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Research Article

Xylopic acid from *Xylopia aethiopica* (Annonaceae) inhibits morphine tolerance in rats

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ABSTRACT

Traditionally, *Xylopia aethiopica* is used to manage pain disorders such as neuralgia, colic pain, rheumatism and headache. Using animal models, this study aimed to investigate the ability of Xylopic Acid (XA), a kaurene diterpene obtained from *Xylopia aethiopica*, to cause tolerance when administered alone or combined with morphine. Development of withdrawal symptoms on discontinuation was also investigated. Tolerance to morphine was induced in rats through an 8-day regimen of chronic administration of morphine (10 mg/kg; twice daily). Effects of XA alone (100 mg/kg) or XA (10-100 mg/kg) on morphine tolerance and withdrawal syndrome precipitated with naloxone hydrochloride (3 mg/kg) were also assessed. XA's mechanism of action was then explored through drug-receptor binding.

Chronic morphine administration in rats resulted in analgesic tolerance and morphine withdrawal syndrome. Chronic XA administration did not result in tolerance to XA's antinociceptive effect. Development of morphine withdrawal syndrome precipitated by naloxone and morphine tolerance was significantly ($F(12, 60)=29.88$, $p<0.0001$) inhibited by XA. Xylopic acid inhibited development of diarrhea, jumps and weight loss. Pretreatment with α-2-adrenoceptor antagonist, yohimbine, 5HT3 antagonist, ondansetron and muscarinic antagonist, atropine, significantly ($p=0.0042$) blocked the inhibitory effect of XA on withdrawal jumps. Pretreatment with naloxone produced similar effects on withdrawal jumps as XA alone. Drug-receptor binding assays revealed a lack of significant interaction of XA on alpha-2 adrenoceptors (A, B, C) but exhibited significant DOR- selective antagonism similar to naltrindole. This study reveals that xylopic acid significantly inhibits morphine antinociceptive tolerance and withdrawal in rats. This is the first report of xylopic acid's antagonism on delta opioidergic receptors and potential as an inhibitor of chronic morphine tolerance.

Keywords:
Delta opioid receptor, Naloxone, Pain, Xylopia, Xylopic acid

1. INTRODUCTION

Opioids have for many years played an instrumental role in the relief of moderate to severe pain[1-2]. Opioids act through interaction with the superfamily of G-protein-coupled opioid receptors: mμ, kappa, and delta. All subtypes of opioid receptors modulate pain perception, excluding the kappa 2 receptor. All three receptor subtypes inhibit adenylyl cyclase, suppress calcium conductance, activate potassium conductance and inhibit neurotransmitter release[1-2]. The benefits of the antinociceptive effect of opioids have unfortunately been limited due to increased tolerance and dependence upon chronic use[3]. For patients with pain which may or may not be associated with malignancy and in palliative care, the likelihood of developing analgesic tolerance or opioid dependence due to pain therapy is very high[4]. Opioid tolerance usually results in a decreased responsiveness

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to an opioid agonist such as morphine; a dose escalation is usually required in such an instance[5]. Dependence on opioid drugs results in compulsive use of these drugs. Moreover an attempt to abruptly cease consumption commonly triggers withdrawal signs[6]. These signs may be mitigated by administration of drugs like clonidine and methadone[7]. Multilayered mechanisms have been associated with opioid tolerance and dependence such as the generation of free radicals, down-regulation of opioid receptors and activation of the N-methyl-D-aspartate (NMDA) glutamatergic receptor[8-9].

Xylopic acid, a kaurene diterpene from *Xylopia aethiopica* (Annonaceae), possesses many therapeutic benefits. Among these is its antinociceptive effect[10-11]. Woode et al. previously reported xylopic acid's antinociceptive mechanism[11]. Xylopic acid possibly acts by opioidergic, NO-cGMP, serotoninergic, cholinergic, adrenergic, and adenosinergic mechanisms. Opioidergic activity of XA suggests a possibility of development of tolerance, dependence or withdrawal symptoms on chronic use. Given, the common use of *Xylopia aethiopica* (*X. aethiopica*) in the Ghanaian traditional society, our study sought to investigate xylopic acid for the possibility of tolerance on chronic use as well as its effect on tolerance to morphine and withdrawal syndrome in animal models.

