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Anti-nociceptive and anti-inflammatory activities of *Tetracarpidium conophorum* seed lectin

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ABSTRACT

The anti-nociceptive and anti-inflammatory activity of the lectin purified from *Tetracarpidium conophorum* seeds were investigated.

The lectin was purified from the phosphate buffered saline crude extract of the seeds using gel filtration and affinity chromatography techniques. Hemagglutination assay and hapten-sugar inhibition test were carried out to ascertain the blood group and sugar specificities of the lectin respectively. Anti-nociceptive and anti-inflammatory activities of the lectin were evaluated (in-vivo) with standard models including formalin licking test, carrageenan-induced paw edema test and peritonitis model.

A yield, purification fold and subunit molecular weight of 27%, 17 and 34 kDa respectively were obtained for the purified lectin from *T. conophorum* seed (*TcSL*). The lactose/galactose specific *TcSL* showed significant inhibition ($p < 0.05$) of nociception as measured by paw licking time upon pain induction by formalin and inflammation as measured by paw swollenness induced by sub-plantar injection of carrageenan. The lectin also significantly reduced carrageenan-induced leucocyte migration to the peritoneum in a dose-dependent manner.

The study concluded that *TcSL* showed anti-nociceptive and anti-inflammatory activities and thus poised to be a good bio-active peptide for the design of novel anti-inflammatory therapy.

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Introduction

Inflammation though normally a defensive strategy to quell and eliminate threats emanating from physical trauma, noxious chemicals, microbiological agents, environmental pollutions etc. [1] may become extremely exaggerated and over-sustained resulting in no apparent benefit but rather severe adverse consequences. Prolonged experience of pain could be-

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come insufferable as observed in arthritis, various autoimmune diseases and hypersensitivities. Perhaps the most grievous is tumorigenesis which is favored by chronic inflammation [2].

Anti-inflammatory drugs, broadly in the class of synthetic glucocorticoids, steroidal and non-steroidal anti-inflammatory drugs (NSAIDS) have been linked to gastrointestinal disorders, adverse cardiovascular side effects [3], acute renal failure [4], osteoporosis, insulin resistance and diabetes [5]. There is therefore the need to search for novel compounds of natural origin with potential anti-inflammatory properties. Plants' roots, stems, flowers, fruits and seeds are repertoire of such indispensable bio-active agents, including the exploitable class of proteins called lectins, which could be used as safer alternatives. Lectins are carbohydrate recognizers, imbued with carbohydrate-recognition domains which can be region-and cell-surface specific and capable of reversible interactions with glyco-moieties [6]. This distinguishing attribute could be pivotally exploited in events underlying inflammation especially in white blood cells communication with interactive carbohydrate recognition proteins and various cell surface adhesion molecules involved in migration, homing and trafficking of leucocytes during inflammation. Adhesion molecules are in fact mainly lectins or lectin-like compounds, thus the adhesion family have been given the generic name LEC–CAM, that is, lectin cell adhesion molecules [7].

The transmigration of leucocytes into the site of inflammation (extravasation) which is totally dependent on carbohydrate recognition and adhesion proteins, prolongs and leads to chronic inflammation. Thus, a check on the process opens a therapeutic solution window to inflammation [8]. Transmigration relies on sequential events involving adhesion molecules like selectins to accomplish leukocyte passage in the direction of a chemotactic gradient [9]. Adopting an exogenous lectin to interfere with crucial pro-inflammatory processes and impede influx of white blood cells into inflammation sites, thus quelling chronic inflammation is a new paradigm.

T. conophorum commonly referred to as African walnut is a climbing shrub (10–20 feet long) belonging to the *Euphorbiaceae* family. It is widely and principally cultivated for its nuts or seeds which are consumed as delicacy in Nigeria [10]. The plant is indigenously acclaimed to be medicinally effective in improving fertility in males. Walnuts extracts have also been shown to possess anticancer, antidiabetic and antimicrobial activities [11–13].

