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Research Article

Zanthoxylum zanthoxyloides Lam. Chewing Stick Elicits Salivation in Male Wistar Rats Via a Muscarinic-Dependent Pathway

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Abstract
A recent study has documented empirical evidence that *Zanthoxylum zanthoxyloides* (Lam.) Watermann root stimulates salivation in healthy adult humans. The mechanism underlining the *Z. zanthoxyloides*-stimulated salivation was however not known. This study was therefore designed to determine the role of adrenergic, muscarinic and calcium channels on *Z. zanthoxyloides*-induced salivation in male Wistar rats. Twenty male Wistar rats (120-130 g) were randomly grouped into four (n=5) as: *Z. zanthoxyloides* (Z), *Z. zanthoxyloides*+Atropine (ZA), *Z. zanthoxyloides*+Prazosin (ZP) and *Z. zanthoxyloides*+Nifedipine (ZN). Cotton swab soaked in juiced *Zanthoxylum zanthoxyloides* root was rubbed into the oral cavity of anaesthetized rats in Z to stimulate saliva. Rats in ZA, ZP and ZN were pretreated with atropine (2 mg/kg), prazosin (2 mg/kg) and nifedipine (20 mg/kg), respectively, before introduction of *Z. zanthoxyloides*. Saliva volume, flow rate, amylase activity and total protein were determined using standard methods. All experiments were conducted in triplicates. *Z. zanthoxyloides* significantly increased production of saliva (0.49±0.036ml) when compared with the unstimulated animals that produced no visible saliva throughout the observation period. Treatment with atropine in ZA (0.095±0.002ml) significantly decreased saliva production when compared with Z. There was however no significant difference in the saliva volume of ZP (0.35±0.032 ml) and ZN (0.445±0.75ml) when compared with Z. Salivary flow rate increased in similar pattern with saliva volume in all the groups. Salivary amylase activity and total protein were significantly reduced in ZA compared with Z while they were not different in the ZN and ZP groups. *Z. zanthoxyloides* increases salivation through a mechanism that may involve the muscarinic pathway.

Key Words: *Zanthoxylum zanthoxyloides*, Adrenergic receptor, Muscarinic receptor, Calcium channel, saliva

INTRODUCTION

Traditional dental care practices across the world include the use of chewing sticks which tends to vary across different cultures (Wu *et al*., 2001). Twigs, split stems and roots of several plant species are chewed as oral cleanser, plaque removal, and strengthener for the teeth and gum (Blay, 2004) thus, the usefulness of chewing stick in oral hygiene is acceptable by a vast majority. In fact, the World Health Organization in 1987 encouraged the use of chewing sticks obtained from plant roots or stems and this was reaffirmed in the year 2000 Consensus Report on Oral Hygiene (Wu *et al*., 2001). The fact that chewing sticks are natural, less toxic with majority having LD_{50} above 5000 mg (Alaribe *et al*., 2022), readily available and cheaper alternative to chemical-based toothpaste made them to be appealing among low-income populations (Femi-Oyewo, 2021). Clinical trials comparing the tuft made by chewing them with toothbrush found them effective with greater mechanical cleansing of the oral tissue (Malik *et al*., 2014; Taha *et al*., 2022).

Zanthoxylum zanthoxyloides (Lam.) Watermann root, known as Candlewood in English and Orín àta in Yorùbá, is a popular chewing stick among Nigerians (Medhi *et al*., 2013). It has antibacterial properties (Shittu *et al*., 2019), used as antisickling agent in sickle cell anemia patients (Ouattara *et al*., 2009) and reputed to have high fluoride content (Obontu *et al*., 2012) and retention ability (Emeke *et al*., 2019). The roots usually give a warm, pungent and benumbing effect on the palate when chewed, and its decoction is used as a mouthwash and for sore throat (Elujoba *et al*., 2005). The characteristic aromatic warm taste with attendant profuse salivation is believed to be beneficial to the elderly and those with sore gums and other oral disease conditions (Adesina, 2005). Recently, a study involving apparently healthy adults documented that it is capable of stimulating saliva production up to 600 % of the basal value and when the amount of saliva produced was compared to that produced by chewing sugarless gum, it was found that *Z. zanthoxyloides* had a profound stimulatory effect that was beyond the mechanical act of chewing (Shittu *et al*., 2024). Since the study eliminated the mechanical stimulatory effect of chewing on saliva production, the mechanism by which *Z. zanthoxyloides* caused the increased salivation is yet to be discerned.

