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Original Article

THE EFFECT OF BROMELAIN MICROCAPSUL FORMULATION ON LEUKOCYTE AND TNF-α LEVEL IN MALE WHITE MICE INDUCED BY H5N1 VACCINE

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ABSTRACT

Objective: Bromelain is a sulfhydryl proteolytic enzyme extracted from the pineapple plant (*Ananas comosus*. L), which has various activities, including as an immunomodulator. Microencapsulation of bromelain is a process by which a layer surrounds bromelain to produce microcapsules to increase its activity. This research intends to see the effect of bromelain microcapsule formulation on total leukocytes count, leukocyte percentage, and the levels of TNF-α in male white mice exposed to the H5N1 Vaccine.

Methods: Experimental animals were divided into three groups, specifically a negative control group given Na CMC 0.5%, the comparison group given 200 mg/kgBW bromelain enzyme, and the test group given 200 mg/kgBW bromelain microcapsules for seven days orally. On the eighth day, the total amount and the percentage of leukocytes and the levels of TNF-α were counted. The data were analyzed by two-way ANOVA and Duncan's multiple range test ($p<0.05$).

Results: The study showed that the administration of 200 mg/kgBW bromelain microcapsule group significantly reduced total leukocyte count and increased the segmented neutrophil compared to the bromelain group ($p<0.05$). However, there was no significant correlation between the two groups in reducing monocyte, lymphocyte, eosinophil, and TNF-α levels ($p>0.05$).

Conclusion: It can be concluded that providing bromelain microcapsules can reduce the total amount of leukocytes and increase the segmented neutrophil in male white mice exposed to the H5N1 Vaccine.

Keywords: Bromelain, Microcapsules, Leukocytes, TNF-α

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INTRODUCTION

Pineapple (*Ananas comosus* L.) belongs to the Bromeliaceae family. Almost all parts of the pineapple plant have benefits for humans. Pineapple contains carbohydrates, protein, mother, phosphorus, iron, vitamins A, B, and C, and the bromelain enzyme. Bromelain is a sulfhydryl protease enzyme that is distributed in pineapple plant tissue with an optimum pH of 7 and an optimum temperature of 55 °C [1]. Bromelain can be used in the food, beverage industry and in the pharmaceutical field. Bromelain belongs to a proteolytic enzyme that catalyzes the breakdown of proteins into amino acids [2]. Bromelain is an amorphous powder that is easily oxidized and hydrolyzed. Bromelain has anti-inflammatory properties, helps digestion, and interferes with the growth of cancer cells [3].

Bromelain has optimal enzyme activity at a pH of 4.0 to 8.0. Although bromelain's proteolytic activity is reduced in artificial gastric fluid, it remains largely stable for the first four hours. Decreased proteolytic activity of bromelain is followed by deactivation and degradation of gastric acid and gastric enzymes. According to Chobotova *et al.* (2010), after bromelain was encapsulated as a controlled drug delivery system, bromelain was absorbed into the human intestine without losing activity [4]. Bromelain is unstable in the stomach, where the stomach has an acidic pH ranging from 1-3, which can cause the bromelain protein to coagulate [2].

To maintain the stability of the bromelain enzyme, the microencapsulation method was used. Microencapsulation is a process by which a layer surrounds small particles or droplets to produce microcapsules. The idea of microencapsulation is to create a barrier between the probiotic cells and their surrounding environment. The inside of the microcapsule is called the core, internal phase, or content. At the same time, the wall is called a shell, coating, or membrane. The microcapsule form may be an aggregate or a single particle form and usually has a broad range of 1-5000 µm [5]. The advantage of microencapsulation is to protect unstable and sensitive materials from the environment. The method that can be used in the preparation of microcapsules is the solvent evaporation emulsification method. The tools used in this method are relatively simple than other methods [6] and able to provide a more optimum effect of reducing postoperative pain and swelling [7].

Avian influenza, sometimes known as bird flu, is the sickness caused by infection with avian (bird) influenza (flu) Type A viruses sub-type H5N1. These viruses may infect both wild and domesticated birds and mammals. All around the world, wild aquatic birds normally coexist with them [8]. In humans, the infection begins with a viral infection of the epithelial cells of the airways. This virus then reproduces itself fast, however, can lead to the lysis of epithelial cells and desquamation of the epithelial layer in the respiratory tract. With the replication of the virus, it can stimulate the formation of proinflammatory cytokines, including IL-3, IL-6, and TNF-α which then enter the systemic circulation and in turn will be able to cause systemic influenza symptoms such as fever, malaise, myalgia, etc. In conditions of a decreased immune system, the virus will be able to pass and enter the blood circulation and into other body organs [9].

