ANTI-NOCICEPTIVE PROPERTIES OF THE ETHANOLIC EXTRACT OF FRUITS OF *XYLOPIA AETHIOPICA* (DUNAL) A. RICH (ANNONACEAE) AND ITS MAJOR CONSTITUENT, XYLOPIC ACID.

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST. This work has not been submitted for any other degree.

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ABSTRACT

The fruits of *Xylopia aethiopica* are traditionally used to treat malaria, fibroid (uterine), fungal infection, rheumatism, arthritis, amenorrhea, boil, haemorrhoids and flatulence whilst the crushed seeds are applied topically on the forehead in the treatment of headache and neuralgia.

TLC and HPLC analyses of the extract revealed the presence of several compounds. The isolated xylopic acid produced a single spot in a number of solvent systems including petroleum ether: ethyl acetate (1:9) and hexane: ethyl acetate (1:9) and a single peak in HPLC analysis certifying the level of purity of the compound

The extract and xylopic acid exhibited antinociception in all the pain models used. In the acetic acid-induced writhing test, the extract (30 – 300 mg kg^{-1}) ($F_{3,28}$=14.37, P<0.0001) and xylopic acid (10 – 100 mg kg^{-1}) ($F_{3,28}$=20.56, P<0.0001) significantly reduced abdominal writhes induced with acetic acid in mice with the highest dose of the extract inhibiting visceral nociception by 98.8 ± 0.8%. The highest dose of xylopic acid also inhibited visceral nociception by 93.8 ± 1.4%. Morphine (1 – 10 mg kg^{-1}) ($F_{3,28}$=9.77, P=0.00016) and diclofenac (1 - 10 mg kg^{-1}) ($F_{3,28}$=4.04, P=0.0165) used as controls in this model similarly exhibited significant antinociceptive activities in this test.

The extract (30 – 300 mg kg^{-1}) ($F_{3,28}$=6.93, P=0.0012) significantly attenuated mechanical hyperalgesia in the Randall-Selitto test with maximum possible effect of 110 ± 16.17% at the highest dose used. Similarly xylopic acid (10 – 100 mg kg^{-1}) significantly ($F_{3,28}$=4.86, P=0.0076) attenuated mechanical hyperalgesia with a maximum possible effect of 94.58 ± 21.6% at the highest dose in the same test.

The extract and xylopic acid were both relatively more effective in the Hargreaves thermal paw withdrawal test, (extract: $F_{3,64}$= 8.10, P=0.0338, xylopic acid: $F_{3,64}$= 7.11, P=0.03) compared to the tail flick test (extract: $F_{3,64}$= 6.47, P=0.045, xylopic acid: $F_{3,64}$= 19.5, P<0.0001).

For the acute and chronic musculoskeletal pain tests morphine was most efficacious. The extract was also more efficacious than xylopic acid. Xylopic acid was however more potent in reducing both the chronic muscle and knee pain whereas the extract was more efficacious in the chronic skeletal pain model.

The extract, xylopic acid and pregabalin ameliorated vincristine-induced neuropathic pain. Common symptoms experienced by patients with neuropathic pain such as mechanical hyperalgesia, tactile and cold allodynia were measured using Von Frey filaments and cold water. The extract ($F_{3,28}$=5.12, P=0.006), xylopic acid ($F_{3,28}$=3.72, P=0.0229) and pregabalin ($F_{3,28}$=5.92, P=0.0029) produced tactile anti-allodynia. Similar effects were observed in the Von Frey intermediate and mechanical hyperalgesia as well as cold allodynia tests.

In the formalin test the extract and xylopic acid inhibited both neurogenic and inflammatory phases of pain. The antinociception of the extract, xylopic acid and morphine involved the inhibition of opioid, NO-cGMP, 5-HT$_3$, adenosine and muscarinic pathways Further determination of the mechanism of antinociception of xylopic acid carried using binding assay revealed the binding of xylopic acid to μ-opioid receptors with an enhancement of endogenous opioid binding. Capsaicin-sensitive C-fibres-glutamatergic-nociceptive pathway was found to participate in the antinociception of extract and xylopic acid.

Tolerance to morphine antinociception on the opioid receptors developed after chronic treatment for eight days but failed to develop to the extract and xylopic acid. Also morphine tolerance did not cross-generalize to the extract and xylopic acid. In order to elucidate the drug—drug interaction between xylopic acid/morphine and xylopic acid/diclofenac in combination administrations, isobolographic analysis was performed. The experimental ED$_{50}$'s (Z$_{mix}$) of xylopic/morphine combination were smaller than their corresponding theoretical ED$_{50}$'s (Z$_{add}$) in both phases of the formalin test indicating synergism. Isobolographic analysis of xylopic acid/diclofenac combination carried out also indicated potentiation of the combination as the experimental ED$_{50}$ lay below the line of additivity.

The degree of potentiation calculated as interaction index indicated that the combination synergized to produce antinociception.

In summary these findings provide scientific data for the use of the fruit of *X. aethiopica* in the treatment of painful conditions and co- administration of xylopic acid/morphine and xylopic acid/diclofenac may be said to be beneficial as their various side effects may be reduced due to lower doses used with potentiation of their therapeutic effects.

ACKNOWLEDGEMENT

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ABBREVIATIONS

5 – HT	5-hydroxytryptamine
AIDS	Acquired immunodeficiency syndrome
AMPA	α-Amino-3-hydroxyl-5-methylsoxazole-4-propionic acid
COX	Cyclooxygenase
C RP	C – reactive protein
DNA	Deoxyribonucleic acid
ED_{50}	Dose of drug which elicits 50% of the maximum response
ERK	Extracellular signal-regulate
Extract/XAE	70% ethanolic extract of *Xylopia aethiopica*
GAFCO	Ghana agro food company
i.p.	Intraperitoneal
1CR	Imprinting control region

IASP	International association for the study of pain
IKK	IκB kinase
IL	Interleukin
KNUST	Kwame Nkrumah University of Science and Technology
LBP	Lipopolysaccharide-binding protein
L-NAME	N^G-nitro-arginine methyl ester
MOR	Mu opioid receptor
MPE	Maximum possible effect
NE	Norepinephrine
NIK	*NF-κB-inducing kinase*
NMDA	N-methyl D-aspartate
NO	Nitric oxide
NSAID	Nonsteroidal antiinflammatory drugs
p.o	Per os
PG	Prostaglandin

PWT	Paw withdrawal threshold
SERT	Serotonin transporter
SNRI	Serotonin and noradrenaline reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TNF	Tissue necrosis factor
WHO	World health organization
XA	Xylopic acid
Zadd	Theoretical ED_{50}

Chapter 1

1.1 GENERAL INTRODUCTION

The use of herbs for the treatment of diseases can be dated to pre-historic times. Medicinal application of natural products can be traced back to at least 5000 years. Traditional medicine has been practiced throughout the world well before the introduction of western medicine. Herbs have been used for healing over centuries, while Western medicine has a relatively short history of a few hundred years (Goldman, 2001). Records show that ancient Egyptian medicine in 1000 B.C. cultivated garlic, opium, castor oil, coriander, mint, indigo, and other herbs for medicine. The Old Testament also mentions the cultivation and use of herb including mandrake, vetch, caraway, wheat, barley, and rye (Girish and Shridhar, 2007). At present, there are more than 85,000 plant species that have been acknowledged for medical use globally (Balunas and Kinghorn, 2005). The World Health Organization (WHO) estimates that almost 75% of the world's population has therapeutic experience with herbal remedies (Liu and Wang, 2007). This is principally because of a belief that herbal remedies may have fewer side effects and can enhance the effects of conventional agents or be an alternative treatment, (Desai and Grossberg, 2003). In Ghana, it is anticipated that there is one traditional doctor to approximately four hundred (400) people as opposed to one allopathic or orthodox doctor to every twelve thousand (12,000) people. The fruit of *Xylopia aethiopica* are used to treat several human diseases including pain (Igwe *et al.*, 2003). Plants have served as sources of therapeutic agents. Several compounds such as aspirin (from *Salix alba*), morphine (from *Papaver somniferum*) used to treat pain were derived from plants (Balunas and Kinghorn, 2005). The fruits of *Xylopia aethiopica* are used to treat various painful conditions. The fruits contain among several compounds kaurenoic acid and xylopic acid (fig. 1.2). Kaurenoic acid has been reported to possess anti-inflammatory as well as analgesic properties (Block *et al*., 1998). Interestingly there is no scientific report on the analgesic properties of xylopic acid although it is closely related to

kaurenoic acid. In the light of the above, ethanolic fruit extract of *Xylopia aethiopica* and its major constituent xylopic acid were investigated for their anti-nociceptive properties.

XYLOPIA AETHIOPICA

Figure 1.1 Dried Fruits of *Xylopia aethiopica*

Botanical source: from dried fruits of *Xylopia aethiopica* of family Annonaceae.

Local names;   Twi----- Hwenteaa (slender nose)

                Hausa –Kimba

                Ewe-----Etso

                Fante ---Hwentia

1.1.1   Plant description

Xylopia aethiopica is a composite name derived from the Greek word 'xylon pikron' meaning 'bitter wood' and aethiopica refers to its Ethiopian origin. It is a slim, tall, evergreen, aromatic tree 15–30 m high and about 60–70 cm in diameter with straight stem. It has grey-brown, smooth or finely vertically fissured and peeling easily bark.

The leaves are simple, elliptical, alternate, oblong to ovate, cuneate or slightly rounded at the base, measuring 4-10 cm long and 2-4 cm broad with lateral nerves and entire margin (Orwa *et al.*, 2009).

 The tree bears fruit of about a dozen narrow carpels which are stalkless with white to pale-yellowish brown wood. The fruit appear greenish with 20-30 capsules which turn red when ripe and black when dried. In West Africa, fruiting occurs in December-March and June-September and harvesting time runs from February to May and again from August to October. The fruits are harvested with the inflorescence. Typical fruit yields are about two to three metric tons per annum per hectare (Orwa *et al.*, 2009).

The plant grows rapidly during the first three years. The trees are planted eight meters apart. In Ghana, it has been successfully intercropped with other staple food items in the first four years (Orwa *et al.*, 2009).

1.1.2   *Geographical distribution*

The plant occurs as fringing and deciduous in forests and sometimes cultivated in homes and gardens. The plant is widely distributed in Ghana, Democratic Republic of Congo, Ethiopia, Kenya, Mozambique, Nigeria, Senegal, Tanzania and Uganda (Orwa *et al.*, 2009).

1.1.3   *Uses of Xylopia aethiopica*

 Various parts of *Xylopia aethiopica* are used to treat diverse diseases which can be grouped as follows:

Fruits: The fruits are used to treat malaria, fibroid (uterine), fungal infection, amenorrhea, syphilis, rheumatism, arthritis, boil, haemorrhoids, flatulence, bronchitis, hypertension, diabetes, dysentery (Sofowara, 1978; Iwu *et al.*, 1992; Nnodim *et al.*, 2011) and female infertility (primary and secondary) (Igwe *et al.*, 2003; Tatsadjieu *et al.*, 2003; Ghana Herbal

Pharmacopoeia, 2007). The fruits are smoked and inhaled to treat cough, stomach ache, dizziness, amenorrhoea, bulimia (eating disorder), lumbago and neuralgia. It is also used a calmative, purgative, repulsive to pain, and in the treatment of boils and skin eruptions. The crushed, powdered fruit mixed with shea butter and coconut oil is used as creams, cosmetic products and perfumes (Tatsadjieu *et al.*, 2003; Ghana Herbal Pharmacopoeia, 2007; Orwa *et al.*, 2009).The dried fruits are also important as flavourings to prepare local soups in West Africa.

Seeds: The crushed seeds are applied topically on the forehead to treat headache and neuralgia. Again the seeds are taken as a decoction or concoction or chewed and swallowed in the treatment of haemorrhoids' numbness, epilepsy and anaemia (Igwe *et al.*, 2003).

The seeds were used by traditional medicine practitioners and traditional birth attendants to induce placental discharge postpartum. Due to its (*X. aethiopica*) traditional usefulness after child birth, it was employed in government hospitals in Ghana for its abortifacient properties (Igwe *et al.*, 2003).

Leaves: The leaves are used to treat dyspepsia, laryngitis, splenomegaly and headache (Igwe *et al.*, 2003).

Stem bark: The stem bark is used for dysentery and female infertility (primary and secondary). The stem serves as a good source of firewood. The wood is used as a general purpose timber in tool handles, beds, oxen yokes, knife sheaths and spear handles (Tatsadjieu *et al.*, 2003; Orwa *et al.*, 2009).

Roots: The odoriferous roots of the plant are employed in tinctures, administered orally to expel worms and other parasitic animals from the intestines or in teeth rinsing and mouth wash extracts against toothaches and as dressing for the treatment of cancer (Asekun and Adeniyi, 2004; Orwa *et al.*, 2009).