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The regulation of salivary secretion involves a network of signaling pathways to ensure proper coordination and stimulation of saliva within the salivary glands. Adrenergic receptors are activated by catecholamines to either stimulate or obstruct salivation (Elverdin *et al*., 1990), muscarinic receptors are activated by acetylcholine to induce salivation (Baum,1993) while Calcium channels are key regulator of salivary acinar cell function through modulation of their various ion channels and Ca^{2+} pumps (Ambudkar, 2011). The recent findings that *Z. zanthoxyloides* stimulated saliva to as much as 600 % over it basal value (Shittu *et al*., 2024) had opened a wide gap in literature with respect to the probable mechanism by which it acts. Does it act by recruiting a single pathway i.e. the adrenergic pathway, the muscarinic pathway or calcium channel modulation? Or does it act by recruiting more than one if not all? These are pertinent questions that are imperative to be answered. This study was therefore designed to investigate the role of adrenergic, muscarinic, and calcium channels on *Z. zanthoxyloides*-induced salivation in male Wistar rats.

MATERIALS AND METHODS

Twenty male healthy Wistar rats weighing between 120 and 130 g were obtained from the College of Medicine Central Animal house, University of Ibadan. They were housed in plastic cages at the Department of Physiology animal house under standard conditions of room temperature, humidity and light. The rats had free access to standard feed and water in their cages and the top of the cages were made of wire mesh in order to allow free flow of air. They received humane care throughout the experimental period with the ethical recommendations for care and use of laboratory animals based on the NIH guideline 121-85 on the use of laboratory animals. The animals were acclimatized for a period of two (2) weeks before commencement of the study.

Preparation of *Zanthoxylum Zanthoxyloides* juice: The roots of *Z. zanthoxyloides* were obtained from Ibadan metropolis. It was identified at the Department of Botany, University of Ibadan, Ibadan and it was deposited in the University herbarium (No.195). The roots were washed, chopped into smaller sticks, air-dried for two weeks and stored in an airtight plastic container at room temperature until needed.

Daily, before commencement of each experiment, the bark of Zanthoxylum zanthoxyloides root was peeled off and blended into fine particles. One gram of the blended root was soaked in 4 ml distilled water for 10 minutes to absorb the water, and then transferred into mortar and pestle to extract the juice which is about 3 ml when drained by squeezing out after matching. The juice extracted was then used for the experiment. This approach of extracting the juice is to mimic the chewing extractions that normally occur when man uses *Z. zanthoxyloides* to brush their teeth.

Experimental design: After the period of acclimatization, the animals were divided into four groups (n=5 each) as follows: **Group 1 (Z):** The animals were anesthetized with thiopental (50 mg/kg, ip), firmly secured on dissecting board and laid supine to facilitate access to their oral cavity. The teeth and oral cavity were brushed for 2 minutes with cotton swab soaked in the juiced *Z. zanthoxyloides*. The cotton swab method of stimulating saliva was based on the method of Bellagambi *et al*. (2020). The mouth was dabbed by fresh cotton swab and saliva pool was monitored and collected for 10 minutes.

Group 2 (ZA): Following anesthesia and securing on a dissecting board, rats in this group were pretreated with atropine (2 mg/kg, i.p.) fifteen minutes before the introduction of *Z. zanthoxyloides* and the procedure carried out in the Z group was repeated.

Group 3 (ZP): Following anesthesia and securing on a dissecting board, rats in this group were pretreated with prazosin (2 mg/kg, i.p.) 15 minutes before the introduction of *Z. zanthoxyloides* and the procedure carried out in the Z group was repeated.

Group 4 (ZN): Following anesthesia and securing on a dissecting board, rats in this group were pretreated with nifedipine (20 mg/kg) 15 minutes before the introduction of *Z. zanthoxyloides* and the procedure carried out in the Z group was repeated.

A control experiment was carried in anasthetized rats. They were secured firmly and cotton swab soaked in distilled water was used to brush their oral cavity as it was done for the Z group to obtain unstimulated saliva. They were observed for 10 minutes also. No visible saliva pool was seen in these rats throughout the observation period.

