Anti-allodynic and Anti-hyperalgesic effects of an ethanolic extract and xylopic acid from the fruits of *Xylopia aethiopica* in murine models of neuropathic pain

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INTRODUCTION

Fruits of *Xylopia aethiopica* (Dunnal) A. Rich (Family: Annonaceae), popularly called African pepper in West Africa, is used for the treatment of rheumatism, headache, neuralgia, and colic pain.[1]

The fruit of *X. aethiopica* contains kaurenoic and xylopic acid (XA) which are kauranes, a class of diterpenes. Biological activities of kauranes include antimicrobial, cytotoxic, antiparasitic, insect antifeedant, anti-HIV, and anti-inflammatory activities.[2,3] The anti-inflammatory activity of the kauranes has been shown to involve the impairment of inflammation signaling through inhibition of NF-κB activity.[4,5] Kaurenoic acid (ent-kaur-16-en-19-oic acid), an ent-kaurene diterpene, has several biological activities including analgesia,[6] diuretic, vasorelaxant, anti-inflammatory, and antipyretic effects in rodents.[7,8] Xylopic acid [15β-acet oxy-(β)-kaur-16-en-19-oic acid; Figure 1] and its epimer, acetylgrandifloric acid [15α-acetoxy-(β)-kaur-16-en-19-oic acid], also exhibit antibacterial activity.[9] Some studies have also shown that XA and the ethanol fruit extract of *X. aethiopica* (XAE) have a low toxicity profile.[7,10] A recent study from our laboratory established the analgesic properties of XAE and its major diterpene XA in vincristine-induced neuropathic pain.[11,12]

Neuropathic pain normally results from a number of metabolic, toxic, or traumatic insults to the central or
peripheral nervous system and accounts for enormous morbidity and societal cost in both health care expenditure and lost work.\textsuperscript{[13]}  Neuropathic pain responds poorly to many classical analgesics and is costly to manage.\textsuperscript{[14]-[16]}  Moreover, with the available treatments, a small number of patients experience some pain relief with most patients being resistant to available analgesics and are persistently in pain.\textsuperscript{[16]-[18]}  Over the years, the survival rate of cancer patients have increased but this comes with the cost of patients developing peripheral neuropathy and subsequent neuropathic pain which is related to chemotherapeutic treatment.\textsuperscript{[19]}  Consequently, there is still a considerable need to explore novel treatment modalities for neuropathic pain management especially chemotherapy-induced neuropathic pain.

The analgesic properties of XAE and its major diterpene, XA as well as its possible mechanisms has been recently reported.\textsuperscript{[11,12]}  In the present study, an animal model of vincristine-induced neuropathic pain was used to evaluate the effect of XAE and XA in neuropathic pain.\textsuperscript{[20,21]}

**MATERIALS AND METHODS**

*Collection of plant material, preparation of XAE and isolation and purification of XA (15\(^\beta\)-Acetoxy-(-) - kaur-16-en-19-oic Acid).*

Dried fruits of *X. aethiopica* were collected from the Botanical Gardens (06°41’6.39”N; 01°33’45.35”W) of Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana, between the months of August and December 2008. The fruits were authenticated by Dr. Kofi Annan of the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST. A voucher specimen (No. FP/09/77) has been kept at the herbarium of the Faculty.

Preparation of XAE and isolation and purification of XA (15\(\beta\)-Acetoxy-(-) - kaur-16-en-19-oic Acid) were as previously described.\textsuperscript{[11,12,22]}

**Animals**

Sprague-Dawley rats (150-200 g) of both sexes were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana, and housed in the animal facility of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST). The animals were housed in groups of six in stainless steel cages (34×47×18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema, Ghana), given water *ad libitum*, and maintained under laboratory conditions (temperature 24-25°C, relative humidity 60-70%, and 12 h light-dark cycle). All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985, revised 1996). All protocols used were approved by the Departmental Ethics Committee.

**Drugs and chemicals**

Pregabalin (Lyrica\textsuperscript{®}) was purchased from Pfizer Pharmaceuticals, New York, USA and vincristine sulfate from Health Biotech Limited, Chandigarh, India.

