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EFFECT OF GALAM LEAF EXTRACT AND TRI-CALCIUM SILICATE CEMENT ON NEUTROPHIL CELLS IN WISTAR RAT PULP

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ABSTRACT
Background: *Pulp capping is a method of sealing the pulp chamber in teeth with cavities that extend to the pulp. Direct pulp capping involves applying a material directly onto the exposed pulp tissue. Tri-calcium silicate cement (Ca3SiO5) is commonly used due to its nanoparticle mineral composition, but it can cause continued inflammation. Galam leaf (Melaleuca cajuputi) is known for its anti-inflammatory properties, owing to secondary metabolites like alkaloids, flavonoids, polyphenols, and saponins, potentially compensating for the drawbacks of tri-calcium silicate cement.* ***Purpose:*** *To determine the effect of combining Galam leaf extract (Melaleuca cajuputi subsp. Cumingiana (Turz) Barlow) with tri-calcium silicate cement as a direct pulp capping material on neutrophil cell count in the pulp of Wistar rats (Rattus norvegicus).* ***Methods:*** *This pure experimental study used a posttest-only control design. Thirty-six Wistar rats were divided into nine groups: one received a combination of 100% Galam leaf extract and tri-calcium silicate cement; one positive control group received tri-calcium silicate cement alone; and one negative control group received direct placement with glass ionomer cement (GIC). The neutrophil cell count was assessed on days 1, 2, and 3. Results: Two-way ANOVA results indicated a significant effect based on treatment and time ($p < 0.05$). Further analysis with the Post Hoc Bonferroni test ($p < 0.05$) revealed differences in neutrophil cell counts across nearly all groups on days 1, 2, and 3.* ***Conclusion:*** *The combination of 100% Galam leaf extract and tri-calcium silicate cement significantly reduced neutrophil cell counts compared to both positive and negative control groups on days 1, 2, and 3, indicating anti-inflammatory effect.*

Keywords : *direct pulp capping, galam leaf extract, reversible pulpitis, tri-calcium silicate cement*
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INTRODUCTION

The Basic Health Research Data (Riskesdas) reached 45.3%, and in South Kalimantan, it reached 46.9%.[1] Dental caries is a multifactorial disease, one of the causes of which is bacteria. Dental caries result in cavities on the tooth surface. Mechanical trauma during the treatment of caries on the tooth surface can lead to the exposure of the pulp tissue. Exposed pulp tissue can cause pulpitis.[2]

Reversible pulpitis is an inflammatory condition in which the inflammation subsides and the pulp returns to normal once the cause is removed.[3,4] Pulpitis triggers an inflammatory response from the pulp and the formation of reparative dentin.[4,5] The pulp inflammatory response is triggered by immune cells and leukocytes. Immune cells release cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and prostaglandin E2 (PGE2), which will trigger the activation, increase, and migration of neutrophil cells to the site of inflammation, marking the beginning of the inflammatory phase. Neutrophil cells are responsible for phagocytosis during the first day of their migration to the site of inflammation. By the third day, neutrophil cells decrease and are replaced by monocyte cells that migrate to the site of

inflammation and transform into macrophages.[6,7,8] The inflammatory response in cases of exposed pulp can be treated using the pulp capping treatment method.

Pulp capping is a treatment method for covering the roof of the pulp. Direct pulp capping is the treatment involving the application of a material directly on top of the pulp tissue in cases where the tooth has a cavity that extends to the pulp.[3,9] The materials used to treat cases of exposed pulp must meet ideal requirements, one of which is the ability to maintain pulp vitality.[5]

Tricalcium silicate cement (Ca3SiO5) is a nanoparticle mineral cement created as a pulp capping material to enhance physicochemical and chemical properties. Tricalcium silicate cement (Biodentine; Septodont) has the advantage of stimulating reparative dentin formation, has better odontogenic potential compared to calcium hydroxide, and can produce silicon ions that gradually reduce the number of inflammatory cells.[5,10] The drawback of tricalcium silicate cement (Ca3SiO5) is that this material can cause continued inflammation following application, on the 7th and 14th days.[5,11–13]

The Galam plant (Melaleuca cajuputi) is a plant that grows in swampy/peaty areas. This plant, especially its leaves, can be utilized to treat ailments such as sore throat and toothache.[14] Galam leaves have the effect of accelerating wound healing because they contain secondary metabolite compounds from the alkaloid, flavonoid, polyphenol, and saponin groups. The total phenol content is 8.3615 ppm, and the flavonoid content is 0.3438 mg QE/g.[15] Galam leaf extract also exhibits very strong antioxidant activity and good antibacterial effects.[14,16,17] he combination of galam leaf extract and tricalcium silicate cement will be applied to Wistar rats (Rattus norvegicus) in experimental studies.

