ISSN: 0974-3618 (Print)
ISSN: 0974-360X (Online)

Research Journal of Pharmacy and Technology

RJPT

An International Peer-reviewed Journal of Pharmaceutical Sciences

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ISA: Indian Science Abstracts
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Research Journal of Pharmacy and Technology

ISSN

0974-360X (Online)

0974-3618 (Print)

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Online Since: Thursday, Nov 24, 2022 [*Views:* 15117]

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DOI: *10.52711/0974-360X.2022.00836 (https://www.doi.org/10.52711/0974-360X.2022.00836)*

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DOI: *10.52711/0974-360X.2022.00838 (https://www.doi.org/10.52711/0974-360X.2022.00838)*

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Research Journal of Pharmacy and Technology

ISSN
0974-360X (Online)
0974-3618 (Print)

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ISSN 0974-3618 (Print)
0974-360X (Online)

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RESEARCH ARTICLE

Effect of Glutaraldehyde Concentration Variation on Diclofenac Sodium Scaffolds as Cross-Linking Agent

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ABSTRACT:

Chitosan and gelatin were used as polymer scaffolds for cartilage tissue engineering. The scaffold was used as a biodegradable drug delivery system for diclofenac sodium to treat cartilage defects on osteoarthritis (OA). The materials were composed of diclofenac sodium, chitosan, gelatin, and cross-linking agent-glutaraldehyde (GTA) were form as scaffold. The purpose of this study to investigate the effect of GTA concentration variations (0.00%; 0.25%; 0.50%; 1.00%; 2.50%) on characteristics and the release of diclofenac sodium from chitosan-gelatin scaffold. The scaffolds were made by using the pre-freezing method with a temperature of -56 ± 5°C for 24 hours and characterized by porosity, pore size, swelling, degradation, toxicity test, and diclofenac sodium released from chitosan-gelatin scaffolds at pH and temperature body. The results showed, the addition of GTA increased the swelling ratio from 195.79 ± 7.04% to 793.49 ± 6.92% and minimized weight loss up to 50.98 ± 0.82%, percentage of living cells >60%, optimal porosity at 106.94 ± 9.38 % with pore size 135.48 ± 89.70 µm, diclofenac sodium as sustained release drug completed in 542 hours and the release was following zero-order kinetic. Chitosan-gelatin scaffold is a potential candidate for cartilage tissue engineering and drug delivery system for diclofenac sodium.

KEYWORDS: Drug Delivery System, Glutaraldehyde, Scaffold, Diclofenac Sodium, Chitosan, Gelatin.

INTRODUCTION:

Osteoarthritis (OA) is chronic condition that causes pain, functional restrictions, fatigue, increased healthcare use, and substantial societal expenses[1]. OA primarily affects articular cartilage, which degrades over time due to a decrease of chondrocytes or excessive production of extracellular matrix-degrading protease enzymes (MMPs and IL-1)[2]. The pharmacological therapy of NSAIDs which are more commonly prescribed in Indonesia for OA patients, is Na-diclofenac[3]. The mechanism of action of diclofenac sodium by inhibiting inflammatory factors has a powerful effect as an analgesic and effectively reduces the pain[4]. Diclofenac sodium works by inhibiting both COX-1 and COX-2.

Received on 08.06.2021 Modified on 21.12.2021
Accepted on 19.04.2022
Research J. Pharm. and Tech 2022; 15(11):4974-4980.
DOI: 10.52711/0974-360X.2022.00836

Prolonged systemic use of diclofenac sodium is reported to cause several side effects in 30% of patients, including ulceration, gastrointestinal, elevated liver enzymes, thrombocytopenia, and impaired renal function[5].

Diclofenac sodium is widely formulated for local treatment and designed to reduce side effects due to systemic use[6]. The topical formulation of diclofenac sodium has disadvantages such as a high partition coefficient of 13.4 (Log P = 1.13) making it difficult to penetrate through the skin[7]. Scaffolds with natural polymers can be formulated to form a local diclofenac sodium delivery system, as research conducted by Iglesias (2018)[8] shows that natural polymers such as chitosan can be used as a delivery system for diclofenac sodium. Scaffold act as a temporary extracellular matrix, providing support to cells as well as the newly produced tissue[9] stimulate the regeneration and growth of tissue.

