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Research Article

Ocimum gratissimum L. Leaf Enhances Relaxation Response to Acetylcholine without Inhibiting the Intrinsic Myogenic Tone in Wistar Rats' Corpus Cavernosum Smooth Muscle

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Abstract
Ocimum gratissimum (OG) has been used as an aphrodisiac agent with no report on its effect in the modulation of corpus cavernosum relaxation process, hence this study. The effects of OG on acetylcholine responses were assessed in 12 Corpus Cavernosa Smooth Muscle (CCSM) obtained from 6 male Wistar rats. In an organ bath set-up, the CCSM were incubated in Krebs' solution (control, n = 6) or Krebs' solution containing OG (60 µg/mL, n = 6). The tissues were pre-contracted with potassium chloride (KCl, 60 mM), followed by graded doses of acetylcholine (10^{-9} to 10^{-5} M) and responses were recorded using an isometric force transducer and a data acquisition system connected to a computer. Mechanisms involved in the OG effects were then assessed by co-incubation with L-NAME, nifedipine, or Fasudil. Data were analysed using ANOVA at α0.05. Increased maximum relaxation response to acetylcholine was observed in OG-incubated CCSM (64.4±2.6%) compared with the control (47.6±3.4%). Co-incubation of OG with L-NAME (52.3±3.2%), nifedipine (56.8±2.9%) or Fasudil (52.3±1.6%) significantly reduced the relaxant effect of OG. It was therefore concluded that *Ocimum gratissimum* leaf enhanced the relaxation response of corpus cavernosum to acetylcholine via modulation of calcium ion sensitivity. The intrinsic myogenic tone of the tissues was not inhibited by the relaxant-promoting activity of *Ocimum gratissimum* leaf.

Key Words: *Ocimum gratissimum, corpus cavernosum, relaxation, Acetylcholine*

INTRODUCTION

Erectile dysfunction is described as the failure to achieve or maintain a penile erection that is sufficient for satisfactory sexual performance (Salonia *et al*., 2012). Its prevalence could be as high as 46.9% among Italians, 42.5% among Germans and 41% among the Chinese (Goldstein *et al*., 2020). In South-western Nigeria, erectile dysfunction ranging from mild to severe was found to occur in 58.9% of men aged 30-80 years in Ogbomoso town, Nigeria (Oyelade *et al*., 2016) and 55.1% of men aged 18-70 years attending a primary health care facility in Ibadan, Nigeria (Adebusoye *et al*., 2012). Generally, the prevalence is continuously increasing, with the projection of about 322 million men being affected worldwide by the year 2025, translating to about 111% increase from 1995 (Goldstein *et al*., 2020).

One of the widely used treatments for erectile dysfunction is phosphodiesterase-5 (PDE-5) inhibitors (Kumar *et al*., 2022), which inhibit the corpus cavernosum smooth muscle contraction by blocking the metabolism of cGMP. The efficiency of these drugs in modulating erectile activity is well proven. Still, contraindications like cGMP accumulation and hypotensive episodes in patients with a history of myocardial infarction, stroke, arrhythmias or congestive heart failure are major limitations to their usage (Dong *et al*., 2011; Vlachopoulos *et al*., 2013; Sharma and Kumar, 2017). Furthermore, prolonged and painful penile erection, which does not subside after sexual intercourse, and otherwise known as priapism (Burnett, 2003), has been associated with the use of PDE-5 inhibitors (Sur and Kane, 2000; Schifano *et al*., 2022). A window for new drug discovery to treat erectile dysfunction is thus widely open (Milenkovic *et al*., 2018).

Ocimum gratissimum is a widely consumed plant with medicinal properties, such as antifungal (Faria *et al*., 2006), antioxidant (Akinmoladun *et al*., 2007; Shittu *et al*., 2016) and antidiabetic (Shittu *et al*., 2018) activities. Pande and Pathak (Pande and Pathak, 2009) reported its potential in penile erectile function. However, the mechanisms involved in this action were not elucidated. This study was therefore designed to investigate the mechanisms involved in the activity of *Ocimum gratissimum* corpus cavernosum of Wistar rats in-vitro.