All the animals recovered from anaesthesia, allowed to rest for 3 days and the procedures were repeated twice again to give a cumulative 3 times experimentation in all groups.

Saliva samples collection: All experimental procedures and sampling were carried out three times at different days between the hours of 8.00 and 10.00 am. After brushing the oral cavity of each animal in the various groups, the mouth was dabbed with dry cotton swab immediately and the saliva was collected as reported by Lasisi *et al*. (2014). The salivary lag time was determined as the onset of salivation was characterized by increased tingling of the tongue and pooling of saliva in the mouth. The pooled saliva was collected for 10 minutes into 2 ml graduated sample bottle, the total volume was divided by the time of collection to obtain the salivary flow rate. The samples were immediately transferred into Styrofoam box loaded with ice blocks to maintain their biological integrity and stored in – 20 0 C before determination of salivary amylase activity and protein content.

Determination of alpha amylase activity: Salivary amylase activity was determined using the Bernfeld (1955) method. The method relies on the ability of amylase to hydrolyse starch into maltose which forms colour complex with 3,5-dinitrosalicylic acid (DNS) that can be quantified spectrophotometrically. Briefly, 500µL of cooked (1% w/v in phosphate buffer, pH 6.9) was pipette into a tube, 500µL of saliva was added and incubated for 5 minutes at 37 0C. After the incubation, the reaction was stopped by addition of 500µL of DNS reagent (containing 3,5-dinitrosalicylic acid and sodium potassium tartrate in a strong alkaline solution). The tube was then placed in boiling water for 15 minutes for colour development, it was cooled over ice and 4500 µL of distilled

water was added. The absorbance of the solution was read at 540 nm against a reagent blank. The amylase activity was then obtained from a standard curve plotted by serial dilution of 0.2 % maltose treated with DNS reagent.

Statistical analysis: All the obtained data were expressed as mean ± standard error of mean (SEM). Inferential statistics was done using one-way analysis of variance (ANOVA) followed by Tukey posthoc test at $p < 0.05$. Analysis was done using Graph Pad prism 9.0.0.

RESULTS

Effect of atropine, nifedipine and prazosin on *Z. zanthoxyloides* stimulated salivary volume in male Wistar rats: As shown in figure 1, *Z. zanthoxyloides* caused significant increase in saliva production (0.49 ± 0.036 ml) when compared with the unstimulated animals which produced no saliva for the 10 minutes observation period. The observed increase in saliva production was significantly reduced when the animals were pre-treated with atropine before introducing Z (0.095 ± 0.002 ml) when compared with the Z only group (0.49 ± 0.036 ml), $P < 0.05$. Nifedipine (0.445 ± 0.75 ml) and Prazosin (0.35 ± 0.032 ml) had no significant effect on saliva volume produced by *Z. zanthoxyloides* stimulation (0.49 ± 0.036 ml).

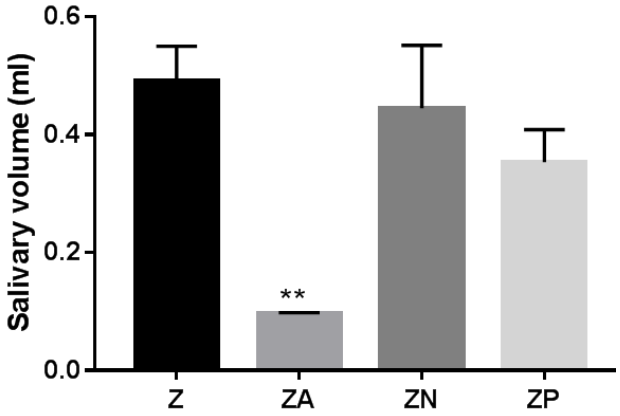

Figure 1
Effect of atropine, nifedipine and prazosin on *Zanthoxylum zanthoxyloides* stimulated salivary volume in male Wistar rats. **P <0.05