**Vincristine-induced neuropathic pain**

Vincristine sulfate was dissolved in saline and stored as a stock concentration of 1 mg l\(^{-1}\) at 4°C. The animals received intraperitoneal (i.p.) injection of vincristine at a final concentration of 0.1 mg kg\(^{-1}\) day\(^{-1}\) during two cycles of five consecutive working days (i.e. days 1-5 and days 8-12 with 2 days off). This dose was chosen because it produces hyperalgesia with no significant motor deficit.\textsuperscript{[21]} On day 15, baseline response was measured in the Randall-Selitto test, Von Frey test (4 g, 8 g and 15 g) and cold allodynia (cold water at 4.5°C). The animals were later treated with XAE (30-300 mg kg\(^{-1}\) \(p.o\)), XA (10-100 mg kg\(^{-1}\) \(p.o\)), pregabalin (10-100 mg kg\(^{-1}\) \(p.o\)), or saline. Three sets of experiments were then performed in order to evaluate the effects of XAE, XA, and pregabalin in vincristine-induced neuropathic pain.

**Experimental design**

**Assessment of tactile allodynia**

To evaluate the effect of XAE, XA, and pregabalin on static tactile allodynia, animals were placed in a restrainer. Tactile allodynia was assessed using von Frey filaments (IITC Life Science Inc. Model 2888, Woodland Hills, CA, USA) with bending forces of 4 g. Chemotherapy-induced responses to 4 g are best described as tactile allodynia (pain induced by a normally innocuous stimulus) because normal rats never withdraw from this stimulus.\textsuperscript{[23,24]} In ascending order of force, each filament was applied to the mid-plantar
% inhibition = \left( \frac{AUC_{\text{control}} - AUC_{\text{treatment}}}{AUC_{\text{control}}} \right) \times 100

Differences in AUCs were analyzed using one-way ANOVA with drug treatment as a between subject factor. Further comparisons between vehicle- and drug-treated groups were performed using the Holm-Sidak’s post hoc test.

Doses for 50% of the maximal effect (ED$_{50}$) for each drug were determined by using an iterative computer least squares method, with the following nonlinear regression (three parameter logistic) using the equation

\[ Y = \frac{a + (b - a)}{1 + 10^{\left(\log ED_{50} - X\right)}} \]

Where X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape.

The fitted midpoints (ED$_{50}$) of the curves were compared statistically using F test[26,27] GraphPad Prism for Windows version 6 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED$_{50}$ determinations. P < 0.05 was considered statistically significant.

RESULTS

Tactile allodynia

Intraperitoneal injection of vincristine for 2 weeks produced a marked, prolonged dynamic tactile allodynia in rats. One hour after the various drug treatments, tactile allodynia was measured using Von Frey filament of 4 g and the control animals showed increased response to tactile allodynia compared to the treated animals. XAE andXA increased the latency to paw response ($F_{3,25} = 6.03, P = 0.0060; F_{3,25} = 4.19, P = 0.0228$, respectively) with the highest doses used achieving a tactile anti-allodynia of 61.3 ± 11.4% and 65.8 ± 23.8%, respectively [Figure 2a and b]. Pregabalin significantly and dose-dependently inhibited tactile allodynia ($F_{3,25} = 7.14, P = 0.0029$): With the highest dose of pregabalin (100 mg kg$^{-1}$) producing an anti-allodynic effect of 62.4 ± 8.93% [Figure 2c]. XA was more potent and efficacious than pregabalin and XAE. Pregabalin was, however, more potent and efficacious than XAE [Table 1 and Figure 6].

Intermediate hyperalgesia with Von Frey 8 g

Von Frey filaments of 8 g was used to assess the effect of XAE, XA, and pregabalin on mechanical hypernociception, intermediate to tactile allodynia, and mechanical hyperalgesia. One hour after the various drug treatments, pain was measured using Von Frey filament

Assessment of intermediate and mechanical hyperalgesia with Von Frey

Intermediate and mechanical hyperalgesia were assessed with Von Frey filaments of 8 and 15 g, respectively. Responses to 15 g are best described as hyperalgesia (heightened pain response from a normally painful stimulus) because normal rats withdraw from this stimulus 5-10% of the time. The responses to 8 g are intermediate.[23,24] The percentage overall responses to Frey filaments of 8 and 15 g were measured as described earlier.
of 8 g. The control animals showed increased response to pain compared to the treated animals. XAE, XA, and pregabalin produced anti-hyperalgesia ($F_{3,28}^1 = 3.20, P = 0.0515$; $F_{3,28}^1 = 4.05, P = 0.0272$; $F_{3,28}^1 = 2.59, P = 0.0889$; figure 3 upper panel) in this test. The highest doses of XAE, XA, and pregabalin produced possible anti-hyperalgesic effect of $454.6 \pm 11.5\%$, $66.46 \pm 10.3\%$, and $53.8 \pm 11.4\%$ (Figure 3 upper panel), respectively. XA was more potent and efficacious than pregabalin and XAE. Pregabalin was also more potent and efficacious than XAE [Table 1 and Figure 6].