MATERIALS AND METHODS

Six male Wistar rats (150-170 g) were used to obtain twelve corpus cavernosum smooth muscle strips used for this study. The animals were kept in well-aerated plastic cages covered with wire mesh in the animal house of the Department of Physiology, College of Medicine, University of Ibadan, Ibadan, Nigeria. They were given standard pelletized rat feed (TopFeed®, Ibadan, Nigeria) and water *ad libitum*. Acclimatisation was allowed for 2 weeks before the commencement of the study. All experimental protocols and

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handling complied with the University of Ibadan (UI-ACUREC/2017/046) on animal use and handling for experiment which were in accordance with the NIH publication No. 85–23 guidelines.

System Set-up, tissue mounting and experimental procedure: An experimental set-up comprising of organ bath (model 4400; Ugo Basile, Varese, Italy), force transducer (model 7004; Ugo Basile, Varese, Italy) and a data acquisition system (model 17400; Ugo Basile, Varese, Italy) connected to a computer was used to monitor and record the contractile and relaxation responses of the isolated corpus cavernosum.

The organ bath filled with Krebs Heinseleit solution was turned on 15 minutes prior to the experiment to heat up the bath temperature to 37°C which was maintained by an in-built thermostat throughout the experiment. The air inlet was opened to allow constant aeration with 95% oxygen and 5% carbon dioxide air mixture. The corpus cavernosum was tied at the two ends by a tiny silk thread with the thread being looped on one end and connected to the organ chamber, while the thread on the other end was attached to the force transducer connected to the data acquisition system which was earlier launched on the computer. An initial tension of 2 g was applied to the corpus cavernosum strip then allowed to stabilise for 90 minutes in the set-up. During the stabilisation period, the tissues were stimulated thrice with 10^{-7} M phenylephrine for 5 minutes at an interval of 30 minutes to ascertain the integrity of the tissues for the experiment (Salahdeen *et al*., 2015)

The stabilised tissues were separately incubated in media containing blank Krebs Heinseleit solution (control, n = 6) and aqueous extract of *Ocimum gratissimum* leaf (OG, n = 6). They were pre-contracted with potassium chloride (60 mM) and relaxed with graded doses (10^{-9} M to 10^{-5} M) of acetylcholine. The tissues were washed and allowed to stabilise for mechanism elucidation. The tension on the tissue upon pre-contraction and the tension at each dose of acetylcholine were used to calculate the percentage relaxation response to acetylcholine (Cartledge *et al*., 2000; Zhang *et al*., 2004) as shown below:

$$\text{Response to acetylcholine (\%)} = \frac{\text{Tension at precontraction} - \text{Tension at the current Acetylcholine dose}}{\text{Tension at precontraction}} \times 100$$

The mechanism involved in the effects of aqueous extract of *Ocimum gratissimum* on corpus cavernosum relaxation response *in-vitro* was investigated by introducing pathway inhibitors including Nitro-L-Arginine Methyl Ester (L-NAME, Sigma Aldrich, Germany), nifedipine (Krishat Pharmaceutical company), or Fasudil to the control and OG media. The effects of *Ocimum gratissimum* leaf on the relaxation response to acetylcholine in the presence of pathway inhibitors were then compared with their corresponding responses in the absence of the inhibitors.

Statistical Analyses: The mean responses of penile smooth muscle to the varied doses of acetylcholine were used to generate the tissue response curve. The response at the highest dose represented the maximum relaxation response while the area under the curve was calculated to show the cumulative response of the tissues to acetylcholine. The data were analysed using one-way ANOVA and Fisher's LSD. $p<0.05$ was considered statistically significant. All data computation and analysis were done using Graph Pad Prism® version 7.0.

RESULTS

Responses of corpus cavernosum strip to acetylcholine in phenylephrine and potassium chloride induced contractions: The effects of *Ocimum gratissimum* on graded doses and cumulative response to acetylcholine in phenylephrine pre-contracted and potassium chloride pre-contracted tissues are shown in Figure 1a and Figure 1b respectively. *Ocimum gratissimum* significantly increased the response to acetylcholine in potassium chloride pre-contracted strips while the response in phenylephrine pre-contracted strips were not different compared with their respective controls.

