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Research Article

The Analysis of Proanthocyanidins Cacao Peel Extract (Theobroma cacao L.) Potential on The Expression of TNF-α and COX-2 on Periodontitis Rat

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ABSTRACT
Analyze the potency of proanthocyanidins extract of cacao peel extract to inhibit TNF-α and COX-2 expression in rat gingival sulcus fluid induced by Porphyromonas gingivalis. 3 groups in this study, namely negative control, positive control, and treatment. In all groups, on the 0th day, gingival sulcus fluid was taken, then the rats were induced by Porphyromonas gingivalis once every 3 days for 2 weeks; then in the negative control group a placebo gel was applied and the treatment group was applied proanthocyanidin gel of cacao peel extract every day for 7, 14, and 28 days and serial gingival crevicular fluid collection was carried out on days 7, 14, and 28. The expression of TNF-α and COX-2 in the gingival crevicular fluid was observed using the ELISA method. The statistical test used was Anova. There were a significant difference in TNF-α and COX-2 expressions ($p < 0.05$). There was a decrease in the expression of COX-2 in rat gingival sulcus fluid induced by Porphyromonas gingivalis and given 10% cacao peel extract. Proanthocyanidin in the cacao peel extract has the potential to reduce TNF-α and COX-2 expression in periodontitis rats.

Keywords : proanthocyanidins, periodontitis, cacao peel extrac, TNF-α, COX-2.

INTRODUCTION

Cacao peel only used as animal feed and if they are not used properly, they will increase the amount of waste from the cacao peel, even though from a medical perspective, the cacao peel has many ingredients that can be isolated and utilized [1]. One of the largest ingredients is polyphenols in the form of catechins (37%), anthocyanins (4%), and proanthocyanidins (58%) [2]. Proanthocyanidin, the largest polyphenol type of cacao peel extract, can be used as an immunomodulatory, anti-cancer, antioxidant, antibacterial, and anti-inflammatory agent [3].

Proanthocyanidin, the largest polyphenol type of cacao peel extract, can be used as a natural alternative for healing inflammation, such as periodontal disease [4]. Several studies have shown that proanthocyanidin can inhibit the growth of Porphyromonas gingivalis (P. gingivalis) and biofilm formation, also has anti-inflammatory activity by reducing the production of proinflammatory mediators (IL-1 and TNF-α) and can inhibit the secretion of IL-8 and chemokine (C-C motif) ligand 5 (CCL5) exposed by P. gingivalis [5,6].

P. gingivalis is a species that is closely related to the formation process of chronic periodontitis, the number is found about 85% in periodontal tissue that is inflammed [7]. The damage that occurs in periodontal tissue is caused by various virulence factors of P. gingivalis such as lipopolysacharide, capsule, fimbriae, and gingipains [8].

Virulence factors possessed by P. gingivalis bacteria can cause inflammation by releasing proinflammatory cytokines (IL-1, IL-6, and TNF-α). These proinflammatory cytokines activate transcription of the COX-2 gene [9]. Proinflammatory cytokines can cause inflammation of the periodontal tissue through the arachidonic acid metabolic pathway [10]. Cyclooxygenase is an enzyme that has an important role in the metabolic process of arachidonic acid to produce inflammatory mediators, especially COX-2. COX-2 is one of the enzymes responsible for the **synthesis of** inflammatory mediators, namely thromboxane A2

(TXA2) and prostglandin E2 (PGE2). COX-2 is not expressed continuously, its expression increases when it receives inflammatory stimuli such as lipopolysaccharides, hormones, growth factors and proinflammatory cytokines, which will cause an increase in inflammatory mediators. Continuous increase of inflammatory mediators can cause tissue damage [11].