These characteristics make scaffold an excellent candidate for medication and molecular delivery to specific tissues[10].

Natural polymer such as gelatin and chitosan have been used in medical application such as antiviral, antimicrobial, wound healing, analgesic, antitumor, antioxidant, and bone cartilage tissue engineering[11-13]. Gelatin and chitosan have been shown as non-toxic, inexpensive, and favorable for its biocompatibility and biodegradability[14-16]. Scaffold as a drug delivery system containing a hydrophilic polymer matrix (collagen and chitosan) and diclofenac sodium as an active ingredient is given a cross-linking treatment, causing physical and chemical changes as evidenced by the formation of three-dimensional materials[17-18]. Cross-link agents are used to stabilizing the bonds between polymers to form scaffolds more firmly in order to decrease the porosity and prolonged the release of active ingredients[19].

GTA is the most commonly used cross-link agent, inexpensive, and efficient in stabilizing gelatin and chitosan molecules[20]. GTA increases the strength and water resistance of the scaffold matrix[21], and also reduces its cytotoxic effects by low concentration[22]. Based on research from Feigal (1990) [23] shows that the maximum non-toxic concentration of GTA is 2.50%. This research was conducted to determine the characteristics and release of diclofenac sodium from chitosan-gelatin scaffold with a ratio of cross-link agent GTA 0%, 0.25%, 0.50%, 1.00%, and 2.50%.

MATERIALS AND METHODS:

Materials:

Materials used in making scaffolds include aquadem, diclofenac sodium from KALBE Co., Ltd; gelatin type B (CARTINO Co., Ltd); Chitosan from shrimp shells; NaOH; acetic acid, GTA (KGTAA Frankfurter Co., Ltd), PEG-400, and saline phosphate buffer pH 7.40 ± 0.0; BHK-21 (Baby Hamster Kidney)-21 fibroblast cells, Eagle media; FBS (Fetal Bovine Serum); MTT assay solution; dimethylsulfoxide, UV-Vis spectrophotometer UV-VIS, SEM TM pore diameter test equipment, ELISA readers, 37° C incubators (Memmert, Germany), Microplate 96 well, culture bottles (Roux, Schott, Duran, Germany).

Methods:

Scaffold preparations consist of natural polymeric materials, which serve as a base with additives to optimize the scaffold as a diclofenac sodium delivery system. Scaffold components as shown in Table 1.

Table 1. The formula for diclofenac-gelatin-chitosan scaffolds

Ingredient	Function	Concentration (% b/v)				
		I (%)	II (%)	III (%)	IV (%)	V (%)
Chitosan	Base	4	4	4	4	4
Gelatin	Base	4	4	4	4	4
PEG 400	Plastisizer and cosolven	0.50	0.50	0.50	0.50	0.50
Diclofenac sodium	Drug	1	1	1	1	1
NaOH	Neutralizer	0.50	0.50	0.50	0.50	0.50
Acetic Acid	Solvent	1	1	1	1	1
Glutaraldehyde	Cross-link Agent	0	0.25	0.50	1.00	2.50
Aquadem	Solvent	Qs	qs	qs	qs	qs

Scaffold Preparations:

Scaffold preparations consist of natural polymeric materials, which served as a base with excipients to optimize the scaffold as a diclofenac sodium delivery system. Added scaffold formula as shown in Table 1 with 1% acetic acid 50mL and 2 grams of chitosan to 200 ml beaker glass, stirred it on a hot plate until homogeneous. 2 grams of gelatin were added into 100 mL beaker glass with 50 ml hot aquadem (40-50 °C) stirred until homogeneous. Mix chitosan and gelation while stirring on a hot plate. Next, 1gram diclofenac sodium was dissolved in a 0.5% PEG-400 solution ad 100 mL and added to chitosan-gelatin mixture. Then, 0.5 ml of GTA mixed into every 16 mL of the total mixture while stirring for 1-2 hours then molded the preparations in a 1 × 1 cm cube container. Freeze the mixture and washed using 0.5% NaOH, then rinsed with aquadem and dried. In the last stage, the mixture was dried using a freeze drying at -56 °C for 24 hours.