Studies on the phytochemical constituents of *T. conophorum* have reported the presence of alkaloids, tannins, oxalates and phytates. The seed is a rich source of proteins, fibres, oil and carbohydrate, phytosterols, polyphenols, phytoestrogens and omega-3 fatty acids [10]. The seeds have been shown to be rich in lectin with specificities for D-galactose and lactose [14], a 34 kDa monomeric protein [15]. Our previous studies have also delved into the physicochemical characterization and toxicity of the lectin [16]. The present focus is centered on animal models for investigation of possible anti-inflammatory activities of the lectin in terms of the inhibitory effect on nociception (extreme sensation of pain), swollenness or edema and leucocyte migration into inflammatory sites.

Materials and methods

Materials and reagents

Sodium dihydrogen orthophosphate, disodium hydrogen phosphate, sodium chloride, sodium azide, glacial acetic acid are products of BDH Chemical Limited, Poole, England. Formalin, carrageenan, chloroform, Acrylamide, TEMED, methylenebisacylamide (MBA), methanol. Sugars; glucose, mannitol, maltose, galactose, mannose, sorbose, fructose, N-acetylglucosamine and lactose are of Sigma Chemical Company, St Louiz, Mo, USA. Sephadex G-200, Sepharose 4B are products of Pharmacia Fine Chemicals, Upsalla, Sweden. All other chemicals used were of analytical grade.

Blood collection

Human blood groups (A, B, O) were obtained from apparently healthy donors and rabbit erythrocytes from rabbits purchased from College of Health Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria.

Experimental animals

Wistar rats averagely weighing 120 g were obtained from the College of Health Sciences, Obafemi Awolowo University. The animals were acclimatized to ambient conditions (temperature 27.2 ± 4.4 °C and a light/dark cycle (12/12 h) for at least two weeks and fed with feeds and water ad libitum. All experimental procedures were carried out in strict accordance with the European Commission Directive (86/609/EEC) for guidelines in the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of the Obafemi Awolowo University, Ile-Ife.

Methods

Extraction and purification of the lectin

Crude extract of the lectin was prepared by homogenization of *T. conophorum* seed powder in phosphate buffered saline (PBS, pH 7.2) for 24 h. The resultant supernatant of subsequent centrifugation at 1147 x g for 30 min were pooled together as the crude extract. The crude extract was purified as previously described by Kuku et al. [16] with the use of gel filtration chromatography on Sephadex G-200 and affinity chromatography on a lactose – Sepharose 4B column. Non- Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis was carried out to ascertain the purity of the lectin sample.

Table 1
Hapten Inhibition Test of *T. conophorum* Lectin.

Sugar	Hemagglutination titre
Control (no sugar)	2^{12}
Fructose	2^{12}
Mannose	2^{12}
Mannitol	2^{12}
N-acetylglucosamine	2^{12}
Sorbose	2^{12}
D-Glucose	2^{11}
D-Galactose	2^{1}
Lactose	2°

Each experiment contained of 100 μl of serially diluted crude lectin in a U-shaped microtitre well. 50 μl 0.2 M sugar solution and 50 μl of 4% suspension of rabbit erythrocytes were added to each well. Positive control was without sugar while negative control did not contain extract and sugar.

Hemagglutination assay and sugar specificity test

Hemagglutination assay and sugar specificity test was carried out according to the method of Wang et al. [17].

In-vivo antinociceptive activity of T. conophorum *lectin (*TcL*)*

Formalin licking test was carried out as described by Dubuission and Dennis [18] and as modified by Tjølsen et al. [19]. The animals were grouped into six (five rats in a group), four groups of rats received sub-cutaneous injection of the graded doses of the lectin, while the control group and the standard group received normal saline (3 ml/kg) and diclofenac sodium (10 mg/kg) respectively. Nociception was induced 30 min later with 1.5% formalin solution (20 µl) injected into the right hind paw of the rats. Time spent licking the paw (taken as a measure of nociception) was recorded. The first 5 min (first phase) was taken as corresponding to a direct chemical stimulation of nociception while 15–30 min was taken as a late phase reaction to nociception.

