

Evaluation of Antiallergy Potentials of *Z. tessmannii* Root Extracts via *In vitro* Inhibition of Stressed Rat Peritoneal Mast Cell Degranulation

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ABSTRACT

Mast cells are important cells that mediate allergic response of the immune system. They release of preformed bioactive substances such as histamine is essential for signalling the multiple events of allergy vasodilation and mucus hypersecretion. The traditional uses of *Zanthoxylum tessmannii* have been reported, including the treatment of rheumatism, inflammatory disorders, and snakebite. However, there is a dearth of information on the anti-allergy potentials of the plant. In this study, the qualitative phytochemical compositions of aqueous (A.E), ethanol (E.E), ethyl acetate (EA.E), and dichloromethane (DCM.E) extracts of *Z. tessmannii* roots were carried out using standard methods while the anti-allergy activities of the aqueous and dichloromethane extracts and fractions of *Z. tessmannii* roots were evaluated by inhibition of mast cell degranulation. The phytoconstituents of all extracts screened revealed the presence of alkaloids, flavonoids, tannins, saponins, steroids, triterpenes, and cardiac glycosides. The anti-allergy evaluation however, revealed that all extracts and fractions of the plant exhibited a negative % inhibition of mast cell degranulation following hypotonic and thermal stimulation. Results showed significantly ($p < 0.05$) increased mast cell degranulation activities and did not protect the mast cells.

Keywords – Allergy, anti-allergy, degranulation, histamine, mast cells, roots, *Zanthoxylum tessmannii*.

Aims Research Journal Reference Format:

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1. INTRODUCTION

Allergic disorders are increasingly becoming a global epidemic and mast cells are one of the cells that play prominent roles in the allergic response of the immune system. The mast cells are located throughout the body, especially in the skin, the gastrointestinal and respiratory tracts. In rodents, they are found in the peritoneal and thoracic cavities and are located in all vascularised tissues, except the central nervous system and the retina [1]. These typical locations allow the mast cells to function as

first responders following harmful extraneous invasions [2].

As granulated cells, mast cells contain several pre-formed mediators of the allergic and inflammatory responses. When the mast cells are activated, a process of degranulation results, leading to the release of the mediators synthesised *de novo* [3]. These mediators, including histamine, proteases, leukotrienes, prostaglandin D₂ [2], trigger smooth muscle contraction, vasodilation, increased vascular permeability, and mucous hypersecretion [4]. Furthermore, the release of these mediators allows the mast cells to play crucial roles in allergic episodes such as [5]. Consequently, the inhibition of mast cell degranulation is a model used to evaluate the antiallergy properties of medicinal plants.

The *Zanthoxylum* genus has been employed all over the world in the treatment of multiple diseases in both humans and animals, such as herpes, skin ulcer [6], asthma, malaria, toothache, mouth ulcer, and sore throat, [7, 8] as well as rheumatism, inflammatory disorders, and snakebite [9]. Among this genus, *Zanthoxylum tessmannii*, a species that grows in the rain forest of West/Central Africa, is commonly used as a toothbrush or chewing stick and also used for treating tumours, swellings, inflammation and gonorrhoea [6]. In another study, the anti-inflammatory activity of the aqueous and dichloromethane extracts of *Z. tessmannii* roots was demonstrated with the aqueous extract reported as the most active fraction [10]. However, its antiallergy potentials have not been previously reported. Hence, this study was designed to evaluate the antiallergy potential of *Z. tessmannii* roots via the inhibition of mast cell degranulation.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Material

Fresh roots of *Zanthoxylum tessmannii* were collected from Alagbe, Ipetu-Ijesha, Osun state (7°28'43" N 4°53'59" E), Nigeria, with the help of a local hunter, Mr. Samuel. The plant roots, leaves and seeds were collected for identification in the month of February 2018 and were then identified and authenticated at the IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Osun State in Nigeria.

2.2 Preparation of the *Z. tessmannii* Root Extracts

The cleaned root samples were air-dried at room temperature until they are free from moisture. The dried roots were cut into tiny bits and then ground to powder using a grinding mill. Approximately 130 g of the powdered *Z. tessmannii* root was extracted exhaustively by maceration for 72 hours in respective polar (aqueous and ethanolic) and non-polar (dichloromethane and ethyl acetate) solvents. The *Z. tessmannii* root solutions were separately decanted, filtered with a muslin cloth, and filtered with a vacuum filter. Each solution was concentrated using a rotary evaporator at 40°C except for the aqueous solution of the extract that was freeze-dried to get the crude extracts. The extracts were stored in a desiccator till further use.