1.1.4 Some identified compounds in the fruits of X. aethiopica

The fruits of *X. aethiopica* have been shown to contain about 98 compounds (Konan *et al.*, 2009) and among them are essential oil, volatile oil, resin, arocene, rutheroside fat, bitter principles, alkaloids, glycosides, saponins, tannins, mucilages, xylopic acid (fig. 1.2) and kaurenoic acid. Carbohydrate, protein and free fatty acid have been identified in the fruit and these confer on the fruit some nutritional values (Igwe *et al.*, 2003).
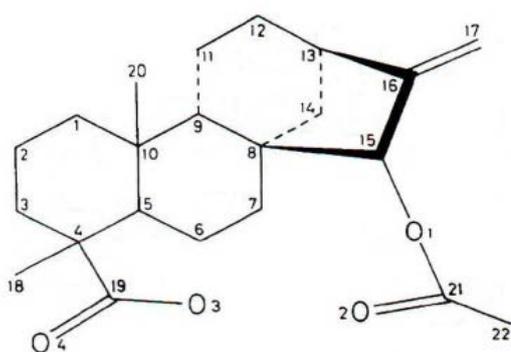
Molecular weight: 360.487 g/mol

Molecular formula: $C_{22}H_{32}O_4$

Figure 1.2 Structure of Xylopic acid

1.1.5 Bioactivities of fruit extracts of Xylopia aethiopica and xylopic acid

Antimicrobial properties

• Xylopic acid and other diterpene isolates have been reported to exhibit antimicrobial action against gram positive and negative bacteria and *Candida albicans* (Boakye-Yiadom *et al.*, 1977).

• Antimicrobial and antifungal activity of ethanolic extract of the fruits hve also been demonstrated (Boakye-Yiadom *et al.*, 1977)

• Xylopic acid and acetylgrandifloric acid (ent-kaur-16-en-15 alpha-acetyloxy-19-oic) an epimer of xylopic acid (ent-kaur-16-en-15 beta-acetyloxy-19-oic) are reported to exhibit antibacterial activity (Davino *et al.*, 1989).

Insecticidal

Hexane and alcoholic extracts of *X. aethiopica* have been shown to posses termite anti-feedant activity (Lajide *et al.*, 1995).

Cardiovascular effects

Extract of the fruits, xylopic acid and kaurenoic acid have been reported to posses diuretic and vasorelaxant properties in rodents (Somova *et al.*, 2001).

Analgesic and Anti inflammatory effects

- Kaurenoic acid from the extract has been reported to exhibit analgesic and anti-inflammatory activities in rodents (Ekong and Ogan, 1968; Ogan 1971; Block *et al.*, 1998).
- The fruit of *Xylopia aethiopica* has been shown to possess antioxidant properties (Nnodim *et al.*, 2011).

Ocular and hypoglycaemic effects

The fruit of *Xylopia aethiopica* has been demonstrated to act as hypolipidemic and hypoglycaemic agent (Nnodim *et al.*, 2011).

Aqueous fruit extract of *Xylopia aethiopica* has been shown to reduce intraocular pressure in human subjects (Uzodike and Onuoha, 2010). In the light of the above the fruits of *Xylopia aethiopica* were screened for possible effects against pain

1.2   Pain

Pain is an unpleasant experience that is primarily associated with tissue damage or described in terms of tissue damage or both (Merskey, 1994). It forms part of most diseases and it is usually the major factor of the disease that alerts the patient to seek medical treatment (Schim and Stang, 2004). Pain is subjective and that makes the patient the only one to describe the intensity and quality of the pain (Clancy and Mcvicar, 1992). Pain impacts negatively on quality of life affecting several aspects of health and well-being

including relationships, cognitive abilities and the capacity to work (Wilhelm *et al.*, 2009). Despite the frequency of pain symptoms, individuals often do not obtain satisfactory relief of pain and the reason for this is attributable to inappropriate or insufficient use of existing therapies (Mcmahon and Koltzenburg, 2006; Chen and Tang, 2011). Pain experienced by patients may be due to the demand for the activation of recuperative motivational system needed for restoration of one's health (Mcnally, 1999). The result of this is the activation of recuperative motivational system by pain that leads to promoting rest and body care at the same time inhibiting other types of motivation such as hunger, thirst, and exploration. The behavioural changes produced by sickness are similar to those produced by pain. Indeed there is a clear parallel between the functional significance of pain and febrile illness. Thus, just as analgesia may be an important component of an animal's defensive behavioural repertoire so too may pain be an important component of an animal's recuperative behavioural repertoire. In fact pain is now considered the fifth vital sign, thus making pain assessment equally important as obtaining a patient's temperature, pulse, blood pressure, and respiratory rate (Chapman, 2005).

1.2.1 Classifications of pain

Pain can be classified as Nociceptive or Non Nociceptive pain (Kamal and Abdulrahman, 2002). Nociceptive pain can further be divided into Somatic and Visceral pain while non nociceptive pain can be divided into neuropathic pain and sympathetic pain. Nociception is defined as the neural processes of encoding and processing noxious stimuli. This activity is initiated by nociceptors, (also called pain receptors), that can detect mechanical, thermal or chemical changes, above a set threshold. Once stimulated, a nociceptor transmits a signal along the spinal cord to the brain (Loeser and Treede, 2008).

1.2.2 Nociceptive pain

Nociceptive pain is the type of pain that arises from stimulation of nociceptors from somatic (body wall) and visceral (internal organs) structures. Nociceptive pain, similar to neuropathic pain can occur both in the acute and chronic form. The sensory input is mediated via nociceptors such as 5-HT_3, bradykinin and vanilloid receptors coupled to excitatory, cation-permeable ion channels. This causes sensitization of nocisponsive peripheral afferent fibres by the engagement of intracellular transduction systems including the activation of adynylyl cyclase and phospholipase C leading to a further increase in the intensity and duration of nociceptive impulse discharges (Mcnally, 1999). Higher brain centres such as periaqueductal gray matter, raphe nucleus and anterior cingulated cortex have been associated with nociception (Millan, 1999; Loeser and Treede, 2008).

1.2.2.1 Acute Pain

Acute pain is short lasting and usually manifests in ways that can be easily described and observed. It can be described as fulfilling physiological roles by its inherent protective nature (Millan, 1999). The alerting function of acute pain reflects the phasic activation of sensors (nociceptors) by potentially dangerous stimuli exceeding the physiological range but just below tissue damaging threshold. The response to this alert may manifest as sweating or increased heart rate. The sensation of pain of acute form is one of the vital functions of the nervous system. If the causes are not eliminated, it persists and becomes chronic generating fear and anxiety (Ito *et al.*, 2001). Intensity of acute pain is from mild to severe and lasts less than 3 to 6 months (Calvino *et al.*, 1992; Schim and Stang, 2004; Abdel-Salam and El-Batran, 2005).

1.2.2.2 Chronic Pain

Chronic pain is defined as pain lasting for more than 6 months. It is much more subjective and not as easily described as acute pain. It persists beyond the point at which healing

should occur and effectively treating this type of pain poses a great challenge for physicians (Deleo, 2006). This type of pain is described as pathological (Millan, 1999) and arises from several disease states such as musculoskeletal pain and cancer pain. Chronic, unremitting pain in deep tissues that results from cancer, cancer chemotherapy or musculoskeletal disorders adversely affects a disproportionately large portion of the population. Approximately 14 million people have cancer worldwide (Kehl *et al.*, 2003). More than 70% of patients in advanced stages of this disease experience chronic pain (World Health Organization, 1996) and more than 36% of patients with cancer experience pain sufficiently severe to interfere with normal function (Kehl *et al.*, 2003). Non-malignant musculoskeletal pain relatively affects even larger share of the population and is one of the most frequent symptoms for which medical assistance is sought (Kehl *et al.*, 2003).

1.2.3   *Mechanism of nociception*

1.2.3.1   *Peripheral transmission of pain and wind-up*

Tissue or nerve injury leads to the production and release of a number of chemical mediators including bradykinin, serotonin and prostaglandins, which can originate locally or from cells that infiltrate the site of injury. A mixture of these agents known as inflammatory 'soup' contributes to changes in vascular permeability, resulting in erythema and oedema. The inflammatory 'soup' also sensitizes peripheral nociceptors primarily C-fibres by initiating a cascade of events that change ionic conductance of the peripheral nociceptor terminal. The nociceptive input is further processed spinally (Millan, 1999; Ito *et al.*, 2001).

1.2.3.2   *Central transmission of pain*

The central analgesia system is mediated by 3 major components: the periaqueductal grey matter, the nucleus raphe magnus and the nociception inhibitory neurons within the dorsal horns of the spinal cord, which act to inhibit nociception-transmitting neurons also located

in the spinal dorsal horn. Pain transmission through the spinal cord involves the lateral spinothalamic tract pathway. The lateral spinothalamic tract has two pathways for nociceptive information to reach the brain: the neospinothalamic tract for "fast spontaneous pain" and the paleospinothalamic tract for "slow increasing pain". The neospinothalamic tract is responsible for fast pain which travels via type Aδ fibers to terminate on the dorsal horn of the spinal cord where they synapse with the dendrites of the neospinothalamic tract. The axons of these neurons travel up the spine to the brain and cross the midline through the anterior white commissure, passing upwards in the contralateral anterolateral columns and then terminate on the ventrobasal complex of the thalamus and synapse with the dendrites of the somatosensory cortex. The paleospinothalamic tract is involved in slow pain transmission via slower type C fibers to laminae II and III of the dorsal horns, together known as the substantia gelatinosa. Impulses are then transmitted to nerve fibers that terminate in lamina V, also in the dorsal horn, synapsing with neurons that join fibres from the fast pathway, crossing to the opposite side via the anterior white commissure, and travelling upwards through the anterolateral pathway. These neurons terminate throughout the brain stem, with one tenth of fibres stopping in the thalamus and the rest stopping in the medulla, pons and periaqueductal grey of the midbrain tectum (Millan, 1997; Millan, 2002; Kivell and Prisinzano, 2010).

1.2.4   Modulation of nociceptive processing

1.2.4.1   Descending Facilitation

Descending pathways exerts both inhibitory and facilitator actions in the dorsal horn. The neurotransmitters found in the spinal cord may exert multiple actions in the dorsal horn as a function of the type of neurone targeted and the receptor activated. In chronic pain states, impairment of the descending pain inhibitory system gives rise to an enhanced descending pain facilitatory system. This is chiefly mediated via the glutamatergic pain system. Moreover, descending inhibitory and facilitatory pathways to the dorsal horn may even be derived from the same structure. There is evidence that descending facilitatory mechanisms

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exert excitatory actions both on the terminals of nociponsive peripheral afferent neurons, as well as on intrinsic dorsal horn neurones (Jones, 1992; Millan, 1997).

1.2.5 *Descending inhibition*

Descending pathways originating in the brainstem and other cerebral structures play an important role in the modulation and integration of nociceptive messages in the dorsal horn. Serotoninergic, noradrenergic and, to a lesser extent, dopaminergic networks comprise major components of these descending mechanisms. The descending pathways generally inhibit nociception by modulating the release of neurotransmitters from nociceptive terminals. Further activation of descending pathways directly and indirectly inhibits nocisponsive peripheral nerve. This action is mediated through the inhibition of excitatory interneurones and the excitations of inhibitory interneurones (Fields and Basbaum, 1994; Lopez-Garcia and King, 1996).

1.2.6 *Models of nociceptive pain*

To gain deeper insight and understanding into the mechanism of pain in order to develop safer and more effective analgesics, various animal models have been developed to elucidate the mechanisms of these painful states and to evaluate analgesics for exploring effective treatment. These animal models have been often extended to behavioural analyses of knockout mice in combination with the evaluation of pro- and anti-nociceptive agents (Millan, 1999). The models can be grouped into acute, inflammatory, persistent and neuropathic pain models. Some acute models of animal nociceptive test discussed below are the hot plate test, Tail flick test and acetic acid-induced writhing test.

The hot plate test employs heat source of stimulus in pain induction. The paw of the animal is heated by contact with a hot plate (50–56 ° C) and the latency time to paw licking or withdrawal is measured. This test is similar to the Tail flick or Tail withdrawal thermal test of nociception. In this test the distal portion of the tail is heated by a radiant heat source and the latency to tail flick is recorded (Kamal and Abdulrahman, 2002).

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The Randall–Selitto test employs the application of linearly increasing pressure (range: 0–450 g) between the third and fourth metatarsals of the hind paw via a blunt perspex cone until the rat vocalises or withdraws the paw. This value is recorded as the threshold for withdrawal from a mechanical stimulus (Ito *et al.*, 2001).

The Acetic acid writhing test also considered as a visceral pain model employs the intraperitoneal injection of 0.9% acetic acid into rodents and the frequency and/or duration of stretch responses is counted for 30 min (Ito *et al.*, 2001).