Anti-inflammatory activity of TcL

Carrageen-induced paw edema test was carried out according to the method of Winter et al. [20] as described by Amir and Kumar [21]. Rats were pre-treated with normal saline (3 ml/kg), T. conophorum lectin (3 mg/kg, 6 mg/kg, 12 mg/kg, and 12 mg/kg incubated with 0.2 M lactose), diclofenac (10 mg/kg) and were challenged by sub-planar injection of 0.1 ml of 1% carrageenan dissolved in carboxylmethyl cellulose (1%) into right hind paws to induce edematous swellings in the paws. The paw volume was measured for 4 h at 1 h interval post-assault, using micrometer screw gauge. The percentage inhibition of the swollenness was taken as an indication of anti-inflammatory potential of the lectin and was calculated formulas follows:

Peritonitis model to evaluate leucocyte migration

Animals were pre-treated with the graded doses of *TcL*, normal saline and standard drug, administered intravenously 30 min before the administration of inflammatory stimuli effected by the intra-peritoneal injection of carrageenan to induce leucocytes migration to the peritoneum. Four hours later, animals were anaesthetized, the peritoneal cavity was opened and washed with sterile saline and the peritoneal fluids were collected into heparinized bottles maintained at 4oC. The peritoneal fluids were immediately subjected to total and differential leukocyte (lymphocyte, granulocyte and monocyte) counts using an auto-analyzer. Results were expressed as means ± S.E.M of the number of cells x 103/ml of peritoneal fluid.

Statistical analysis

All the data collected in each of the model of investigation including the formalin licking tests, carrageenan paw swollenness, and peritonitis model were compared statistically with analysis of variance (ANOVA) generated with graph pad prism.

Results and discussion

Hemagglutinating activity, sugar specificity and purification of the lectin

The hemagglutination assay affirmed the presence of a strong hemagglutinating lectin, capable of agglutinating human blood groups and rabbit erythrocytes; such lectins are termed pan-agglutinins. The lectin's hemagglutinating activity was inhibited by D-galactose and lactose while none of the other tested sugars had significant effect on the hemagglutinating activity of the lectin (Table 1), corroborating previous study which classified the lectin as a galactose/lactose-binding lectin [16]. The high affinity of the lectin for lactose informed the use of a two-step purification of the lectin with gel filtration on Sephadex G-200 and affinity chromatography on lactose-Sepharose 4B (Figs. 1 and 2). Non SDS - PAGE ascertained the purity of the lectin with only one distinct band observed after affinity chromatography (Fig. 3).

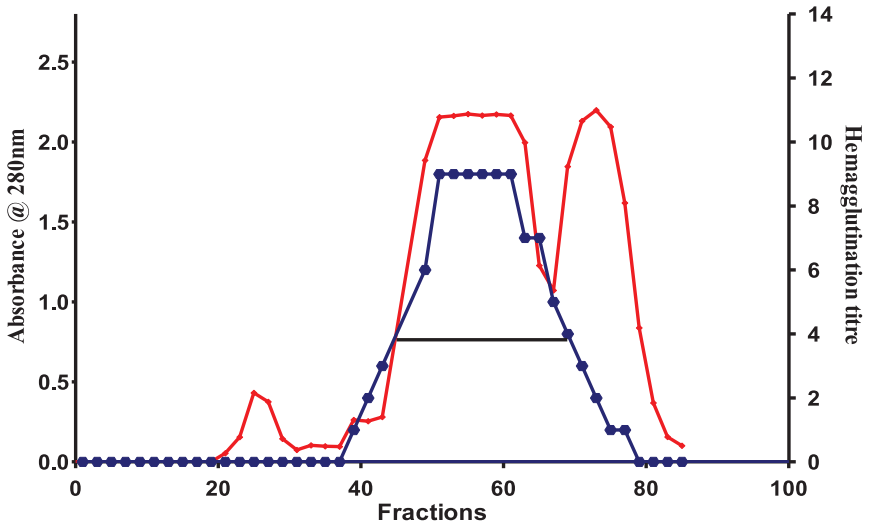

Fig. 1. Gel Filtration Chromatography of Crude Extract of *T. conophorum* Seeds on Sephadex G-200.
Crude extract (20 mg) was layered on Sephadex G-200 column (2.5 × 40 cm), previously equilibrated with PBS, pH 7.2. Fractions of 4 ml were collected at the flow rate of 20 ml/h, elution was monitored at 280 nm and the fractions were assayed for haemagglutinating activity.