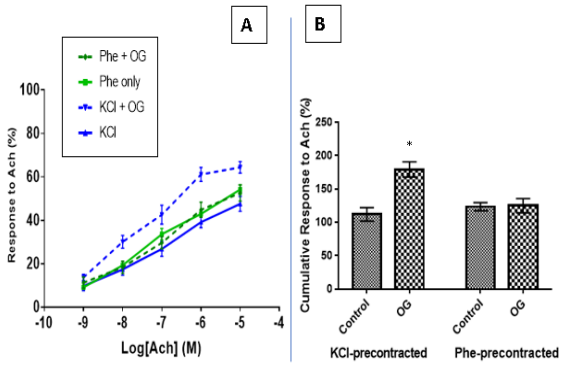

Figure 1:
(A) Responses of corpus cavernosa smooth muscle to graded dose of acetylcholine (B) Cumulative response of corpus cavernosa smooth muscle to Acetylcholine
OG = *Ocimum gratissimum*; KCl = Potassium Chloride; Phe = Phenylephrine *p<0.05 (OG vs Control).

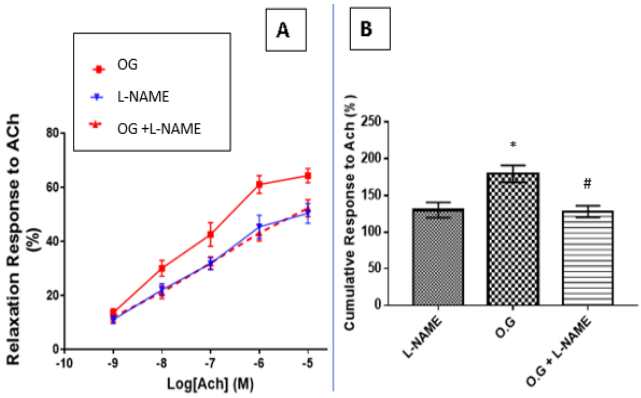

Figure 2:
(A) Response of corpus cavernosum to acetylcholine in the presence of *Ocimum gratissimum* or Nitric oxide inhibitor (L-NAME) (B) Cumulative response of corpus cavernosum to acetylcholine in the presence of *Ocimum gratissimum* or Nitric oxide inhibitor (L-NAME)
Ach = Acetylcholine; OG = Occimum gratissimum *p<0.05 (OG vs L-NAME) #p<0.05 (OG + L-NAME vs L-NAME)

Response of corpus cavernosum to acetylcholine in the presence of *Ocimum gratissimum* and L-NAME: Figure 2a-b represents the graded doses and cumulative dose responses of corpus cavernosum to acetylcholine in the presence and absence of nitric oxide inhibitor, L-NAME in potassium chloride induced contraction. The response to acetylcholine in

tissues incubated with *Ocimum gratissimum* only was significantly higher compared with those incubated with L-NAME only. The effect of OG on the responses of corpus cavernosum to acetylcholine was significantly reduced in the presence of L-NAME.

Response of corpus cavernosum to acetylcholine in the presence of *Ocimum gratissimum* and nifedipine: The responses to acetylcholine in the presence or absence of nifedipine are shown in figure 4a-b while figures 3c-d show the responses to calcium chloride in the presence or absence of nifedipine. The response to acetylcholine in tissues incubated with *Ocimum gratissimum* was not different from the response observed in tissues incubated in nifedipine alone. Co-incubation of nifedipine with *Ocimum gratissimum* significantly decreased the maximum response to acetylcholine compared with the tissues incubated in *Ocimum gratissimum* only ($p<0.05$). Co-incubation of nifedipine with *Ocimum gratissimum* had no effect on the response to calcium chloride in calcium free medium compared with the tissues incubated in *Ocimum gratissimum* only. There was no significant difference in the response to calcium chloride across all groups.