**Table 1: ED$_{50}$s and E$_{max}$ values for *X. aethiopica* extract, xylopic acid, and pregabalin in the various models**

<table>
<thead>
<tr>
<th>Model</th>
<th>Xylophia extract</th>
<th>Xylopic acid</th>
<th>Pregabalin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED$_{50}$</td>
<td>E$_{max}$</td>
<td>ED$_{50}$</td>
</tr>
<tr>
<td>Tactile allodynia</td>
<td>34.4±1.9</td>
<td>66.6</td>
<td>7.1±1.4</td>
</tr>
<tr>
<td>Intermediate hyperalgesia</td>
<td>54.8±2.2</td>
<td>53.7</td>
<td>24.9±1.8</td>
</tr>
<tr>
<td>Mechanical hyperalgesia</td>
<td>39.7±2.1</td>
<td>52.7</td>
<td>14.7±1.9</td>
</tr>
<tr>
<td>Cold allodynia</td>
<td>63.9±1.9</td>
<td>55.2±1.9</td>
<td>47.2±1.8</td>
</tr>
<tr>
<td>Randall-Selitto</td>
<td>62.0±2.0</td>
<td>24.1</td>
<td>21.1±1.5</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.E.M. (n=7-8). The values were obtained from experiments shown in Figures 2-6. ED$_{50}$s were obtained by least-square nonlinear regression as described above.

**Mechanical hyperalgesia using Von Frey 15 g**

Baseline mechanical hyperalgesia taken on day 15 using the Von Frey hairs of 15 g revealed that both hind paws exhibited marked static mechanical hyperalgesia. XAE (30-300 mg kg$^{-1}$, p.o.) produced a significant ($F_{3,28}^1 = 4.05, P = 0.0256$) and dose-dependent inhibition of static mechanical hyperalgesia (Figure 3 lower panel). The highest dose of XAE increased the latency to mechanical hyperalgesia by $48.3 \pm 32.5\%$ (Figure 3 lower panel). Significant ($F_{3,28}^1 = 16.56, P < 0.0001$) and dose-dependent inhibition of static mechanical hyperalgesia was produced after XA (10-100 mg kg$^{-1}$, p.o.) administration, with the highest dose increasing the latency to static mechanical hyperalgesia by $63.4 \pm 25.5\%$ (Figure 3 lower panel). The administration of pregabalin (10-100 mg kg$^{-1}$) significantly ($F_{3,28}^1 = 14.98, P < 0.0001$) and dose-dependently inhibited mechanical hyperalgesia and a maximum anti-hyperalgesia of $72 \pm 25.1\%$ (Figure 3 lower panel) being achieved at the highest dose administered. Pregabalin was more efficacious than XA and XAE. XA was also more efficacious than XAE [Table 1 and Figure 6].

**Figure 2:** Effect of (a) XAE (30-300 mg kg$^{-1}$, p.o.), (b) XA (10-100 mg kg$^{-1}$, p.o.), and (c) pregabalin (10-100 mg kg$^{-1}$, p.o.) on the time course of vincristine-induced neuropathic pain (tactile allodynia, von Frey 4 g) in rats. Each point represents mean ± S.E.M (n = 8). $P$ von Frey P von Frey 4 P von Frey 4 g in rats. Each point represent-way repeated measures ANOVA followed by Holm-Sidak's post hoc. The box-and-whisker plots (insets) depict AUCs derived from the respective time course curves. The plots show the 25th and 75th percentiles, the median (horizontal line within the box), and the 10th and 90th percentiles (whiskers). $P$ (whisker$^{\text{R}}$P whiskers). way ANOVA followed by Holm-Sidak post hoc.
pregabalin and XAE. Pregabalin was also more efficacious than XAE [Table 1 and Figure 6].