2.3 Phytochemical Analysis of *Z. tessmannii* Root Extracts

The aqueous, ethanolic, dichloromethane and ethyl acetate extracts of *Z. tessmannii* were subjected to qualitative phytochemical tests, based on chemical reactions with observable colour change, foam or precipitate formation for various phytoconstituents [11, 12].

2.3.1 Test for Flavonoids

Each extract of *Z. tessmannii* root was dissolved separately in 5.0 ml of distilled water and filtered. Few drops of ethanolic potassium hydroxide solution were then added to 1ml of the filtrate. The formation of suspension or precipitation indicated a positive test for flavonoids.

2.3.2 Test for Tannins

The extracts of *Z. tessmannii* root (0.05g) were dissolved separately in 20ml of distilled water in a test tube and filtered. Few drops (2-3) of 0.1% ferric chloride in glacial acetic acid solution was then added to the filtrate. The mixture was then examined for the formation of a brownish-green or black precipitate.

2.3.3 Test for Alkaloids

An acidic solution of each of the extracts of *Z. tessmannii* root was prepared by weighing 0.05g into three test tubes, and 10ml of 10% (v/v) HCl was added. The reaction mixture was then heated and filtered. Then, to each filtrate, 1.0 ml of Mayers reagent, Wagner's reagent and Drangedorff reagents were added, respectively. The mixtures were observed for colour change, turbidity or formation of a precipitate. An equal volume of 10% (v/v) HCl was used as parallel control.

2.3.4 Test for Saponins

To 0.05g of each of the extracts of *Z. tessmannii* root in a test tube, 2.0 ml of distilled water was added, vigorously shaken and noted for froth. Afterwards, the test tube was warmed gently at about 70°C for 10 mins and shook again. The appearance and persistence of froth indicated the presence of saponins.

2.3.5 Test for Steroids

Acetic anhydride (2 ml) and dilute sulphuric acid (2 ml) were added to 0.5g of the extracts of *Z. tessmannii* root in separate tubes and shaken. A colour change from violet or light brown to deep, viscous brown indicated the presence of steroids.

2.3.6 Test for Phlobatannins

Each extract (0.5 g) was dissolved in 10 ml of water in a test tube and then filtered. The filtrate (1 ml) was shaken with 10% HCl and then observed for the deposition of red precipitate, which indicated a positive test for phlobatannins.

2.3.7 Test for Cardiac Glycosides

The extracts of *Z. tessmannii* root (0.5g) were extracted separately with 2 ml of chloroform and then filtered into a clean test tube. Concentrated sulphuric acid (H₂SO₄) was added carefully to form a layer. The presence of a reddish-brown colour ring at the chloroform/sulphuric acid interphase indicated the presence of a steroidal ring or glycone of the cardiac glycosides.

2.3.8 Test for Terpenoids

Each extract of *Z. tessmannii* root (0.5 g) was gently warmed with 5 ml of chloroform and filtered. Concentrated acid (2 ml) was added carefully to the filtrate, and a reddish-brown colouration formed at the interphase indicated the presence of triterpenes.

2.3.9 Test for Xanthoproteins

To 0.05g of each extract, 1 ml of concentrated nitric acid is added, heated and cooled with the subsequent addition of ammonia solution. The appearance of a reddish-brown precipitate indicated the presence of xanthoproteins.

2.4 Thin-Layer Chromatography (TLC) Analyses of the Phytochemicals Present in *Zanthoxylum tessmannii* Root Extracts

The presence of alkaloids, flavonoids, tannins, saponins, steroids, triterpenes, and cardiac glycosides in all the respective root extracts of *Zanthoxylum tessmannii* was analysed using thin-layer chromatography (TLC silica gel 60 F₂₅₄) [13, 14]. The extracts were separately spotted manually using a capillary tube on pre-coated silica gel F TLC plates (5 × 5 cm; 3 mm thickness). The spotted plates were put into a solvent system to detect the suitable mobile phase (Table 1). After separating the phytochemical constituents, the spraying reagents such as Dragendorff reagent, 1% ethanolic AlCl₃, 70% H₂SO₄, 1% ferric chloride (FeCl₃), and vanillin sulphuric acid reagent were used to identify the respective compounds. After development in the tank, the plates were dried, and the components were observed under UV light (254 and 365 nm).

