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Cytokine Inhibition of Il-6 and TNF-α Accounts for The Amelioration of Cotton Pellet-Induced Tissue Granuloma Formation in Sprague-Dawley Rats by The Aqueous Ethanolic Stem Bark Extract of *Bombax costatum* Pellegr and Viullet (Bombacaceae)

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ABSTRACT

Background: Medicinal plants are known to possess certain biological activities. *Bombax costatum* is used traditionally to treat pain, wounds, and other diseases. **Aim:** This study aims to investigate the antiproliferative effect of 70 % aqueous ethanol extract of *Bombax costatum* P.V. stem bark on cotton pellet tissue granuloma formation. **Methods:** The effects of *Bombax costatum* (EBC) extract (10-100 mg/ kg, *p.o*) were studied in cotton pellet-induced granuloma tissue formation. Either normal saline (1 ml), dexamethasone (3.0 mg/ kg, *p.o*), or EBC (10-100 mg/ kg, *p.o*) was administered. Furthermore, the effect of *Bombax costatum* (EBC [10-100 mg/ kg, *p.o*]) on serum cytokine levels of IL-6 and TNF-α was assessed using ELISA kit assay analysis. Haematological analysis and spleen weight analysis was also carried out. **Results:** EBC (10-100 mg/ kg, *p.o*) decreased the serum cytokine levels of IL-6 and TNF-α significantly from 38.6±3.28 pg ml^{-1} and 85.21±4.11 pg ml^{-1} to 26.3±1.80 pg ml^{-1}, 24.22±1.92 pg ml^{-1}, 17.99±2.08 pg ml^{-1}, and 70.35±2.45 pg ml^{-1}, 63.19±4.05 pg ml^{-1}, 49.89±2.33 pg ml^{-1} at 10, 50 and 100 mg/ kg respectively in a dose-dependent when compared to the control respectively (P<0.05).**Conclusion:** The aqueous ethanolic stem bark extract of *Bombax costatum* attenuated cotton pellet-induced granuloma tissue formation and thus, exhibited inhibitory effects on the inflammatory response.

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1.0 INTRODUCTION

Inflammation is the body's response to injury caused by infections, chemical or thermal stimuli, or autoimmune disorders [1]. An inflammatory response is a complex reaction triggered by an array of mediators through a series of cellular pathways [2]. While acute inflammation is self-limiting, a dysregulated inflammatory response can develop with chronic inflammation, which can lead to asthma, cardiovascular disease, rheumatoid arthritis, and other immune-inflammatory diseases, as well as organ failure [3]. Presently, inflammatory diseases are treated using conventional drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs), steroidal (glucocorticoids), and disease-modifying anti-rheumatoid drugs (DMARDs). The usage of these drugs beyond their therapeutic benefits is associated with several adverse effects including gastrointestinal tract disorders, obesity, and immunosuppression [4]. It is therefore imperative to search for a newer and alternative source of managing inflammatory diseases.

The plant *Bombax costatum* Pellet &. Vuilet. (Bombacaceae) is also known as the red-flowered silk cotton tree. It is widely distributed in the savanna dry woodlands from Senegal to central Africa, from Guinea across Ghana, and Nigeria to southern Chad. This plant has been used traditionally to treat numerous diseases including trichomoniasis, amoebiasis, oedema, and skin diseases; the root is used to treat epilepsy; leaves to manage fever, leucorrhoea, diarrhea, convulsions, and jaundice [5]. However, there is no literature reported on the effects of the stem bark of *Bombax costatum* extract on the inflammatory response to support its traditional use in the management of inflammatory disorders. Consequently, as part of our continuous research into selected medicinal plants, the present study is aimed at evaluating the antiproliferative effects of the aqueous ethanolic stem bark extract of *Bombax costatum* P.V on cotton pellet-induced tissue granuloma formation in Sprague-Dawley rat. The most appropriate strategy for testing the efficiency of medications against the proliferative phase of inflammation is to use cotton pellets to induce granuloma development [6].