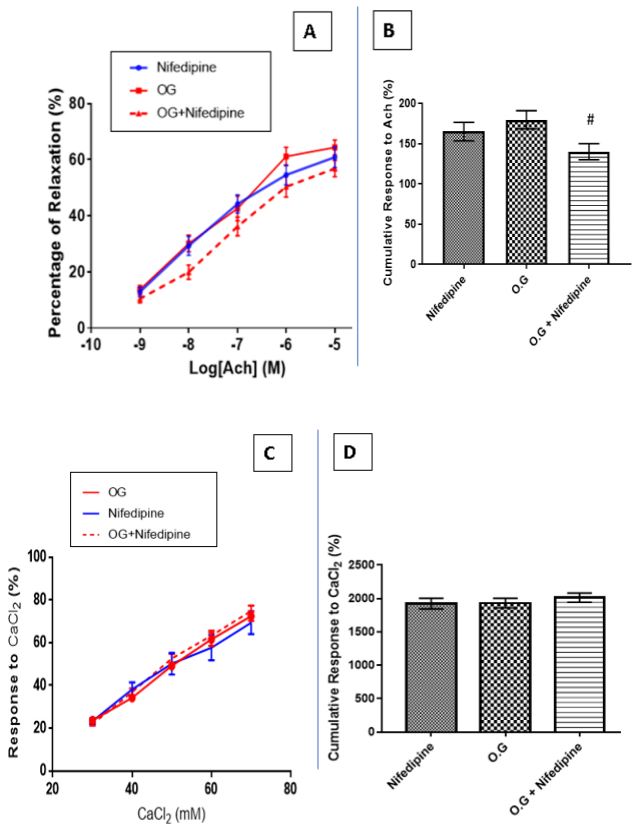

Figure 3:
(a) Response of corpus cavernosum to acetylcholine in the presence of *Ocimum gratissimum* or calcium channel inhibitor (Nifedipine) (b) Cumulative response of corpus cavernosum to acetylcholine in the presence of *Ocimum gratissimum* or calcium channel inhibitor (Nifedipine) (c) Response of corpus cavernosum to calcium chloride in the presence of *Ocimum gratissimum* or calcium channel inhibitor (Nifedipine) (d) Cumulative response of corpus cavernosum to calcium chloride in the presence of *Ocimum gratissimum* or calcium channel inhibitor (Nifedipine).

OG = *Ocimum gratissimum* #p<0.05 (OG + Nifedipine vs O.G)

Response of corpus cavernosum to acetylcholine and calcium chloride in the presence of rho-kinase inhibitor: The response to acetylcholine was significantly reduced when *Ocimum gratissimum* was co-incubated with fasudil compared to *Ocimum gratissimum* alone (Figure 4a-b). Also, the response to calcium chloride was significantly reduced in tissue co-incubated with *Ocimum gratissimum* and fasudil compared with Ocimum gratissiumum alone (Figure 4c-d).