2.5 Preparation of Rat Peritoneal Mast Cells (RPMCS)

Rat peritoneal mast cells (RPMCs) were isolated using modifications of methods previously described by Moon *et al.*, (2005) and Meurer *et al.*, (2016). In brief, rats were anaesthetised with diethyl ether. A forceps was used to raise the sternum and 1 mL air, and 20 mL of Tyrode buffer B (NaCl, glucose, NaHCO₃, KCL, NaH₂PO₄) containing 0.1% gelatin (Sigma) was injected slowly into the peritoneal cavity to prevent the disintegration of vessels and leaking of blood into the peritoneal cavity. The rat was placed in one hand, and the body was carefully shaken several times. This proceeding allowed the cells to detach from tissue and transit into the buffer solution. The peritoneal cavity was then carefully opened, and a Pasteur pipette was used to aspirate the fluid containing peritoneal cells. Then the peritoneal cells were centrifuge for 5 min at 1,200 rpm at 4°C.

According to the method described by Moon *et al.*, (2005), the supernatant was gently removed, and the cell pellet was resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells (i.e., macrophages and small lymphocytes). In brief, peritoneal cells suspended in 2 ml of Tyrode buffer B were layered onto 4 ml of 2.25 × 10² mg/ml metrizamide (density 1.12 g/ml; Sigma) and centrifuged at room temperature for 15 min at 400×g. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were then washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% bovine serum albumin) containing calcium. Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

Table 1 Solvent Systems used for the TLC of Root Extracts of *Z. tessmannii*

S/N	Secondary metabolites	Solvent system	Solvent ratio	Spraying reagent
1	Alkaloids	EtOAc:MeOH:H ₂ O	10:01:04	Dragendoeff
2	Flavonoids	EtOAc acid Acetone:Fomic acid	4.5:4.5:1.0	1%Ethanolic AlCl ₃
3	Tannins	EtOAc:Fomic acid:MeOH	3.3:0.8:0.2	1%FeCl ₃
4	Saponin	CF:MeOH	1.2:0.2	5% Vanillin H ₂ SO ₄
			7.2:2.9	70%H ₂ SO ₄ 70% Acetic acid in ethanol
6	Triterpenes	DCM	100%	
7	Cadiac glycosides	H ₂ O:MeOH: EtOAc	8:11:81	

DCM-Dichloromethane
Hex- Hexane

EtOAc- Ethylacetate
MeOH-Methanol

2.5.1 Methods of Stimulating Mast Cell Degranulation

Mast cell degranulation was stimulated by a modification of the methods described by Moon *et al.*, (2005) and Zhang *et al.*, (2012). RPMC suspensions (2 x 10⁵ cells/ml) were preincubated for 10 min at 37 °C for stabilisation before applying mechanical and thermal stress. For measurements on suspensions of intact cells, the cells were preincubated with 0.25 mg/ml and 0.5 mg/ml of aqueous and dichloromethane of the extracts (Aq.E and DCM.E) and fractions (Aq.F and DCM.F) respectively for 30 minutes. Then mechanical stress was applied by incubating for 20 min with hypoosmotic solution (with Tyrode buffer A containing 100 mM NaCl instead of Tyrode buffer A containing 130 mM NaCl). For thermal stimulation of the RPMC, the chamber was perfused with preheated isosaline solution. The temperature was monitored and maintained at 56°C for thermal stimulation of degranulation for 20 min. The cells were also preincubated with 0.25 mg/ml and 0.5 mg/ml of aqueous and dichloromethane extracts (Aq.E and DCM.E) and fractions (Aq.F and DCM.F) respectively before thermal stimulation.