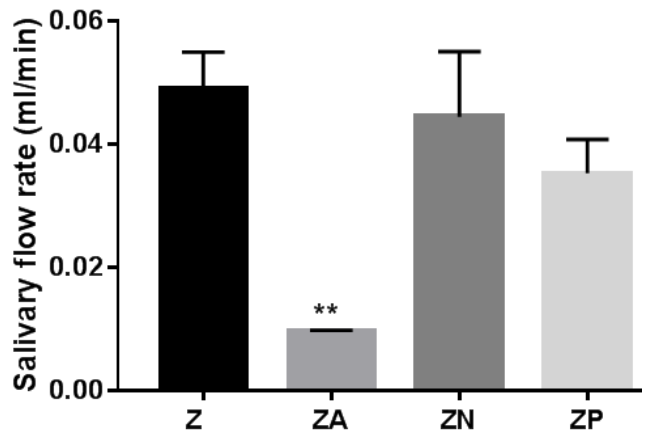

Figure 2:
Effect of atropine, nifedipine, and prazosin on *Zanthoxylum zanthoxyloides* stimulated salivary flow rate in male Wistar rats. **P <0.05

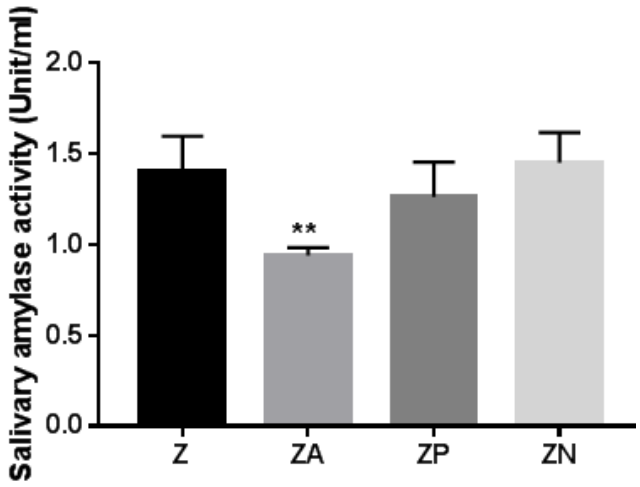

Figure 3:
Effect of atropine, nifedipine and prazosin on *Zanthoxylum zanthoxyloides*- stimulated salivary amylase in male Wistar rats. **P <0.05

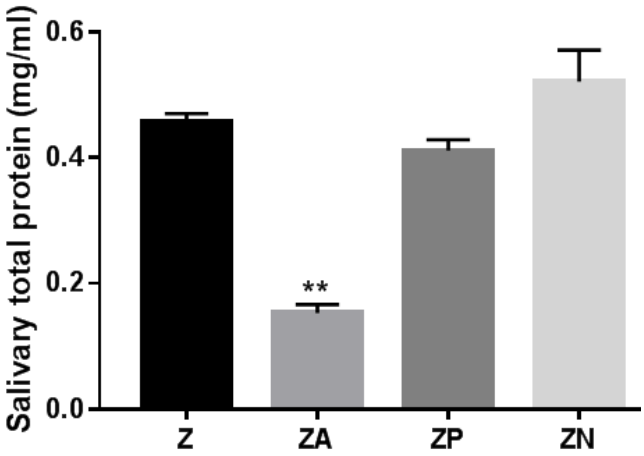

Figure 4:
Effect of atropine, nifedipine, and prazosin on *Zanthoxylum zanthoxyloides* stimulated total protein in male Wistar rats. **P <0.05

Effect of atropine, nifedipine and prazosin on *Z. zanthoxyloides* - stimulated salivary flow rate in male Wistar rats: As shown in figure 2, *Z. zanthoxyloides* caused a significantly increase salivary flow rate (0.049 ± 0.003 ml/min) when compared with the unstimulated animals that produced no saliva within the observation period of 10 minutes. The observed increase in salivary flow rate was significantly reduced when the animals were administered Atropine before introducing *Z. zanthoxyloides* (0.009 ± 0.00 ml/min). Nifedipine (0.044 ± 0.007 ml/min) and Prazosin (0.035 ± 0.003 ml/min) had no significant effect on the *Z. zanthoxyloides* stimulated salivary flow rate, $P < 0.05$.