The direct pulp capping material tricalcium silicate (Ca3SiO5) can cause ongoing inflammation, while galam leaf extract contains anti-inflammatory compounds such as flavonoids that can inhibit cyclooxygenase-2 (COX-2) enzyme and lead to decreased production of Prostaglandin E2 (PGE-2), which inhibits proinflammatory cytokines and induces activation and migration of neutrophils in the inflammatory phase.

Research on galam leaf extract in relation to oral health has not been extensively studied. Therefore, it is necessary to conduct research on the effect of the combination of galam leaf extract (Melaleuca cajuputi subsp. Cumingiana (turz) Barlow) and tricalcium silicate cement (Ca3SiO5) as a direct pulp capping material on the number of neutrophil cells in the dental pulp of Wistar rats (Rattus norvegicus).

METHODS

This research has been approved by the ethics committee of the Faculty of Dentistry, ULM, approval number 134/KEPKG-FKGULM/EC/XI/2023. The study utilizes a true experimental research method with a post-test only control group design, and sample selection is conducted using simple random sampling techniques.

The sample used in this study consists of 36 Wistar rats. The inclusion criteria for this study are male rats, healthy condition, weighing between 250-300 grams, and aged 3-4 months. The exclusion criteria are rats that are deceased and Wistar rats appearing unhealthy (lethargic and inactive). The samples are divided into 9 treatment groups: the group of rats given galam leaf extract and tricalcium silicate cement on day 1, the group of rats given galam leaf extract and tricalcium silicate cement on day 2, the group of rats given galam leaf extract and tricalcium silicate cement on day 3, the group of rats given tricalcium silicate cement on day 1, the group of rats given tricalcium silicate cement on day 2, the group of rats given tricalcium silicate cement on day 3, the group of rats directly capped with glass ionomer cement (GIC) on day 1, the group of rats directly capped with glass ionomer cement (GIC) on day 2, and the group of rats directly capped with glass ionomer cement (GIC) on day 3.

Galam Leaf Extract

The galam leaves used are sourced from Gambut Subdistrict, South Kalimantan. The criteria for selecting leaves include being green in color, fresh, whole leaf shape, free from pests, and the fourth leaf from the top to the base of the branch. The galam leaves are cleaned and weighed 1 kg, then sliced, dried using a vacuum oven dryer at 60°C for two hours, and blended. The leaves, now in the form of powdered crude drugs, are sieved and filtered to obtain fine powder weighing 151 grams. The galam leaf powder is dissolved using 70% ethanol solvent. The solution is then homogenized using a hot plate with magnetic stirrer for 3 hours at 60°C. It is then filtered using filter paper, followed by evaporation using an evaporator. Subsequently, it is placed on a water bath until obtaining 88 grams of concentrated extract.[4,18]

Combination of Galam Leaf Extract and Tricalcium Silicate Cement

The tricalcium silicate cement with the trade name Biodentine will be used for the preparation of the combination. Biodentine is homogenized by mixing 0.18 ml of liquid into a capsule containing 0.7g of powder and placed in a mixer for 30 seconds. The consistency of Biodentine will be in the form of a paste once homogenized. The combination is obtained with a ratio of 1:1, which involves mixing 0.7g of Biodentine paste and 0.7g of galam leaf

extract, stirred using a Biodentine spatula until evenly mixed and thick.