2.5.2 Measurements of Histamine Release

In order to illustrate mast cell degranulation by physical stimulation, the histamine released from RPMC into Tyrode buffer A was measured according to the methods described by Zhang *et al.*, (2012). Briefly, the RPMCs were divided into eleven groups: untreated (negative) and positive controls, and cells treated by hypotonicity or heat with and without extracts or fractions (stimulations are as described earlier). The cells were incubated for 30 min. The cell solutions (with the density of 2×10^5 /ml) were centrifuged at 3000 revolutions per minute for 5 min at 4°C, and the supernatants will be collected. To aliquots of 1 ml supernatant 0.5 ml NaOH (0.4 M) and 0.1 ml o-phthalaldehyde (0.05 %) were added to stabilize the fluorescence. After 10 min the samples were neutralised by adding 0.5 ml HCl (0.1 M). The cell pellets were then resuspended in 2 ml Tyrode buffer A and boiled at 100 °C for 5 min. Lysis solution was collected after the centrifugation as described before for the supernatant. Fluorescence intensity in cell supernatants and lyses solutions was determined by a fluorescence spectrometer (Hitachi, F-4500) (ex=350 nm, em= 440 nm). Released histamine was calculated as the ratio of fluorescences (F) = (Fsupernatant/(Fsupernatant + Flyses)) x 100

2.5.3 Solvent-solvent Partitioning

Solvent-solvent partitioning of the aqueous extract (the most active extract) of *Z. tessmannii* root was carried out with a modification of the method described by Rédei *et al.*, (2017). Briefly, the most active extract was partitioned successively with four solvents of different polarities (2 × 500 ml each). The aqueous extract was concentrated to 300 ml using a lyophiliser and then partitioned with n-hexane with gentle shaking. The supernatant was decanted and replaced with fresh n-hexane and decanted. The residue of the aqueous extract was suspended sequentially in both dichloromethane, ethyl acetate and butanol as done with n-hexane. The fractions collected were filtered and freeze-dried to obtain the aqueous (Aq.F) and dichloromethane (DCM.F) fractions respectively. These resulting fractions were then analysed for their antiallergic activities as described previously.

2.6 Statistical Analysis

Results within the groups were expressed as mean ± SEM for triplicate independent experiments. The statistical significance of the data was analysed with a one-way ANOVA followed by the Post hoc-tukey analysis. P-values of less than 0.05 was considered to be statistically significant [19, 20].

3. RESULTS AND DISCUSSION

3.1 Phytochemical Constituents of *Z. tessmannii* Root Extracts

The preliminary qualitative phytochemical screening of the crude extracts (aqueous and dichloromethane) of *Z. tessmannii* roots revealed the presence of the following phytoconstituents, alkaloids, flavonoids, tannins, saponins, steroids, triterpenes, and glycosides were ascertained in all the crude extracts (Table 2). Conversely, while phlobatannins were present in aqueous and ethyl acetate crude extract, xanthoproteins were observed as not present in only the ethyl acetate crude extracts. Phytochemical analysis of the various crude extracts (aqueous, ethanolic, ethyl acetate and dichloromethane) of *Z. tessmannii* root revealed the following secondary metabolites, alkaloids, flavonoids, tannins, saponins, steroids, triterpenes, and glycosides.

The presence of these phytoconstituents indicates that *Z. tessmannii* root is a rich source of bioactive compounds and could yield products of plant origin with pharmacological significance if properly screened.

Table 2 Xanthine Oxidase Inhibitory Activity of the Crude Extracts of *Z. tessmannii* Roots

S/N	Phytoconstituents	Tests	Aqueous	Ethanol	Ethyl acetate	Dichloro-methane
1	Alkaloids	Mayer's, Wagner's and Dragendorff's tests	+	+	+	+
2	Flavonoids	Alkaline reagent test	+	+	+	+
3	Tannins	Ferric chloride test	+	+	+	+
4	Saponin	Froth formation test	+	+	+	+
5	Steroids	Liebermann-Burchard test	+	+	+	+
6	Triterpenes	Salkowski test	+	+	+	+
7	Cardiac glycosides	Keller killiani test	+	+	+	+
8	Phlobatannins	HCl test	+	-	+	-
9	Xanthoproteins	Bontrager's test	+	+	-	+

+ denotes presence

- denotes absence

3.2 TLC Profiles of Various Crude Extracts of *Z. tessmannii* Root

Plant-based products are essential ingredients in traditional medicine systems. Since antiquity, numerous plants and their derived products have effectively treated and managed several disorders including allergies [21]. Various scientific studies have shown the presence in indigenous plants of metabolites with anti-allergic potentials [8]. TLC investigations help to reveal the presence of phytochemicals in plant extracts that may possess important activities to form the basis of therapeutic potentials.