Based on the description above, a study was conducted to see the effect of the bromelain microcapsule formulation as an anti-inflammatory to reduce the total amount of leukocytes, percentage of leukocytes, and TNF-α level when given orally in mice induced by H5N1 vaccine.

MATERIALS AND METHODS

Tools

The tools used in this study were standard laboratory glassware, magnetic stirrer, centrifuge, infrared spectrophotometer (Perkin Elmer FT IR Spectrophotometer Frontier), analytical balance (Ohaus PA214), hemocytometer (Assistant-Germany), object and cover glass, moisture balance, microscope with optilap

Materials

The materials used in this study were bromelain enzyme powder (Shaanxi Fheng (FH) Biotechnology Co., Ltd, China.), Hydroxypropyl

methylcellulose (HPMC), acetone, Paraffin Liquid, Tween 80 and Aquadest, Giemsa solution, Turk solution, vaccine H5N1 (PT. Caprifarmindo Laboratories).

Bromelaine microcapsule preparation

The test preparation was made in the form of microcapsules containing pure bromelain enzyme. The design formula used in this study were seen in table 1. Bromelain microcapsules were made by the solvent evaporation method. First, HPMC microcapsules were prepared by dissolving them with acetone in a glass beaker (M1). The solution contains dissolved pure bromelain powder HPMC (M1). In another glass beaker, put paraffin liquid and tween 80 (M2). Put tween 80 and paraffin liquid in a different glass beaker (M2). For 4 h, the emulsion was stirred at room temperature using a magnetic stirrer at a speed of 700 rpm. The formed microcapsules were collected by pouring in six ways, then washed with n-hexane two times until all the paraffin were washed off. After being cleaned with n-hexane, microcapsules are dried in an oven for two hours at a temperature of 40 to 50 °C.

Table 1: Bromelain enzyme microcapsule formula design

Material	Formula		
	F1	F2	F3
Bromelain enzyme powder	2	2	2
Hidroxypropyl methylcellulose (HPMC) (g)	1	2	3
Acetone (ml)	30	30	30
Paraffin liquid (ml)	60	60	60
Tween 80 (ml)	1.2	1.2	1.2
N-Heksan (ml)	60	60	60

Note: F1 (Formula Design 1), F2 (Formula Design 2), F3 (Formula Design 3) of Bromelain Enzyme Microcapsule

Bromelain microcapsule evaluation

The evaluation of microcapsules included the weight of the obtained microcapsules, FT-IR spectroscopic analysis, particle size analysis, determination of water content, determination of bromelain powder content in microcapsules, determination of drug loading percent, entrapment efficiency, and dissolution profile.

Organolectic evaluation

Organoleptic evaluation of microcapsules includes evaluation in terms of shape, color and smell.

Evaluation of the obtained microcapsule weight

The weight of the microcapsules obtained was weighed with an analytical balance on each formula.

FT-IR spectroscopic analysis

NaCMC, bromelain, and the manufacture of bromelain microcapsules were all subject to analyses. Fourier Transform Infrared (FT-IR) equipment was used to measure the infrared absorption of standard bromelain microcapsules, which were in powder form. A clear disk was covered with compressed bromelain microcapsules, which were subsequently scanned using an FT-IR instrument.

Particle size analyzer analysis

Particle size can be analyzed using PSA, which uses the dynamic light scattering technique. Dynamic scattering theory is a technique used to measure particle size from a few nanometers to a few microns. The concept is those tiny particles in suspension move in random patterns.

Evaluation of determination of water content

The water content of the microcapsules was measured using a moisture balance meter. Each microcapsule with a different formula was weighed as much as 1 g and then placed in a sample pan, then the temperature was set at 105 °C, and then measured the water content.