Animal models of inflammatory pain use agents capable of inducing inflammatory response. Intraplantar injection of carrageenan or complete Freund's adjuvant (CFA) model is such model. Chronic inflammatory conditions produce a state of hyperalgesia that is evident from a few hours to days after peripheral injection of chemical irritants such as carrageenan, dextran, formalin, yeast, turpentine, CFA, and mustard oil (Shivkar and Kumar, 203; Northover and Subramanian, 1962; Di Rosa *et al.*, 1971). Carrageenan is also often used for production of the hyperalgesic state in the animal following intraplantar injection (Winter and Risley, 1962; Ito *et al.*, 2001). The hyperalgesic state is assessed by applying thermal or mechanical stimulus to the inflamed and normal paws.

The Formalin test also considered as a persistent model of animal nociception employs injection of a dilute solution of formalin (1–5%) into the hind paw. This produces two distinct phases of pain-like behaviour. The first results from activation of primary afferent fibres, while the second is believed to represent both inflammation-evoked sensory activity and facilitatory processes in the spinal cord resembling prolonged injury-induced hyperalgesia states (Millan, 1999; Ito *et al.*, 2001; Kamal and Abdulrahman, 2002).

1.2.7   *Non nociceptive pain*

Neuropathic pain refers to the pain caused by damage to nervous tissue. Pain elicited by peripheral nerve injury is sometimes used synonymously with `neuropathic' pain although it also incorporates the `central' pain associated with damage to the CNS (Millan, 1999).

Patients with neuropathic pain frequently report sensory abnormalities including burning sensations, exaggerated responses to noxious stimuli, pain sensations resulting from innocuous stimuli (allodynia) and spontaneous pain episodes (dysesthesia). Neuropathic pain can also alter the patient's quality of life by interfering with emotional well-being (Benbouzid *et al.*, 2008). Because of its severity, chronicity and resistance to some classical analgesics neuropathic pain is a challenge in clinical practice (Backonja and Stacey, 2004; Irving, 2005; Martin *et al.*, 2007). Of the symptoms of this pain type mechanical allodynia comprises the most striking perturbation of sensation. Also allodynia to cold may be pronounced, in particular in sympathetically maintained painful states. This pattern of altered sensitivity in neuropathic pain clearly differs to the primary (heat) hyperalgesia of nociceptive pain, in which cooling of the site of injury may even reduce pain, and more closely resembles the secondary hyperalgesia which accompanies tissue damage in stroke (Millan, 1999).

1.2.8 *Models of neuropathic pain*

Several chemotherapy-induced peripheral neuropathy models have been developed and are used to study the underlying mechanisms of neuropathic pain. Although all of these models feature some degree of direct neuronal damage, they differ in the time course and pathomechanisms that are associated with the development of hyperalgesia. Examples include pain associated with traumatic injury—phantom limb pain after amputation (Bennett and Xie, 1988; Seltzer *et al.*, 1990; Kim and Chung, 1992; Decosterd and Woolf, 2000), chemically induced nerve damage—neuropathy caused by cancer chemotherapies. An example of the chemotherapy-induced neuropathic pain is vincristine-induced neuropathy. The development of vincristine-induced neuropathy is a formidable clinical problem as it is stubbornly resistant to existing pharmacotherapy, such as the NSAIDs, opioids and steroids (Higuera and Luo, 2004).

Other experimental animal models for neuropathic pain, such as diabetic neuropathy, inflammatory neuropathy, nerve-injured neuropathy and neuropathic herpes pain share

common characteristics with vincristine-induced neuropathy. For experimental animal models for diabetic neuropathy, streptozotocin-induced diabetic animals are used as well as spontaneous diabetic BB/Wor rat rodents (Courteix *et al.*, 1998; Zhang *et al.*, 2002). There are three major experimental animal models for the nerve-injured neuropathy, Seltzer model, Bennett model and Chung model. In Seltzer model also known as the partial ligation model, the half of sciatic nerve is tightly ligated, while the whole sciatic nerve is loosely and constrictively ligated in Bennett model also known as the chronic constriction injury model. In contrast, the segmental spinal nerve (especially L5 or L6) is ligated in the Chung model (Mizoguchi *et al.*, 2009).
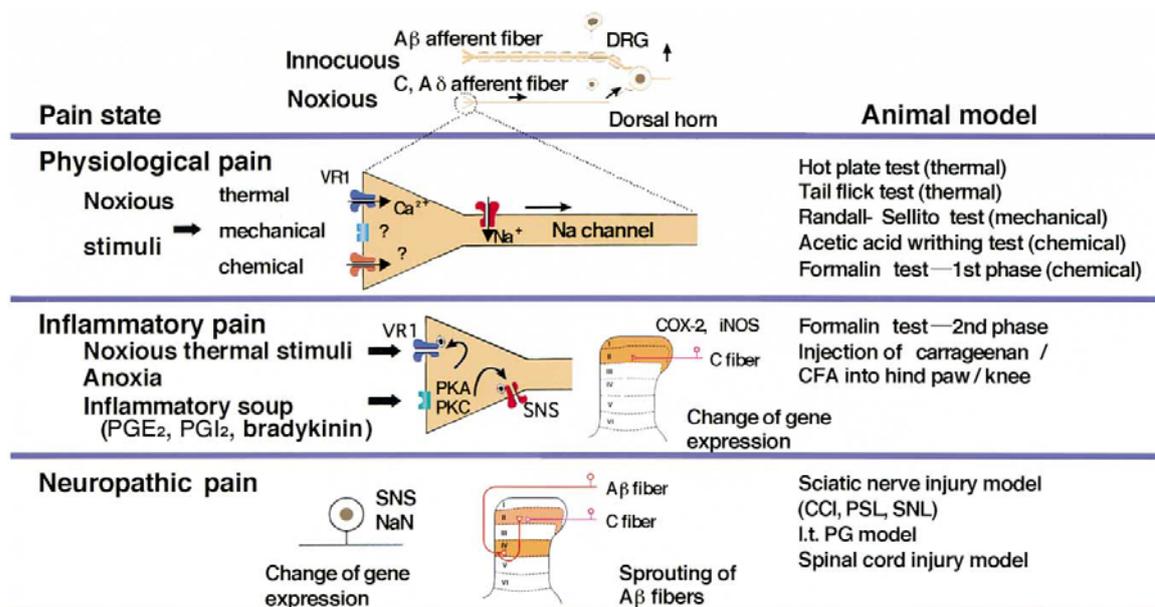
Figure 1.3 Summary of pain states and animal models.

1.2.9 Cross talk between the immune system and pain states

Most of the ongoing immune surveillance in healthy peripheral nerves is performed by immune cells that reside within the nerve itself (Moalem and Tracey, 2006). Resident immunocompetent cells include Schwann cells, fibroblasts, endothelial cells, dendritic cells, macrophages, and mast cells. Immunocompetent refers to cells that can respond to

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inflammation, infection, and/or trauma by the production and release of inflammatory mediators classically thought of as immune-derived. In healthy nerves, these cells are "resting" in the sense that they provide active surveillance of the nerve's microenvironment but are not releasing proinflammatory mediators as they do upon activation. With the exception of circulating activated T lymphocytes (Watkins *et al.*, 2007), blood-borne immune cells have relatively limited access to peripheral nerves under normal circumstances, due to the blood–nerve barrier (Olsson, 1990).This scenario changes dramatically upon trauma to peripheral nerves inflammation. Upon activation by nerve trauma or inflammation, a number of these immune cells release chemo attractant cytokines called "chemokines" (e.g., macrophage inflammatory protein-2 and monocyte chemo attractant protein-1 [MCP-1]) that recruit neutrophils and macrophages from the damage myelin, and disrupt the blood–nerve barrier, thus further facilitating the movement of immune products into damaged nerve. In addition, some activated resident immune cells release degradative enzymes and acids in response to nerve trauma that exposes peripheral nerve proteins (e.g., P0, P2). Nerve proteins such as P0 and P2 are responded to as "non-self" as they are normally buried within the myelin sheath and not detected by immune cells. Once released, immune derived enzymes and acids attack myelin and disrupt the blood–nerve barrier, again allowing increased access of the nerve to blood-borne immune cells (Olsson, 1990). Clinically relevant peripheral nerve damage can occur as a result of antibody attack, complement activation, and T-lymphocyte attack, in addition to frank trauma and inflammation. Immune cells activated as a result of partial nerve injury importantly contribute to the resultant exaggerated pain state as well as axonal hyper excitability and Wallerian degeneration (Moalem and Tracey, 2006; Watkins *et al.*, 2007). Nerve injury leads to an immediate activation of calpain, a calcium-activated protease in Schwann cells and associated myelin. Calpain, in addition to destroying myelin, causes a rapid burst of proinflammatory cytokine and chemokine release from injured Schwann cells that, in turn, leads to the recruitment of circulating neutrophils and macrophages to the injury site and further proinflammatory cytokine and chemokine release (Moalem and Tracey, 2006; Watkins *et al.*, 2007). Driven by TNF and IL-1, the extracellular protease matrix metalloproteinase-9 (MMP-9) is induced in

Schwann cells and macrophages, contributing to neuropathic pain, neurovascular permeability, immune cell recruitment, demyelination, and degeneration. The anti-inflammatory cytokine IL-10 also rapidly falls after injury, further disrupting the balance between pro- and anti-inflammatory cytokine influences. Both trauma-induced Wallerian degeneration and enhanced pain (Moalem and Tracey, 2006; Watkins *et al.*, 2007) have been associated with the activity of macrophages recruited to the site of injury. Simply delaying macrophage recruitment to the site of nerve injury delays both the development of neuropathic pain and Wallerian degeneration. Conversely, actively attracting macrophages to the injury site enhances neuropathic pain. Activated macrophages have been found to persist for years within human painful neuromas, suggestive that they may perseveratively influence pain from peripheral nerve damage (Durrenberger *et al.*, 2006). Recent data have also provided evidence that, in addition to macrophages, mast cells, T lymphocytes, neutrophils, and schwann cells all contribute to neuropathic pain at the site of nerve injury. Also, products produced by immune cells beyond proinflammatory cytokines have now been implicated, including reactive oxygen species, NO, growth factors, prostaglandins, activation of the complement cascade, and mast cell products. Tryptase released from mast cells activates protease-activated receptor-2. This tryptase receptor modulates the function of transient receptor potential vanilloid-1 (TRPV1) channels, lowering their threshold for activation from 42 °C to well below body temperature (Moalem and Tracey, 2006; Watkins *et al.*, 2007). As functional TRPV1 channels are expressed mid axon, this provides a mechanism whereby mast cell degranulation could directly increase spontaneous activity and excitability of sensory neurons. This may have clinical relevance as mast cells remain elevated at sites of nerve trauma long after injury and mast cell degranulation/release has been proposed to contribute to neuroma pain in humans (Watkins *et al.*, 2007). Further support for the importance of immune cells in neuropathic pain comes from the study of clonidine, an alpha2-adrenoceptor agonist. While alpha-2 adrenoceptors are absent from normal nerves, levels elevate in injured nerve due to expression by recruited macrophages, lymphocytes, and other immune cells (Watkins *et al.*, 2007).

1.2.10 *Management of pain*

Various techniques have been employed in pain management including pharmacotherapy and non pharmacotherapy procedures. The correct diagnosis and proper treatment of pain is an important public health concern. Millions of people in the world with severe acute and chronic pain suffer because of the ignorance of doctors and the lack of a standardized scientific approach to pain management as reported in the WHO normative guidelines on pain management, Geneva, June, 2007.

Pharmacological management of pain is the mainstay of pain therapy. Almost half of individuals who suffer from pain choose a non prescription analgesic as their initial choice for pain relief. Up to one in five Americans take an over-the-counter or prescription analgesic on a daily basis (Berry *et al.*, 2001). Treatment of pain with analgesic is in three steps as recommended by WHO. The first step in this approach is the use of acetaminophen, aspirin, or another Non-steroidal Anti-inflammatory drug for mild to moderate pain. Adjuvant drugs to enhance analgesic efficacy, treat concurrent symptoms that exacerbate pain, and provide independent analgesic activity for specific types of pain may be used at any step.

When pain persists or increases an opioid such as codeine or hydrocodone is added (not substituted) to the NSAID. Opioids at this step are often administered in fixed dose combinations with acetaminophen or aspirin. When higher doses of opioid are necessary, the third step is used. At this step separate dosage forms of the opioid and non-opioid are used.

Pain that is persistent or is of moderate to severe intensity from the outset should be treated by increasing the dosage or with more potent opioids. Drugs such as codeine or hydrocodone are replaced with more potent opioids like morphine, hydromorphone and methadone. Commonly prescribed drugs for pain are the NSAIDs. The primary mechanism of action of NSAIDs is inhibition of the enzyme cyclooxygenase (COX) which blocks prostaglandin synthesis. Acetaminophen, another nonopioid, appears to act mostly via a central mechanism (Kamal and Abdulrahman, 2002). All nonopioids have anti-

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inflammatory, antipyretic, and analgesic effects, but the anti-inflammatory effect of acetaminophen is essentially negligible. The analgesic effect of NSAIDs is prompt (minutes to hours), whereas the anti-inflammatory effect may take longer (1-2 weeks or longer) (Field and Cassel, 1997). This latter effect can indirectly relieve some pain by reducing tissue swelling. NSAIDs relieve a variety of types of acute and chronic pain (e.g., trauma, postoperative, cancer, arthritis pain) and are especially effective for certain types of somatic pain. Acetaminophen and NSAIDs, alone, often relieve mild pain, and some NSAIDs relieve certain types of moderate pain (Besson and Chaouch, 1987).