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Preparation of plant extract

The stem bark of *Bombax costatum* was collected from Nkawkaw in the Eastern Region of Ghana between February and April 2019. The bark was subsequently authenticated at the Department of Herbal Medicine, Kwame Nkrumah University of Science and Technology, and a voucher specimen KNUST/HM1/2019/WP006 was deposited in the herbarium of the Department of Herbal Medicine, KNUST. Aqueous ethanol extract of the stem bark of the plant was prepared by air-drying the freshly chopped stem bark for 14 days, after which it was milled into a coarse powder using a hammer mill (DF-15, DADE, 15 kg/h-110v, HXJQ, China). Subsequently, 500 g of the powder was extracted by cold maceration in 2.0 L of 70% ($^{v}/_{v}$) ethanol for 72 h, after which the supernatant was decanted and filtered. The filtrate was then concentrated at 60 °C under low pressure in a rotary evaporator (RE-LA-5, LAB-GENI, China), to obtain a dark-brown liquid which was evaporated to dryness in an oven (MOV-112PE, HXJQ, Panasonic, China) at 60 °C over 24 h. The semisolid concentrate was stored in a desiccator to remove excess moisture. A final percentage yield of 8.76 % ($^{w}/_{w}$) was obtained. The extract was freshly reconstituted in 2% $^{w}/_{v}$ tragacanth mucilage (dissolved in normal saline) before administration, herein referred to as aqueous ethanol extract of *Bombax costatum* (EBC).

2.1.2 Animals

Sprague Dawley rats (200-250 g) of both sexes were purchased from the Center for Plant Medicine Research, Mampong-Akwapim, Ghana. All animals were housed in the Animal Facility of the Department of Pharmacology, KNUST. Following the Animal Welfare Regulations and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002), all animals used in this study were humanely handled throughout the experimental period. The study was conducted with the approval of the Department of Pharmacology, KNUST Ethics Committee. The animals were randomly grouped (n=5), housed in stainless steel cages (34 cm × 47 cm × 18 cm) with softwood shavings as bedding, and were fed with a normal commercial pellet diet (GAFCO, Tema, Ghana). All animals were given access to water *ad libitum*. The animals were allowed enough time to acclimatize to the new environment and were maintained at a room temperature of 26 ± 2 °C in a 12 h

light-dark cycle. Each animal was used only once and at the end of each experiment, all animals were euthanized.

2.1.3 Chemicals and Reagents

Dexamethasone (Pharm-Inter, Brussels, Belgium), IL-6 and TNF-α ELISA kits (Boster, CA, USA).

2.2 Method

2.2.1 Cotton pellet tissue granuloma formation in rats

The antiproliferative effect of EBC was assessed using cotton pellet-induced granuloma tissue formation in rats. Inflammation was induced using sterilized cotton pellets with a method earlier described by Swingle and Shideman (1972). Briefly, rats (200-250 g, n=5) were divided into six (6) groups, allowed to fat overnight, and anesthetized by intraperitoneal injection of pentobarbital (20 mg/ kg) in the groin region of the skin shaved and disinfected with 70 % ethanol. An incision was made at the groin area and using blunt forceps, subcutaneous tunnels were formed into which sterilized cotton pellets (40 ± 1 mg) were implanted bilaterally on both sides. The incisions were closed subsequently with sutures. After recovering from anesthesia, rats were treated starting from day 0 with;

Group I (naïve) received only normal saline (1 ml, *p.o*, daily) for 7 days.

Group II (negative control) received 1 ml of normal saline daily for 7 days.

Group III was treated with Dexamethasone (3 mg/ kg, *p.o*, daily) for 7 days.

Group IV-VI (EBC-treated group) received 10-100 mg/ kg (daily, *p.o*) of EBC for 7 days.

The rats were monitored for 7 days and sacrificed on the 8^{th} day by cervical dislocation. The pellets were removed (along with granular tissue formed around) and freed from extraneous tissue, weighed immediately for wet weight. Then, the pellets were dried in an incubator at 60°C for 24 h to obtain a constant dry cotton pellet weight. The exudate weight (mg) was determined as the difference between the wet and dry weights of cotton pellets. The granuloma formation is considered the mean weight of granuloma tissue formed by subtracting ((40 ± 1 mg) from the dry weight of each cotton pellet. The percentage mean change of granuloma formation was calculated using the formula:

$$\% \text{ change of granuloma formation} = \left[\frac{Wc-Wt}{Wc}\right] \times 100$$

Where Wc is the difference in pellet weight of the control group and Wt is the difference in pellet weight of the treated group.