Effect of atropine, nifedipine and prazosin on salivary amylase activity in *Z. zanthoxyloides* exposed male Wistar rats: *Z. zanthoxyloides* (1.399 ± 0.03 unit/ml) caused significant increase in salivary amylase activities which blocked by atropine pretreatment (0.941 ± 0.029 unit/ml) while nifedipine (1.264 ± 0.135 unit/ml) and prazosin (1.452 ± 0.096) had no significant effect on salivary amylase activity produced by *Z. zanthoxyloides*, $P < 0.05$, Figure 3.

Effect of atropine, nifedipine and prazosin on salivary total protein level in *Z. zanthoxyloides* exposed male Wistar

rats: As shown in figure 4, salivary total protein produced by Zanthoxylum zanthoxyloides was significantly decreased by atropine pre-tereatment while prazosin and nifedipine had no effect.

DISCUSSION

Salivation is produced by intraoral cavity stimuli and can be increased by mechanical actions within the oral cavity through chewing or sucking that triggers the taste fibres related to the mechanoreceptors present in the oral cavity (Penderson *et al*., 2018). The wide margin between saliva produced by chewing *Z. zanthoxyloides* and that produced by chewing sugarless gum in an earlier study (Shittu *et al*., 2024) indicated that *Z. zanthoxyloides* had a profound stimulatory effect that was beyond the mechanical act of chewing. Therefore, the current study investigated the probable mechanism by which *Z. zanthoxyloides* stimulates saliva secretion.

The cotton swab method used to introduce *Z. zanthoxyloides* into the oral cavity of the rats in this study is a standard method used by several studies that stimulated saliva production by citric acid in rats (Götrick and Tobin, 2004; Götrick *et al*., 2009; Ogawa *et al*., 2014) and humans (Huang *et al*., 2022). The observed saliva pooled after introducing *Z. zanthoxyloides* is a novel finding which within the scope of our literature search has not been documented in rats by any other researcher albeit numerous report of its traditional use in human to promote salivation (Adesina, 2005; Elujoba *et al*., 2005). *Z. zanthoxyloides* stimulates saliva production in the rats up to 0.49 ± 0.003 ml within the 10 minutes observation period. Such salivary secretagogue activity had earlier been documented in several other plants like *Salvia miltiorrhiza*/ Danshen (Wei *et al*., 2015), *Capsicum annuum* (Halawa *et al*., 2016) and Ixeris dentata (Bhattarai *et al*., 2017) that stimulated saliva production through different mechanisms.

The observed significant decreased the saliva volume produced by *Z. zanthoxyloides* in the atropine-pretreated rats in the current study suggests a role for the muscarinic receptor in the *Z. zanthoxyloides*-elicited salivation. Plant-based saliva secretagogue, pilocarpine obtained from *Pilocarpus microphyllus*, which incidentally belong to the Rutaceae family with *Zanthoxylum zanthoxyloides*, stimulates saliva production via the muscarinic pathway (Narita *et al*., 2019). Muscarinic stimulation of saliva is associated with increased expression of Aquaporin 5, a water-specific channel localized in the apical, basal and lateral membranes of acinar cells of rat submandibular glands which are important in their secretory activities (Matsuzaki *et al*., 1999; Delporte C, Steinfeld, 2006). Although Aquaporin 5 was not determined in the current study, *Ixeris dentate* (Bhattarai *et al*., 2017; 2018) and pilocarpine (Asari *et al*., 2009) which stimulate salivation via the muscarinic pathway have been shown to increase aquaporin 5 expression.

The salivary amylase activity and total protein response to muscarinic receptor blockade matched the decreased salivation produced by *Z. zanthoxyloides*. Calcium channel activity plays significant role in exocytotic release of enzymes and proteins (Eich *et al*., 2018) while alpha adrenergic receptor stimulates protein laden saliva (Aps and Martens, 2005). However, the observed no effect of nifedipine or prazosin on amylase activity and total protein observed in saliva stimulated by Zanthoxylum zanthoxyloides in the current study indicate the none involvement of calcium channel activity and alpha adrenergic receptor.

In conclusion, *Zanthoxylum zanthoxyloides* increases salivary volume, flow rate, salivary amylase and total protein in male Wistar rats through a mechanism that majorly involves muscarinic pathway. The relationship between *zanthoxylum zanthoxyloides*-induced salivation and salivary glands aquaporins will be viable future study to elucidate the findings of the current study.

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