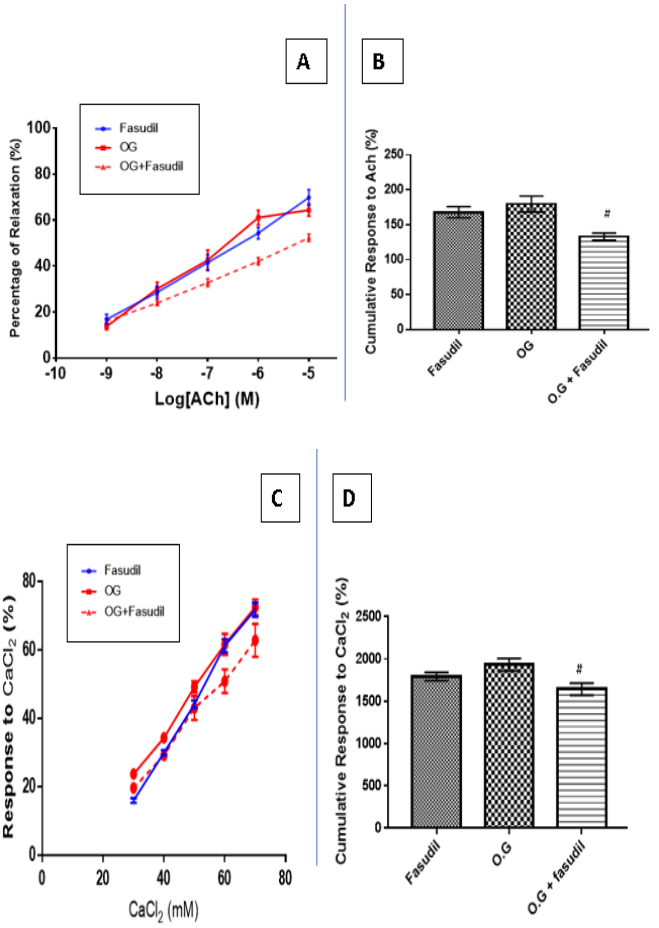

Figure 4:
(a) Response of corpus cavernosum to acetylcholine in the presence of *Ocimum gratissimum* or Rho-kinase inhibitor (Fasudil) (b) Cumulative response of corpus cavernosum to acetylcholine in the presence of *Ocimum gratissimum* or Rho-kinase inhibitor (Fasudil) (c) Response of corpus cavernosum to calcium chloride in the presence of *Ocimum gratissimum* or Rho-kinase inhibitor (Fasudil) (d) Cumulative response of corpus cavernosum to calcium chloride in the presence of *Ocimum gratissimum* or Rho-kinase inhibitor (Fasudil).
OG = *Ocimum gratissimum* #p<0.05 (OG + Fasudil vs OG)

DISCUSSION

This study investigated the mechanisms involved in the erectogenic properties of *Ocimum gratissimum* leaf extract. The molecular mechanism underlying penile erection, a cumulative activity of two interconnected pathways -the calcium-dependent pathway and Rho-A kinase pathway, both of which are characterised by different stages of signal transduction (Mas, 2010) - were explored in this study by observing the responses of corpus cavernosum to smooth muscle contractile modulatory agents in the presence or absence of *Ocimum gratissimum*.

The observed increase in relaxation responses to acetylcholine by corpus cavernosum incubated with *Ocimum gratissimum* showed that the extract could enhance the process of penile erection. This corroborates the aphrodisiac potential of *Ocimum gratissimum* that has been previously reported (Pande and Pathak, 2009; Kankara *et al*., 2015)

As observed in this study, the effects of *Ocimum gratissimum* on the relaxation process were more pronounced in potassium chloride-induced contraction than in phenylephrine. This may corroborate the work of Ratz *et al*. (2005), who reported a complexity in the contraction induced by G-protein coupled receptor agonists like phenylephrine. It was shown that the force of contraction elicited by phenylephrine was not proportional to the change in intracellular calcium ion level; hence, the use of potassium chloride as a simple tool to study the mechanisms involved in smooth muscle contraction and relaxation processes.

The downstream effect of nitric oxide is to induce smooth muscle relaxation. It may function to prepare the smooth muscle for subsequent contraction, hence the link between NO/cGMP/calcium-dependent pathway and rho-kinase modulated calcium sensitization pathway (Sopko *et al*., 2014). When nitric oxide is synthesized, it simultaneously increases the rhoA protein expression as it increases cGMP production. However, the expressed rhoA protein is continuously phosphorylated by cGMP, hence smooth muscle relaxation becomes the dominant effect of nitric oxide (Sasaki *et al*., 1993; Narumiya1 *et al*., 1996; Priviero *et al*., 2010). The decreased responses observed in the presence of L-NAME and *Ocimum gratissimum* Linn showed that *Ocimum gratissimum* was not involved in the production of nitric oxide in this study.

The insignificant difference observed in the relaxation response to acetylcholine between the tissues incubated in nifedipine compared with the tissues incubated in *Ocimum gratissimum* alone showed that *Ocimum gratissimum* can play a role in the restriction of calcium ion entry through L-type voltage-gated calcium channel, similar to nifedipine. This corroborates the report from other studies that *Ocimum gratissimum* inhibits the transport of calcium ions through voltage-operated calcium channels (Interaminense *et al*., 2007).