Total tissue granuloma formed during the 7 days was determined as the area under the time-course curve (AUC). The percentage inhibition of the total tissue granuloma was calculated using the formula:

$$\% \text{ Inhibition of tissue granuloma} = \left[\frac{AUC\ control - AUC\ treated}{AUC\ control}\right] \times 100$$

The effect of EBC on cotton pellet tissue granuloma formation was assessed by the following clinical parameters; haematological study, spleen weight/ 100 g of body weight, and serum cytokine levels of IL-6 and TNF-α using ELISA.

2.2.1.1 Haematological analysis

Blood samples were collected into EDTA tubes before and after the experiment for whole blood count using an automated heam analyzer (HP-HEMA6500A, Zhengzhou Hepo International Trading Co. Ltd, Henan, China).

2.2.1.2 Spleen weight/ 100 g of body weight of rats

Animals were sacrificed by cervical dislocation and the spleens were removed. The weight of the spleen for each rat in the treatment groups was measured and recorded.

2.2.1.3 Serum cytokine of IL-6 and TNF-α levels determination

Blood samples were collected into vacuum container gel and activator tubes and allowed to clot at room temperature. The blood samples were then centrifuged (model: 5415C, Medizin & Labortechnik GmbH, Schnakenberg, Germany) at × 1000 g for 15 min. Sera formed were aliquot into Eppendorf tubes and stored at -20 ℃ before analysis. Serum levels of the cytokines (IL-6 and TNF-α) were measured in duplicates with an appropriate rat ELISA kit per the protocol outlined by the manufacturer.

2.3 DATA ANALYSIS

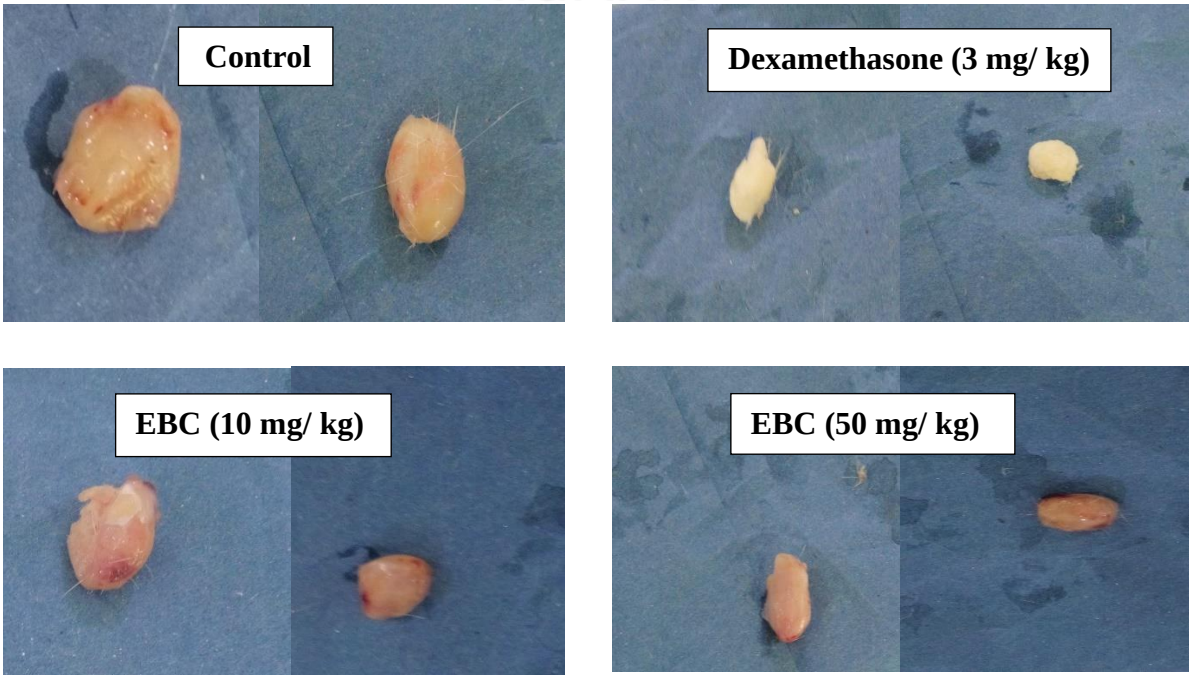
All data are presented as the mean ± SEM (n=5). All data were analyzed using one-way ANOVA followed by Dunnett's *post hoc* test. $P<0.05$ was considered statistically significant.