**Cold allodynia**

Baseline cold allodynia was measured from both hind paws on day 15 using cold water at a temperature of 4.5°C. XAE (30–300 mg kg⁻¹ p.o.) produced a significant (F(3,28) = 18.09, P < 0.0001) and dose-dependent inhibition of cold allodynia [Figure 5a] which was demonstrated as increased latency of paw withdrawal. The highest dose of XAE increased the latency of paw withdrawal to cold allodynia by 110.6 ± 5.13% [Figure 5b]. XA (10–100 mg kg⁻¹ p.o.) also produced a significant (F(3,28) = 16.69, P < 0.0001) and dose-dependent inhibition of cold allodynia [Figure 5b]. The maximum time of paw withdrawal afterXA administration was increased by 91.8 ± 5.6% [Figure 5b] which occurred at the highest dose. The administration of pregabalin (10–100 mg kg⁻¹) inhibited cold allodynia (F(3,28) = 18.04, P < 0.0001); the highest dose produced anti-allodynic effect of 111.4 ± 5.19% [Figure 5c]. XAE was more efficacious than pregabalin and XA. Pregabalin was also more efficacious than XA [Table 1 and Figure 6].

**DISCUSSION**

The results of the current study show clearly that XAE, XA, and pregabalin ameliorated vincristine-induced tactile and cold allodynia, as well as mechanical hyperalgesia.
Systemically administered vincristine is known to destroy Schwann cells and neurons of the dorsal root ganglion. This causes recruitment of macrophages which in turn cause the release of inflammatory cytokine IL-6, which elicits neuroinflammation and activates Janus kinase (Jak) - transcription-3 (STAT3) pathway (Jak-STAT3 pathway), leading to neuropathic pain. The p38 MAP kinase pathway also contributes to the development and maintenance of neuropathic pain in the CNS when it is activated by proinflammatory cytokines such as TNF-α. [28-30]

Castrillo et al. (2001) proposed that kaurene diterpenes, of which XA is an example, inhibit p38 and/or ERK1 and ERK2 pathways leading to inhibition of NF-κB activation. [24]

This mechanism may contribute, at least in part, to the antinociception of XAE and XA in this experiment.

Changes in the PNS and CNS caused by systemic administration of vincristine leads to spontaneous activity of C- and Aδ-fibers resulting in spontaneous pain and abnormal sensations both peripherally and centrally. [31] It has also been demonstrated that small diameter C- and Aδ-fibers are mainly involved in the response to cold and intense mechanical stimuli whereas large Aβ-fibers (low threshold fibers) response to tactile stimuli. [32,33] With XAE and XA inhibiting vincristine-induced tactile and cold allodynia as well as mechanical hyperalgesia as demonstrated in this study, it is likely that XAE and XA may be inhibiting pain stimuli propagation in the
Degenerated unmyelinated and myelinated C-, Aδ-, and Aβ-fibers

Neuropathic pain states, especially the cancer pain type, are known to be opioid resistant due to downregulation of mu-opioid receptors in dorsal spinal cord. This has been identified to be mediated through the activation of NMDA receptors and protein kinase A.[34] Pregabalin is effective both experimentally as shown in this study and clinically in the management of neuropathic pain. This is because it is an antagonist on α2-δ1 Ca2+ channel subunit of N-type voltage-dependent calcium channels. Inhibition of calcium channels prevents neuronal excitability and other cellular enzymatic cascade reactions that lead to pain sensation.[35,36] Kaurenoic acid and XA have been reported to exert calcium channel-blocking effects.[10] It is therefore possible that XAE (containing kaurenoic acid and XA) and XA, among other mechanisms, may have blocked pain in this model by inhibiting calcium channels similar to pregabalin leading to inhibition of pain stimuli propagation. It has also been recently reported that the antinociceptive effect of XAE and XA involved inhibition of NMDA, adrenergic (β and α), and protein kinase A/C pathways.[11] This may therefore also account for the observed anti-hyperalgesia and anti-allodynia of XAE and XA in this neuropathic pain model since NMDA receptor antagonists or protein kinase C inhibitors have been shown to suppress the development of the hyperalgesia and allodynia in neuropathic pain states.[37,38]

**CONCLUSION**

The ethanolic fruit extract of X. aethiopica and its major diterpene xylopic acid have anti-allodynic and anti-hyperalgesic properties in vincristine-induced neuropathic pain.

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