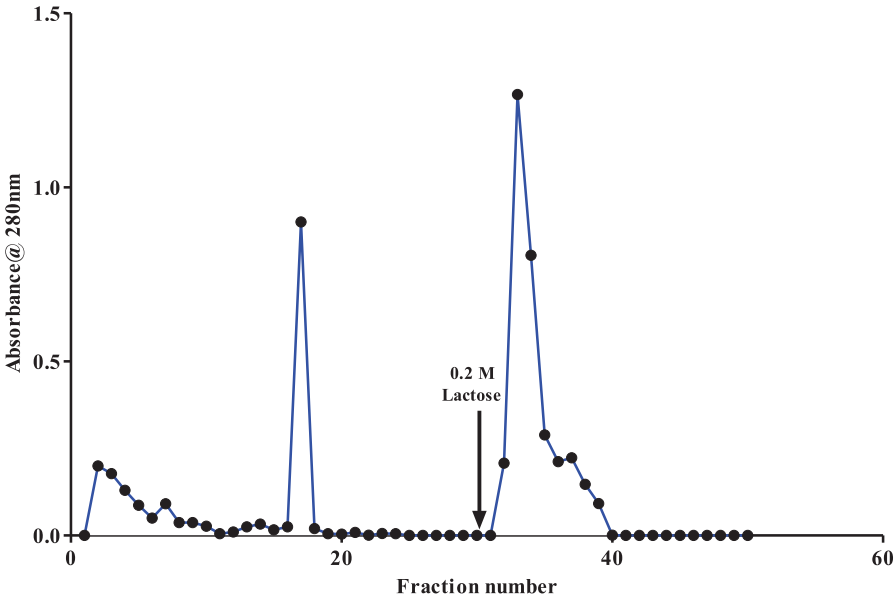

Fig. 2. Affinity Chromatography of Active Gel Filtration Peak on Lactose-Sepharose 4B Column.
The active pooled fractions from gel filtration after dialysis was layered on lactose – sepharose 4B column (10 × 1 cm). The column was first washed with PBS to remove the unadsorbed proteins then the adsorbed lectin was eluted with 0.2 M lactose in PBS. The flow rate was 18 ml/h and fraction size was 1 ml.

Anti-nociceptive and anti-inflammatory activities

Inhibition of formalin-induced nociception

Previous studies have reported that oral administration of *T. conophorum* lectin did not cause any observable toxicity, however, when administered intra-peritoneally (i.p.), the lectin elicited toxicity with a lethal dose, 50% (LD_{50}) of 50 mg/kg [16]. These findings informed the designs of the dosing regimen used for this study. Injection of formalin into the hind paw of Wistar rats induced a biphasic pain response; the first phase is thought to result from direct activation of primary afferent sensory neurons, whereas the second phase has been proposed to reflect the combined effects of afferent input and central sensitization in the dorsal horn [22].

The lectin inhibited the paw licking time of the animals in a dose - dependent manner at the early phase of nociception (i.e. the first five minutes after induction of pain with 1.5% formalin). Inhibition of pain at the early phase was minimal