Evaluations:

a) Organoleptic:

Scaffolds were observed organoleptically for appearance color.

b) Toxicity Test:

MTT assay was used for toxicity test by using BHK-21 fibroblast cells. 200 mg of diclofenac-chitosan-gelatin scaffold with various concentrations of GTA (0%; 0.25%; 0.50%; 1.00%; and 2.5%) were used for MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The examination was carried out by means of a suspension BHK-21 inserted in 96 well plates of 50 µL / well with a range of 2 x 105 cells (except for control). Added 20% DMEM as much as 100 µL/well. 96 well plates containing cells were incubated in a CO2 incubator at 37°C for 24 hours. After 24 hours, the biomaterial that has been prepared was inserted, each sample was replicated five times. After 24 hours, the remaining scaffold contained in the plate was

removed and washed the plate using PBS 150 µL. Added 30 MPL DMEM media and MTT solution of 10 mL/well. Re-incubated for 3 hours, added DMSO 50 µL/well, incubated for 5 minutes, then read using ELISA reader (Thermo Scientific) with a wavelength of 625 nm. The percentage of live cells was measured using the Freshney formula:

$$\% \text{ Living cell} = \frac{\text{OD Treatment} + \text{OD media} \times 100\ \%}{\text{OD control cell} + \text{OD media}} \quad (1)$$

Note: % of live cells = Percentage of live cells count; Treatment = Optical density value of scaffold; Media = Optical density value of scaffold on media; Cell = Optical density value of scaffold on control cells.

c) Porosity Test:

The porosity tests carried out included observing the pore diameter using SEM TM 3000 and the percentage of pore diameter using the liquid media transfer method (Buffer Phosphate pH 7.40 ± 0.05) as previously described[24]. The percentage of porosity was measured using the formula:

$$P = \frac{Wb - Wk}{Vs} \times 100\% \quad (2)$$

Note: P = Percentage of porosity (%); Wk = Weight of dry scaffold (gram); Wb = Weight of wet scaffold (gram); Vs = Scaffold volume cm^3

d) Degradation Test:

Degradation testing carried out by two parameters, swelling ratio, and weight loss. Observations were carried out for 28 days by observing changes in the weight of the preparation soaked in PBS pH 7.40 ± 0.05.

- **Swelling ratio:**

Scaffolds were submerged in 5 ml PBS and incubated at 37ºC for up to 35 days. Scaffolds were then dried in a vacuum oven at 37ºC until they reached a constant mass. After different time intervals (1 h, 12 h, 1d, 7 d, 14 d, 14 d, 21d, 27d, and 35d) samples were removed, and determined the wet weight (mw) and swollen weight (msw). The swelling ratio of the scaffold was defined with the following formula:

$$\text{Swelling ratio} = \frac{mw - msw}{mw} \quad (3)$$

- **Weight loss:**

Scaffolds were submerged in 5 ml PBS and incubated at 37 ºC for up to 42 days. Scaffolds were dried in a vacuum oven at 37 ºC until a constant mass was reached (ms). After different time of intervals (1d, 7 d, 14 d, 14 d, 21d, 27d, 35d, and 42d) samples were washed in a large volume of deionized water to remove buffer salts and dried on filter paper to remove excess water before dried in an oven at 40ºC. The pH value of the PBS was measured after each time point using a pH meter. The final mass recorded (md) and used to calculate the percentage weight loss:

$$\% \text{ Weight Loss} = \frac{ms - md}{ms} \quad (4)$$

e) Dissolution:

Drug release testing was carried out to observe drug loading, release of diclofenac sodium, and kinetic order to release diclofenac sodium phosphate from diclofenac-chitosan-gelatin scaffold. Observations were carried out for 22 days using and reported levels of scaffold preparations submerged in the media of PBS pH 7.40 ± 0.05 using a UV-Vis spectrophotometer.

f) Drug Loading:

test was performed by weighing 260 mL diclofenac-chitosan-gelatin scaffold in each preparation (4 formulas and 1 control replicated three times) dissolved in PBS 5.0 mL in test tube, Perform vortex for 10 minutes. Take 100.0µL and put in 10.0 mL volumetric flask, add the PBS and shake, diclofenac sodium test solution for absorption at the maximum wavelength. The diclofenac sodium concentration was determined by entering the absorbance value of the sample into the regression equation of the sodium diclofenac standard curve y = 3.14.10-2 x + 1,764.10-1. Once the levels are found, they are multiplied by 100 (100x dilution).