The results of the thin layer chromatography showed several bands for all the crude extracts of *Z. tessmannii* roots, thereby confirming the presence of various phytochemicals when viewed with daylight (a), 254 nm (b) and 366nm (c) (Figures 1 - 5). Each of the bands indicates distinct classes of metabolites of the various phytochemicals. The TLC studies showed that all solvents (aqueous, ethyl acetate, dichloromethane and ethanol) used extracted sufficient quantities of metabolites present in *Z. tessmannii* root.

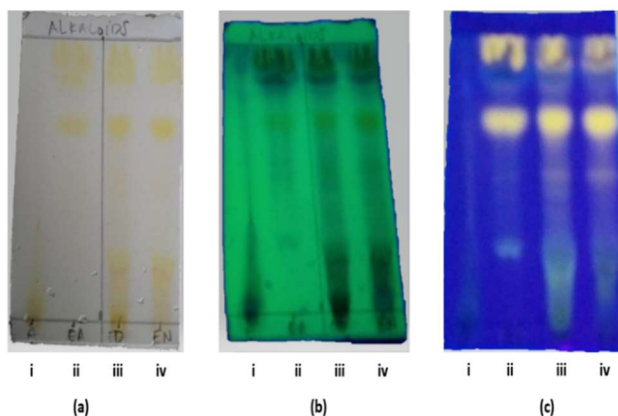


Fig. 1 TLC Chromatogram for alkaloid compounds in root extracts of *Z. tessmannii*.

- i. Aqueous extract
- ii. Ethyl acetate extract
- iii. Dichloromethane extract
- iv. Ethanolic extract

Obtained under a) day light, b) 254 nm, and c) 366 nm

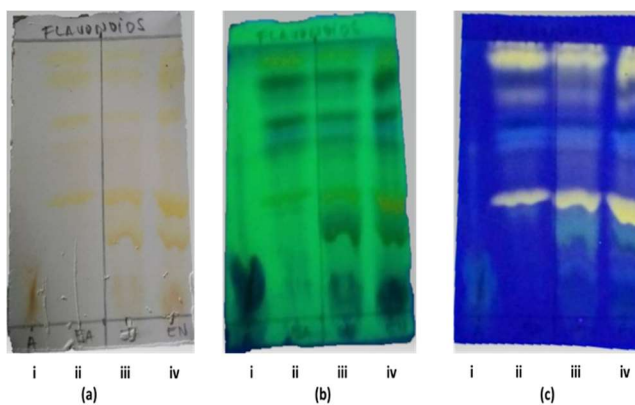


Fig. 2 TLC Chromatogram for flavonoid compounds in root extracts of *Z. tessmannii*.

- i. Aqueous extract
- ii. Ethyl acetate extract
- iii. Dichloromethane extract
- iv. Ethanolic extract

Obtained under a) day light, b) 254 nm, and c) 366 nm

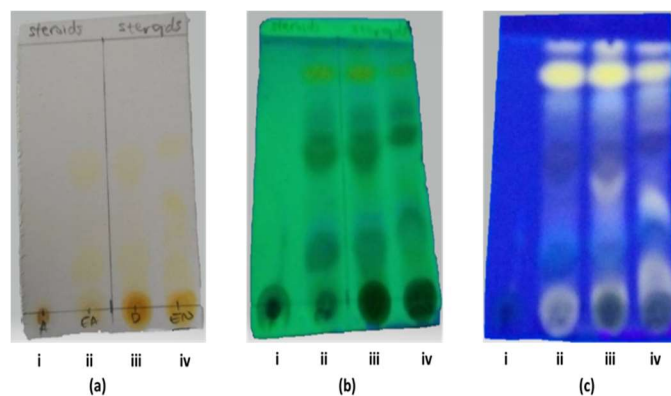


Fig. 3 TLC Chromatogram for steroid compounds in root extracts of *Z. tessmannii*.