2. MATERIALS AND METHODS

2.1. Drugs

Atropine and ondansetron (GlaxoSmithKline, Middlesex, UK), Morphine and Naloxone HCL Injection (Sandoz Canada Incorporated, Boucherville, Canada), Yohimbine (Walter Ritter GmbH, Hamburg, Germany).

2.2. Experimental animals

Rats (200-250 g Male Sprague-Dawley) obtained from the Noguchi Memorial Institute for Medical Research, Accra, Ghana, were kept under laboratory conditions in steel cages with free access to tap water and food. Animals were given seven (7) days to adapt to the laboratory environment before experiments began. Rats were put in experimental groups (n=8) randomly. Faculty Animal Ethics Committee, Kwame Nkrumah University of Science and Technology, granted ethical approval for the study.

2.3. Xylopic acid extraction

The extraction of xylopic acid from *Xylopia aethiopica* was done as reported by Boampong J and colleagues[12]. Fresh unripe fruits of *X. aethiopica* were obtained from the Botanic Gardens of KNUST (06° 41′6.38″ N; 01° 33′ 44.34″ W). *X. aethiopica* has been stored at the KNUST herbarium (Voucher number FP/09/77). The fruits were shade-dried and pulverized. Two (2) kg of powdered sample was soaked for 3 days in 5 L of petroleum ether (40-60°C). The petroleum ether was drained and a rotary evaporator was used to obtain a concentrate of the sample at 50°C. Ethyl acetate was then added to the concentrate to crystalize xylopic acid out of the mixture. The xylopic acid crystals were allowed to stand for three days after which they were flushed repeatedly with petroleum ether (40-60°C) to rid the crystals of all unwanted materials. Purification of xylopic acid fraction using 96%v/v ethanol to obtain 95% purity was done. Purity was determined using HPLC.

2.4. Antinociceptive test

Antinociceptive testing was done using the hot water immersion tail-withdrawal assay as performed by Steimiller CL and colleague[13]. The distal half of the rat's tail was dipped into a water bath containing hot water (Temperature: 50±1.0°C) and tail withdrawal latency measured. Tissue damage was prevented with a cut-off time of ten seconds. In measuring antinociception, an increase in tail withdrawal latency was used. Animals that failed to withdraw their tails were awarded the cut-off latency and the experiment immediately ended. Antinociception was calculated as follows:

Eq. (1): %Antinociception (%MPE)=[(Tb-Ta)/(T0-Ta)×100], where Ta=pre-drug withdrawal latency; Tb=post-drug withdrawal latency; T0=cut-off latency.

2.5. Morphine tolerance induction

Twice daily dosing (7:00 a.m. and 6:00 p.m.) of 10 mg/kg; i.p. morphine was done for eight (8) days. On days 1, 3, 6 and 8, the tail-withdrawal assay was used to measure antinociception 30 min prior to, and after morphine administration. Xylopic Acid was administered either alone (100 mg/kg; p.o.) or together (XA, 10-100 mg/kg; p.o.) with morphine (10 mg/kg; i.p.). On the days of antinociceptive testing, xylopic acid was given after morphine administration and antinociception testing was conducted (Figure 1).