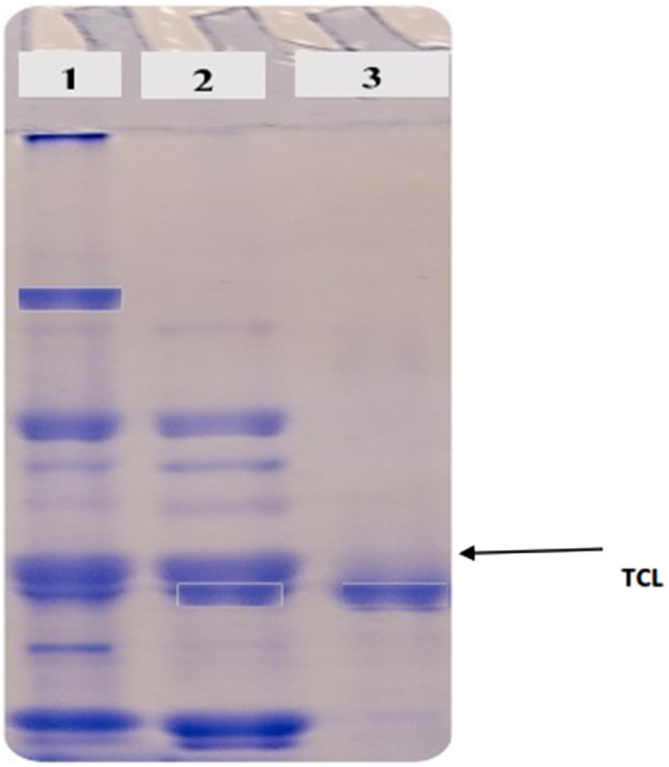

Fig. 3. Electrophoretogram of Non-SDS Polyacrylamide Gel Electrophoresis of Crude, Pooled Gel Filtration and Affinity Chromatography Peaks of *T. conophorum*.
Lane 1: Crude Extract
Lane 2: Gel filtration active peak
Lane 3: Affinity chromatography active peak (TCL).

Table 2
Percentage inhibition of formalin – induced nociception at the early and late phase.

Groups ($n=5$)	Dose (mg/kg)	Early Phase (0–5mins) ±SEM	%Inhibition	Late phase (15–30 min) ±SEM	% Inhibition
Group 1	Normal saline	44.25 ± 8.23	0	142.33 ± 51.2	0
Group 2	0.75	42.50 ± 8.76	3.95	0 ± 0	100
Group 3	1.5	41.50 ± 0.50	6.21	0 ± 0	100
Group 4	3	14.20 ± 11.30	67.91	0 ± 0	100
Group 5	6	12.20 ± 2.15	72.43	0 ± 0	100
Group 6	Diclofenac Na (10 mg/kg)	24.30 ± 6.33	45.08	6.80 ± 4.16	95.22

at lower dosage (0.75 mg/kg and 1.5 mg/kg showing 3.0% and 6.0% inhibition of pain respectively), however, higher doses of the lectin gave significant inhibition of nociception (67.0% and 72.0% inhibition at 3 and 6 mg/kg respectively). At the late phase, 15–30 min after pain induction, the lectin inhibited the pain sensation completely (Table 2). Comparison of the untreated (normal saline) and lectin-treated groups showed significant difference ($p < 0.05$) in the percentage inhibition of pain but there was no significant difference in the pain inhibition observed with the standard drug (diclofenac sodium, 10 mg/kg). Non-steroidal anti-inflammatory drugs mitigate inflammatory pain by inhibiting the formation of pain mediators at the peripheral target sites where prostaglandins and bradykinin are proposed to play a significant role in the pain process [23]. The potentials of lectins to act as anti-inflammatory and anti-nociceptive agents have been reported in recent literature. According to the report of Rivanor et al. [24], lectin from the green seaweed, *Caulerpa cupressoides*, displayed potent anti-inflammatory and antinociceptive effects via a mechanism that is dependent on tumor necrosis factor (TNF-α) and COX-2 inhibition.

Carrageenan-induced paw edema

Inflammation induced by carrageenan originally described by Winter et al. [20] has been established as acute, non-immune, well-researched, and highly reproducible. The inflammatory response is quantified by increase in paw size which is maximal around 4–5 h post-carrageenan injection [25].