- i. Aqueous extract
- ii. Ethyl acetate extract
- iii. Dichloromethane extract
- iv. Ethanolic extract

Obtained under a) day light, b) 254 nm, and c) 366 nm

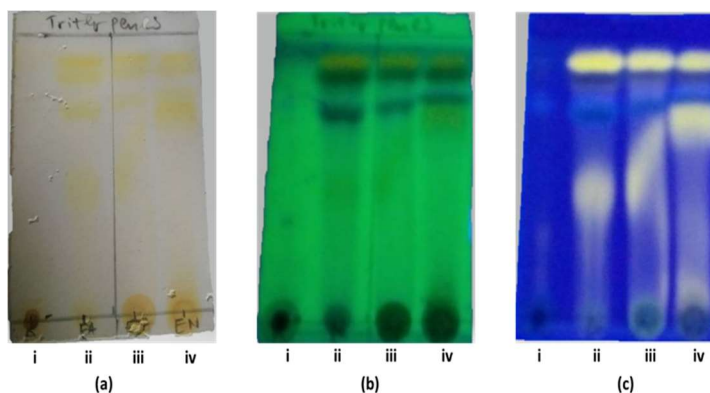


Fig. 4 TLC Chromatogram for triterpene compounds in root extracts of *Z. tessmannii*.

- i. Aqueous extract
- ii. Ethyl acetate extract
- iii. Dichloromethane extract
- iv. Ethanolic extract

Obtained under a) day light, b) 254 nm, and c) 366 nm

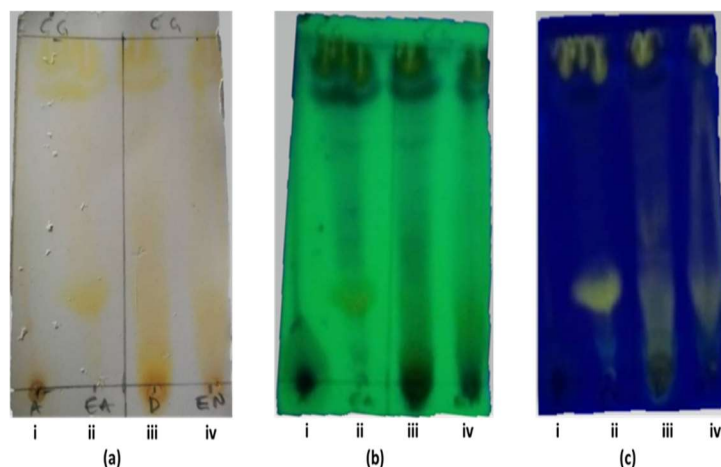


Fig. 5 TLC Chromatogram for cardiac glycoside compounds in root extracts of *Z. tessmannii*.

- i. Aqueous extract
- ii. Ethyl acetate extract
- iii. Dichloromethane extract
- iv. Ethanolic extract

Obtained under a) day light, b) 254 nm, and c) 366 nm

3.3 Anti-Allergic Activities of Crude Extracts and Fractions of *Z. tessmannii* Roots

Mast cells are important participants in the onset and progression of allergic episodes upon the cross-linking of high-affinity IgE receptors (FC ϵ RI) via allergens and release various cytokines, eicosanoids, and secretory granules [22]. Many researchers have reported that mast cell degranulation is one of the critical events during allergen-induced asthma. The resultant release of several bioactive substances synthesised *de novo*, such as histamine, leukotrienes, and various cytokines/chemokines is therefore crucial in activating underlying mechanisms of asthma, such as bronchoconstriction and mucous hypersecretion [23].

The anti-allergy potentials of the aqueous and dichloromethane crude extracts of *Z. tessmannii* roots on rat peritoneal mast cells (RPMCs) exposed to hypotonic and thermal stimulations are presented in Fig. 6. All extracts of *Z. tessmannii* roots at both 0.25 mg/ml and 0.3 mg/ml showed significantly ($p < 0.05$) increased mast cell degranulation activities and did not protect the mast cells.