Evaluation of drug loading and recovery factors

It can be calculated using formula:

$$\%\ \text{loading} = \frac{\text{weight of active substance bromelain enzyme}}{\text{weight of microcapsules}}\ \text{x}100\ \%$$

The percentage of microcapsules yield/recovery factor was calculated using formula:

$$\%\ \text{yield}\ = \frac{\text{M}}{\text{M0}}\ \text{x}\ 100\ \%$$

Where: M= Weight of microcapsules MO= initial weight of bromelain enzyme+initial weight of HPMC

Preparation of test animals

The animals used in this study were male white mice, aged 2-3 mo old, weighed 20-30 g and had never been treated with drugs. Mice were acclimatized for seven days before being used as test subjects in order to regulate the environment, maintain health, and ensure uniform feeding. The dose of bromelain microcapsules used in this study was 200 mg/kgBW. 15 mice were divided into three treatment groups consisting of 5 mice where each group was given a different treatment:

1. Control negative group: Mice were induced by H5N1 vaccine 0.05 ml intraperitoneally on day 1 and then given 0.5% Na CMC for 7 d.

2. Bromelain group: Mice were induced by H5N1 vaccine 0.05 ml intraperitoneally on day 1 and then given bromelain enzyme suspension 200 mg/kgBW for 7 d.

3. Microcapsule bromelain group: Mice were induced by H5N1 vaccine 0.05 ml intraperitoneally on day 1 and then given microcapsule bromelain enzyme suspension 200 mg/kgBW for 7 d.

The research ethics committee of the faculty of medicine at Andalas University gave its ethical permission for the use of the experimental animals with approval number 627/UN.16.2/KEP-FK/2022.

Bromelain microcapsule suspension preparation

According to the effective dose, bromelain microcapsules were weighed, suspended in 10 ml of Na CMC, and then blended until homogenous. Mice were ingested up to 0.2 cc of each mixture using a probe needle.

Calculating the percentage of leukocyte cells

On the eighth day, the tails of the mice were cut and blood smears were created and subsequently dried. After drying, it was dripped with methanol, leaving the blood smear completely coated, and allowed to sit for 5 min. Stain with giemsa and let for 20 min. Wash with distilled water, dry and add immersion oil and observe under a microscope. At a magnification of 1000x, eosinophils, rod neutrophils, segment neutrophils, lymphocytes, and monocytes were counted.

Counting the number of leukocytes

Fresh blood was collected with a leukocyte pipette up to the number 0.5; then the receipt solution was sucked up to the 11 mark then shaken for 3 min; from the leukocyte pipette, 1-2 drops were discarded and one drop was added to the hemocytometer, counting chamber. The liquid is left for 2 min for the leukocytes to settle. In the four corners of the counting chamber, leukocytes were counted.

$$\text{The number of leukocytes} = \text{the number of leukocyte X}\ \frac{20}{0{,}4}$$

Measurement of TNF-α level

Levels of blood are drawn through the guillotine (neck artery). After that, serum was obtained by centrifuging the blood for 30 min at 3000 rpm. The TNF α level was then determined using the serum ELISA technique.

Data analysis

Using SPSS statistical software, the study's data were statistically evaluated using the one-way Analysis of Variance (ANOVA) approach and continued by Duncan's analysis.

RESULTS

This study was conducted to determine the effect of bromelain microcapsules formulation on leukocyte cells and TNF-α in mice. The bromelain samples were examined by organoleptic, FT-IR spectroscopy, particle size distribution, and water content to evaluate whether the microcapsule obtained was in accordance with the standard.

An organoleptic examination was done to evaluate the characteristic feature of bromelain. The organoleptic observations showed that the bromelain enzyme powder was yellow in color and had a distinct odor. This matches the previous study that bromelain enzyme powder has a clear white to yellowish color and a distinctive smell [10]. The evaluation of the weight of the microcapsules was obtained using an analytical balance. The weight of the microcapsules in formula 1, formula 2, and formula 3 were 1.501 g, 2.470 g, and 3.020 g, respectively. The weight of formula 1 meets the requirements for the formation of microcapsules, while in formula 2 and formula 3 the weight obtained is more than the total weight in the formula that should be. This is caused by the presence of another material (liquid paraffin) that is still trapped inside microcapsules.