COX-2 will be secreted in the gingival crevicular fluid in periodontal inflammation. The resulting of secretions can be used as biomarkers in determining the diagnosis and severity of periodontal disease. Detection of the presence of the COX-2 enzyme is easiest by taking gingival crevicular fluid, in patients with periodontitis the amount of gingival crevicular fluid will increase [12]. The advantages using gingival crevicular fluid as a method of analyzing periodontal inflammation are due to easy, inexpensive, and non-invasive method [13].

MATERIALS AND METHODS

Equipment

Tools and materials used in this research among others were analytical scales, oven, measuring flask, erlenmeyer, rotary evaporator, waterbath shaker, falcon tube, test tube, ose, centrifuge, spectrophotometer, stopwatch agar desicator, 1.5 ml eppendorf tube, refrigerator for storing samples at minus 60 C, vortex, yellow tip, micropipette, channel 8 micropipette, 96-well plate, COX-2 ELISA kit, TNF-α, and ELISA reader. Cacao peel extract, CMC-Na, acetone, sterile distilled water, P. gingivalis ATCC 33277 0.05 ml with a concentration of 2x109 CFU / ml, paper point number 15, cotton roll, and Phosphate Buffered Saline (PBS) [14].

Animal preparation

The treatment procedure for experimental animals has received approval (ethical clearance) by the Health Research Ethics Commission of Gajah Mada Faculty of Dentistry, namely No. 0019/KKEP/FKG-UGM/EC/2019. The experimental animals were adapted first for 7 days in a closed cage and given standard food and drink. This aims to obtain uniformity prior to conducting research to control experimental animals.

Extraction proantocyanidin of cacao peel

The cacao peel (Theobroma cacao L.) was cut and dried in the sun to dry, then shaved and blended to get a fine powder. Powdered cacao peel were freed from fat using n-hexane solvent by maceration with a ratio of powder to solvent (1: 5) three times. The fat-free sample was dried. Powder cacao peel 100 gram was put into an erlenmeyer containing 700 ml of 70% acetone solution and 300 ml of distilled water, then stirred until homogeneous and put in a waterbath shaker at 50°C for 20 minutes. The extract solution was separated from the supernatant by centrifugation at 2000 rpm for 10 minutes. The extract solution was then put into a rotary evaporator, after which it was transferred to a petri dish and put in the oven. The petri dish was removed from the oven, then the thick part that is at the bottom of the petri dish was removed and placed in a beaker [15].

Chromatography analysize used HPLC method

Proanthocyanidin analyze was performed using the column HPLC method which was operated at 25°C. The compounds were detected at 200 and 400 nm wavelengths. The mobile phase of HPLC consisted of 2% (v/v) acetic acid in water (eluentA) and 0.5% acetic acid in water and acetonitrile (50:50, v/v; eluent B). The mass spectrophotometric analysis of the extract was carried out by a mass spectrometer in negative ionization mode. The nebulizer pressure was set to 45 psi and the drying gas flow rate was 5 l/min. The flow rates and temperature of the casing gas were 11 l/min and 350°C, respectively. The mass ranges from 50 m/z to 2000. Chromatographic separation was performed on an ODS C18 analytical column (4.6 × 250 mm), using the Agilent 1290 Infinity HPLC system (Agilent Technologies, USA). About 0.3 ml/min of eluent was introduced into the mass detector. Selecting ion monitoring (SIM) was used to select molecular ion isomers from the procyanidins group in the proanthocyanidin extract of the cacao peel for quantification. The Agilent Mass Hunter Workstation was used for data acquisition and processing [16].

Preparation for Proanthocyanidin of cacao peel extract gel

96 ml distilled water was measured with a volumetric flask and poured into the mortar. Then 4 grams CMC-Na were measured by analytical scales and putted into a mortar containing distilled water. Let stand 10-15 minutes, stirring it until expands and forms a yellow gel. The mixture of CMC-Na and distilled water that has become gel was weighed as much as 45 grams and 100% proanthocyanidin of cacao peel extract as much as 5 grams, then it was putted in the mortar and mix until homogeneous to get a gel of proanthocyanidin of cacao peel extract with 10% concentration [17].

