higher percentage of 95%. This shows that the combination of sodium alginate sponge with 12.5% extract of *Hibiscus rosa-sinensis L.* leaves proved to be more non-toxic than the non-combination sodium alginate sponge group.

The addition of 12.5% *Hibiscus Rossa Sinensis L.* leaf extract did not cause any additional cytotoxicity compared to the control group using only sodium alginate, it means the presence of *Hibiscus* extract in the formulation did not increase cytotoxicity or reduce cell viability. In the tests, the control group using only sodium alginate showed a high viable count, and when the *Hibiscus* extract at 12.5% concentration was safe and non toxic to fibroblast cells. This is because sodium alginate as an excipient can maintain the *Hibiscus rosa-sinensis L.* leaf extract during processing because the barrier created by sodium alginate can protect the substance from physical stress and allows it to avoid immunological reactions with the host (Szekalska *et al.,* 2016)

The cell viability percentage in the control group was established as 100%, however, in the hibiscus leaf extract test, the calculated cell viability was not fully 100% but only 95%, suggesting the occurrence of cell death while still remaining within the non-toxic range. This may be due to the limitations of the MTT assay method employed in this research, particularly in discerning sublethal effects on cell mortality at low levels. These limitations might arise from factors such as the concentration of MTT reagent used, duration of cell incubation with MTT, type of culture medium utilized, elimination of cell supernatant post-incubation, the wavelength selected for optical density measurement, and the experimental treatments (Ghasemi *et al*., 2021)

The results of the cytotoxicity study in the combination group of sodium alginate sponge with 12.5% extract of *Hibiscus rosa-sinensis L.* leaves proved not to decrease also due to the antioxidant flavonoids, tannins, saponin, and alkaloid activities in the extract. Antioxidants have an important role in protecting cells from damage by free radicals and have been shown to inhibit the production of pro-inflammatory cytokines induced by sodium alginate so that the two can balance each other (Szekalska *et al.,* 2016; Anand & Sarkar, 2017; Taherkhani, Suzuki, & Castell, 2020)

5. Conclusion

The combination of *Hibiscus rosa-sinensis L*. leaf extract and sodium alginate in the drug development demonstrates non-cytotoxic efficacy.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare no conflict of interest. This work is intended for the public domain and to advance the field of biomedical science

References

[1] Alfaridz, F. and Musfiroh, I. (2020) 'Interaksi Antara Zat Aktif dan Eksipien dalam Sediaan Farmasi', Majalah Farmasetika, 5(1), pp. 23–31. doi: 10.24198/mfarmasetika.v5i1.25755.

[2] Anand, A., & Sarkar, B. (2017). Phytochemical screening and antioxidant property of anthocyanins extracts from hibiscus rosa-sinensis. In Applications of Biotechnology for Sustainable Development (pp. 139–147). Springer Singapore.

[3] Anusha, G. et al. (2020) 'Asian Journal of Pharmaceutical Research and Development', Asian Journal of Pharmaceutical Research and Development, 8(6), pp. 77–80.

[4] Bainbridge, P. (2013). Wound healing and the role of fibroblasts. Journal of Wound Care, 22(8), 407–412.

[5] Buckner, C. A. et al. (2016) 'We are IntechOpen , the world ' s leading publisher of Open Access books Built by scientists , for scientists TOP 1 %', Intech, 11(tourism), p. 13. Available at: https://www.intechopen.com/books/advanced-biometric-technologies/liveness-detection-in-biometrics.

[6] Carmona, F., & Pereira, A. M. S. (2013). Herbal medicines: Old and new concepts, truths and misunderstandings. Revista Brasileira de Farmacognosia, 23(3), 379–385.

[7] Ghasemi, M., Bagheri, S., Salari, R., & Beheshti, F. (2021). The mtt assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. International Journal of Molecular Sciences, 22(23), 12827.

[8] Guo, Y., Wang, M., Liu, Q., Liu, G., Wang, S., & Li, J. (2023). Recent advances in the medical applications of hemostatic materials. Theranostics, 13, 161–196.