The evaluation of Fourier Transform Infrared (FTIR) spectroscopy analysis was carried out to identify functional groups in the compound. FT-IR spectroscopic analysis is a reliable technique to detect interactions between drug compounds and carriers in microcapsules. The presence of a new transmittance peak or a shift in the position of a transmitter peak at a specific wave number often indicates as an interaction like hydrogen bonding [11]. The results on the bromelain enzyme powder showed the N-H strain band at 3,275.13 cm^{-1}, the C-H functional group stretched at 2,935.66 cm^{-1}, the intensity of the strain band at C=O with a height of 1,663.71 cm^{-1}, and the C-N functional group. band stretching at the height of 1,531.48 cm^{-1}. In Hidroxypropyl methylcellulose (HPMC), the results of the stretching of the O-H band (intermolecular hydrogen bonds) at 3.448 cm^{-1}, the C-H functional group stretching at the height of 2,904.80 cm^{-1}, the functional group C=O at the stretching band at the height of 1,654.28 cm^{-1}, and the C-O-C group is at the peak of 1,051.20 cm^{-1}, the results of the FTIR characterization of formula 1, formula 2, and formula 3 which are a physical mixture of the bromelain enzyme and HPMC, show the similarity of bands functional group; however, there is a slight shift in wave number, it is still within the range of the functional group. This proves that there is no chemical interaction between the bromelain enzyme compound and the HPMC polymer.

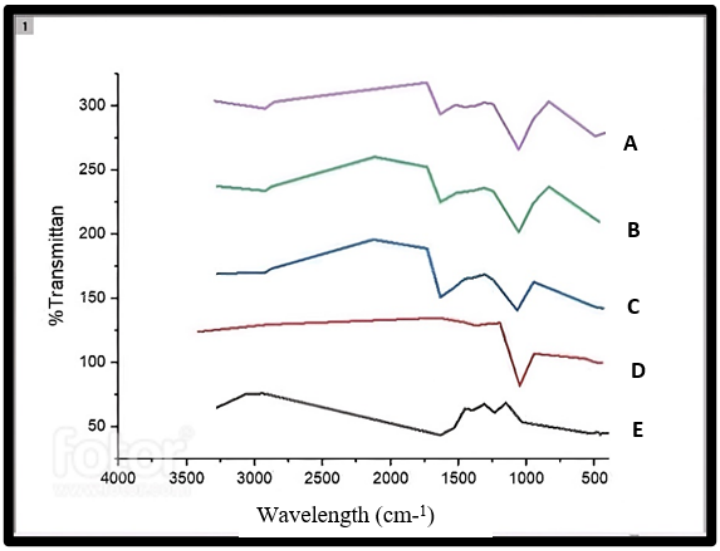

Fig. 1: Spectroscopy FT-IR of (A) Formula 3; (B) Formula 2; (C) Formula 1; (D) Hidroxypropyl Methylcellulose (HPMC); and (E) Bromelain

Evaluation of the particle size distribution of microcapsules in each formula were carried out using a Particle size analyzer (PSA). The result show that the mean particle size and mid value of F1= 0.118 µm and 0.122±0.221 µm; F2 0.148 µm and 0.154±0.220 µm; and F3 0.097 µm and 0.101±0.225 µm. Microcapsule particle size distribution is influenced by HPMC, which is used to form the microcapsule wall. The greater the amount of HPMC used, the greater the thickness of the microcapsule wall formed, so the larger the size of the microcapsule produced.

Moisture balance was used to evaluate and determine the water content of the microcapsules in each composition. The amounts of water found in formulas 1, 2, and 3 were, respectively, 4.28%, 5.75%, and 6.08%. The amount of coating applied affects the water content of the microcapsules. This is due to the fact that HPMC contains more water than the active ingredient does. A good matrix must have a water concentration of 3-5%. In conclusion, only formula 1 water content meets the requirements because it is less than 5%, while in formulas 2 and 3 the content of water is 5.75% and 6.08%; this may happen because the heating process is not optium.

Determination of the bromelain content in the microcapsules in each formula obtained the results of % loading at Fl=66.62%, F2=40.48%, F3= 33.11%. Formula 1 data is bigger than formula 2 and 3 due to the difference in the amount of HPMC used. While the recovery factor (% yield) at F1 = 100%, F2= 123.5%, and F3=120.8 %. Data from formula 2,3 shows that the recovery exceeds 100%. This is due to the presence of liquid material (liquid paraffin), which is still trapped in the microcapsules, while formula 1 shows a 100% recovery, which means that the microencapsulation process gets good results. After the microcapsule formulation was made and evaluated, the microcapsules with the best evaluation results were selected and continued with the test on mice. Based on this study, formula 1 microcapsules was choose to be tested on the animal.