g) Determination of the cumulative percentage of release:

The release of diclofenac sodium from the chitosan-gelatin scaffold was carried out in a dissolution medium using PBS solution pH 7.4 ± 0.05. The release test used a water bath at 37 ± 0.5ºC and replicated three times. 300 mg ± 10 mg of diclofenac-chitosan-gelatin scaffold put into a test tube containing 5 mL PBS with a pH of 7.4 ± 0.05. Then put the samples into a water bath at 37 ± 0.5ºC, then 100.0 µL samples were taken at various times (1 h, 2 h, 4 h, 5 h, 6 h, and continuously taken one time/day for 22 days). Each sample previously taken was replaced by PBS with the same amount (100.0 µL). The sample was then observed for absorption with a UV-Vis spectrophotometer at a wavelength of 276 nm. The actual concentration obtained by calculating the dilution of 100.0 µL of media in each sample, the following correction factors are used in the wuster formula:

$$Cn = C'n + \frac{a}{b} \sum_{s=1}^{N-1} Cs \quad (5)$$

Note:

Cn : Actual concentration after correction (mg / L)

C'n: Concentrations measured by spectrophotometer

(mg / L)
Cs : The concentration measured by a spectrophotometer from a previous sample (mg / L)
a : Sample volume taken (mL)
b : Media volume dissolution (mL)

Calculated % drug cumulative of diclofenac sodium released from chitosan-gelatin scaffold by using the following formula:

$$\% \text{ Drug Cumulative} = \frac{\text{Mass of sodium in the media}}{\text{Mass of sodium diclofenac weighed}} \times 100 \quad (6)$$

h) Determination of the Release Kinetics Model:
The release data from the steady-state point was calculated by kinetic models (zero-order, first-order, and Higuchi) to determine the release kinetics model. The model chosen was the model with the correlation coefficient (R2) closest to 1[25].

RESULTS:

1. Organoleptic:

The results showed diclofenac-chitosan-gelatin scaffold had a different appearance. The scaffold's appearance was darker by increasing on GTA concentration (Figure 1)

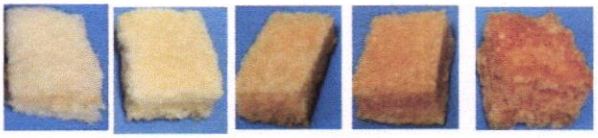

Figure 1. Color changes of diclofenac-chitosan-gelatin diclofenac scaffold

2. Toxicity:

The results of diclofenac-chitosan-gelatin scaffold at various GTA concentrations showed the percentage of living cells for all formulations was more than 60% (Figure 2 and Table 2).

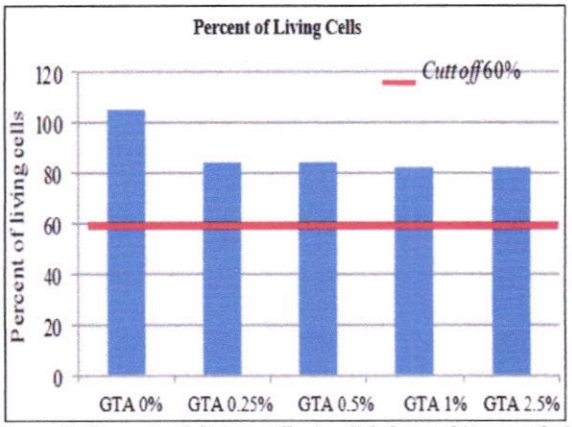

Figure 2. Percent of living cells in diclofenac-chitosan-gelatin scaffold

Table 2: The results of Living Cell Percentage of Diclofenac-Chitosan-Gelatin

n	Media	Cell Control	Formula				
			GTA 0%	GTA 0,25 %	GTA 0,5 %	GTA 1%	GTA 2,5 %
1	0,088	0,121	0,140	0,094	0,093	0,082	0,079
2	0,068	0,121	0,116	0,086	0,076	0,088	0,085
3	0,076	0,123	0,191	0,086	0,097	0,083	0,082
4	0,185	0,155	0,145	0,123	0,110	0,107	0,109
5	0,083	0,126	0,119	0,080	0,094	0,085	0,087
Mean	0,100	0,129	0,142	0,094	0,094	0,089	0,088
SD	0,048	0,015	0,030	0,017	0,012	0,010	0,012
% Living cell			105,672*	84,555	84,642	82,461	82,199