All graphs were plotted using GraphPad Prism for windows version 7.0 (GraphPad Software, San Diego, CA, USA).

3.0 RESULTS

3.1 Effect of EBC on cotton pellet tissue granuloma formation

Chronic inflammation was induced by implanting two sterilized cotton pellets weighing 40 ± 1 mg bilaterally in the subcutaneous layer of the groin region of each rat. The cotton pellets were removed on day 8 after the observation period and weighed immediately (Fig. 1). In the study, the exudate amount (Fig 1) recorded for the control was 4.27±0.15 mg (Table 1). EBC (10-100mg/ kg) significantly *(P<0.05)* reduced the mean exudate amount (Fig. 1) to 2.63±0.18 mg, 2.46±0.11 mg, and 2.18±0.19 mg at 10, 50, and 100 mg/ kg when compared to the control respectively (Table 1) with corresponding percentage inhibition of 36.75 %, 45.32 % and 54.82 % at the same doses (Table 1). The granuloma tissue formed at the end of the study was 2.00 ± 0.05 mg (Table 1). From the study, EBC (10-100mg/ kg) significantly *(P<0.05)* decreased the granuloma tissue formation to 1.45±0.05 mg, 1.19±0.15 mg, and 1.00±0.05 mg at 10, 50, and 100 mg/ kg when compared to the control respectively (Table 1) with respective percentage inhibition of 34.09 %, 45.71 % and 54.55 % at the same doses (Table 1).



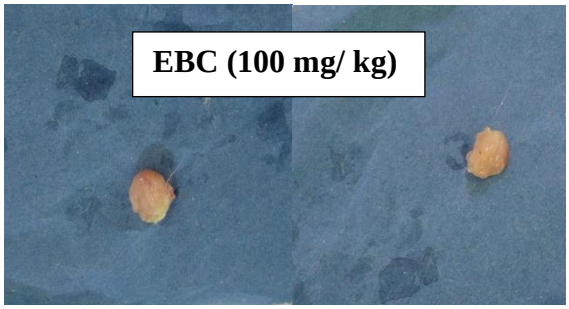


Figure 1. Photographs of cotton pellets removed from rats with various treatment groups in cotton pellet-induced granuloma tissue formation. Sterilized cotton (40 ± 1 mg) was implanted bilaterally into the subcutaneous tissue of the abdomen of Sprague-Dawley rats (200-250 g, n=5). Cotton pellets were removed and weighed immediately for wet weight.

Table 1. Effect of EBC on granuloma tissue formation in rats.

GROUP	Exudate weight/ (mg)	% Inhibition	Granuloma tissue formation (dry weight)/ mg	% Inhibition
Control	4.27±0.15	-	2.00 ± 0.05	-
Dexamethasone				
3.0 mg/ kg	1.99±0.30**	53.49 %	0.88±0.10***	60.10 %
EBC				
10 mg/ kg	2.63±0.18*	38.35 %	1.45±0.05*	34.09 %
50 mg/ kg	2.46±0.11*	42.41 %	1.19±0.15**	45.71 %
100 mg/ kg	2.18±0.19**	49.02 %	1.00±0.05***	54.55 %

Data presented as mean ± SEM (n=5). *P<0.05* compared to the control-treated group (One-way ANOVA followed by Dunnet *post hoc* test).

3.2 Haematogical analysis of EBC effects on cotton pellet tissue formation

Whole blood assessment for the treatment group was done on both days 0 and 8 of the observation period. In the study, EBC (10, 50, and 100 mg/ kg, *p.o*) showed no significant *(P<0.05)* changes in the haematological parameters (on day 0) when compared to the control (Table 2). On the 8th day, however, EBC significantly *(P<0.05)* decreased WBC levels at the inflammatory site from 3.01±0.05 (×10^3/ µL) to 5.29±0.58, 4.9±0.12 and 4.3±0.29 (×10^3/ µL) at 10, 50, and 10 when compared to the control group respectively (Table 2).