Preparation of P. gingivalis suspention

After making the culture media, one ose of P. gingivalis with 33277 ATCC pure was inoculated in each petridish, then incubated for 2x24 hours.[18] The suspension was made by taking 1 ose P. gingivalis bacteria from the culture preparation and it dissolving in 1 cc of saline/PZ solution in the tube. The reaction after that was homogenized by centrifuge and measured at a concentration of 2x109 CFU/ml according to the Mc Farland 0.5 standard using a spectrophotometer [14].

Applied of P.gingivalis and proanthocyanidins

The negative control, positive control, and treatment groups were injected with 0.05 ml of P. gingivalis ATCC 33277 at a concentration of 2x109 CFU/ml in the distobuccal and distopalatal of gingival sulcus of maxillary first molar. The injection was repeated once every 3 days for 2 weeks [18]. After obtaining the periodontitis rat model, then taking the rat gingival sulcus fluid on day 0 for the negative control and treatment group. Applying placebo gel for the negative control group, metronidazole for the positive control group and proanthocyanidin extract gel of cacao peel 10% for treatment group, daily for 28 days. Gingival crevicular fluid was taken on days 7, 14, and 28. Placebo gel and proanthocyanidin extract gel of cacao peel 10% was applied to the distobuccal and distopalatal gingival sulcus area of the maxillary first molar using a plastic filling instrument [19].

Measurement of TNF-α and COX-2 expression

Teeth were cleaned with cotton to control saliva, then gingival crevicular fluid (GCF) samples were taken with paper point number 15 for 30 seconds. The paper point was positioned horizontally in the area of the gingival groove in the distobuccal part of the maxillary first molar. Taking GCF should be done carefully so did not make a injury to the gingival groove area which in turn will cause contamination. Paper points were inserted into 0.5 mL eppendorf tube and stored at -20°C, until the ELISA test was performed. Before the ELISA test, the eppendorf tube was added with 50 µL 0.02 M of Phosphate Buffer Solutions (pH 7.0-7.2), followed by 2000-3000 RPM centrifugation at room temperature 18-25°C for 20 minutes, after that the ELISA test was carried out with the TNF-α and COX-2 ELISA kit. Then the test results are read using an ELISA reader with a wavelength of 450 nm for a maximum of 30 minutes after giving the stop solution [20].

Statistical analysis

The data obtained were analyzed using the Statistical Package for the Social Sciences (SPSS). Kolmogorov-Smirnov test used to test for normality and Levene's test for homogeneity. One-way analysis of variance (ANOVA) would be carried out to compare the TNF-α expression between each treatment group, followed by Least Significant Different (LSD). Groups differences were significant if the p value was < 0.05.

RESULTS AND DISCUSSION

TNF-α expression

The expression of TNF-α was presented in **Table 1.** There were significant difference of TNF-α expression on 0, 7, 14 and 28 days (p= 0.000). On 0 day, the most expression was proanthocyanidins group (268.452 ± 4.83), negative control group higher than positive control group (267.609 ± 3.74); on the 7 day, the most expression of TNF-α was negative control group (267.419 ± 3.62), positive control group higher than proantocyanidins group (239.560 ± 4.57); on the 14 day, the most expression of TNF-α was negative control group (227.384 ± 6.51), proantocyanidins group higher than positive control group (195.120 ± 7.456); and on the 28 day, the most expression of TNF-α was negative control group (216.402 ± 2.45), proanthocyanidins higher than positive control (175.772 ± 5.86).

Table 1 The TNF-α expression in the rat GCF (U/l) and Anova test.