Moderate to severe pain that does not respond to non opioids are treated with opioids as stand alone or with NSAIDs. Opioids play a major role in the treatment of acute pain (e.g., trauma, postoperative pain), breakthrough pain, cancer pain, and some types of chronic non cancer pain. Opioids bind to opioid receptors in the central and peripheral nervous system to inhibit the transmission of nociceptive input from the periphery to the spinal cord, activate descending inhibitory pathways that modulate transmission in the spinal cord, and alter limbic system (Ferreira and Nakamura, 1979; Price *et al.*, 1989).

Antiepileptic drugs such as pregabalin, gabapentin, carbamazepine, phenytoin and valproic acid are types of adjuvant analgesic approved for the management of trigeminal neuralgia, migraine prophylaxis and neuropathic pain especially lancinating (i.e., episodic shooting, stabbing, or knife-like) pain from peripheral nerve syndromes. They act to reduce pain by reducing membrane excitability and suppress abnormal discharges in pathologically altered neurons (Berry *et al.*, 2001).

1.3   JUSTIFICATION OF THE STUDY

Pain accompanies almost every disease state and it is usually the major factor of the disease that alerts the patient to seek medical treatment (Schim and Stang, 2004). Pain is the most common reason for medical appointments. Pain results in an estimated 40 million hospital visits annually costing an estimated $100 billion each year in health care loss in United States. This has been attributed to the lack of effective analgesics or their inappropriate

usage (Kivell and Prisinzano, 2010). Pain is a leading cause of medically related work absenteeism and results in more than 50 million losses of work days per year in the United States. About 25% of the population in industrialized nations suffers from chronic pain severe enough that they miss days of work. Poorly treated pain can lead to metabolic disorders by altering the release of multiple hormones (e.g., ACTH, cortisol, catecholamines, insulin) manifesting clinically as weight loss, fever, increased respiratory and heart rate. On the cardiovascular system poorly managed pain can lead to unstable angina, myocardial infarction and deep vein thrombosis. On the Respiratory system, poorly managed pain leads to decreased air flow due to involuntary (reflex muscle spasm) and voluntary ("splinting") mechanisms that limit respiratory effort manifesting clinically as Pneumonia and Atelectasis (National Pharmaceutical Council, 2001). Individuals with chronic pain often face long-term or permanent unemployment or underemployment. Such loss can be recovered if analgesics of improved quality and less costly are developed .This is because NSAIDs, steroids and opioids, most prescribed drugs for pain have limited use due to their side effects (Kivell and Prisinzano, 2010). This underscores the need to search for newer therapeutic agents which are relatively safer and have improved efficacy. It is undisputable that plants have served as great stores of these active principles (therapeutic agents) and for that reason many plants are used extensively as drugs though without scientific evidence of safety or efficacy (Johnson *et al.*, 2007). Interestingly, the predominant pathophysiological processes involved in pain are subject to modulation by several compounds or metabolites obtained from plants (Duwiejua and Zeitlin, 1993). One of such therapeutically useful medicinal plant in Ghana and other parts of Africa is the fruit of *Xylopia aethiopica* which is used traditionally to relieve arthritic and colic pain, rheumatism, headache and neuralgia (Igwe *et al.*, 2003). Despite its local use as an analgesic, there is little scientific evidence to ascertain these claims. Interestingly, the traditional use of some herbs may just be based on community knowledge of existence and application of such herbs but not necessarily as a result of scientific validation of the herbs (Zhang, 2000) and the traditional use of *X. aethiopica* as analgesic is not an exception. This project therefore seeks to investigate the possible antinociceptive effect of ethanolic extract of the fruit of *X. aethiopica* thereby providing scientific evidence to the traditional use of

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the fruit as analgesic and further isolate and evaluate the antinociceptive potential of xylopic acid: the major compound in the fruit (Adosraku and Oppong, 2011) on which most of the properties of the fruits such as antibacterial, anti fungal, hypotensive and diuretic properties have been conferred. Dose and side effect determination can easily be carried out subsequently for xylopic acid and the extract thus standardizing the drug. Standardization and quality control of herbal products and herb-related investigations have been shown to proceed separation and determination of the active chemical constituents of the herbal product (Zhao *et al.,* 2006). This in effect will improve patient acceptability of the drug (xylopic acid) because of its plant origin, its use as a delicacy and predictable side effects.

1.3.1   *Aim of the study*

The aim of the study was to determine the antinociceptive activity of fruit extract of *Xylopia aethiopica* and xylopic acid and to provide scientific evidence to the use of the fruits as an analgesic.

1.3.2   *Specific objectives of the study*

To evaluate the antinociceptive activity of the ethanolic extract of the fruit of *Xylopia aethiopica* and xylopic acid using:

➢ Formalin test (Wilson *et al.*, 2002).
➢   Acetic acid-induced writhing test (Amresh *et al.*, 2007).
➢   Carrageenan-induced mechanical hyperalgesia model (Randall and Selitto, 1957).
➢   Musculoskeletal pain models (Radhakrishnan *et al.*, 2003; Sluka *et al.*, 2006).
➢ Vincristine –induced neuropathic pain model (Thibault *et al.*, 2008).
➢   To investigate the mechanism(s) of action of the ethanolic fruit extract of *Xylopia aethiopica* and xylopic acid using the formalin test (Wilson *et al.*, 2002), glutamate and capsaicin induced—nociception (Wilhelm *et al.*, 2009), and epinephrine, bradykinin, and prostaglandin E_2 -induced mechanical hyperalgesia (Meotti *et al.*, 2006).
➢   To study the drug – drug interaction between xylopic acid-morphine and xylopic acid-diclofenac co administration using isobolographic analysis (Miranda *et al.*, 2007).

Chapter 2

MATERIALS AND METHODS

2.1 PLANT COLLECTION AND EXTRACTION

The dried fruits of *Xylopia 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 at the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST. A voucher specimen (FP/09/77) has been kept at the herbarium of the Faculty.

2.1.1 *Preparation of 70% ethanolic extract of Xylopia aethiopica (XAE)*

Dried fruits of *X. aethiopica* (2 kg) were pulverized into fine powder. A quantity of 0.36 kg of the powdered material was placed in cylindrical jars and macerated with 70% ^{v}/v ethanol for three days. The filtrate was concentrated using a rotary evaporator at a temperature of 60 °C. This resulted in a greenish solid mass of ethanol extract of *Xylopia aethiopica* with a percentage yield of 34.8% (^{w}/w).

2.1.2 *Extraction of xylopic acid (15β-Acetoxy-(-) - kaur-16-en-19-oic Acid)*

The extraction process was similar to that as described by Ekoag and Ogan (1968). A quantity of the fruit (2 kg) was pulverized and 0.36 kg of the fine powder placed in cylindrical jars. This was soaked with 5 L of petroleum ether (40-60 °C) and allowed to stand for three days. The petroleum ether extract was collected and concentrated using a rotary evaporator at a temperature of 50 °C. Ethyl acetate (5 ml) was added to the concentrate to facilitate crystallization of xylopic acid. Crystals (xylopic acid) formed after the concentrate had been allowed to stand for five days were washed with petroleum ether (40-60 °C).

The xylopic acid obtained was purified by recrystalization in 96% ethanol. The resulting concentrated solution was filtered while hot and crystals of xylopic acid were

deposited after the solution cooled and stood for five days. The yield of the isolated/purified xylopic was 1.41% (^{w}/w).

The constituent(s) of the extract and xylopic acid were determined by thin layer chromatography. A number of solvent systems including ethyl acetate: petroleum ether (2:18) and hexane: ethyl acetate (1:9) were prepared and allowed to stand for 30 minutes. After dissolving the extract and xylopic acid in chloroform they were spotted on thin plates and allowed to stand in the tank until 10 cm mark was attained by the solvent front. Both thin plates were then sprayed with anisaldehyde.

Purity of the isolated xylopic acid was determined with high performance liquid chromatography (HPLC). The chromatograph consisted of LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied Biosystems) and Shimadzu CR501 Chromatopac. Phenomenex Hypersil 20 micron C18 200 × 3.20 mm column was used. The mobile phase consisted of methanol and water (9:1) eluted isocratically at 0.5 ml min^{-1}. Portions of 20 µl of a suitable concentration of pure xylopic acid were loaded and injected unto the column after dissolving in the mobile phase at 60 ^{o}C. The eluent was monitored at 206 nm. Portions of the extract and xylopic acid were loaded and injected. The peak(s) were noted as component(s) of the extract and xylopic acid.

Melting point determination was carried out on the crystals as well as the solubility of the crystals in petroleum ether, ethanol, methanol, ethyl acetate and chloroform. The purity and structure of the isolated xylopic acid were confirmed by the method of HPLC, mass spectroscopy and nuclear magnetic resonance (Adosrako and Oppong, 2011).

2.2   RESULTS

2.2.1   *Thin layer chromatography*

The extract revealed several spots which indicate the presence of several compounds (Plate 1a). On the contrary, xylopic acid revealed a single spot indicating the presence of a single compound (Plate 1b). The spot for xylopic acid occurred at 5.3 ± 0.01 for both the isolated xylopic acid and the xylopic acid present in the extract after the solvent front had been allowed to travel a total distance of 10 cm.

Solvent front

Start line

(a)   (b)
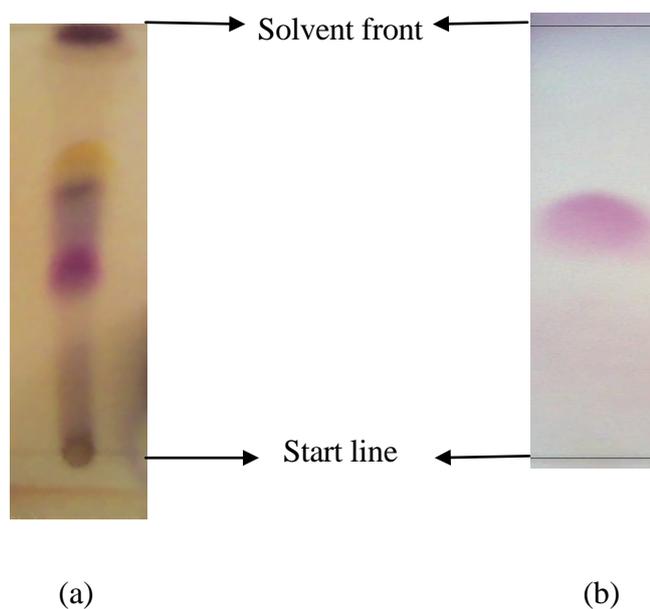
Plate 1 TLC results of (a) extract revealing several spots indicating the presence of several compounds and (b) xylopic acid showing a single spot indicating the presence of a single compound.

2.2.2   *High performance liquid chromatography and determination of some properties of xylopic acid*

HPLC was further used for the determination of the purity of the isolated xylopic acid. Several peaks were observed after loading the extract indicating the presence of several compounds in the fruits (Fig. 2.1a). A single peak was observed for xylopic acid indicating the presence of a single compound (Fig. 2.1b). The purity of xylopic acid was determined to be 96%. Mass spectroscopic and nuclear magnetic resonance analyses indicated that the compound isolated was xylopic acid (Adosrako and oppong, 2011)

Melting point of the crystals (xylopic acid) was determined to be 260-261 °C. The crystals were sparingly soluble in petroleum ether, ethanol, methanol, ethyl acetate and soluble in chloroform.

a

b
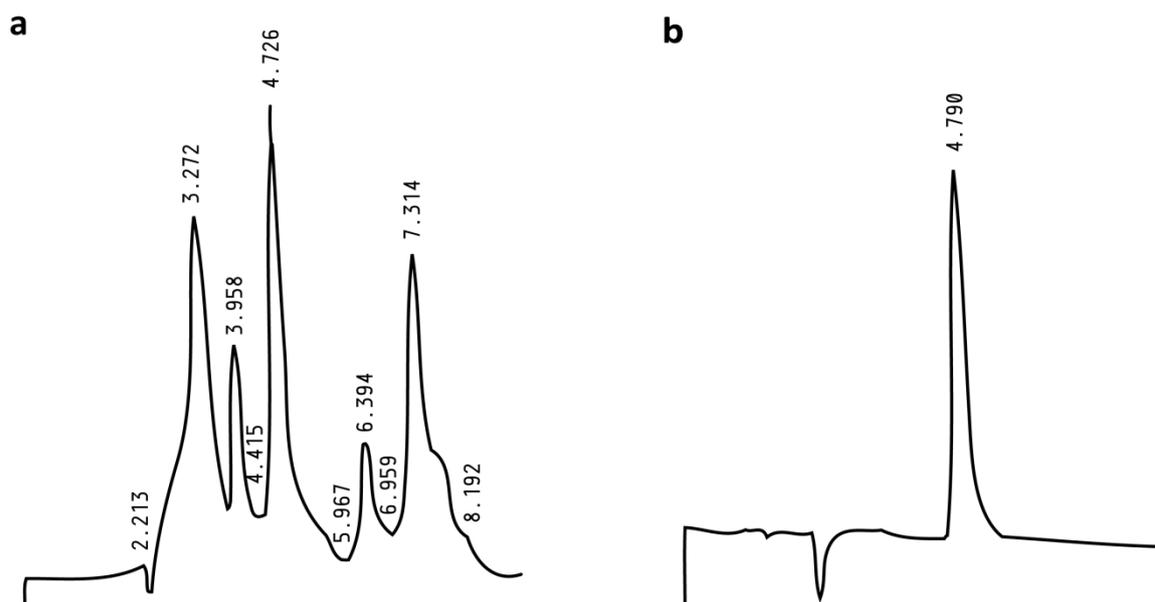
Figure 2.1 HPLC finger print of (a) extract showing several peaks of the various compounds in the extract and (b) xylopic acid showing a single peak.