Table 2. Effect of EBC on haematological parameters in cotton pellet-induced granuloma formation in Sprague-Dawley rats.

		DAY 0				DAY 8		
	WBC ($\times10^3$/ µL)	HCT %	RBC ($\times10^{-6}$/ µL)	HGB (g dL^{-1})	WBC ($\times10^3$/ µL)	HCT %	RBC ($\times10^{-6}$/ µL)	HGB (g dL^{-1})
Control	5.83±0.15	53.73±2.52	9.13±0.54	14.97±0.22	7.40±1.25	38.20±7.50	6.20±0.43	11.77±0.73
Dexamethasone								
3.0 mg/ kg	5.50±0.23	52.47±2.21	8.01±0.21	14.74±0.15	3.01±0.05***	38.90±1.44	7.21±0.14	13.07±0.15
EBC								
10 mg/ kg	6.76±0.50	49.70±1.55	8.23±0.36	14.27±0.12	5.29±0.58*	42.10±2.19	7.66±0.34	12.43±0.98
50 mg/ kg	6.40±0.53	52.03±1.74	8.04±0.15	14.73±0.28	4.91±0.12*	38.50±0.50	7.59±0.45	12.07±0.44
100 mg/ kg	5.61±0.32	48.63±1.16	8.31±0.31	14.57±0.23	4.36±0.29**	40.50±2.86	7.54±0.56	12.53±1.05

Cotton pellets (40 ± 1 mg) were implanted subcutaneously (bilateral) in the groin regions of rats. Either drug vehicle, dexamethasone (3.0 mg/kg, *p.o*, daily) or EBC (10-100 mg/ kg, *p.o*, daily) was administered daily for 7 consecutive days. The blood sample was collected on the 8th day and a full blood count was carried out. Data presented as mean ± SEM (n=5) ($P<0.05$) compared to the control-treated group using one-way ANOVA followed by Dunnet *post hoc* test.

3.3 Spleen weight/ 100 g of body weight of rats

The weight of the spleen/ 100 g of body weight of the rats was measured in each treatment group. In the study, the total weight of the spleen/100 g of body weight for the control was 0.93 ± 0.02 g (Table 3). BCE (10-100mg/ kg) reduced the total spleen weight significantly to 0.81 ± 0.01 g, 0.79 ± 0.01 g, and 0.73 ± 0.02 g at 10, 50, and 100 mg/ kg compared to the control respectively (Table 3).

Table 3. Effect of EBC on spleen weight/ 100 g of body weight of rats on granuloma tissue formation in Sprague-Dawley rats.

Groups	Spleen weight/ 100 g of body weight
Control	0.93 ± 0.02
Dexamethasone (3.0 mg/ kg)	0.67 ± 0.03 ***
EBC	
10 mg/ kg	0.81 ± 0.01 **
50 mg/ kg	0.79 ± 0.01 **
100 mg/ kg	0.73 ± 0.02 ***

Cotton pellets (40 ± 1 mg) were implanted subcutaneously (bilateral) in the groin regions of rats. Either drug vehicle, dexamethasone (3.0 mg/kg, *p.o*, daily) or EBC (10-100 mg/ kg, *p.o*, daily) was administered daily for 7 consecutive days. The blood sample was collected on the 8th day and a full blood count was carried out. Data presented as mean ± SEM (n=5). *P<0.05; **P<0.01; ***P<0.001 compared to the control group using one-way ANOVA followed by Dunnet's *post hoc* test.