Groups	TNF-α expression (Mean ± SD)				p
	0 day	7 days	14 days	28 days	
NC	267.609 ± 3.74	267.419 ± 3.62	227.384 ± 6.51	216.402 ± 2.45	*0.000
PC	229.585 ± 4.51	239.560 ± 4.57	193.694 ± 19.087	148.350 ± 8.82	
PA	268.452 ± 4.83	215.036 ± 7.310	195.120 ± 7.456	175.772 ± 5.86	

Note : NC = negative control

PC = positive control
PA = proanthocyanidins

The difference of TNF-α expression between group was presented in Table 3. On the 0 day, there were difference between negative and positive control group (0.00), also between positive control and proanthocyanidins group (0.00); on the 7 days, there were difference between negative and positive control group (0.00), between negative control and proanthocyanidins group (0.00), also between positive control and proanthocyanidins group (0.00); on the 14 days, there were difference between negative and positive control group (0.00); on the 28 days, there were difference between negative and positive control group (0.00), between negative control and proanthocyanidins group (0.00), also between positive control and proanthocyanidins group (0.00).

COX-2 expression

The expression of COX-2 was presented in **Table 2**. There were significant difference of COX-2 expression on 0, 7, 14 and 28 days (p= 0.000). On 0 day, the most expression was negative control group (71.740±3.56), proantocyanidins group higher than positive control group (56.577±1.070); on the 7 day, the most expression of TNF-α was negative control group (113.393±12.527), positive control group higher than proantocyanidins group (97.897±6.931); on the 14 day, the most expression of TNF-α was negative control group (194.094±6.592), positive control group higher than proantocyanidins group (78.397±2.134); and on the 28 day, the most expression of TNF-α was negative control group (55.924±1,706), positive control group higher than proantocyanidins group (54.081±2,644).

Table 2 The COX-2 expression in the rat GCF (U/l) and Anova test.

Groups	COX-2 expression (Mean ± SD)				p
	0 day	7 days	14 days	28 days	
NC	71.740 ± 3.56	113.393 ± 12.527	194.094 ± 6.592	55.924 ± 1,706	*0.000
PC	52.666 ± 0.00	97.897 ± 6.931	78.397 ± 2.134	54.081 ± 2,644	
PA	56.577 ± 1.070	92.811 ± 2.662	74.019 ± 4.594	52.695 ± 2,175	

The difference of COX-2 expression between group was presented in Table 4. On the 0 day, there were difference between negative and positive control group (0.04); on the 7 day, there were difference between negative control and proanthocyanidins group (0.02); between negative control and proanthocyanidins group (0.00), also between positive control and proanthocyanidins group (0.00); on the14 day, there were difference between negative and positive control group (0.00), also between negative control and proanthocyanidins group (0.00).

Table 3 The result of different test of The TNF-α expression by LSD test (p).

	0 day			7 days			14 days			28 days		
Groups	NC	PC	PA	NC	PC	PA	NC	PC	PA	NC	PC	PA
NC		*0.00	0.80		*0.00	*0.00		*0.00	0.07		*0.00	*0.00
PC			*0.00			*0.00			0.79			*0.00
PA												

Table 4 The result of different test of The COX-2 expression by LSD test (p).

	0 day			7 days			14 days			28 days		
Groups	NC	PC	PA	NC	PC	PA	NC	PC	PA	NC	PC	PA
NC		*0,04	0,10		0,09	*0,02		*0,00	*0,00		0,83	0,72
PC			0,66			0,57			0,62			0,87
PA												

The damage that occurs in periodontal tissues is caused by various virulence factors from P. gingivalis such as lipopolysacharides, capsules, fimbriae, and gingipains [8]. The presence of lipopolysaccharides will stimulate the formation of inflammatory mediators through the cyclooxygenase (COX) pathway [10]. COX is an enzyme that useful for accelerating the synthesis of prostaglandins from arachidonic acid. Arachidonic acid is an unsaturated fatty acid found in the phospholipid bilayer [21]. The inflammatory stimulus causes the activation of phospholipase A2 which causes the release of arachidonic acid from the cell membrane to the cytosol. Arachidonic acid metabolism through the cyclooxygenase (COX) pathway will produce prostaglandin E2 (PGE2) and thromboxane A2 (TXA2) [22].