2.6. Induction of morphine withdrawal syndrome

Intraperitoneal injections of morphine were administered twice daily for 7 days as follows: 2.5 mg/kg on days 1 and 2, and doubled every subsequent day. Forty (40) mg/kg was given on day 6 and 50 mg/kg on day 7. Xylopic acid (10-100 mg/kg) was dosed 20 min before morphine to investigate possible effects on the occurrence of morphine withdrawal syndrome. On day 7, 3 mg/kg of intraperitoneal naloxone hydrochloride was administered 5 h following the last administration of morphine. Subsequent to administration of naloxone,

	Days							
Groups	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Vehicle	Saline twice daily	Saline twice daily	Saline twice daily	Saline twice daily	Saline twice daily	Saline twice daily	Saline twice daily	Saline twice daily
Morphine-only	Morphine twice daily	Morphine twice daily	Morphine twice daily	Morphine twice daily	Morphine twice daily	Morphine twice daily	Morphine twice daily	Morphine twice daily
XA-only	XA twice daily	XA twice daily	XA twice daily	XA twice daily	XA twice daily	XA twice daily	XA twice daily	XA twice daily
XA-Morphine	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily
	Tail withdrawal test		Tail withdrawal test			Tail withdrawal test		Tail withdrawal test

Figure 1. Scheme for morphine tolerance induction.

	Days						
Groups	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Vehicle	Saline twice daily	Saline twice daily	Saline twice daily	Saline twice daily	Saline twice daily	Saline twice daily	Saline twice daily
Morphine-only	Morphine twice daily	Morphine twice daily	Morphine twice daily	Morphine twice daily	Morphine twice daily	Morphine twice daily	Morphine twice daily
XA-only	XA twice daily	XA twice daily	XA twice daily	XA twice daily	XA twice daily	XA twice daily	XA twice daily
XA-Morphine	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily	XA + Morphine twice daily
							Naloxone 5 h after last treatment

Figure 2. Scheme for induction of morphine withdrawal syndrome.

animals were individually put into a transparent observation chamber and observed for occurrence of withdrawal signs (Figure 2). Both quantitative (increased frequency of jumps and abdominal contractions) and qualitative (diarrhea, weight loss) signs were noted according to work by de Corde A and colleagues[14]. Activity of XA (30 mg/kg) was blocked with selected antagonists yohimbine (2.5 mg/kg; p.o.), atropine (5 mg/kg; p.o.), Ondansetron (0.5 mg/kg; p.o.), naloxone (2 mg/kg; i.p.) towards determination of its mechanism of action. XA (30 mg/kg) was selected as it was the median effective dose for xylopic acid.

2.7. Drug receptor binding

For primary binding assays, compounds were sampled at a single concentration (10 μM) as well as quadruplicates (0.16, 0.32, 0.63, 1.25, 2.5, 5.0 and 10.0 nM). Compounds which exhibited a minimum of 50% inhibition at 10 μM were then tagged for secondary radioligand binding assays to determine equilibrium binding affinity at specific targets. In secondary binding assays, compounds were tested at 11 concentrations (0.1, 0.3, 1, 3, 10, 30, 100, 300 nM, 1, 3, 10 μM) and in triplicates.

Ki determinations and receptor binding profiles were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract #HHSN-271-2018-00023-C (NIMH PDSP). The NIMH PDSP is directed by Bryan L. Roth at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscoll at NIMH, Bethesda MD, USA. For experimental details please refer to the PDSP web site https://pdsp.unc.edu/ims/investigator/web/.

2.8. Data analysis

Data were stated as mean±S.E.M and significant differences between means calculated by one-way analysis of variance (ANOVA) and two-way ANOVA followed by Tukey post hoc test. In all instances, $p<0.05$ was considered significant. Statistical analyses were carried out with GraphPad Prism® Version 8.0 (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1. Xylopic acid extraction

Isolated xylopic acid purity was determined using HPLC. The resultant single peak for xylopic acid indicated a single compound was present (Figure 3).

3.2. Morphine tolerance induction

As shown in Figure 4, there was a decrease in antinociceptive effect after chronic administration of morphine. This was taken as a measure of tolerance onset. Concomitant administration of morphine with XA (30 and 100 mg/kg) significantly ($F(12, 60)=29.88$, $p<0.0001$) inhibited morphine tolerance. Treatment with XA only showed antinociceptive effect without tolerance.