Animal Experimental Procedure

The animal experimental treatment is conducted at the Biochemistry Laboratory of the Faculty of Medicine, Lambung Mangkurat University. Wistar rats (Rattus norvegicus) are first acclimatized to the laboratory conditions for one week. Each rat is provided with standard feed and water, then divided into groups of 4 rats each, with a total of 36 male Wistar rats assigned to each group number. Male Wistar rats are anesthetized intramuscularly with a solution of Ketamine and xylazine.[4,18]

The teeth used in the rats are the upper jaw first molars because they have a structure and anatomical shape similar to human molar teeth. Class 1 cavity preparation is performed on the occlusal surface of the upper left first molar tooth, perpendicular to the axis of the tooth, until the pulp is exposed. The preparation depth is the size of the bur head (0.5-1mm) using a handpiece with a round bur (diameter 0.84mm) at medium speed until reaching the pulp chamber. The preparation depth is estimated to be 0.5-1mm. After perforation, the cavity is irrigated with sterile saline solution and dried with sterile cotton pellets. Any resulting bleeding is stopped using a sterile paper point.[4,18]

Each treatment group 1, 2, and 3 (n=12) is applied with a combination of galam leaf extract (Melaleuca cajuputi subsp. Cumingiana Barlow) at a concentration of 100% and tricalcium silicate cement. Positive control groups 3, 4, and 5 (n=12) are given tricalcium silicate cement. Negative control groups 6, 7, and 8 (n=12) are directly capped with GIC. The material is applied to the pulp surface using a ball applicator (applicator tip diameter 0.63mm), and the cavity is restored with glass ionomer cement (GIC) applied with a plastic filling instrument and smoothed using a ball applicator, pressing it until the cavity is adequately filled and ensuring it does not cause traumatic occlusion. [4,18]

Preparation of Histopathological Specimens

The specimen collection is carried out after sacrificing the Wistar rats. The collection is done at the interdental bone of the upper left first molar region of the jaw. Tissue sections are placed in a fixation solution (10% buffered formalin) for approximately 4 days at room temperature. Subsequently, the tissues undergo the decalcification process using a 2% nitric acid solution for approximately 10 days at room temperature, followed by rinsing with flowing water. Afterward, the tissues proceed to the processing stage, which is carried out for approximately 18 hours. Once all stages are completed, the tissues are sectioned using a microtome to a thickness of approximately 5µm parallel to the long axis of the tooth.[4,18]

The tissue sections that have been cut are floated on warm water in a water bath at a temperature of 40º-50º C to prevent shrinking, then placed on object glass slides and labeled. Subsequently, paraffin is melted by placing the object glass slides on a hotplate, and the specimens are ready for staining.[18]

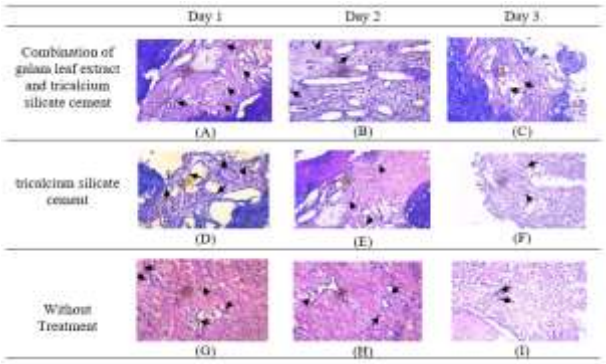
The specimens are stained using hematoxylin-eosin (HE) staining to observe the presence or absence of neutrophil cells within the dental pulp. If good staining results are obtained, the specimens are dried at the bottom using tissue paper, labeled, and the object glass slides are covered with cover slips. Subsequently, they are observed under a light microscope.[18]

Handling of Experimental Animals After Tissue Collection

After tissue collection from the experimental animals, the carcasses are handled by first cleaning them, then wrapping them in cloth, and burying them at a depth of approximately 25-75cm.[18]

RESULTS

Table 1. Histopathology of Neutrophil Cells with Hematoxylin Eosin Staining in Dental Pulp Inflammation of Wistar Rats



Descriptions:

A: histopathology of neutrophil cells given a combination of galam leaf extract and tricalcium silicate cement on day 1.

B: histopathology of neutrophil cells given a combination of galam leaf extract and tricalcium silicate cement on day 2.

C: histopathology of neutrophil cells given a combination of galam leaf extract and tricalcium silicate cement on day 3.

D: histopathology of cells given tricalcium silicate cement on day 1.

E: histopathology of cells given tricalcium silicate cement on day 2.

F: histopathology of cells given tricalcium silicate cement on day 3.

G: histopathology of neutrophil cells directly capped with GIC on day 1.

H: histopathology of neutrophil cells directly capped with GIC on day 2.

I: histopathology of neutrophil cells directly capped with GIC on day 3.