3. Porosity:

The results of observations of pore diameter (Figure 3) and porosity percentage shown in Table 3 showed the optimal addition of GTA at a concentration of 0.50% formed pore diameter and porosity percentage diclofenac-chitosan-gelatin at 135 ± 89.70 µm and 106.94 ± 9.38%.

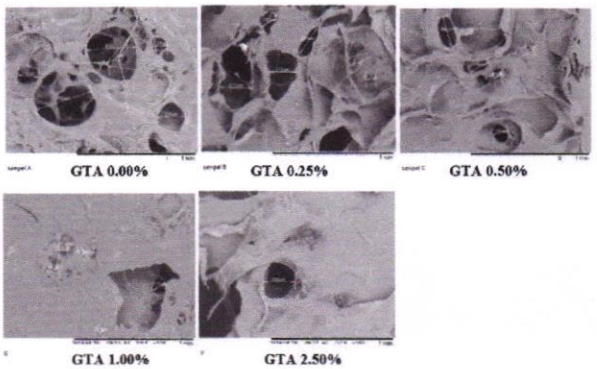

Figure 3. Observation of pore diameters diclofenac-chitosan-gelatin scaffold at magnification 100x

Table 3. The results of observations of pore diameter and porosity percentage on scaffold diclofenac-chitosan-gelatin.

	Min-Max pore diameter (µm)	Pore Diameter average ± SD (µm)	Porosity Percent average ± SD (%)
Blanko (GTA 0%)	176-493	285.40 ± 166.58	23.64 ± 0.78
GTA 0.25%	81-271	191.40 ± 80.04	36.51 ± 3.85
GTA 0.50%	49.1-265	135.48 ± 89.70	106.94 ± 9.38
GTA 1.00%	20.4-117	60.26 ± 36.43	88.56 ± 3.71
GTA 2.50%	266 ~	>266	99.16 ± 4.47

4. Degradation:

The degradation results showed that scaffold without the addition of GTA decreased in weight to 87.14 ± 0.28% in 42 days. Scaffold with the addition of GTA has a % weight loss of 15.83 ± 5.01% to 50.98 ± 0.92% (Figure 4).

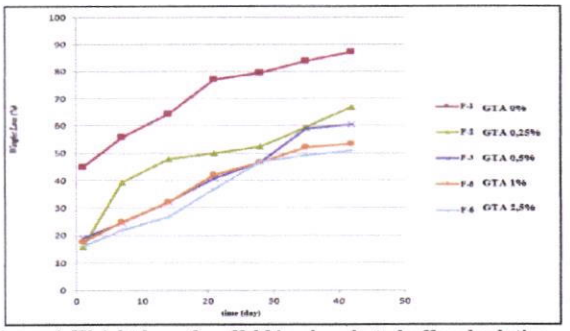

Figure 4. Weight loss of scaffold in phosphate-buffered solution.

The swelling test results showed that scaffold without GTA has a swelling ratio of 13.09 ± 0.96% to 214.35 ± 11.76%. Scaffold with the addition of GTA has a swelling ratio of 195.79 ± 7.04% -793.49 ± 6.92%. The maximum swelling ratio for each formula is on the 7th day except on Formula 5 and 6.

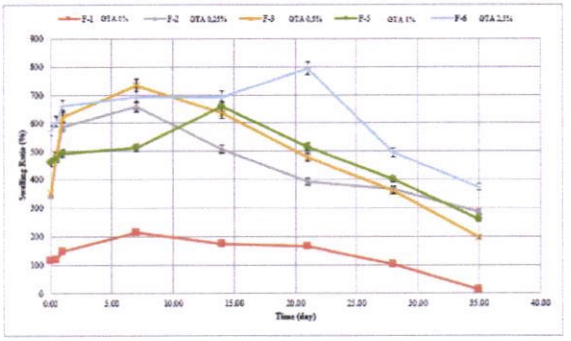

Figure 5: The swelling ratio of scaffold in phosphate-buffered solution

5. Dissolution:

The results from the dissolution test showed the drug loading from the scaffold showed an increase in levels in the capacity of the diclofenac sodium bond along with the addition of GTA concentrations. In addition, the total released times of diclofenac sodium from scaffold within 542 hours and determination of the order of kinetics on Table 4 showed that all formulas followed zero-order kinetics.