3.4 Effect of BCE on serum IL-6 and TNF-α levels

Bilateral implantation of sterilized cotton pellets in the subcutaneous layer of the groin region of the rat's induced inflammation. Stimulation of certain antibodies activates the migration of plasma cells such as neutrophils, which activate the release of cytokines such as IL-6, TNF-α, interferon γ, and other mediators. In the study, serum cytokine levels of IL-6 and TNF-α were determined using ELISA. In the study, the mean serum levels of IL-6 and TNF-α for the control group were 41.79±2.18 pg ml^{-1} and 90.81±6.07 pg ml^{-1} respectively (Fig. 2 A and B). EBC (10-100mg/ kg) exhibited a significant *(P<0.05)* reduction of the mean serum levels of IL-6 (41.79±2.18 pg ml^{-1}) to 26.43±4.35 pg ml^{-1}, 22.85±2.92 pg ml^{-1} and 18.88±2.46 pg ml^{-1} at 10, 50 and 100 mg/ kg in a dose-dependent manner when compared to the control respectively (Fig. 2A). Similarly, EBC reduced the mean serum levels of TNF-α (90.81±6.07 pg ml^{-1}) significantly *(P<0.05)* to 43.51±8.21 pg ml^{-1}, 36.91±14.07 pg ml^{-1} and 27.42±12.19 pg ml^{-1} at 10, 50 and 100 mg/ kg when compared to the control group respectively (Fig. 1B).

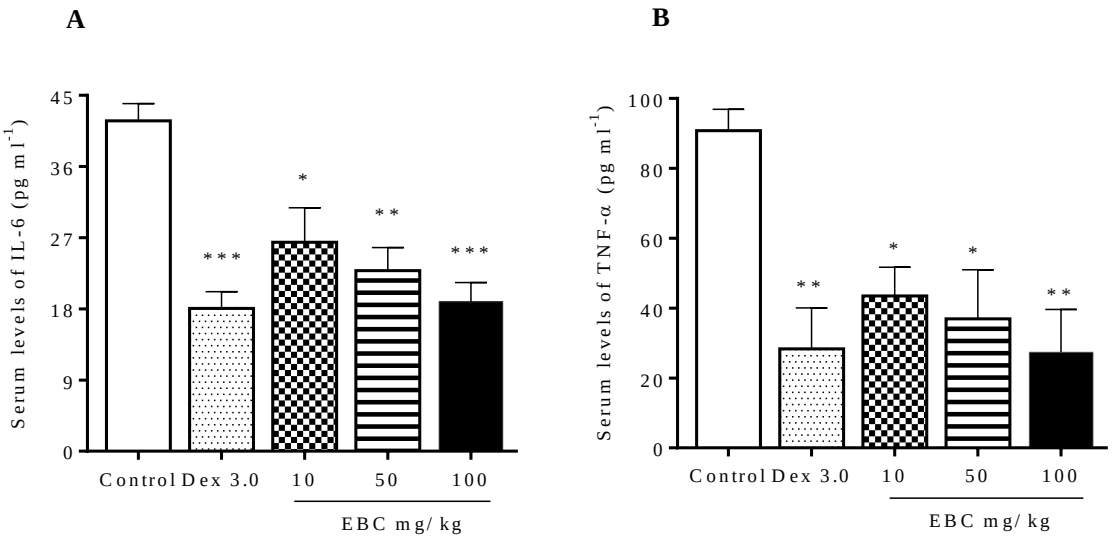


Figure 2: Effect of EBC on the total serum cytokine levels of IL-6 (A) and TNF-α (B) in cotton pellet-induced tissue granuloma formation in Sprague-Dawley rats. Data presented as mean ± SEM (n=5). P<0.05 compared to the control-treated group (One-way ANOVA followed by Dunnet *post hoc* test).

4.0 DISCUSSION

In this study, the aqueous ethanolic stem bark extract of *Bombax costatum* was regarded as a potential antiproliferative agent based on its use in folkloric medicine for the management of several diseases. The antiproliferative effect of *Bombax costatum* on cotton pellet-induced granuloma tissue inflammation was scientifically investigated and validated.

It has been reported that a key characteristic model of chronic inflammatory response is cotton pellet-induced granuloma tissue formation [7]. The cotton pellet-induced granuloma tissue formation test is an inflammatory model widely used to assess the antiproliferative activity of agents capable of managing chronic inflammation [8]. The implantation of sterilized cotton pellets subcutaneously in rats undergoes three stages of the inflammatory response: 1) the first stage, a transudative stage, occurs during the first 3 h in which leaked fluid from blood vessels containing low protein caused by increased vascular permeability, 2) and exudative stage that occurs between 3-72 h post-implantation of the cotton pellet and 3) the final stage is the proliferative stage that occurs between 3-6 days resulting in the formation of granuloma tissue due to the release of inflammatory mediators [9]. The proliferative phase is quantified as the increase in dry weight of granuloma tissue formation [2], While the moist wet weight of the pellets correlates with the transudative phase [10]. The

cotton pellet is known to stimulate the immune system to produce antibodies and proinflammatory mediators including cytokines such as interleukins (IL-1, IL-6) and tumor necrosis factor-alpha (TNF-α).