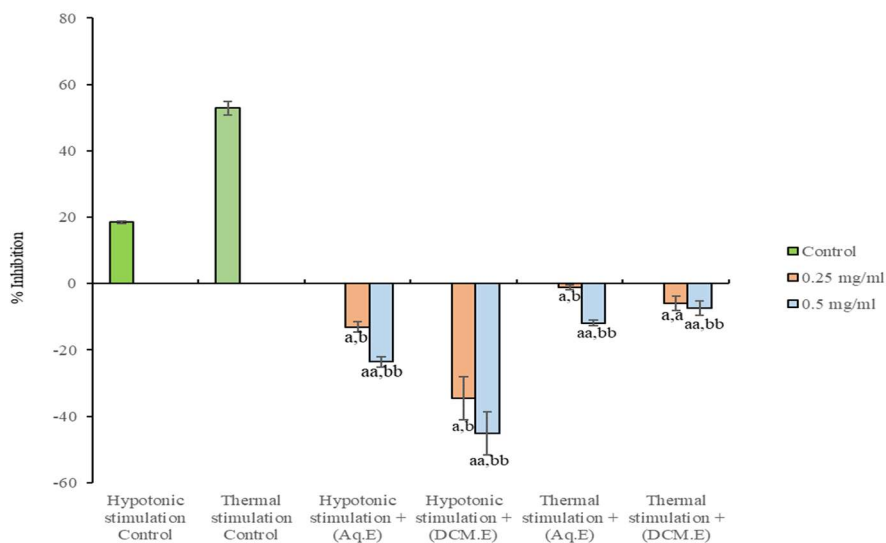


Fig. 6 % Inhibition of histamine release from RPMCs preincubated with various concentrations of *Z. tessmannii* root extracts before respective hypotonic and thermal stimulation.

Each value represented the mean \pm SEM of 3 readings. a,b denotes significant differences ($p < 0.05$) when compared with hypotonic and thermal controls at 0.25 mg/ml respectively and aa,bb denotes significant differences ($p < 0.05$) when compared with hypotonic and thermal controls at 0.003 mg/ml respectively. Aq.E – Aqueous Extract; DCM.E – Dichloromethane Extract.

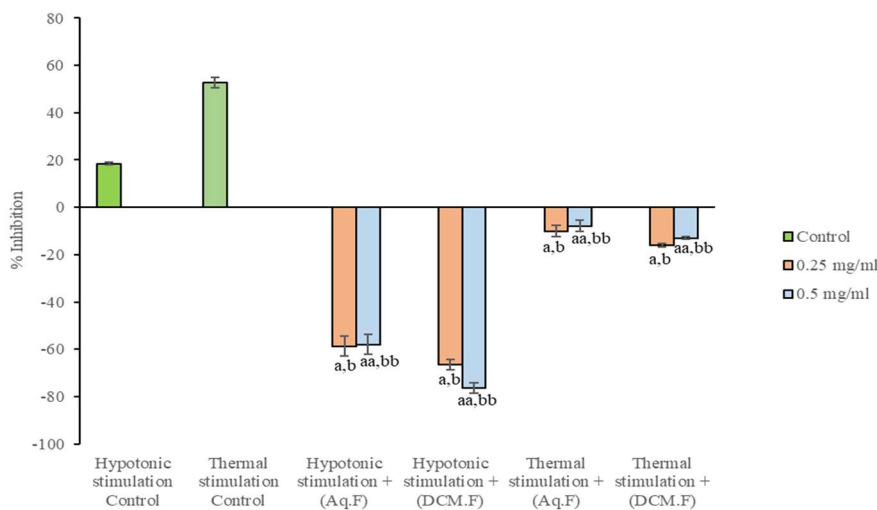


Fig. 7 % Inhibition of histamine release from RPMCs preincubated with various concentrations of *Z. tessmannii* root fractions before respective hypotonic and thermal stimulation.

Each value represented the mean \pm SEM of 3 readings. a,b denotes significant differences ($p < 0.05$) when compared with hypotonic and thermal controls at 0.25 mg/ml respectively, and aa, bb denotes significant differences ($p < 0.05$) when compared with hypotonic and thermal controls at 0.003 mg/ml, respectively. Aq.F – Aqueous Fraction; DCM.F – Dichloromethane Fraction

Similarly, all fractions of *Z. tessmannii* roots did not inhibit the degranulation of mast cells exposed to hypotonic and thermal stimulation (Fig. 7). Additionally, all the fractions showed significantly ($p < 0.05$) increased mast cell degranulation activities and, as such, did not show any protection for the mast cells. However, control cells that were not incubated with the fractions showed positive inhibitions. Conversely, *Zanthoxylum piperitum* was reported to elicit the attenuation of allergy via the inhibition of mast cell degranulation.

4. CONCLUSION

This study revealed that extracts and fractions of *Z. tessmannii* roots do not inhibit, but rather appear to enhance mast cell degranulation. Hence, it is implied that *Z. tessmannii* roots do not possess antiallergy activity, but on the contrary, may possess elicit allergic reactions.

Therefore, it is recommended that further studies be carried out to identify the bioactive constituents in *Z. tessmannii* roots that may be allergenic.

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