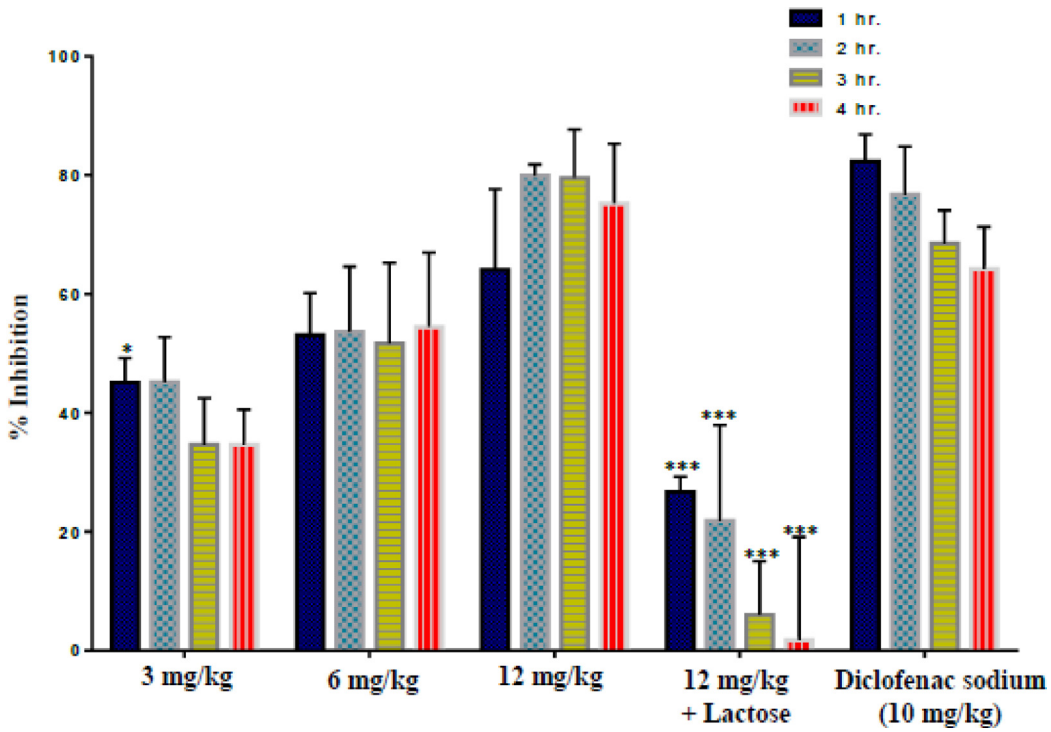

Fig. 4. Percentage Inhibition of Rats Paw Swollenness.
Percentage inhibition of swollenness was calculated taking the control group (i.e. administered normal saline) as having zero inhibition (i.e. 100% edema). The calculated% Inhibition exhibited direct proportionality to lectin dose.
* represent significant difference at $p < 0.05$ in comparison to the standard drug i.e. diclofenac sodium (10 mg/kg)
*** represent significant difference at $p < 0.001$ in comparison to diclofenac sodium (10 mg/kg).

In this study, paw edema was rapidly induced in the rats' hind paw by the sub-plantar injection of carrageenan indicating the induction of inflammation. Inflammation peaked in the control group, (administered normal saline prior to carrageenan injection) with an average of 52.7% increase in the rat paw volume by the second hour (Fig. 4). The lectin reduced the rats' paw edema, in a dose dependent manner, with the highest dose (12 mg/kg) exhibiting a remarkable minimal edema in the 2nd hour (10.6%) compared to the control. Effectiveness at inhibiting swollenness was comparable with the standard drug. Incubating the lectin (12 mg/kg) with 0.2 M lactose before administration reduced the lectin's anti-inflammatory effect significantly ($p < 0.001$).

Carrageenan-induced edema has been classified as bi-phasic; the early phase (1–2 h) of the carrageenan model is mainly mediated by histamine, serotonin, and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorph nuclear cells, and prostaglandins produced by tissue macrophages [26–28]. The lectin acted comparably well with diclofenac sodium (10 mg/kg) which is a standard inhibitor of cyclooxygenase (COX-2). It could be opined therefore that the lectin's anti-inflammatory activities may be tied to possible modulatory potential on prostaglandins secretion. Inferably, the lectin's anti-inflammatory activities seem to be dependent on the specific carbohydrate recognition and binding ability of the lectin as shown with the loss of the anti-edema activity of the lectin (Fig. 4) even at the highest dose (12 mg/kg), when incubated with lactose prior to administration.

Peritonitis model for leucocytes migration

Total leucocyte count of the peritoneal fluid. Inflammatory stimuli are followed by recruitment, sensitization and activation of leucocytes transmigrated to the site of inflammation [9]. The peritoneum has been described as a natural hub for leucocytes which rapidly populate the site upon appropriate induction. The peritonitis model thus used the peritoneum as a central hub to study leucocyte migration upon induction of inflammation in rats challenged with carrageenan.