3.3. Induction of morphine withdrawal syndrome

Administration of naloxone induced diarrhea in morphine only-treated rats. Vehicle only-treated rats, in contrast, did not exhibit diarrhea. Concurrent morphine administration with 100 mg/kg XA decreased the number of rats with diarrhea significantly ($p<0.01$).

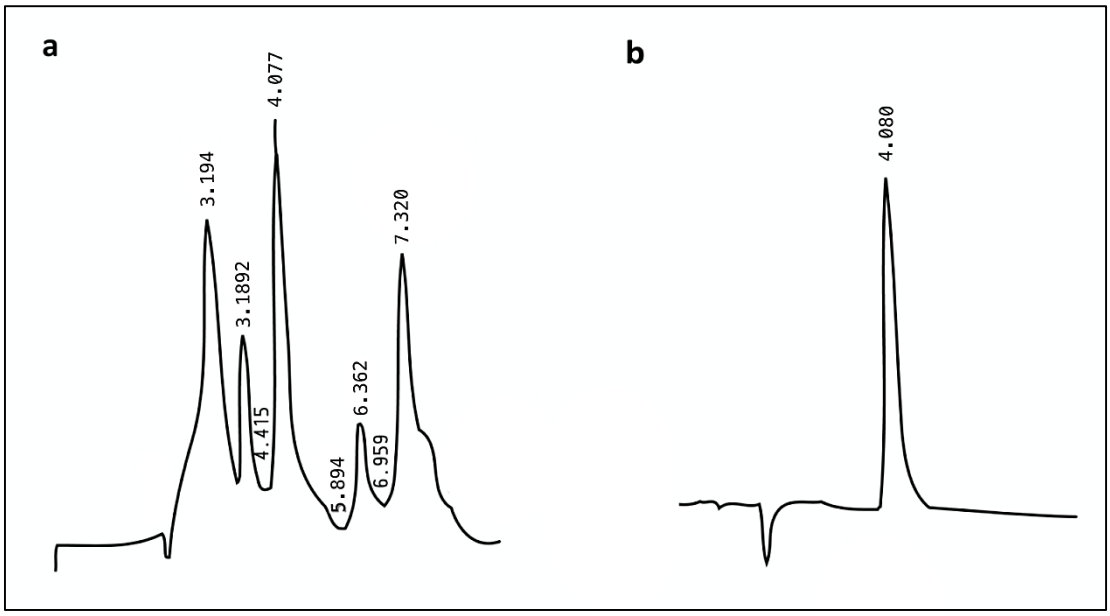

Figure 3. HPLC chromatogram of a) Xylopia crude extract b) xylopic acid.