Prostaglandin E2 causes increased vasodilation and endothelial permeability resulting in increased infiltration of inflammatory cell. PGE2 is the most type prostaglandin in the pathogenesis of periodontitis [23]. Increased proinflammatory cytokines (IL-1 and TNF-α) and PGE2 can stimulate bone resorption by increasing osteoclast formation. Proinflammatory cytokines and PGE2 will also inhibit the formation of osteoprotegerin (OPG) which functions to inhibit osteoclast formation, resulting in increased osteoclast formation and bone resorption [24].

The results of this research showed the treatment group with 10% proanthocyanidin extract gel had the lowest TNF-α and COX-2 expression. This was caused by the proanthocyanidin in the cacao peel extract having anti-inflammatory and antibacterial properties [25]. Proanthocyanidin activity in inhibiting COX-2 is probably by inhibiting the activity of proinflammatory cytokines. This was supported by several previous studies showing that proanthocyanidin can inhibit the growth of P. gingivalis and reduce COX-2 expression through inhibition of inflammatory cytokines activity [26].

According to research conducted by La, proanthocyanidin can inhibit the invasion of P. gingivalis and inhibit the activity of virulence factors such as gingipain [5]. Gingipain functions to activate proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) which can trigger inflammation.7 Based on research by Lee et al. (2017), proanthocyanidin from grape seeds can also decrease the expression of IL-1β, IL-6 and TNF-α [26]. Proanthocyanidin from cranberries can decrease the expression of IL-8 and CCL-5 from the oral epithelial cells induced by P. gingivalis [5].

Previous study showed that there was an increase in COX-2 mRNA expression after induced by IL-1β for nine hours and decreased significantly after proanthocyanidins administration [27]. Lee et al. explained that proanthocyanidins from cacao has an antiinflammatory effect by inhibiting proinflammatory cytokine activity. His study also explained that there was increased activation of IL-4 (anti-inflammatory cytokines) and decreased expression of IL-8 (proinflammatory cytokines) [26].

The study also showed that metronidazole gel which was used as a positive control could reduce COX-2 expression and there was a significant difference in the average COX-2 expression on days 7, 14, and 28. The decrease was due to metronidazole gel are bactericid against anaerobic gram-negative bacteria such as P. gingivalis by interfering for bacterial DNA synthesis. The mechanism of metronidazole eliminate these bacteria is by entering the bacteria and reducing it to a polar product which produces 2-hydroxymethyl metronidazole which will bind to bacterial DNA and disrupt its helical structure, then inhibit the synthesis of nucleic acids and result in bacterial cell death [28].

According to Lu et al. in vitro, mangosteen alpha of mangosteen peel was shown to be able to reduce lipopolysaccharide (LPS) induction against pro-inflammatory cytokine TNF-α and IL-4 by inhibition of oncostatin M gene expression in the MAPK pathway in the cell culture U937 [27]. The decreased of IL-1 and TNF-α expression will decrease COX-2 expression due to the signal inhibition of IL-1 and TNF-α for the release of phospholipids from the cell membrane, whereas COX-2 expression in the negative control group is higher. The amount of mRNA and gingival COX-2 protein in subjects with chronic periodontitis was higher than in healthy ones [29]. This is reinforced by the results of Mesa et al. study that COX-2 expression in patients with gingivitis or periodontitis was higher than in healthy gingiva [30].

Research conducted by Mori et al. demonstrated that P. gingivalis gingipain can induce COX-2, wherein COX-2 mRNA levels are greatly increased after 2 hours and can still be detected at 6 hours and 12 hours after exposure by Gingipain, lipopolysaccharides, capsules, and fimbriae. P. gingivalis can regulate inflammatory

cytokines such as IL- 1β, IL-6, IL-8, and TNF- α. IL-1β is an inflammatory cytokine that can increase COX-2 expression in some cells [31].