[9] Gunawan, R. B., Aisyah, R., & Sutrisna, E. M. (2016). Effect of 70% Ethanol Extract of Hibiscus Leaves (Hibiscus rosa-sinensis L.) in Shortening Bleeding Time and Clotting Time in Male Swiss Mice. The Effect of 70% Ethanolic Extract Of Hibiscus rosa-sinensis L. Leaf In Reducing Bleeding Time And Clotting Time In Male Swiss Mice.

[10] Gupta, P., Suryarao, S., Suryawanshi, H., Kumari, U., Oswal, D., & Professor, A. (2022). Drug Development: Stages Of Drug Discovery And Development Process.

[11] Handa, S. S., Khanuja, S. P. S., Longo, G., & Rakesh, D. D. (2008). Extraction Technologies for Medicinal and Aromatic Plants.

[12] Hermarana, K., Jeyashree, K. V, Babu, M. M., & Kannan, M. (2014). Preliminary bioactive compounds screening and antibacterial activity of methanolic extract of Hibiscus rosasinensis against selected skin pathogens. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 5(2), 1210–1218.

[13] Jadach, B., Świetlik, W., & Froelich, A. (2022). Sodium alginate as a Pharmaceutical Excipient: Novel Applications of a Well-known Polymer. Journal of Pharmaceutical Sciences, 111(5), 1250–1261.

[14] Jayasinghe, G. D. T. M., Jinadasa, B. K. K. K., & Sadaruwan, N. A. G. (2022). Pathway of sodium alginate synthesis from marine brown algae, Sargassum wightii from Sri Lanka. Discover Food, 2(1).

[15] Kamiloglu, S., Sari, G., Ozdal, T. and Capanoglu, E. (2020) 'Guidelines for cell viability assays.' Food Frontiers. John Wiley and Sons Inc, 1(3) pp. 332 - 349.

[16] Korkmaz, S., Parmaksiz, A., Sait, A., & Omurtag Korkmaz, B. İ. (2022). The analysis of the effects of propolis products as food supplements on the viability of baby hamster kidney (BHK-21) and murine macrophage (RAW 264.7) cells by spectrophotometric MTT assay. Journal of Advances in VetBio Science and Techniques.

[17] Kumar, D., & Dureja, H. (2013). Pharmaceutical Excipients: Global Regulatory Issues. Indonesian J. Pharm, 24(4), 215–221.

[18] López-García, J., Lehocký, M., Humpolíček, P., & Sáha, P. (2014). HaCaT Keratinocytes Response on Antimicrobial Atelocollagen Substrates: Extent of Cytotoxicity, Cell Viability and Proliferation. Journal of Functional Biomaterials, 5(2), 43–57.

[19] Moghadam, E. T. et al. (2020) 'Current herbal medicine as an alternative treatment in dentistry: In vitro, in vivo and clinical studies', European Journal of Pharmacology, 889(June), p. 173665. doi: 10.1016/j.ejphar.2020.173665.

[20] Osterberg, R. E., & See, N. A. (2003). Toxicity of Excipients-A Food and Drug Administration Perspective. International Journal of Toxicology, 22, 377–380.

[21] Quan, W., Li, P., Wei, J., Jiang, Y., Liang, Y., Zhang, W., … Ouyang, Q. (2022). Bio-Multifunctional Sponges Containing alginate/Chitosan/Sargassum Polysaccharides Promote the Healing

[22] Reker, D. et al. (2019) 'Inactive ingredients in oral medications', Science Translational Medicine, 11(483), pp. 1–14. doi: 10.1126/scitranslmed.aau6753.

[23] Taherkhani, S., Suzuki, K. and Castell, L. (2020) 'A short overview of changes ininflammatory cytokines and oxidative stress in response to physical activity and antioxidant supplementation.' Antioxidants. MDPI pp. 1–18.

[24] Tønnesen, H. H. and Karlsen, J. (2002) 'alginate in drug delivery systems.' Drug Development and Industrial Pharmacy pp. 621–630.