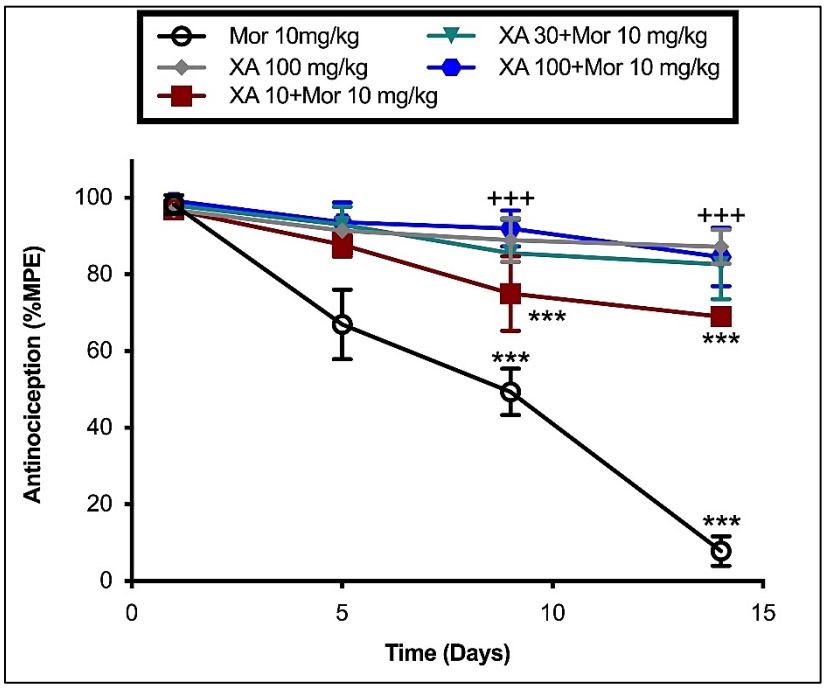

Figure 4. Xylopic Acid (XA; 10-100 mg/kg) effect on development of morphine tolerance in rats (n=8). Points represent mean±S.E.M. +++ $p<0.001$ as compared with morphine-injected rats on the same day, ***$p<0.001$ indicates significant difference vs %MPE obtained on the first day.

Table 1. Withdrawal signs exhibited by animals after naloxone hydrochloride administration in morphine-dependent rats.

Treatment Groups	Withdrawal Signs (number of animals with sign/number observed)	
	Diarrhea	Weight Loss
Veh	0/8	0/8
Mor	8/8***	8/8***
XA	0/8+++	2/8+
Mor+XA10	6/8*	7/8***
Mor+XA30	4/8*+	3/8+
Mor+XA100	1/8++	2/8+

*p<0.05, **p<0.01, ***p<0.001 significantly different compared with Veh group.
+p<0.05, ++p<0.01, +++p<0.001 in comparison to morphine-only group, one-way ANOVA followed by Tukey Post Hoc test.

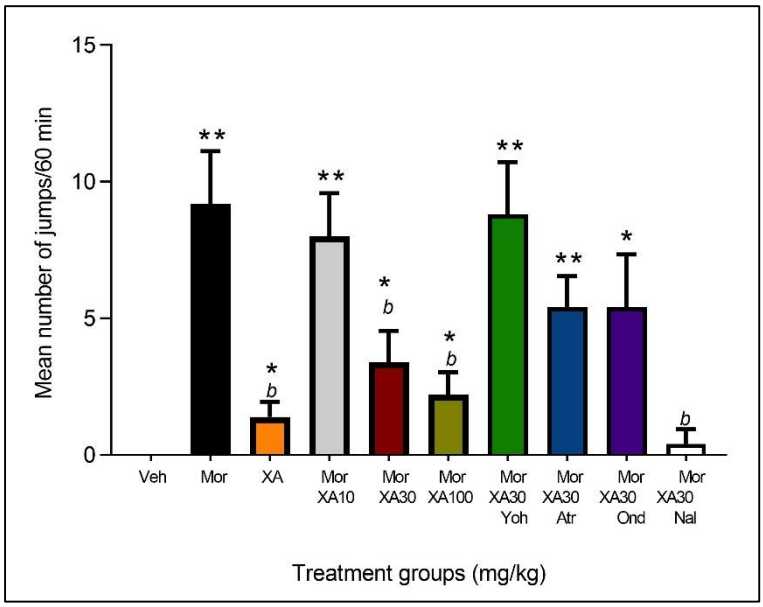

Figure 5. Effect of XA (10-100 mg/kg), naloxone (2 mg/kg), yohimbine (2.5 mg/kg), atropine (5 mg/kg), Ondansetron (0.5 mg/kg) on naloxone-induced jumps in rats (n=8). Results represent mean±S.E.M. *p<0.05, **p<0.01 vs. control rats. b p<0.05, in comparison with morphine-treated animals.