Consequently, this results in the formation of granuloma tissue and the proliferation of lymphocytes [11, 12]. Furthermore, it has been shown that during angiogenesis, produced inflammatory cells deliver more oxygen and nutrients to the site of inflammation, assisting in the creation of granuloma tissue [13]. Thus, suppression of the proliferative phase is an indication of a possible decrease in the production of fibroblasts, cell infiltration, interleukins, kinins, synthesis of collagen, and monopolysaccharides during granuloma tissue formation [9, 14]. In the study, *Bombax costatum* extract significantly exhibited a reduction of both the dry weight and wet weight of granuloma tissue formation at the site of inflammation, which is an indication of its inhibitory effects on both the transudative and proliferative stages of chronic inflammation and this is consistent with previous reports [8, 15]. Furthermore, *Bombax costatum* extract significantly downregulated the gene expression of the proinflammatory cytokines, IL-6 and TNF-α, and suppressed cell infiltration. This was supported by our earlier findings in this study where *Bombax costatum* extract significantly decreased serum levels of IL-6 and TNF-α, and this is in agreement with other previous reports [16].

To explain the blood-related effects of plant formulations, haematological parameters are critical for assessment [17]. The evaluation of these blood indicators presents any clinical risk associated with haematological alterations and quickly indicates metabolic modification of the body that gives essential data on the body's response to injury or infection [18, 19]. In this study, there were elevated levels of WBC migration to the inflammatory site which is reported to be a biomarker of inflammation [20]. From the study, *Bombax costatum* extract significantly reduced the levels of WBC at the site of inflammation suggestive of its inhibitory effects against inflammation and proliferation of the endothelium [15].

It has also been reported that spleen enlargement is a characteristic feature of cotton pellet-induced granuloma tissue formation due to its phagocytic character [20]. *Bombax costatum* extract in this study showed a significant decrease in the spleen weight relative to the enlarged spleen weight of the control. Thus, *Bombax costatum* extract exhibited its inhibitory activity on the phagocytic nature of the spleen during the inflammatory response and this is consistent with previous reports [20].

Conclusion

In conclusion, this study has established the antiproliferative effect of aqueous ethanolic extract of the stem bark of *Bombax costatum* as shown by its inhibitory effects on granuloma tissue formation. The restorative action of the extract on haematological parameters during the inflammatory response and its antiproliferative effects from these findings strongly support its folkloric usage in the management of inflammation. Further quantitative chemical evaluation is underway to determine the active secondary metabolic phytoconstituents that are responsible for the anti-inflammatory activities.

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DATA AVAILABILITY

All data regarding this study is available upon request.

Author Contribution

MAA, EO, and GO conceptualized and executed the study. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. Cook, A. D., Christensen, A. D., Tewari, D., McMahon, S. B., & Hamilton, J. A. (2018). Immune cytokines and their receptors in inflammatory pain. *Trends in immunology*, *39*(3), 240-255.
2. Patil, K. R., & Patil, C. R. (2016). Anti-inflammatory activity of bartogenic acid-containing fraction of fruits of *Barringtonia racemosa* Roxb. in acute and chronic animal models of inflammation. *Journal of traditional and complementary medicine*, *7*(1), 86-93.
3. Ricciotti, E., & FitzGerald, G. A. (2011). Prostaglandins and inflammation. *Arteriosclerosis, thrombosis, and vascular biology*, *31*(5), 986–1000.