The research conducted by Tamura et al. showed that cells induced by IL-1β increased the expression of mRNA and protein COX-2 and PGE2 [32]. Interleukin-1 and TNF-α produced by macrophages will also cause the release of phospholipids from the gingival epithelial cell membranes, fibroblasts, mast cells, neutrophils, macrophages, lymphocytes so that arachidonic acid metabolism occurs by the action of the enzyme phospholipase A2. The cyclooxygenase (COX) is an enzyme synthesized from arachidonic acid metabolism, it play in catalyzing 2 stages of prostaglandin biosynthesis and exists in 2 forms, namely COX-1 and COX-**2.** The COX-1 plays in the homeostasis process, while the number of COX-2 increased when inflammation occurs and plays a role in prostaglandin synthesis, especially PGE2.

The changes of COX-2 expression based on time, in the proanthocyanidins group COX-2 increased on day 7 and decreased slowly until day 28. Meanwhile, COX-2 levels in the negative control group continued to increase until day 14 and decreased drastically until day 28. The enhance of COX-2 expression was probably due to the negative control group still experiencing an inflammatory process, where COX-2 is an important enzyme for the synthesis of inflammatory mediator precursors. Based on the research of Paulasilva et al., it was shown that in the periodontal tissue of rats induced by P. gingivalis, there was fluctuation in COX-2 expression, where the highest expression of COX-2 occurred on day 14 [33].

Based on the results of the study, the highest COX-2 expression was in the negative control group, and there was a significant difference in the average COX-2 expression in the negative control group on days 0, 7, 14 and 28. Based on research conducted by Mesa et al., COX-2 expression in patients with gingivitis and periodontitis was higher than in patients without periodontal disease.[31] Morton and Dongari also reported that COX-2 expression was found in mononuclear cells, endothelial cells, gingival fibroblasts, and epithelial cells in inflamed gingiva [34].

CONCLUSIONS

The proanthocyanidin contained in the cacao peel extract (Theobroma cacao L.) has a potential to reduce TNF-α and COX-2 expression in periodontitis rats.

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The Analysis of Proanthocyanidins Cacao Peel Extract (Theobroma cacao L.) Potential on The Expression of TNF-a and COX-2 on Periodontitis Rat
YANI CORVIANINDYA RAHAYU, AGUSTIN WULAN SUCI DHARMAYANTI, YOLANDA EKA PUTRI, ANIS IRMAWATI

Segmentation And Calculation Of Volume Of Tumor On Mri Using Efficient Feature Extraction Techniques
NISHA JOSEPH, D MURUGAN, BASIL JOHN THOMAS, RAMYA A

The Dynamics of Fatwa among a Diverse Community
M.USMAN , MUDOFIR, LAYYIN MAHFIANA, MUH.NASHIRUDIN

Simwos: Improving Semantic Similarity Between Gene Ontology Terms Based On Pfam Clans And Pathway Analysis
ANOOJA ALI, VISHWANATH R HULIPALLED, S.S.PATIL

Study On Impact Of Social Networking Sites On The Performance Of Employees In The Banking Sector.
DR. SARVJEET KAUR CHATRATH, AMANJOT KAUR, HARPREET SINGH

Precautionary Measures And The First Wave Evolution Of Covid-19: A Comparison Study
A.HAJ ISMAIL, T.JWAID, E.DAWI, A.ABDELKADER

Effect Of Butanol Extract Pomegranate Peel (Punica Granatum) Gel As Antioxidant On Shear Bond Strength Of Nanofilled Composite Restoration In Enamel After Bleaching In Office: In Vitro Study
SALLY SALSALINA, TRIMURNI ABIDIN, DENNIS

Pre and Postnatal Developmental Study of Cornea and lens in Rabbits (Oryctolagus cuniculus)
AZHAR SALEEM KHALAF, EKTIFFA S. KHAYOON, ALI FARIS RESHAG