2.3 Discussion

Thin layer chromatography analysis of the extract revealed several spots indicating the presence of several compounds in the extract. On the contrary, a single spot was observed for xylopic acid indicating the presence of single compound. High performance liquid chromatographic analysis of the extract revealed several peaks. These may correspond to any of the various compounds such as essential oil, volatile oil, resin, rutheroside fat, bitter principles, alkaloids, glycosides, saponins, tannins, sterols, carbohydrate, protein, free fatty acid, mucilage, kaurenoic and xylopic acid (Somova *et al.*, 2001; Igwe *et al.*, 2003) which have been reported to be present in the fruits. Xylopic acid, showed a single peak indicating the purity of the isolated xylopic acid.

2.3.1 **Conclusion**

The isolated xylopic acid can be said pure and had a melting point of 260-261 ° C (Ekoag and Ogan, 1968). The crystals were sparingly soluble in petroleum ether, ethanol, methanol, ethyl acetate and soluble in chloroform.

Chapter 3

ANTI-NOCICEPTIVE EFFECTS OF THE EXTRACT AND XYLOPIC ACID

3.1   INTRODUCTION

Nociception is one of the vital functions of the nervous system and by its inherent aversive nature, provides warning information about the occurrence or threat of injury (Rustoen *et al.*, 2008). Nociception resulting from tissue damage affects the total well being of patients (Gilchrist *et al.*, 1996; Millan, 1999) and patients often do not get satisfactory treatment. The question about the safety and side-effect profiles of the currently used analgesics make their clinical use problematic (Jage, 2005). The antinociceptive effect of extract of *X. aethiopica,* a fruit used in managing pain traditionally, and the major constituent, xylopic acid were therefore evaluated in animal models of nociception.

3.2   MATERIALS AND METHODS

3.2.1 Animals

Sprague-Dawley rats (250–300 g) and ICR mice (20-25 g) 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 ($34 \times 47 \times 18$ cm^3) with soft wood shavings as bedding. The animals were fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum* and maintained under laboratory conditions. 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.

3.2.2 Drugs and chemicals

Diclofenac sodium and morphine hydrochloride served as reference drugs. Diclofenac sodium was purchased from Troge Medical GmbH, Hamburg, Germany; morphine hydrochloride was obtained from Phyto-Riker, Accra, Ghana; carrageenan sulphate, formalin and acetic acid were purchased from BDH, Poole, England.

3.2.3 Acetic acid-induced writhing test

The test was carried out as described earlier (Koster *et al.*, 1959; Tang *et al.*, 2007). Thirteen groups of female mice (n=8) received vehicle (10 ml kg^{-1} of 0.9 % NaCl i.p.), the extract (30-300 mg kg^{-1} of tween 80 emulsion *p.o.*), xylopic acid (10-100 mg kg^{-1} of tween 80 emulsion *p.o.*), morphine (1-10 mg kg^{-1}, i.p.) or diclofenac (1-10 mg kg^{-1}, i.p.) 60 min (*p.o.*) or 30 min (i.p.) before intraperitoneal administration of acetic acid (0.6 %: 10 ml kg^{-1}). Drug solutions were prepared such that not more than 1 ml of extract or xylopic acid was given orally and not more than 0.5 ml of the standard drugs were injected intraperitoneally. All drugs were freshly prepared. Mice were then placed individually in a testing chamber (Perspex chamber, 15×15×15 cm). A mirror inclined at 45° below the floor of the chamber allowed a complete view of the mice. Injection of acetic acid induced a nociceptive behaviour, writhing, an exaggerated extension of the abdomen combined with the outstretching of the hind limbs. Responses were captured (30 min) for analysis by a camcorder (Everio[TM], model GZ-MG1300, JVC, Tokyo, Japan) placed directly opposite the mirror and attached to a computer. Tracking of the behaviour was done using the public domain software JWatcher[TM], Version 1.0 (University of California, LA, USA, and Macquarie University, Sidney, Australia, available at http://www.jwatcher.ucla.edu/) to obtain the frequency and duration of writhes per 5 min, starting 5 min after acetic acid administration. A nociceptive score was determined for each 5-min time block by multiplying the frequency and duration of writhes. These data were expressed in a time course which enabled the observation of changes in the writhing induced.

3.2.4   Formalin- induced nociception

The formalin test was carried out as described previously (Dubuisson and Dennis, 1977; Woode *et al.*, 2009). Each male mouse was assigned and acclimatized to one of 20 formalin test chambers (Perspex chamber 15×15×15 cm) for thirty minutes before formalin injection. Ten groups of male mice were then pre-treated with vehicle, the extract (30-300 mg kg^{-1}, *p.o.*), xylopic acid (10-100 mg kg^{-1}, *p.o.*) or morphine (1-10 mg kg^{-1}, i.p.) 60 min (*p.o.*) or 30 min (i.p.) before intraplantar injection of 10 μl of 5% formalin. The animals were immediately returned individually into the testing chamber and their nociceptive behaviours captured (1 h) for analysis in the same way as described previously in the writhing test above. The pain response was scored for 1 h, starting immediately after formalin injection. A nociceptive score was determined for each 5-min time block by measuring the amount of time spent biting/licking of the injected paw. Tracking of the behaviour was done using public domain software JWatcher^{TM}, Version 1.0. The average nociceptive score for each time block was calculated by multiplying the frequency and time spent in biting/licking. Data were expressed as the mean ± SEM of scores between 0–10 min (first phase) and 10–60 min (second phase) after formalin injection.

3.2.5   Carrageenan-induced mechanical hyperalgesia

Mechanical nociceptive thresholds were measured in the Randall-Selitto test (Randall and Selitto, 1957; Villetti *et al.*, 2003; Stohr *et al.*, 2006) by using an analgesimeter (Model No.15776, Ugo Basile, Comerio, Varese, Italy)). The analgesimeter was used to apply a linearly-increasing pressure, by means of a blunt perspex cone, to the dorsal region of the right hind paw of the rat until the rat vocalized or withdrew the paw. Rats received two training seasons before the day of testing. Pressure was gradually applied to the right hind paw and paw withdrawal thresholds (PWTs) were assessed as the pressure (grams) required eliciting paw withdrawal. A 250 g cut-off point was chosen as the maximum weight to apply to prevent any tissue damage to the paw of the rats. A change in hyperalgesic state was calculated as a percentage of the maximum possible effect (% MPE). The maximal possible effect (% MPE) was calculated according to the formula:

$$\% MPE = 100 \text{ x } (PWT - CT)/ (250g - CT)$$

Where PWT: paw withdrawal threshold and CT: control threshold.

On the test day, a baseline measurement was taken. Thirteen groups of animals (n=8) received carrageenan (100 µl of a 20 mg ml^{-1} solution) into the right hind paw 2.5 h before vehicle, the extract (30-300 mg kg^{-1}, *p.o.*), xylopic acid (10-100 mg kg^{-1}, *p.o.*), morphine (1- 10 mg kg^{-1}, i.p.) and diclofenac (1-10 mg kg^{-1}, i.p.) treatments and PWT were taken again at 3.5, 4, 4.5, 5, 5.5 and 6 h post-carrageenan.

3.2.6 Evaluation of the analgesic effect of extract and xylopic acid in Hargreaves thermal hyperalgesia test

3.2.6.1 Thermal paw withdrawal test

Possible anti-hyperalgesic effect of the extract and xylopic acid was assessed by the Hargreaves model of thermal hyperalgesia (Galbraith *et al.*, 1993). Hind paw and tail sensitivity to a noxious thermal stimulus was measured by means of radiant heat source using the IITC Model 336 Paw/Tail Stimulator Analgesia Meter (Woodland Hills, CA., USA). Male mice were individually placed in plexi glass cages on a clear glass platform and allowed to acclimatize for 15 min to the testing environment. The test head of the paw stimulator was used to present a focused beam of radiant light on to the mid plantar region of the right hind paws. The idle intensity (intensity of light innocuous to the mice) of the light was set at 10% of the maximum intensity and it was used to accurately direct the beam of light to the appropriate region of the hind paw: the active intensity of the light was set at 50% maximum. The thermal nociceptive stimulus was manually directed under the foot pad before and after the intra plantar injection of carrageenan (100 µl of a 2% solution) in to the right hind paw. A timer was set to automatically turn off the light source when the animal withdrew the tail or paw, and the paw withdrawal latencies (PWLs) and tail withdrawal latencies (TWLs) measured were defined as the time required for the paw or tail to show an abrupt withdrawal. A cut-off time of 25 s was chosen as the maximum time the animal's paw/ tail will be stimulated with the light in order to prevent tissue damage. Baseline measurements were taken followed by administration of carrageenan into the right hind paw one hour post treatment with vehicle, the extract (30-300 mg kg^{-1}, *p.o.*), xylopic acid (10-100 mg

kg^{-1}, *p.o.*), morphine (1-10 mg kg^{-1}, *i.p.*) and diclofenac (1-10 mg kg^{-1}, *i.p.*), (n=8). PWLs were taken again hourly for four hours.

3.2.6.2 Tail-flick test

Tail-flick latencies were determined with the IITC Analgesia Meter (Model 336, IITC Life Science Inc., Woodland Hills, CA, USA). A focused beam of radiant light (Active intensity: 50 % of maximum) was delivered to the mice's tail (Galbraith *et al.*, 1993; Fecho *et al.*, 2005). Basal reaction times of male mice (ten groups, n=8) were taken before the administration of vehicle, the extract (30-300 mg kg-^{1}, *p.o.*), xylopic acid (10-100 mg kg^{-1}, *p.o.*) or morphine (1-10 mg kg^{-1}, i.p.). Tail-flick reflex latencies were then measured at 1 h, 2 h, 3 h and 4 h. A cut-off time of 25 s (the maximum time allowed to stimulate the tail of the mice with light) was chosen in order to prevent tissue damage. The analgesic effects exerted by drugs were calculated, from the tail-flick latencies, as a percentage of the maximum possible effect (% MPE) using the following formula: *[(T2-T1)/(T0-T1) × 100],* where T1 and T2 are the pre- and post-drug latencies, respectively, and T0 is the cut-off time.

3.2.7 Skeletal hyperalgesia

The analgesic effect of the extract and xylopic acid were evaluated in acute and chronic skeletal pain models as described by Skyba (2005) with modifications.

3.2.7.1 The effect of the extract and xylopic acid in acute skeletal pain

Acute skeletal hyperalgesia was measured by taking the baseline compression thresholds of the ipsilateral knees with an analgesimeter (IITC Life Science Inc. Model 2888, Woodland Hills, CA, USA). This was done by compressing the knee of the rat until the animal withdrew the limb forcefully or vocalized. The maximum compression force applied at withdrawal was recorded as the baseline compression threshold for the knee joint of the corresponding limb. Rats were then injected with 100 μl of a mixture containing 3% kaolin and 3% carrageenan intra articularly into the left knee joint. Acute skeletal pain was established after 12 hours in ipsilateral limbs. Ten groups of male rats (n=8) received vehicle, extract (30-300 mg kg^{-1} *p.o.* 1 h), xylopic acid (10-100 mg kg^{-1}

p.o. 1 h) or morphine (1-10 mg kg^{-1} i.p. 30 min) after primary hyperalgesia confirmation and compression thresholds were taken again hourly for 5 hours.