Table 1. the histopathological image (HPA) shows neutrophil cells on day 1 at a magnification of 400x. According to the cell count on that day, the group given a combination of galam leaf extract and tricalcium silicate cement had the highest number of neutrophil cells, ranging from 18-20 cells, compared to the positive control group given tricalcium silicate cement, which had 14-16 cells. The lowest number of neutrophil cells was found in the negative control group with GIC, totaling 11-13 cells.

Based on the cell count on day 2, the group given a combination of galam leaf extract and tricalcium silicate cement had the lowest number of neutrophil cells, ranging from 16-18 cells, compared to the positive control group given tricalcium silicate cement, which had 11-13 cells. The lowest number of neutrophil cells was found in the negative control group with GIC, totaling 11-12 cells.

On day 3, the group given a combination of galam leaf extract and tricalcium silicate cement had the lowest number of neutrophil cells, ranging from 1-3 cells, compared to the positive control group given tricalcium silicate cement, which had 3-6 cells. The highest number of neutrophil cells was found in the negative control group with GIC, totaling 6-8 cells.

Figure 1. Average Number of Neutrophil Cells in Dental Pulp Inflammation of Wistar Rats

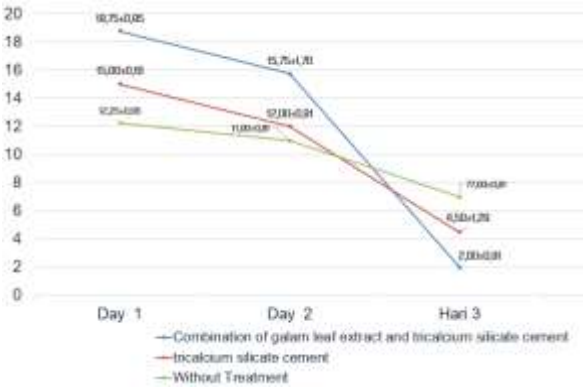


figure 1. illustrates the mean and standard deviation of the number of neutrophil cells in Wistar rats (Rattus norvegicus). The highest number of neutrophil cells on day 1 was found in the group treated with a combination of galam leaf extract and tricalcium silicate cement with a mean value of (18.75±0.95), compared to tricalcium silicate cement (positive control) with (15.00±0.16), and untreated (negative control) with (12.25±0.95).

The average number of neutrophil cells on day 2 showed a decrease with the group treated with a combination of galam leaf extract and tricalcium silicate cement having a mean value of (5.75±1.70) compared to tricalcium silicate cement (positive control) with (12.00±0.81), and untreated (negative control) with (11.00±0.81).

The average number of neutrophil cells on day 3 showed a further decrease compared to the other two days, with the group treated with a combination of galam leaf extract and tricalcium silicate cement having a mean value of (2.00±0.81) compared to tricalcium silicate cement (positive control) with (4.50±1.29), and glass ionomer cement (negative control) with (7.00±0.81).

Table 2. Post-hoc Bonferroni Test of Neutrophil Cell Count Based on Treatment and Day.

		Day 1			Day 2			Day 3		
		KEDGTCS	TCS	GIC	KEDGTCS	TCS	GIC	KEDGTCS	TCS	GIC
Day 1	KEDGTCS		0.001*	0.000*	0.013*	0.000*	0.000*	0.000*	0.000*	0.000*
	TCS			0.032*	1.000	0.013*	0.032*	0.000*	0.000*	0.000*
	GIC				0.002*	1.000	1.000	0.000*	0.000*	0.000*
Day 2	KEDGTCS					0.000*	0.000*	0.000*	0.000*	0.000*
	TCS						1.000	0.000*	0.000*	0.000*
	GIC							0.000*	0.000*	0.000*
Day 3	KEDGTCS								0.077	0.000*
	TCS									0.077
	GIC									

Descriptions:
* : Indicates significant difference
KEDGTCS : Combination of galam leaf extract and tricalcium silicate cement
TCS : tricalcium silicate cement
GIC : *Glass ionomer cement*

Table 2. presents the results of the Post-hoc Bonferroni test based on treatment groups and treatment days, namely the combination of galam leaf extract and tricalcium silicate cement, tricalcium silicate cement (positive control), and untreated (negative control) on day 1, day 2, and day 3. The results indicate significant differences ($p<0.05$) in almost all pairwise comparisons between treatment groups and treatment days. However, there were also non-significant differences ($p>0.05$) observed in some comparisons, including the untreated (negative control) group on day 1 and day 2 ($p=1.000$), tricalcium silicate cement (positive control) group on day 2 and the untreated (negative control) group on day 2 ($p=1.000$), the combination of galam leaf extract and tricalcium silicate cement group on day 3 and tricalcium silicate cement (positive control) group on day 3 ($P=0.077$), and tricalcium silicate cement (positive control) group on day 3 and the untreated (negative control) group on day 3 ($P=0.077$).