Isolation and Amplification of Emm Gene From Streptococcus Pyogenes Isolated from Iraqi Children.
NADA ZAIDAN KHALAF, ASRA'A ADNAN ABDUL-JALIL, LAITH MOSLIH NAJEEB

Allergic Conjunctivitis and Its Complications Among Patients Attending Al-Nahrain Eye Specialty Center in Ramadi City
, THAKIR M. MOHSIN, ZEINA M. ALSABTI, OMAR MUAYAD AL-NAQEEB

Microbiological Contaminant Isolation and Detection in Cosmetics Sold in Iraq
MUNTAHA A.H.NASIR, QUTAIBA A.QASIM

In Silico Identification of Potential Inhibitors Against Sars-Cov-2 Protease
VAJIHEH ESKANDARI

Development of The Stable, Reliable, Fast and Simple RP-HPLC Analytical Method for Quantifying Diphenhydramine-Hcl (DPH) In Pharmaceuticals
H.N.K.AL-SALMAN, ERFAN A.S.ALASSADI, RAJAA HUSSEIN FAYADH, HUSSEIN H.HUSSEIN, EKHLAS QANBER JASIM

Physiochemical Effects of Nano Particles from Welding Fume on Rumaila Oil Field Welders by Using FESEM- EDS
RAFID A.DOOLAB

Implementation of Blended Learning Based-Model in English For Specific Purposes for The Islamic University: A Case Of UNISNU, Jepara, Indonesia
HARYANTO , PURWANTO, GIYOTO

Evaluation Model of Learning in Inclusive Settings for Students with Disability in INTIS Elementary School Yogyakarta
ALEX YUSRON AL MUFTI, NAHIYAH JAIDI FARAZ, MUKMINAN, ANIS FITRIYAH, SUBAIDI

The Reconstruction of Marriage Law in Classic Fiqh Perspective: The Case of Unregistered Marriage in Indonesia
MUNASIR , SA'DULLAH ASSA'IDI

Implementation of Digital Curriculum at Madrassa Aliyah Balekambang Jepara
ALI AS'AD, PURWANTO, YUSUF ROHMADI

Exploring Marketing Performance Based on Agro Value Co-Creation
SAMSUL ARIFIN, ALI, NURUL KOMARIYATIN

Same Verses Detection Apllication: An Innovative Media For Memorizing Qur'an In Tahfidz Pesantren
AZZAH NOR LAILA, DARNOTO, ANA RAHMAWATI, HERMAN SURJONO, MUHTADI

The Effect of Teacher Motivation and Teacher Work Discipline Towards Costs of Private School Heads in Karanganyar Indonesia
SUTARMAN , SUTARNO JOYOATMOJO, NUNUK SURYANI, ASROWI

The Information Technology Based-Regional Government Innovation for Integrated Civil Servant Management of East Java Provincial Office Indonesia
NURKHOLIS , SOESILO ZAUHAR, MR.KHAIRUL MULUK, ENDAH SETYOWATI

Improving Marketing Performance Through Exploiting Green Product Competencies
NURUL KOMARYATIN, SAMSUL ARIFIN, ALI

Textile Industrial Liquid Treatment with Activated Carbon
DARSINI , AGUS PURWANTO

Empowering Mental Attitude And Mindset Of Business In SMES Through Sales Tournament For Students Of Sragen School Business, Indonesia
JOKO SURYONO, MAHENDRA WIJAYA, HERU IRIANTO, MOHAMAD HARISUDIN

Exploring the Implementation of Human Resources Management for Teachers at

International Journal of Pharmaceutical Research

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Subject Area and Category	Pharmacology, Toxicology and Pharmaceutics Pharmaceutical Science Pharmacology, Toxicology and Pharmaceutics (miscellaneous)
Publisher	Advanced Scientific Research
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ISSN	09752366
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13