Table 4. Correlation coefficient (R2) kinetics model of scaffold diclofenac-chitosan-gelatin.

Formula	Zero Order	First Order	Higuchi
	R^2	R^2	R^2
Blanko	0.9725	0.9426	0.948
GTA 0.25%	0.9561	0.9154	0.9017
GTA 0.50%	0.9561	0.9492	0.9365
GTA 1.00%	0.9561	0.9491	0.9365
GTA 2.50%	0.9561	0.9512	0.9365

DISCUSSION:

Organoleptic results indicated the discoloration caused by the addition of glutaraldehyde that formed a diazotization reaction between the carboxyl groups of glutaraldehyde and amine groups on gelatin and chitosan polymers. Aldehydes reacted to primary amines from gelatin and chitosan to form imine bonds[26-27]. The toxicity results by MTT assay showed that the percentage of living cells from the calculations stated non-toxic preparations if ≥ 60% of living cells[28]. Therefore, all groups in this study showed that diclofenac-chitosan-gelatin scaffold with glutaraldehyde concentration variations wasn't toxic to BHK-21 fibroblast cells.

The pore diameter in the scaffold preparation has a role as a door of chondrocyte cells and nutrients into the scaffold. Meanwhile, porosity is an empty cavity that cells attached to chondrocytes when the addition of chitosan and gelatin as proteoglycans are expected to form new cartilage tissue[29]. Thus, the pore diameter and porosity results have met the requirements pore diameter (100-400 μm[22])[30], and the percentage of porosity for the scaffold as tissue engineering for cartilage is > 80% to make it easier for cells to migrate, proliferate, and differentiate[31].

The degradation study is a critical parameter to determine the degradation rate of scaffold formulation related to drug release pattern. The scaffold formulation was maintained constant until the end of the degradation process. Scaffolds weight loss because the release of polymer and loss of tissue formation lead to leaching of the polymer out of the matrix[32]. Therefore, degradation is likely to occur with the dissolution of the scaffold structure exposed to PBS. The addition of glutaraldehyde in scaffold by 2.5% of concentration found the minimum weight loss. The results showed that the addition of glutaraldehyde reduced scaffold degradation and the highest concentration of glutaraldehyde resulted in the minimum % weight loss.

Cross-link between polymer chains changed the properties of polymer chains into macromolecular networks, with covalent bonds forming complex networks and made the porosity decreased water absorption and caused swelling. Scaffold without the addition of GTA has greater % weight loss than scaffold with the addition of GTA. It showed that the presence of GTA affected the chemical bond of the scaffold to be more stable and the swelling process reduced particles dissolved in PBS. The tightness of the polymer network is increased by crosslinking, which limits the molecular mobility of the chains between the junctions[33]. The liquid penetrated the scaffold wasn't destroyed but expanded in a long time and has not been destroyed until

the 42nd day. The swelling ratio is essential in assessing a material's efficacy to represent the scaffolding water absorption capacity[34]. In this study, the swelling ratio in PBS added by GTA showed an increase in swelling ratio along with the increasing concentration of GTA. The reduction of swelling ratio is caused by the matrix reaching its maximum point to expand and no longer resisted the penetration of water that enters the scaffold structure. The scaffold experienced an erosion that causes the scaffold to dissolve and release diclofenac sodium slowly. The swelling ratio increased little by little from the first hour onwards then slowly decreased, and this is estimated formula has a constant released (sustained-release). The highly connected porous structure of the scaffold allows the scaffold to quickly absorb the water, exhibiting sponge-like properties, perhaps due to the effect of high cross-link density. If the water absorption capacity is good, the scaffold quickly absorbs the biomaterials and fills the damaged tissue cartilage [35].