The total leucocytes count of the peritoneal fluid recovered after intra-peritoneal stimulation of the peritoneum cavity with carrageenan showed the injection of carrageenan into animals successfully induced leucocyte migration and therefore caused an increase in the white blood cells population within the peritoneum cavity with the unchallenged group having a low total leucocyte count (1.33 × 103/ml) while the control group (animals treated with normal saline prior to intra-peritoneal injection of carrageenan) had the highest total leucocyte count of 4.63 × 103/ml. The lectin significantly impeded leucocytes' migration into the peritoneum in a dose-dependent manner. Fig. 5 showed that there was no significant dif-

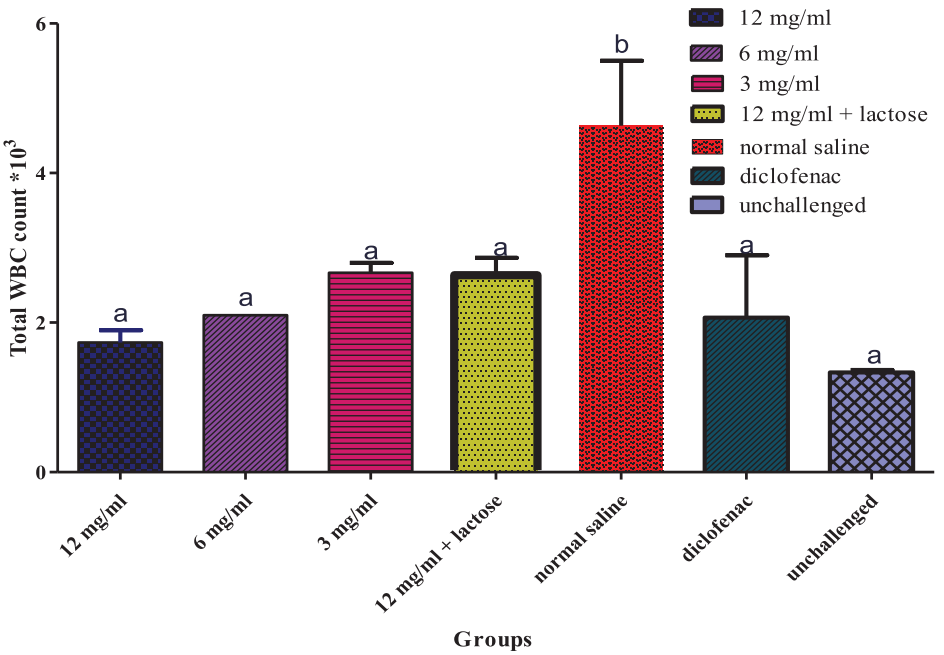

Fig. 5. Dose-dependent Inhibition of Total White Blood Cell Count.
Total white blood cell count increased with reduction in lectin dose and with incubation of lectin with lactose. "a": represent significant difference ($p < 0.05$) in comparison with normal saline group
"b": represent significant difference ($p < 0.05$) in comparison with the un-assaulted group.
The lectin treated groups are significantly different from the normal saline group but showed no significant difference when compared with the un-assaulted groups.

Table 3
Total and Differential Leucocyte Count.

Groups	Total WBC Count x 10^3	Lymphocyte Count x 10^3	Granulocyte Count x 10^3	Monocyte Count x 10^3
3 mg/kg	2.67 ± 0.13	1.33 ± 0.06	0.80 ± 0.10	0.56 ± 0.03
6 mg/kg	2.1 ± 0.00	1.17 ± 0.23	0.67 ± 0.17	0.26 ± 0.06
12 mg/kg	1.73 ± 0.17	0.97 ± 0.03	0.5 ± 0.10	0.26 ± 0.03
12 mg/kg + Lactose	2.63 ± 0.23	1.40 ± 0.26	0.73 ± 0.03	0.50 ± 0.00
Normal saline	4.63 ± 0.87	1.67 ± 0.33	1.96 ± 0.43	1.0 ± 0.10
Diclofenac 10 mg/kg	2.07 ± 0.80	0.67 ± 0.23	0.83 ± 0.36	0.57 ± 0.20
Un-challenged	1.33 ± 0.03	0.53 ± 0.03	0.50 ± 0.0	0.30 ± 0.10