H Index

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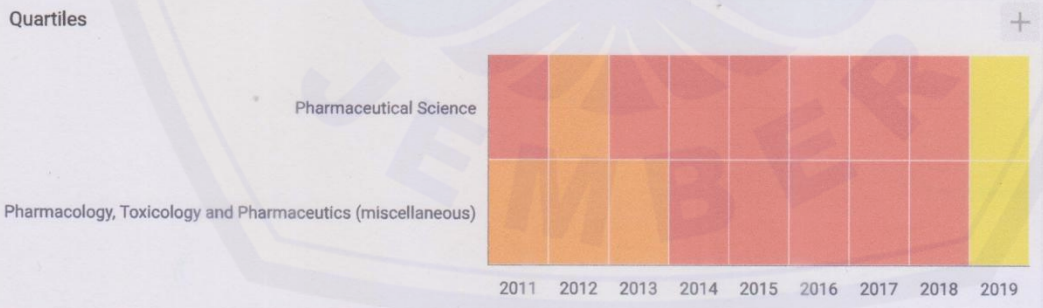

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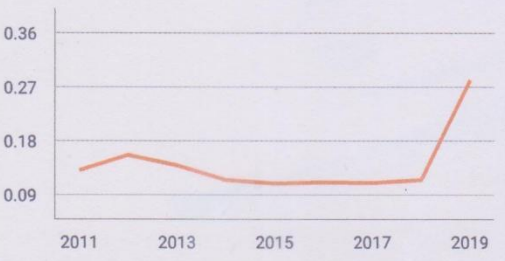

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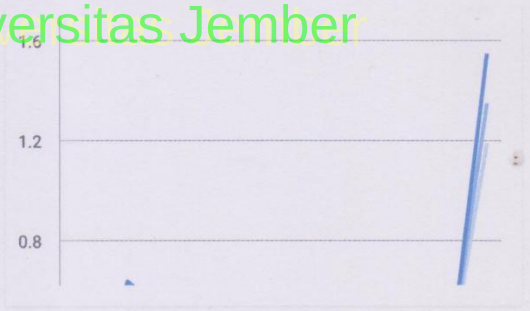

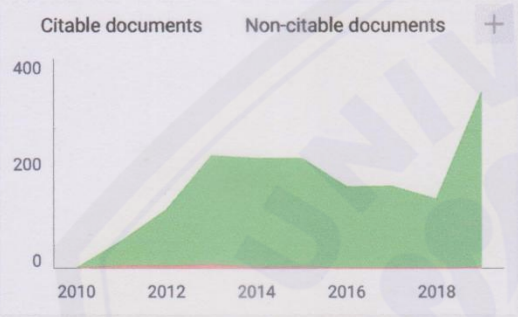

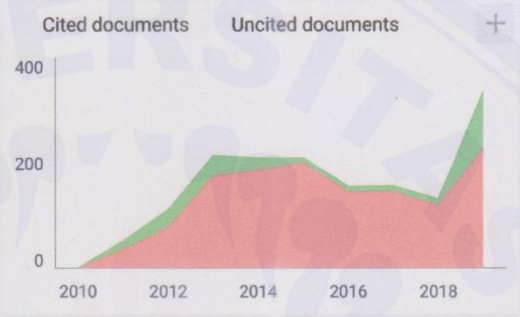

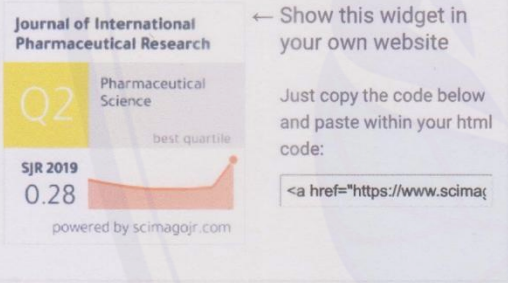

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