The anti-inflammatory effect testing carried out in this study involved specific and non-specific immune responses by

determining the level of TNF-α, the total number of leukocytes using a hemocytometer, and calculating the percentage of leukocyte cell types using the blood smear method. According to the study, bromelain administration can significantly affect mice's total leukocyte count ($p<0.05$). Whereas, based on Duncan's multiple range test shows that the bromelain microcapsule significantly reduces the total leucocyte count compared to the bromelain group (table 1 and fig. 2).

Table 1: Total leukocyte count of the treatment group

Treatment group	Total leucocyte count (cell/mm^3)
Microcapsule Bromelain 200 mg/kgBW	4490±138.74^a
Bromelain 200 mg/kgBW	8550±375.00^b
NaCMC	10720±201.86^c

Note: The mean data with different superscripts in the columns showed a significant difference ($p<0.05$) based on duncan's multiple-range tests analysis

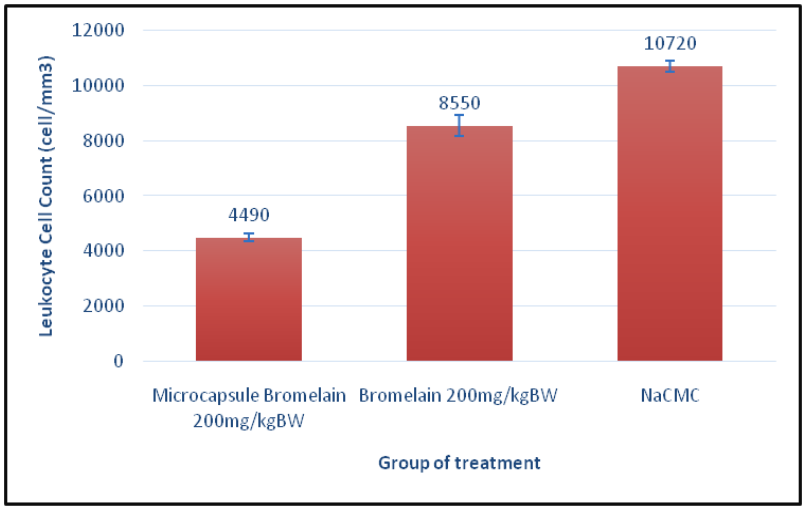

Fig. 2: Graph of total leukocyte cell count of male white mice in microcapsule bromelain, bromelain, and NaCMC treatment group

Unlike the previous result, bromelain microcapsules can significantly reduce eosinophils in mice ($p<0.05$) compared to the NaCMC treatment group. Still, there was no significant reduction of eosinophils between the bromelain and bromelain microcapsule treatment groups ($p>0.05$). The average percentage of eosinophils in the control group, the bromelain, and the bromelain microcapsule treatment group were 5.8%, 3.2%, and 2.8%, respectively (table 2 and fig. 3).

Bromelain microcapsules can dramatically decrease the proportion of stem neutrophils ($p<0.05$) compared to the control group, but there was no significant reduction compared to the bromelain group. The average percentage of stem neutrophils in mice in the control group, bromelain, and bromelain microcapsules group of treatment were 10%, 5.4%, and 4%. Otherwise, bromelain microcapsules enhance segment neutrophil percentage significantly ($p<0.05$) compare to the bromelain group. The average rate of mice segment neutrophils in the control group, the bromelain group, and the bromelain microcapsules, respectively, was 30.2%; 47.6%; 53.6%.

Bromelain microcapsules also can significantly reduce the percentage of lymphocytes ($p<0.05$) compared to the control group with a percentage of 45.8%, 39.6% 36.6% for the control group, bromelain, and bromelain microcapsule. Mice's proportion of monocytes can be greatly decreased by bromelain microcapsules ($p<0.05$) compared to the control group. The average percentage of monocytes in the bromelain group at 200 mg/kg, and bromelain microcapsules at a dose of 200 mg/kg, respectively, was 8.2%; 4.2%; 1.22%. However, for lymphocytes and monocyte, the bromelain microcapsule did not significantly reduce them compared to the bromelain group.