ference ($p < 0.05$) in the leucocyte count derived from the animal groups treated with the lectin prior to carrageenan stimulus and the animals in unchallenged group while there was significant difference ($p < 0.05$) between the pre-assault lectin treated groups (with marked reduction in leucocyte count) and the control (normal saline pre-treated) group. The highest dose of the lectin (12 mg/kg) reduced total leucocyte count to 1.73 × 103/ml which is almost to the level of the un-challenged group (1.33 × 103/ml). This result also showed that lactose was able to inhibit the lectin's leucocyte modulatory action as animal group treated with the lectin (12 mg/kg) incubated with 0.2 M lactose, showed increase in total leucocyte count from 1.73 × 103 to 2.63 × 103 /ml (Table 2). This is suggestive of the fact that any anti-transmigration action of the lectin against white blood cells is dependent on its carbohydrate recognition domain. Lasky [7] highlighted the pivotal importance of some class of lectins like the selectins family to leucocytes rolling and transmigration into inflammatory sites, which is dependent on specific recognition and binding of unique glyco-motifs on the white blood cells. Assreuy et al. [8] described the possible anti-inflammatory activity of lectins corroborative of the possible lectins' competitive blockage of a common leukocyte. Also, since selectin is important in leucocyte transmigration into inflammatory sites [8], interaction of an exogenous lectin with endothelial selectin carbohydrate ligand may deactivate or hinder leucocyte transmigration.
(Table 3).

Comparison of the differential leucocyte count. The differential leucocyte count is a measure of the number of lymphocytes, granulocytes and monocytes present in the total leucocyte mix. The result indicated that lectin impeded all white blood cell types, as the population of all the white blood cell types reduced in a dose dependent manner across board (Fig. 5). Lactose inhibition of the lectin's activity was observed in all the blood cell types (lymphocytes, granulocytes and monocytes).

A larger percentage of the leucocytes population could be attributed to lymphocytes, followed by granulocytes, the lowest white blood cell population in the peritoneal fluid recovered was monocytes. This may be so because, neutrophils recruited at early hours of inflammation are usually cleared from inflammatory sites after the early phase of inflammation while lymphocytes are activated and directed to the sites of inflammation at the late phase to mediate chronic inflammatory response [29].

Conclusion

In all the models of investigations employed in this study, *T. conophorum* seed lectin displayed a remarkable inhibitory proficiency on induced inflammation. Since pain induction by formalin has been tied to tissue damage, the lectin may be able to prevent tissue damaging effects of pro-inflammatory cells and molecules recruited into the site of inflammation (rats' hind paw). Also, the anti-nociceptive and anti-inflammatory activities on the lectin was nullified with lactose, suggesting that the lectin's carbohydrate recognition and binding of glyco-conjugates is also tied to the lectins anti-inflammatory activities. The lectin may be able to prevent chronic inflammation which is usually caused by the extravasation of leucocytes into inflammatory sites and the continued vigorous barrage of inflammatory sites by molecules majorly controlled by immune cells. It can be opined that the lectin may be able to elicit this anti-inflammatory activity via recognition and binding of specific glyco-motifs on those cells, a view which could be subjected to further investigations. Investigating the interaction of the lectin with other inflammatory parameters such as released cytokines, SOD, CAT activity or other pro-inflammatory molecules may be required to corroborate this claim.

Author contributions

A KUKU and N O OMISORE conceived and designed the experiments; O B OLADOKUN and O A OSUKOYA performed the experiments and analyzed the data; A KUKU wrote the paper while all the authors revised and approved the content of the paper.

CRediT authorship contribution statement

Boniface Oladiran Oladokun: Methodology, Data curation, Writing - review & editing. **Omotayo Nusirat Omisore:** Conceptualization, Writing - review & editing. **Olukemi Adetutu Osukoya:** Methodology, Data curation, Writing - review & editing. **Adenike Kuku:** Conceptualization, Writing - original draft, Writing - review & editing.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2019.e00073.

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