Animals that received morphine +30 mg/kg XA exhibited a reduction of diarrheal signs by half. There was no significant reduction of diarrhea signs in Morphine +10 mg/kg XA-treated groups. Results also indicated that naloxone administration led to significant (p<0.001) loss of weight in all rats treated with morphine only compared to the vehicle-treated group. In XA only-treated rats, naloxone administration produced weight loss in two animals. XA at 30 and 100 mg/kg administered concurrently with morphine reduced weight loss precipitated by naloxone, significantly (p<0.05). Concomitant administration of XA (10 mg/kg) with morphine produced no significant effect on weight loss (Table 1).

3.4. Naloxone-induced jumping in morphine-dependent rats

XA (10 mg/kg) could not significantly reduce the mean number of naloxone-induced jumps. At 30 and 100 mg/kg however, a significant (F(2.564, 10.26)=32.63, P=0.0251) reduction in the frequency of jumps was observed when XA was co-administered with morphine (Figure 5). Pretreatment with yohimbine, atropine and ondansetron resulted in significant reversal of withdrawal jumps in rats. Pretreatment with naloxone resulted in a similar effect on withdrawal jumps as XA alone.

3.5. Naloxone-induced abdominal contractions in morphine-dependent rats

Frequency of abdominal contractions after administration of naloxone increased significantly (F(1.771, 7.082)=25.37, P=0.0013) in morphine only-treated groups. Concurrent administration of morphine and XA did not inhibit abdominal contraction induced by naloxone (Figure 6). XA-only administration insignificantly affected naloxone-induced abdominal contracts.

3.6. Radioligand Binding Assay

Primary binding assays (Table 2) showed stimulatory effects of xylopic acid on Kappa and Mμ receptors. It however displayed antagonism on Delta and Sigma 2 receptors. Significant (greater than 50% inhibition) among these interactions was the Delta

Table 2. Mean percentage inhibition (*in vitro*) of xylopic acid on various opioidergic receptors.

Receptor	Mean % Inhibition (*p<0.05)
DOR	66.08±4.3*
KOR	-1.8±-7.792
MOR	-19.39±-4.6
Sigma 2	49.02±1.0
Alpha 2A	-8.518±13.00
Alpha 2B	19.58±9.323
Alpha 2C	-3.023±0.5405
M4	3.125±1.429
M5	8.330±2.428
5HT3	4.293±5.906

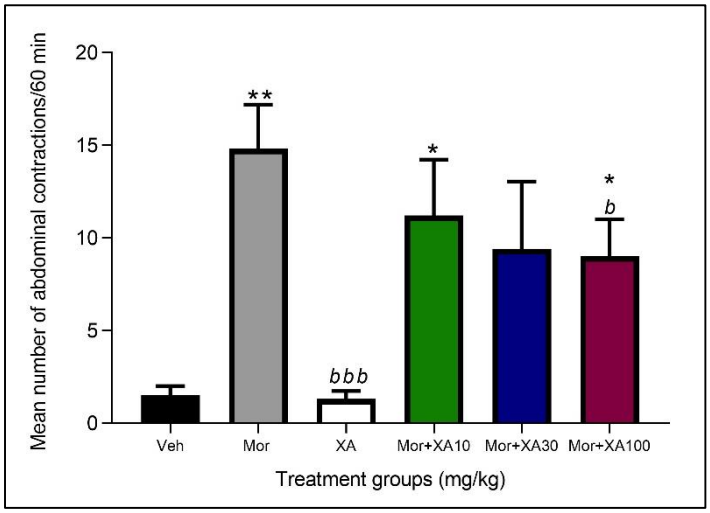

Figure 6. Effect of XA (10-100 mg/kg; n=8) on abdominal contractions induced by naloxone. Results represent mean±S.E.M. *p<0.05, **p<0.01 vs. vehicle-treated rats. b p<0.05, bbb p<0.001, in comparison with morphine-treated animals.