3.2.7.2   *The effect of extract and xylopic acid in chronic skeletal pain*

To evaluate the analgesic effect of the extract and xylopic acid on bilateral hyperalgesia (chronic skeletal pain), baseline compression thresholds of both paws were taken using an analgesimeter (IITC Life Science Inc. Model 2888, Woodland Hills, CA, USA) which is based on the Randall-Selitto test (Randall and Selitto, 1957). Ten groups of rats (n=8) received intra articular injection of 100 µl of a mixture containing 3% kaolin and 3% carrageenan. Animals were allowed 8 days to develop chronic-knee inflammation (Radhakrishnan *et al.*, 2003).

On the test day, chronic skeletal pain was established by measuring compression thresholds for both ipsilateral and contralateral paws. The ten groups of animals then received vehicle, extract (30-300 mg kg^{-1} *p.o.*) xylopic acid (10-100 mg kg^{-1} *p.o.*) or morphine (1-10 mg kg^{-1} i.p.) after bilateral hyperalgesia confirmation and compression thresholds were taken again hourly for 5 hours.

3.2.8 *Muscle hyperalgesia*

The effects of the extract and xylopic were determined in acute and chronic muscle pain models.

3.2.8.1 *The effect of extract and xylopic acid in acute muscle pain*

Assessment of the analgesic effect of the extract and xylopic acid in acute muscle pain was performed by measurement of hind limb grip strength using a grip force analyzer as described by Kehl *et al.,* (2003); Skyba (2005) ; Tillu *et al.*,(2008). The force analyzer measured the amount of tensile force each rat exerted against a wire mesh grid with its hind paws, and gently pulled in a caudal direction. The force each rat applied to the mesh grid was determined by the rat itself. Therefore, the amplitude of force exerted was subject to factors, such as hyperalgesia, which influenced the behavioural performance of each rat. The peak force exerted by the rat before it released its grasp from the wire mesh grid was registered by a force transducer and recorded. Three consecutive hind limb grip force measurements (10 s apart) were obtained for each rat

at each time point; the average of these three measurements was used to represent each rat's grip force for each time point. After baseline grip strength measurements, the animals received 100 μl of 3% carrageenan percutaneously into their left gastrocnemius muscles. Ten groups of male rats (n=8) were then given the extract (30-300 mg kg^{-1} *p.o.* 1 h), xylopic acid (10-100 mg kg^{-1} *p.o.*), morphine (1-10 mg kg^{-1} i.p. 30 min) or vehicle after primary hyperalgesia (acute pain) confirmation at the 12 hour and grip strength measurements were taken again hourly for 5 hours.

Reduction in grip force after percutaneous administration of carrageenan relative to baseline grip force levels provides an index of the reduction in nociceptive threshold and was calculated as % MPE:

% MPE = 100

Groups I–IV   received vehicle.

Groups V–VI   received extract (600 mg kg^{-1}, *p.o.*,) and xylopic acid (200 mg kg^{-1}, *p.o.*) respectively.

Groups VII-IX   received morphine 6 mg kg^{-1}, i.p.

On day 9, these groups were treated in the following manner:

Group I   was treated with vehicle

Groups II–IV   were treated with extract (300 mg kg^{-1}, *p.o*), xylopic acid (100 mg kg^{-1}, *p.o.*) and morphine (3 mg kg^{-1}, i.p.) respectively

Groups   V-VI   received extract (300 mg kg^{-1}, *p.o.*) and xylopic acid (100 mg kg^{-1}, *p.o.*) respectively

Group VII   was treated with morphine (3 mg kg^{-1}, i.p.).

Formalin was injected 60 min for *p.o.* and 30 min for i.p. after the various drugs treatment. To ascertain the possibility of morphine-induced tolerance cross-generalizing with extract or xylopic acid, extract and xylopic acid were administered to animals which had been chronically treated with morphine in the following manner:

Groups VIII – IX   were treated with extract (300 mg kg^{-1}, *p.o.*) and xylopic acid (100 mg kg^{-1}, *p.o.*) administered 60 min before formalin injection.

3.2.10   *Test for catalepsy*

Drugs such as opioids are known to produce catalepsy, a state of decreased responsiveness to external stimuli associated with a waxy rigidity of the extremities which may wrongly be interpreted as analgesia in the grip force test (Tzschentke and Schmidt, 1996). Therefore, the possibility existed, that increased hind limb muscle rigidity would result in increased grip force (i.e. reversing the reduction in grip force): after the various drug treatments and could mistakenly be interpreted as reversal of

hyperalgesia in the grip strength measurement. To distinguish between the anti-hyperalgesic and cataleptic effects of the extract and xylopic acid on grip force measurement and other nociceptive models, possible cataleptic effect of the extract and xylopic acid on grip force in rats that had not received carrageenan was measured. The rats were trained to place the fore limbs on the bar for 12 s for three consecutive days. On the test day, the test was performed by placing the forelimbs of each rat on a metal bar (diameter 1 cm) positioned 5 cm above and parallel to the base of the equipment with its hind paws resting on the base. The total amount of time each rat spent with its forelimbs on the bar in 3 consecutive 12 s trials was summed. To evaluate the effect of catalepsy on grip force, bar test measurements were collected just prior to grip force measurements at each time point. Eight groups of rats (n=8) were tested before and at 60 min after injection of vehicle, extract (30-300 mg kg^{-1} *p.o.*), xylopic acid (10-100 mg kg^{-1} *p.o.*), and 30 min after morphine (40 mg kg^{-1} i.p., a positive control).

3.2.11 Rota-rod

Drugs such as muscle relaxants used as 'adjuvant' in pain management exhibit antinociceptive activity in models such as the acetic acid-induced writhing test making the model non–specific for investigating the antinociception of drugs (Pietrovski *et al.*, 2006). In order to assess whether any of the observed antinociceptive effects of the extract and xylopic acid resulted from sensory blockade or from an impairment of motor function, motor performance of the mice was evaluated in the rota-rod test.

Naive mice were trained on three successive days on the rota-rod (Ugo Basile, model 7600, Comerio, Varese, Italy) at a speed of 25 rpm. On the test day (day 4), seven groups of animals (n=8) received the extract (30-300 mg kg-^{1}, *p.o.*), xylopic acid (10-100 mg kg-^{1}, *p.o.*) or vehicle. The animals were then repeatedly tested for their motor coordination performance on the rota-rod (cut of time 120 s) at 1, 2 and 3 h after drug injection. Impairment of coordinated motor movements was defined as the inability of the animals to remain on the rota-rod for a test period of 120 s (Gareri *et al.*, 2005).

3.3 STATISTICAL ANALYSIS

In all experiments, a sample size of eight animals (n=8) were used. All data are presented as mean ± S.E.M (n=8). Raw data for the tail-flick, musculoskeletal, carrageenan-induced mechanical and thermal hyperalgesia tests were calculated as the percentage change in maximum possible effect (% MPE).The time-course curves were subjected to two-way (treatment × time) repeated measures analysis of variance (ANOVA) with Bonferroni's *post hoc* test. Total nociceptive score for each treatment was calculated in arbitrary unit as the area under the curve (AUC). To determine the percentage inhibition for each treatment, the following equation was used.

$$\% \ inhibition = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}} \right) \times 100$$

Differences in AUCs were analyzed using one-way ANOVA with drug treatment as a between subjects factor. Further comparisons between vehicle- and drug-treated groups were performed using the Newman–Keuls' 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:

$$Y = \frac{a + (b - a)}{\left(1 + 10^{(LogED_{50} - X)} \right)}$$

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}s) of the curves were compared statistically using F test (Miller, 2003; Motulsky and Christopoulos, 2003). GraphPad Prism for Windows Version 4.03 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determinations. $P < 0.05$ was considered statistically significant.

3.4   RESULTS

3.4.1   *Acetic acid-induced writhing assay*

Figure 3.1 shows the effect of various treatments on acetic acid-induced writhing during the 30-min observation period. The extract (30-300 mg kg^{-1}, *p.o.*) significantly ($F_{3,27}$=14.37, *P*<0.0001;Fig. 3.1a) reduced abdominal writhes induced by acetic acid in mice with the highest dose causing a reduction of 98.8 ± 0.8% (Fig. 3.1b). Xylopic acid (10 -100 mg kg^{-1}, *p.o.*) also significantly ($F_{3,28}$=20.56, *P*<0.0001; Fig. 3.1c) and dose-dependently reduced the writhes, with the highest dose causing a reduction of 93.8±1.4% (Fig. 3.1d). Morphine dose-dependently reduced ($F_{3,27}$=9.77, *P*=0.00016; Fig. 3.2a) acetic acid-induced nociception with maximal percentage inhibition of 97.4±2.5 (Fig. 3.2b) at the dose of 10 mg kg^{-1}. Diclofenac also reduced the abdominal writhes dose-dependently and significantly ($F_{3,28}$=4.04, *P*=0.0165; Fig. 3.2c); with the highest dose (10 mg kg^{-1}) producing an inhibition of 90.4±2.6% (Fig. 3.2d). From the ED$_{50}$ values obtained by F-test (Table 3.1), xylopic acid (5.472±0.80 mg kg^{-1}) was more potent than the extract (37.56±7.82 mg kg^{-1}). Morphine was the most potent (0.80±0.26 mg kg^{-1}).
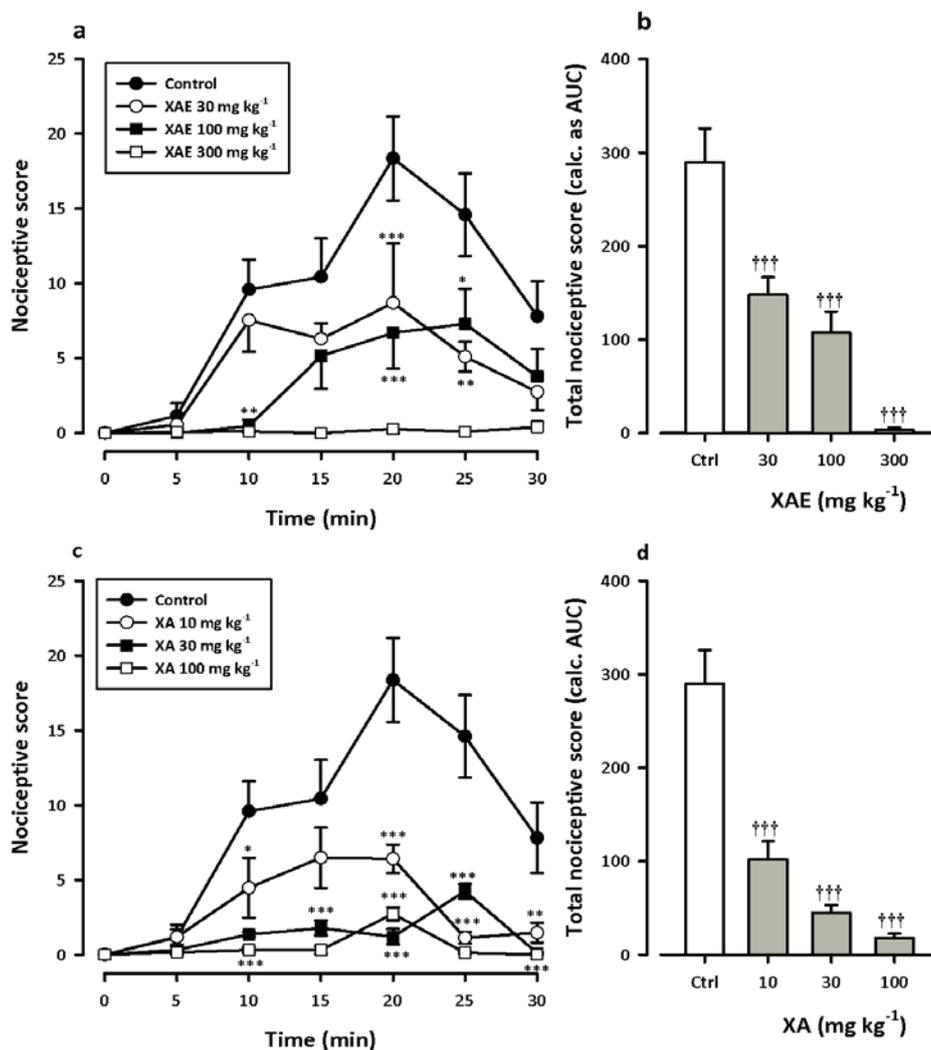
Figure 3.1 Effect of (a) Extract (XAE) (30 – 300 mg kg^{-1}) and (c) xylopic acid (XA) (10 – 100 mg kg^{-1}) on time course curve of acetic acid- induced writhing test in mice. Each point represents Mean ± S.E.M (n = 8); *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (Ctrl) (Two-way repeated measures ANOVA followed by Bonferroni's *post hoc*), (b and d) AUC (total response). Each column in b and d represents the mean ± S.E.M. $^{†††}P \leq 0.001$, (one-way ANOVA followed by Newman-Keuls' *post hoc*).
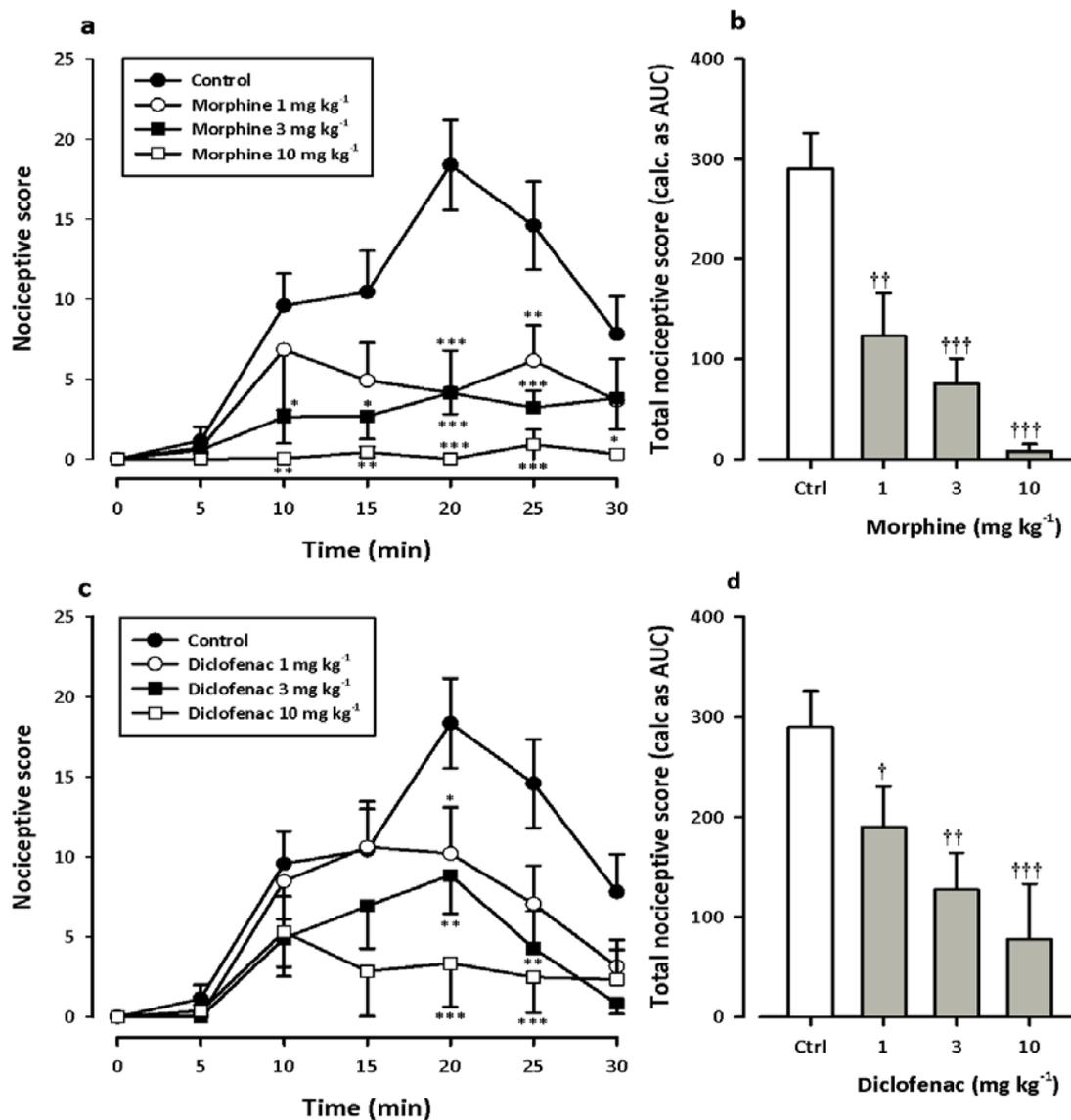
Figure 3.2 Effect of (a) morphine (1 – 10 mg kg^{-1}) and (c) diclofenac (1– 10 mg kg^{-1}) on the time course curve of acetic acid induced -writhing test in mice. Each point represents Mean ± S.E.M (n = 8); *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*) and (b and d) AUC (total response). Each column in b and d represents the mean ± S.E.M. [†]$P \leq 0.05$, [††]$P \leq 0.01$, [†††]$P \leq 0.001$, (one-way ANOVA followed by Newman-Keuls' *post hoc*).