Decreased relaxation response to acetylcholine observed in the tissues co-incubated in nifedipine and OG showed that the OG does not inhibit the intrinsic property of corpus cavernosum to activate myogenic contraction, usually initiated when the relaxation tension of corpus cavernosum is at its maximum (Hill and Meininger, 2012). In this instance, mechano-receptors within the cavernosa tissues sense the threshold when the smooth muscle relaxation becomes excessive and activate muscle contraction via two distinct mechanisms (Ferrier *et al*., 2000). As documented by Ferrier *et al*. (2000), the first mechanism may involve the generation of membrane potential by the cavernosa pacemaker cell to induce voltage-sensitive calcium release. The presence of pacemaker cell activity in the penile smooth muscle, similar to the interstitial cell of Cajal responsible for myogenic contraction in the intestine, was reported by Shafik (2007). According to Ferrier *et al*. (2000), the pacemaker cell generates a membrane potential that activates the extrusion of calcium ion from the sarcoplasmic reticulum back into the cytosol through the ryanodine and inositol triphosphate (IP3) regulated calcium channel, which brings about phasic contraction that culminates in tonic contraction when it is sustained. It has been documented that the type of contraction initiated by these mechanisms is resistant to nifedipine because it occurs independent of L-type voltage-gated calcium channel (Schubert and Brayden, 2005). This raised the inquisitiveness on the role of *Ocimum gratissimum* on the intrinsic electrical activity of penile smooth muscle. This was done by observing the responses of the corpus cavernosum strips to calcium-induced contraction in membrane potential stabilised tissue in calcium-free medium. It has been reported that inclusion of ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) in physiological solution facilitates the stabilisation of tissue membrane potential in smooth muscle experiments (Nakamura, 2019).

The insignificant difference in the response to calcium chloride when nifedipine was co-incubated with *Ocimum gratissimum* compared with *Ocimum gratissimum* alone could indicate that the activities of *Ocimum gratissimum* in facilitating smooth muscle relaxation do not inhibit the membrane potential required to initiate a phasic contraction by the cavernosa pacemaker, even at the highest dose of calcium chloride. In line with the report of Sahin *et al*. (2018), modulators of calcium ion sensitisation, especially through the rho kinase pathway, do not inhibit the intrinsic phasic contraction of smooth muscle. It then becomes pertinent to investigate the activity of *Ocimum gratissimum* on penile tissue sensitivity to calcium ions compared with standard modulators of calcium sensitisation.

Phosphorylation of myosin light chain is a prerequisite for the initiation of smooth muscle contraction, and the enzyme responsible for this is myosin light chain kinase (MLCK) (Gallagher *et al*., 1997; Kamm and Stull, 2001). Hence, calcium ion serves as a second messenger in the process of smooth muscle contraction by binding to calmodulin to form a calcium-calmodulin complex (Gerthoffer *et al*., 1991; Karaki *et al*., 1997). This complex activates myosin light chain kinase to induce the phosphorylation of myosin light chain, which is then followed by the onset of smooth muscle contraction (Murthy, 2006; Rattan *et al*., 2006). The phosphorylated myosin light chain is spontaneously dephosphorylated by myosin light chain phosphatase (MLCP) (Hirano, 2007); hence, the initiated contraction is sustained by rho-kinase, an enzyme that blocks the activity of myosin light chain phosphatase (Mori *et al*., 2009; Puetz *et al*., 2009). Therefore, inhibition of myosin light chain kinase and rho-kinase activity in penile smooth muscle is required for erection.

The reduced responses to acetylcholine when Ocimum gratissmum was co-incubated with other pro-relaxing agents fasudil showed that Ocimum gatissimum promoted the corpus cavernosum response to acetylcholine through inhibition of rho-kinase activity. Furthermore, the significantly reduced sensitivity to calcium chloride in calcium-free Krebs' solution when *Ocimum gratissimum* was co-incubated with rho-kinase inhibitor showed that *Ocimum gratissimum* may continuously promote penile tissue relaxation activity in as much as the membrane potential is kept constant or below the threshold to generate a myogenic contractile response. Undisrupted corpus cavernosa myogenic contraction is an advantage for penile ejaculatory process (Lue and Tanagho, 1987) and transition into the post-ejaculatory penile resolution period during sexual intercourse. However, the time interval to regain erectile potential depends on the extent to which rho-kinase activity is inhibited (Sopko *et al*., 2014) given that the sexual arousal stimulus is kept constant.

CONCLUSION

In conclusion, the erectogenic property of *Ocimum gratissimum* leaf involves the enhancement of the relaxation response of the corpus cavernosum to acetylcholine via modulation of calcium ion sensitivity. The relaxation-enhancing activity does not interfere with the intrinsic myogenic tone of the corpus cavernosum.

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