The dissolution test results showed scaffold with the largest concentration of GTA has the largest drug levels when the drug is forcibly released from the chitosan-gelatin scaffold. GTA extended the drug to release from the scaffold[36] and an increase in crosslinker accompanied by an increase in the active bonding in the polymer chain, causing more drugs trapped in the polymer chain bond[37]. The results of percent cumulative concentration release of diclofenac sodium showed it was in line with the expectations of slow-release modification. The correlation coefficient (R2) data results showed that the formula followed zero-order kinetics because each formula has the highest correlation coefficient (R2) close to 1 than the Higuchi kinetics model and first-order kinetics.

CONCLUSION:

The addition of GTA increased the swelling ratio from 195.79 ± 7.04% to 793.49 ± 6.92%. It minimized weight loss up to 50.98 ± 0.82%, percentage of living cells >60%, optimal porosity at 106.94 ± 9.38% with pore size 135.48 ± 89.70 µm, diclofenac sodium as sustained-release completed in 542 hours, and the release was following zero-order kinetic. Diclofenac-chitosan-gelatin scaffold is a potential candidate for cartilage tissue engineering and drug delivery system of diclofenac sodium.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this study.

ACKNOWLEDGEMENT:

1. International Joint Symposium The 8th Asia Pasific Pharmacy Education Network (APPEN) and the 2th The Halal Pharmaceuticals and Cosmetics (HPC)
2. Indonesian governmental institution of research, technology, and higher education for funding this research

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ISSN

0974360X, 09743618

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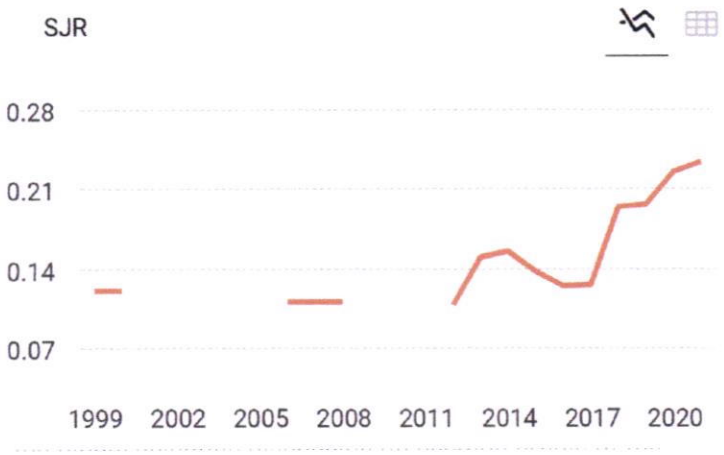
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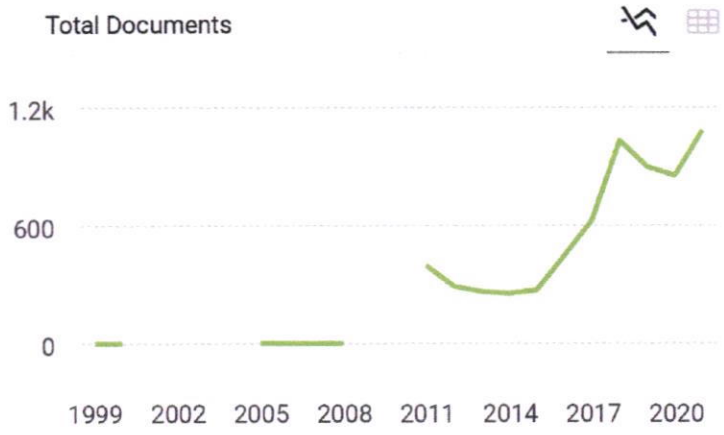
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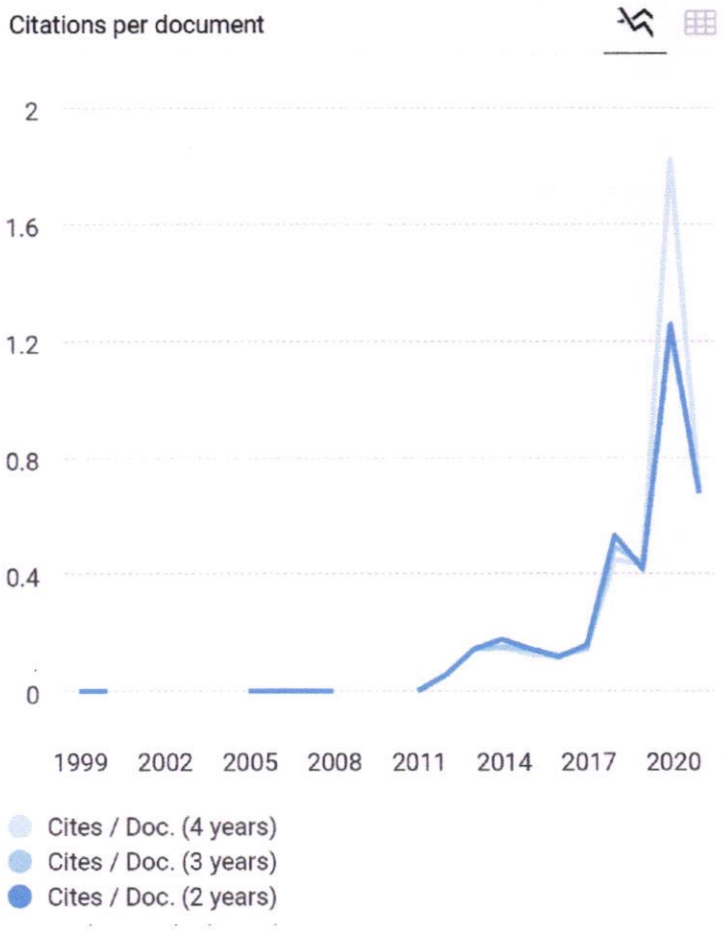
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A **Andi Nafisah** 11 months ago