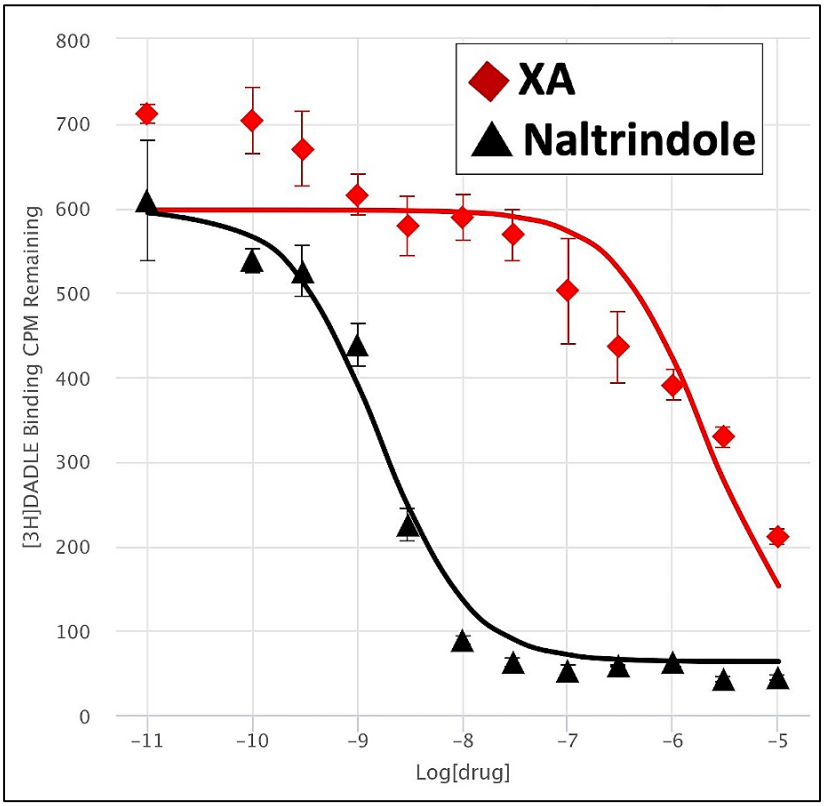

Figure 7. Secondary binding curve of [3H]DADLE on human delta opioid receptor in the presence of Naltrindole and xylopic acid. Results represent mean±S.E.M from three experiments.

Table 3. Secondary binding assay of xylopic acid on delta opioidergic receptors.

Drug	Log Ki	Ki (nM)
Xylopic Acid	-5.97	1073.49
Naltrindole	-9.08	0.84

receptor antagonism (66.08±4.3%). Further secondary binding assay indicated inhibitory action of xylopic acid on delta opioid receptor using naltrindole as standard (Figure 7). Ki value of xylopic acid was 1073.49 nM compared to naltrindole, 0.84 nM (Table 3). Interaction of XA with muscarinic, alpha-adrenergic and serotoninergic receptors were not significant.

4. DISCUSSION

Our results show that XA possesses a significant anti-tolerance effect against morphine-induced antinociception in rats and is not liable to induce tolerance on chronic administration. XA, additionally, alleviated some signs of withdrawal in rats with morphine-dependence. Woode et al., previously demonstrated that tolerance to analgesic or cross-tolerance when combined with morphine does not occur with chronic administration of XA.

The two major theories hypothesized for opioid tolerance involve changes in opioid receptors and adaptations at various levels in the nervous system as well as in the periphery. Nonetheless, these significant changes are initiated by mμ receptor activation.