3.4.2 Formalin-induced nociception

Intraplantar injection of formalin produced a nociceptive behaviour in mice characterized by biting, flinching and licking. The first phase lasted for 10 min and the second phase started at about 15 min and lasted until 1 h. Administration of *X. aethiopica* extract (30-300 mg kg^{-1}, *p.o.*) to the mice produced significant attenuation of formalin-induced nociception in both phases (Phase 1: $F_{3,28}$ =4.29, *P*=0.0131; Phase 2: $F_{3,28}$ =5.18, *P*=0.0056; Fig. 3.3a); with maximal antinociception of 57.3 ± 25.0% and 52.9 ± 34.1% respectively at the highest dose (Fig. 3.3b). Xylopic acid (10-100 mg kg^{-1}) also dose-dependently inhibited both phases of formalin-induced nociception (Phase 1: $F_{3,28}$=7.85, *P*=0.0006; Phase 2 $F_{3,28}$ =7.68, *P*=0.0007; Fig. 3.3c), with the highest dose giving a maximal antinociception of 79.6 ± 17.5 % and 90.2 ± 4.9 % (Fig. 3.3d) in the early and late phases respectively. Morphine (1-10 mg kg^{-1}, i.p.) produced a significant inhibition of both neurogenic phase ($F_{3,28}$ = 22.51, *P*<0.0001) and inflammatory ($F_{3,28}$= 4.76, *P*=0.0083; Fig. 3.3e) phases of the formalin test. Morphine reduced formalin-evoked nociceptive behaviour by maxima of 85.6 ± 12.7 % and 98.3 ± 3% in the early and late phases respectively (Fig. 3.3f).

Comparison of ED_{50}s obtained by F-test (Table 3.1) revealed that both the extract and xylopic acid were more potent in the second phase than the first. Morphine was more potent in the second phase (ED_{50}=0.00039±0.0034 mg kg^{-1}) compared to the first phase (ED_{50}=0.15±0.20 mg kg^{-1}). The potency order was: morphine >xylopic acid> the extract for both phases of the formalin test.
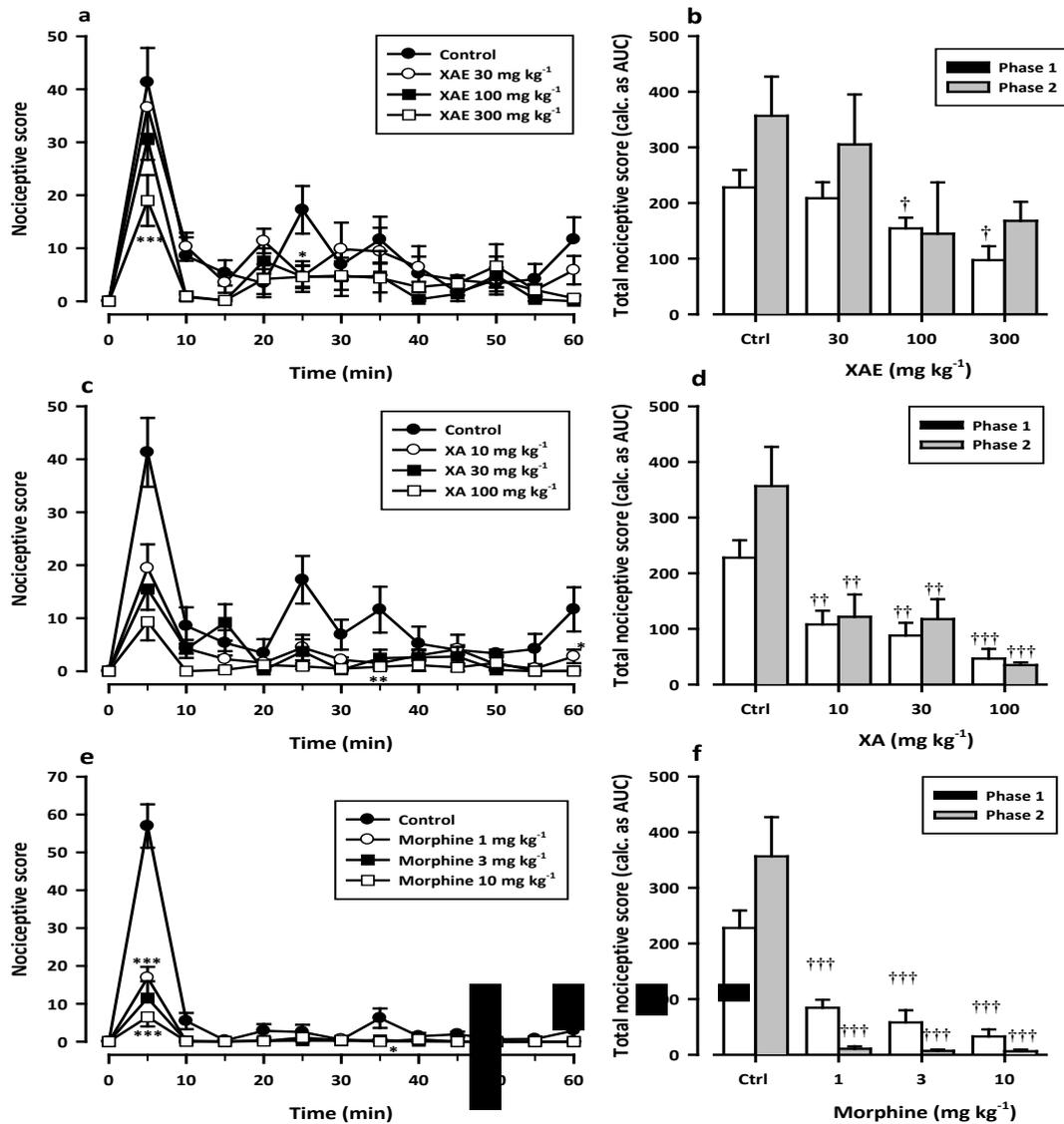
Figure 3.3 Effect of (a) extract (30 - 300 mg kg^{-1} *p.o.*), (c) xylopic acid (10 - 100 mg kg^{-1} *p.o.*) and (e) morphine (1 - 10 mg kg^{-1} i.p.) on time course curve of formalin-induced nociception in mice. Each point represents Mean ± S.E.M (n = 8); *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*) and (b, d and f) the AUC (total response) for phase 1 and phase 2. Each column in b, d and f represent the mean ± S.E.M.; $^{†}P \leq 0.05$, $^{††}P \leq 0.01$, $^{†††}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls' *post hoc*).

3.4.3 Carrageenan-Induced Mechanical Hyperalgesia using Randall- Selitto

Two hours after carrageenan injection, the ipsilateral paw exhibited marked mechanical hyperalgesia and inflammation (Fig. 3.4). A change in hyperalgesic state was calculated as a percentage of the maximum possible effect. the extract (30–300 mg kg^{-1}, *p.o.*) and xylopic acid (10-100 mg kg^{-1}, *p.o.*) administered 2.5 h after carrageenan produced a

significant and dose-dependent attenuation of mechanical hyperalgesia (extract: $F_{3,28}$=6.93, P=0.0012; xylopic acid: $F_{3,28}$=4.86, P=0.0076; Fig. 3.4a-d) with maximal effects at 300 and 100 mg kg^{-1} respectively. Morphine (1-10 mg kg^{-1}, i.p.) and diclofenac (1-10 mg kg^{-1}, i.p.) also dose-dependently attenuated mechanical hyperalgesia with maximal effects at 10 mg kg^{-1} (Morphine: $F_{3,28}$=10.67, P<0.0001; diclofenac: $F_{3,28}$=5.86, P<0.0031 ; Fig. 3.5e-h).