DISCUSSION

The content of galam leaf extract and tricalcium silicate cement shows an increase in the number of neutrophil cells on day 1 and a decrease in neutrophil cells on days 2 and 3. This is due to the addition of galam leaf extract, which contains secondary metabolites such as flavonoids, alkaloids, saponins, and polyphenols with anti-inflammatory activity.[15] These secondary metabolites aid the content of

tricalcium silicate cement, namely silicate ions, Ca2+, and OH, which function to gradually decrease inflammation and induce pulp regeneration.[19]

The release of silicate ions from tricalcium silicate cement affects the release pathway of interleukin-6 (IL-6), resulting in the migration and activation of neutrophil cells in blood vessels, leading to a decrease in the inflammatory phase due to the content of tricalcium silicate cement.[20]

The anti-inflammatory activity of flavonoid secondary metabolites operates by inhibiting the enzyme Cyclooxygenase-2 (COX-2), which affects the reduction in prostaglandin E2 (PGE-2) production. This inhibition results in the decrease of Nuclear Factor-kappaB (NF-κB) transcription, leading to a reduction in proinflammatory cytokines such as Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1 (IL-1), and Interleukin-6 (IL-6), thereby accelerating the healing process.[21–23] Alkaloids acts by inhibiting the neutrophils' ability to migrate towards the inflamed area through chemotaxis, which involves the inhibition of the phosphatidylinositol-3-kinase signaling pathway. Saponine works by inhibiting neutrophil activation through the reduction of oxidative burst like reactive oxygen species and the modulation of proinflammatory enzymes such as myeloperoxidase produced by neutrophils. Meanwhile, polyphenol inhibites the modulation of intracellular signaling pathways like mitogen-activated protein kinase and phosphatidylinositol-3-kinase, which play a role in the activation and function of neutrophil cells.[21,22] This results is in line with the study conducted by Dahlian (2021), which demonstrated that the use of secondary metabolite compounds in plants significantly affects the reduction of neutrophil cell count in the pulp inflammation of Wistar rat teeth.

The group given calcium silicate cement (positive control) showed a more optimal increase in neutrophil cell count on day 1 and a subsequent decrease on days 2 and 3 compared to the untreated group (negative control). The increase on day 1 is attributed to the silicate ions, which accelerate the inflammatory response by enhancing the activation and migration of inflammatory cells, including neutrophils. This leads to a faster and greater increase in neutrophil count compared to what is observed in the negative control group on day 1. The decrease on days 2 and 3 is due to the effects of calcium silicate cement after the inflammatory phase on day 1. It helps regulate the inflammatory response more efficiently, including accelerating the resolution of inflammation, such as inducing apoptosis in neutrophil cells. Consequently, the decrease in neutrophil count on days 2 and 3 could be more optimal than in the negative control group.[20]

The untreated group (negative control) showed an increase in the number of neutrophil cells on day 1 followed by a decrease on days 2 and 3. The increase in neutrophil count on day 1 could occur as a response to the initial inflammatory stimulus. The decrease in neutrophil count on days 2 and 3 is due to several factors. One of them is because they undergo apoptosis or are engulfed by macrophage cells. This mechanism demonstrates a normal inflammatory response occurring due to the absence of any influence from any substance affecting the activation and performance of inflammatory cells, including neutrophils.[24]

In summary, the combination of galam leaf extract (*Melaleuca cajuputi* subsp. Cumingiana Barlow) at a concentration of 100% and tricalcium silicate cement as a direct pulp capping material shows a significant increase in the number of neutrophil cells on the first and second days, followed by a significant decrease in the number of neutrophil cells on the third day, compared to the group treated with tricalcium silicate cement (positive control) and the group treated with direct filling using GIC (negative control).

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