Prolonged opioid activation of mμ receptors is believed to result in changes that lead either to receptor desensitization or receptor down-regulation[15]. However, other mechanisms have been proposed, including actions mediated by the delta opioid receptor. Mμ-opioid receptors (MOR)-delta opioid receptors (DOR) heterodimerization has previously been found to be an important mechanism in the development of opioid tolerance[16]. In the primary binding assay, XA exhibited some activity on mμ, delta, kappa and Sigma 2 receptors; delta receptor binding however was most significant. The secondary binding assay on delta displayed a similarity between the slopes of the standard, naltrindole, and XA. In a study conducted by Reiss D and colleagues[17], the involvement of the opioid receptor delta in opioid tolerance was discussed. Receptor internalization is a key feature of the activation of the delta opioid receptor. Contrasting the mμ opioidergic receptor which can undergo recycling to the surface of the cell upon internalization[18], opioid receptors (delta) are degraded by lysosomes. This receptor inactivation contributes to the onset of opioid tolerance[19]. Investigations in relation to the mechanism of opioid tolerance and dependence have shown an involvement of the delta and mμ receptors. Abdelhamid et al.[20], discovered that naltrindole was able to prevent morphine tolerance in male swisswebster mice. This establishes that delta receptor antagonism is able to attenuate opioid tolerance and could therefore be XA's mechanism of morphine tolerance attenuation.

Release of several neurotransmitters and subsequent activation of the receptor accompany the opioid withdrawal syndrome. Alterations of specific physiological functions are responsible for the individual signs of withdrawal observed[21]. The withdrawal signs investigated were diarrhea, weight loss, mean number of jumps per 60 minutes, and mean number of abdominal contractions[14,22-24]. Research provides evidence suggesting an association of the cholinergic system with biological activity of opiates[25]. Rada and colleagues[26] induced morphine withdrawal in rats and discovered that there was a decline in dopamine and a surge in acetylcholine concentration in the nucleus accumbens. Acetylcholine elevation is responsible for the increase in peristaltic movement, resulting in the diarrhea and abdominal contraction associated with withdrawal syndrome[27]. Many studies have also proven that withdrawal signs are due to the locus coeruleus[28,29]. This involves an elevation in the levels of noradrenaline[30]. The ability of clonidine, an α-2 receptor agonist, to alleviate withdrawal signs confirms the involvement of noradrenaline in the onset of opioid withdrawal[21,26,30]. Motricity, in various studies, has been identified as a sign of withdrawal. In a study by Mavrikaki M and colleagues[21], clothaipine's ability to reduce the frequency of jumps was associated with the inhibition of dopamine receptors produced by this drug. Bläsig J et al.[31] also conducted a study that revealed dopamine played a stimulatory role in the induction of jumping behavior in rats with naloxone-induced withdrawal.

In this study, the morphine group elicited withdrawal signs (diarrhea, weight loss, increased abdominal contractions, elevated frequency of jumps) similar to work done by Bläsig J and colleagues[32]. Co-administration of morphine and high dose XA attenuated all the signs of withdrawal except abdominal contractions. In a prior study, pretreatment of rats with the α-2-adrenoceptor antagonist, yohimbine reversed the antinociception produced by xylopic acid administration[33]. This implies that xylopic acid interacts with the α-2 adrenoceptor or adrenergic pathway and this may be the mechanism by which, like clonidine[30], XA alleviates morphine withdrawal. Yohimbine produced similar actions in this study. However, drug receptor binding failed to establish significant interaction of xylopic acid with the α-2 adrenoceptor. It may therefore be a possibility that xylopic acid may be acting downstream in the

α-2 adrenoceptor cascade.

5. CONCLUSIONS

In conclusion, xylopic acid administration in rat models has been shown to possess significant inhibitory action on withdrawal syndrome induced by naloxone and against tolerance to antinociceptive effects of morphine. The anti-tolerance ability of XA may be associated with delta opioid receptor blockade. XA's ability to alleviate withdrawal may be mediated by interaction with the adrenergic system. Further work may be required to confirm this effect.

Conflict of interest
The authors declare no conflicts of interests.

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None to declare.

Ethics approval
This study was approved by Faculty Animal Ethics Committee, Kwame Nkrumah University of Science and Technology No. FP/A.11/TJ.

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