4. Obese, E., Ameyaw, E. O., Biney, R. P., Henneh, I. T., Edzeamey, F. J., & Woode, E. (2018). Phytochemical screening and anti-inflammatory properties of the hydroethanolic leaf extract of Calotropis procera (Ait). R. Br. (Apocynaceae). *Journal of Pharmaceutical Research International*, *23*(1), 1-11.
5. Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., & Anthony, S. (2009). Agroforestry Database: a tree reference and selection guide version 4. http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp. Assessed 4 January 2022.
6. More, S. K., Lande, A. A., Jagdale, P. G., Adkar, P. P., & Ambavade, S. D. (2013). Evaluation of the anti-inflammatory activity of Solanum xanthocarpum Schrad and Wendl (Kaṇṭakāri) extract in laboratory animals. *Ancient Science of Life*, *32*(4), 222.
7. Sengar, N., Joshi, A., Prasad, S. K., & Hemalatha, S. (2015). Anti-inflammatory, analgesic, and antipyretic activities of standardized root extract of *Jasminum sambac*. *Journal of ethnopharmacology*, *160*, 140-148.
8. Sultana, A., Afroz, R., Yasmeen, O., Aktar, M. T., & Yusuf, M. A. (2019). Anti-Inflammatory Effect of Ethanolic Extract of Carica Papaya Leaves and Indomethacin in Cotton Pellet Induced Granuloma in Animal Model. *Journal of Current and Advance Medical Research*, *6*(1), 2–5.
9. Swingle, K. F., & Shideman, F. E. (1972). Phases of the inflammatory response to subcutaneous implantation of a cotton pellet and their modification by certain anti-inflammatory agents. The Journal of pharmacology and experimental therapeutics, 183(1), 226–234.
10. Kalpana & Upadhyay, A. (2010). Anti-inflammatory evaluation of ethanol extract of leaves of *Haloptelea integrifolia* Planch. *Annals of Biological Research*; 1(2): 185-195.
11. Süleyman, H., Odabasoglu, F., Aslan, A., Cakir, A., Karagoz, Y., Gocer, F., … Bayir, Y. (2003). Anti-inflammatory and antiulcerogenic effects of the aqueous extract of Lobaria pulmonaria (L.) Hoffm. Phytomedicine, 10(6-7), 552–557.
12. Fujiwara, N., & Kobayashi, K. (2005). Macrophages in inflammation. *Current drug targets. Inflammation and allergy*, *4*(3), 281-286.
13. Ghosh, M.N. (2005). Some common evaluation techniques. Fundamentals of Experimental Pharmacology. Hilton & Company, India. pp. 175-190.
14. Pérez, S., Meckes, M., Pérez, C., Susunaga, A., & Zavala, M. A. (2005). Anti-inflammatory activity of Lippia dulcis. Journal of ethnopharmacology, 102(1), 1–4.
15. Subash, A., Veeraraghavan, G., Sali, V. K., Bhardwaj, M., & Vasanthi, H. R. (2016). Attenuation of inflammation by marine algae *Turbinaria ornata* in cotton pellet-induced granuloma mediated by fucoidan-like sulphated polysaccharides. *Carbohydrate polymers*, *151*, 1261-1268.
16. Kumar, R., Gupta, Y. K., & Singh, S. (2016). Anti-inflammatory and anti-granuloma activity of *Berberis aristata* DC. in experimental models of inflammation. *Indian journal of pharmacology*, *48*(2), 155–161.
17. Jorum, O. H., Piero, N. M., & Machocho, A. K. (2016). Haematological effects of dichloromethane-methanolic leaf extract of *Carissa edulis* (Forsk Vahl.) in normal rat models. *Journal of Haematology Thromboembolic Diseases*, *4*(1), 232.
18. Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., Lilly, P., Sanders, J., Sipes, G., Bracken, W., Dorato, M., Van Deun, K., Smith, P., Berger, B., & Heller, A. (2000). Concordance of the toxicity of pharmaceuticals in humans and animals. *Regulatory toxicology and pharmacology. RTP*, *32*(1), 56-67.
19. Fazio, F., Ferrantelli, V., Fortino, G., Arfuso, F., Giangrosso, G., & Faggio, C. (2015). The influence of acute handling stress on some blood parameters in cultured sea bream (*Sparus Aurata* Linnaeus, 1758). *Italian journal of food safety*, *4*(1), 4174.
20. Mishra, N.K., Panda, R.K., Rajakumar, V., Kumar S., Tejonidhi, K., Mishra, G. (2010). Evaluation of Anti-inflammatory activity and dose selection of *Mollugo pentaphylla* by using cotton pellet-induced granuloma in rats. International Journal of Pharmacy & Health Science, 1(3): 155-162.