Table 2: Leukocyte percentage of the treatment group

Treatment group	Percentage of leukocytes (%)				
	Eosinophil	Stem neutrophil	Segment neutrophil	Lymphocyte	Monosit
Microcapsule Bromelain 200 mg/kgBW	2.80±0.83^a	4.00±1.00^a	53.60±2.15^a	36.60±2.70^a	3.00±1.22^a
Bromelain 200 mg/kgBW	3.20±0.84^a	5.40±1.14^a	47.60±2.19^b	39.60±2.30^a	4.20±1.14^a
NaCMC	5.80±0.84^b	10.00±1.58^b	30.20±1.64^c	45.80±1.92^b	8.20±0.84^b

Note: The mean data with different superscripts in the columns showed a significant difference ($p<0.05$) based on duncan's multiple-range tests analysis

Based on this study, bromelain microcapsules can significantly reduce TNF-α levels in mice ($p<0.05$) compared to control but did not give a significant reduction compared to the bromelain group. On average, TNF-α levels in bromelain group mice with a dose of 200 mg/kgBW and bromelain microcapsules at a dose of 200 mg/kgBW were 323.338 ng/l; 273.836 ng/l, 266.718 ng/l respectively (fig. 4).

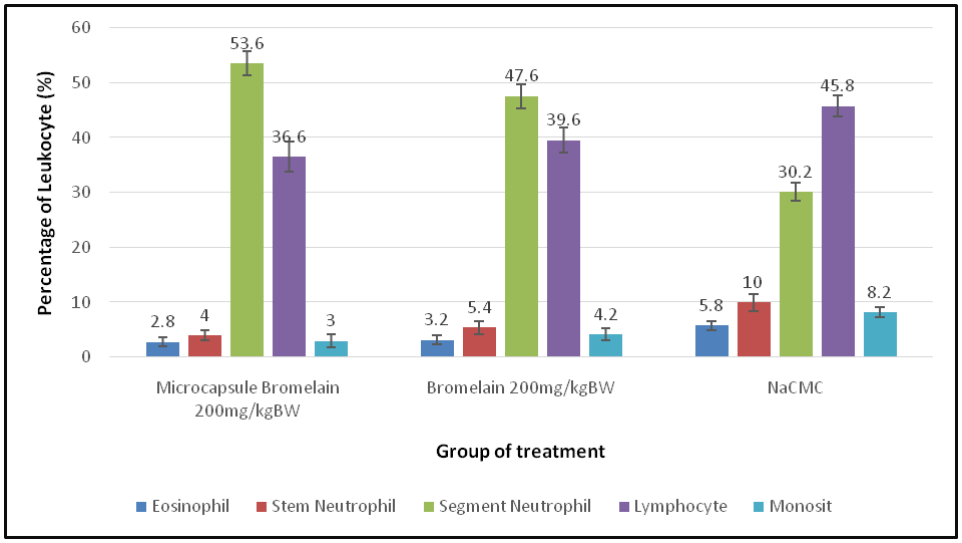

Fig. 3: Graph of the leukocyte cell count percentage in microcapsule bromelain, bromelain, and NaCMC treatment group

Table 3: TNF-α **level of the treatment group**

Treatment group	TNF-α level (ng/l)
Microcapsule Bromelain 200 mg/kgBW	266.71±14.50[a]
Bromelain 200 mg/kgBW	273.83±19.59[a]
NaCMC	323.33±9.62[b]

Note: The mean data with different superscripts in the columns showed a significant difference ($p<0.05$) based on duncan's multiple-range tests analysis