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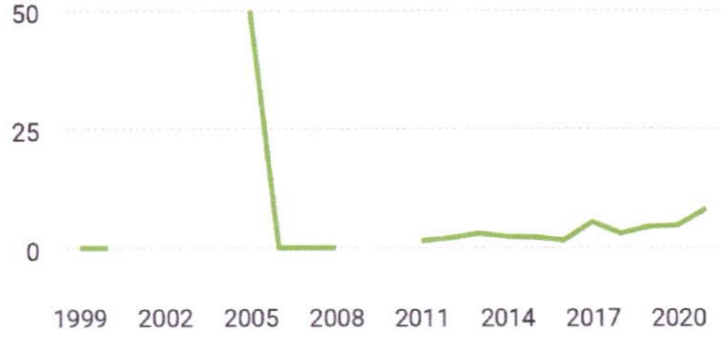

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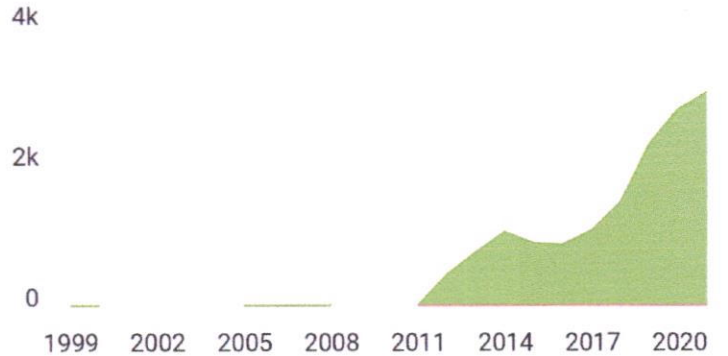

Dr Fatma Bassyouni

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Melanie Ortiz 1 year ago

Dear Fatma,
Thank you very much for your comment.
All the metadata have been provided by Scopus /Elsevier in their last update sent to SCImago, including the Coverage's period data. The SJR for 2020 was released on 17 May 2021. We suggest you consult the Scopus database directly to see the current index status as SJR is a static image of Scopus, which is changing every day.
Best Regards, SCImago Team

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Melanie Ortiz 1 year ago

Dear Vagdevi, Thank you for contacting us.
Unfortunately, we cannot help you with your request, we suggest you visit the journal's homepage or contact the journal's editorial staff , so they could inform you more deeply.
Best Regards, SCImago Team

O **OKEY EDITH N.** 2 years ago

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