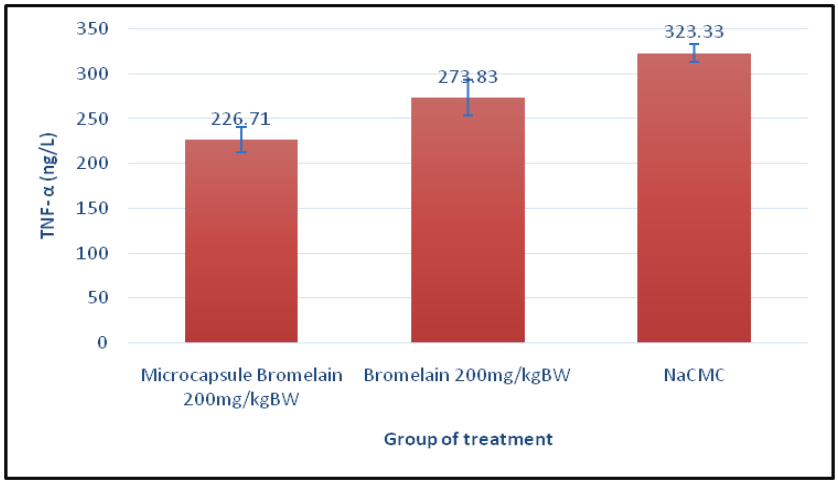

Fig. 4: Graph of TNF-α levels in microcapsule bromelain, bromelain, and NaCMC treatment group

DISCUSSION

The term bromelain refers to a group of proteolytic enzymes or proteases (natural proteolytic enzymatic complex) present in pineapple tissues (*Ananas comosus* var. comosus) and other plant species of the Bromeliaceae family, including the stems, fruit, and leaves. Bromelain will catalyze the breakdown of proteins into amino acids [12]. Multiple pharmacological actions of bromelain include preventing the development of edema and lowering current edema, increasing the absorption of antibiotics, influencing blood coagulation and fibrinolysis, and having anticancer and anti-inflammatory properties [4, 13, 14].

According to this study, bromelain microcapsule can decrease the total leukocyte count and increase the neutrophil segment compared to the bromelain group. The preparation of bromelain microcapsules can protect the compounds from stomach acid and regulate drug release so that it can occur in the small intestine. Bromelain has optimal enzyme activity at a pH of 4.0 to 8.0. The proteolytic activity of bromelain is decreased in artificial gastric fluid because the protein can be coagulated [2]. The microcapsule form can protect unstable and sensitive materials like bromelain from the environment. It is supported by the previous study by chobota that after bromelain was encapsulated as a controlled drug delivery system, bromelain was absorbed into the human intestine without losing activity [4].

This finding is consistent with the earlier studies that the development of targeted delivery for medicinal purposes involves the utilization of bromelain-encapsulating nanoparticles in inorganic substances like silica, synthetic polymers like polyacrylic acid, and

natural polymers like chitosan. According to Bernela *et al.*, bromelain's anti-inflammatory effect was increased when it was encapsulated in katira gum nanoparticles [15-17]. In an *in vitro* test for antioxidant and antiproliferative effects, bromelain nano encapsulated with chitosan provided sustained bromelain release [18]. This study also matches the previous study that describes a successful encapsulation of α2MG into microcapsules that improved both human macrophage phagocytosis and human leukocyte recruitment to inflamed endothelium [10].

In vitro and *in vivo*, bromelain may successfully reduce IL-8-induced neutrophil migration, and it supports the proteolytic elimination of CD128 chemokine receptors as a probable mechanism for this action. More research will need to ascertain how these impacts on neutrophil influx affect the intensity and final development of both acute and chronic inflammatory reactions [14]. The *in vitro* research conducted by Hale *et al.* [19] provides incredibly pertinent information. The combined results of the two experiments provide compelling evidence that the treatment of whole blood leukocytes with bromelain results in a dose-dependent reduction in the expression of 14 of the 59 leukocyte markers under investigation: CD7, CD8, CD14, CD16, CD21, CD41, CD42a, CD44, CD45RA, CD48, CD57, CD62L, and CD128a and CD128b. Studies have also demonstrated that bromelain's ability to inhibit CD antigen expression results from the enzyme's proteolytic characteristics. Bromelain's proteolytic action against leukocyte CDs was significantly reduced when a proteinase inhibitor called 2-macroglobulin (2M), which is often found in plasma, was utilized. However, there was no change in the CDs' bromelain-dependent concentration [10].

Additionally, bromelain has shown that it can reduce NF-kB activity, PGE2, and COX-2 expression. To explain this process, it was proposed that bromelain causes the cleavage of cell surface markers such as CD14. IFN-γ, TNF-α, IL-1, and IL-6 are a few of the secreted inflammatory regulators linked to the NF-kB pathways that react to bromelain. Depending on the situation and microenvironment, these regulators can either trigger immune responses or result in tumor regression [20].

CONCLUSION

It can be concluded that bromelain microcapsules can reduce the number of leukocytes and increase the segment neutrophil compare to the bromelain group.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

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