From the ED$_{50}$ values (Table 3.1); the extract was more potent than xylopic acid but less than diclofenac and morphine.
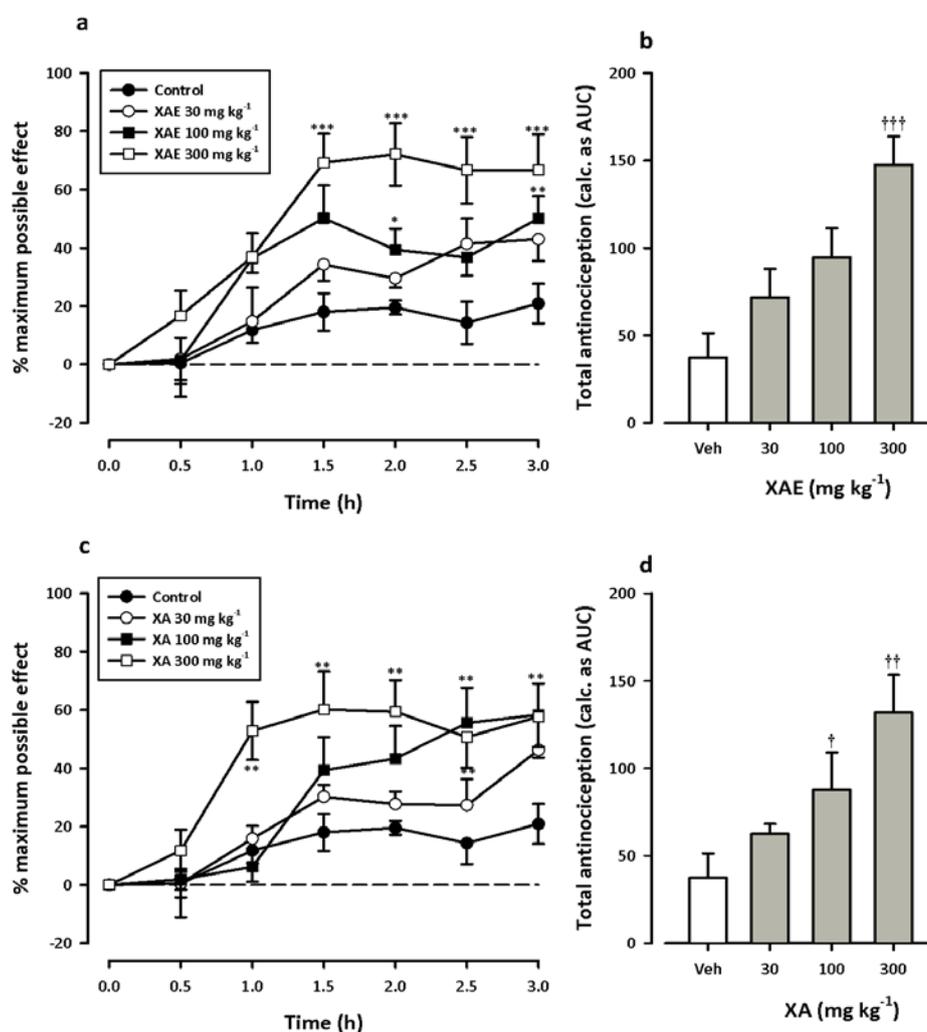
Figure 3.4 Effect of (a) extract (30 - 300 mg kg^{-1} *p.o.*) and (c) xylopic acid (10 – 100 mg kg^{-1} *p.o.*) on the time course curve of carrageenan-induced mechanical hyperalgesia in rat in the Randall- Selitto model and the AUC (b and d). Data is presented as mean ± S.E.M. (n = 7-8); ***P < 0.001; ** P < 0.01; *P < 0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test); †P < 0.05, ††P < 0.01, †††P < 0.001 compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
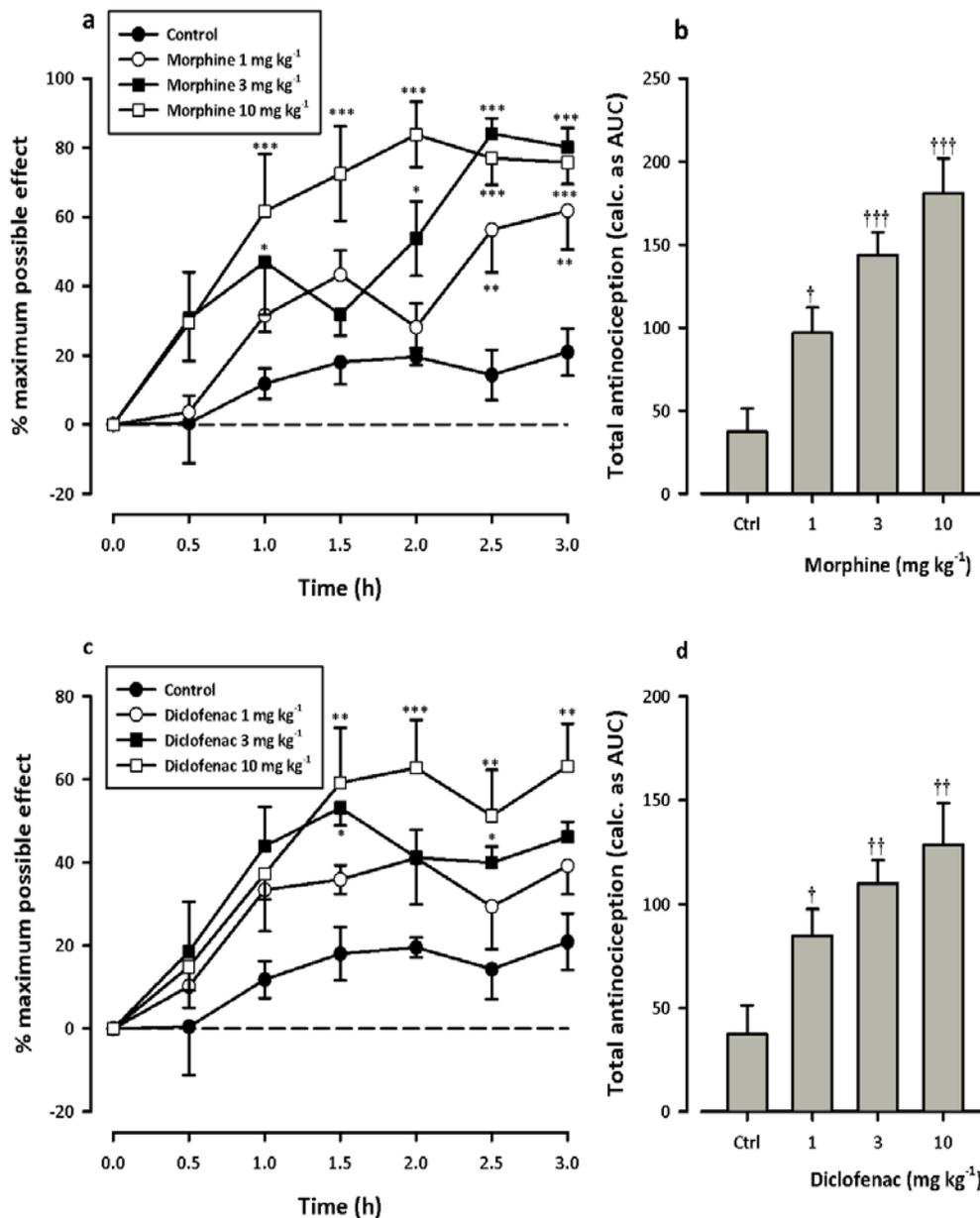
Figure 3.5 Effect of (a) morphine (1-10 mg kg^{-1} i.p.) and (c) diclofenac (1-10 mg kg^{-1} i.p.) on time course curve in carrageenan-induced mechanical hyperalgesia in rat in the Randall- Selitto model and the AUC (b and d). Data is presented as mean ± S.E.M. (n = 8); ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to control (Two-way ANOVA followed by Bonferroni's *post hoc* test); [†]$P \leq 0.05$, [††]$P \leq 0.01$, [†††]$P \leq$ 0.001, compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).

3.4.4   Hargreaves Thermal hyperalgesia

3.4.4.1   Paw withdrawal test

Paw withdrawal latency measured was higher for the tested drugs compared to the control group. The hyperalgesia produced was calculated as % MPE [(extract:

$F_{3,28}$=5.44, P=0.0045; xylopic acid: $F_{3,28}$=13.35, P<0.0001; morphine: $F_{3,28}$=10.24; P=0.0001; Two-way ANOVA (*treatment × time*); Fig 3.7a,c & e]. The hyperalgesia was significantly ($F_{3,28}$= 4.73, P=0.0086) and dose-dependently attenuated by the extract (30-300 mg kg^{-1}, *p.o.*) : with maximal effect at the dose of 300 mg kg^{-1} (Fig. 3.7b). Xylopic acid (10-100 mg kg^{-1}, *p.o.*) also dose-dependently inhibited ($F_{3,28}$= 12.56, P<0.0001; Fig. 3.7d) carrageenan-induced thermal hyperalgesia in the mice. The reference analgesic, morphine (1–10 mg kg^{-1}, i.p.), also showed significant, dose-dependent anti-hyperalgesic activity ($F_{3,28}$= 9.30, P=0.0002; Fig. 3.7f) against the carrageenan-induced thermal hyperalgesia.

From the ED$_{50}$ values (Table 3.1) of the dose-response curves in the test, the extract (46.82±35.92 mg kg^{-1}) was less potent than xylopic acid (27.17±11.15 mg kg^{-1}) and morphine (3.98±2.22 mg kg^{-1}).

3.4.4.2 Tail flick test

All test drugs caused an increase in tail-flick latency, calculated as % MPE [(extract: $F_{3,28}$=3.33, P=0.0336; xylopic acid: $F_{3,28}$=5.89, P=0.003; morphine: $F_{3,28}$=14.70; P<0.0001; Two-way ANOVA (*treatment × time*); Fig 3.6a,c & e]. The extract (30-300 mg kg^{-1}, *p.o.*) caused a significant, dose-dependent, attenuation of thermal nociception ($F_{3,28}$=3.19, P=0.0387) with maximum effect at the dose of 300 mg kg^{-1}(Fig. 3.6b). xylopic acid (10-100 mg kg^{-1}, *p.o.*) also dose-dependently inhibited ($F_{3,28}$=5.16, P=0.0058) thermal pain in the mice (Fig.3.6d). The reference analgesic, morphine (1-10 mg kg^{-1}, i.p.), showed significant, dose-dependent anti-nociception ($F_{3,28}$= 13.23, P<0.0001) with a maximum effect at 10 mg kg^{-1}(Fig. 3.6f).

By comparing the ED$_{50}$ values (Table 3.1) of effects in the tail-flick test, the extract (72.17±76.77 mg kg^{-1}) was less potent than xylopic acid (8.61±6.87 mg kg^{-1}) and morphine (11.67±9.88 mg kg^{-1}). Morphine was the most effective.

Figure 3.6 Effect of extract (30-300 mg kg^{-1} *p.o.*), xylopic acid (10-100 mg kg^{-1} *p.o.*) and morphine (1-10 mg kg^{-1} i.p.) on the time course curve of Hargreaves thermal paw withdrawal test (a, c and e) and the AUC (b, d and f). Data is presented as mean ± S.E.M. (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles respectively. The median is shown as the horizontal line within the box. ****P* < 0.001; **P* < 0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †††*P*<0.001, ††*P*<0.01, †*P*<0.05 compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).

Figure 3.7 Effect of extract (30-300 mg kg^{-1} *p.o.*), xylopic acid (10-100 mg kg^{-1} *p.o.*) and morphine (1-10 mg kg^{-1} i.p.) on the time course curve of tail withdrawal test (a, c and e) and the AUC (b, d and f). Data is presented as mean ± S.E.M. (n = 8). The lower and upper margins of the boxes (b, d, and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Symbol represents outlier. ***P < 0.001; ** *P* < 0.01; *P < 0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †††*P*<0.001, ††*P*<0.01, †*P*<0.05 compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).

Table 3.1 ED$_{50}$ values of drugs in the nociceptive tests.

Test	ED$_{50}$ values (mg kg^{-1})			
	XAE	XA	Morphine	Diclofenac
Acetic acid writing test	37.56±7.8***	5.47±0.8[†††]	0.80±0.2	1.95±0.5
Formalin test	219.40±69.28[a]	13.56±3.88 [a]	0.15±0.2 [a]	-
	149.9±12.5[b]	7.61±2.31[b]	0.00039±0.0034[b]	
Tail-flick test	72.2±76.77***	8.61±6.87[†††]	11.67±9.88	-
Mechanical hyperalgesia test	319.70±65.96	415.4±98.7[†††]	4.37±0.90	10.84±2.62
Hargreaves thermal paw hyperalgesia	46.82±35.92	27.2±11.15[†††]	3.98±2.22	-

ED$_{50}$s ± S.E.M. were obtained by least-square nonlinear regression and comparison was made with F-test as described in material and methods. ***$P < 0.001$ compared to xylopic acid-treated group: [†††]$P<0.001$ compared to morphine-treated group.

[a] First Phase of the formalin test

[b] Second Phase of the formalin test

3.4.5   Skeletal hyperalgesia

The effects of the extract and xylopic acid in acute and chronic skeletal hyperalgesia are described below:

3.4.5.1 Acute skeletal hyperalgesia

Acute knee (skeletal) hyperalgesia induced by injecting mixture containing 3% kaolin and 3% carrageenan was measured using the Randall-Selitto model. The knee

withdrawal reflexes indicative of knee hyperalgesia was significantly ($F_{3,28}$=14.74, P= 0.000006) and dose-dependently increased by the extract (30-300 mg kg^{-1}) administration (Fig. 3.8a). The highest dose of extract produced acute knee anti-hyperalgesic effect of 181.50±11.2% (Fig. 3.8b). Pre-treatment with xylopic acid (10-100 mg kg^{-1}) significantly ($F_{3,28}$=14.41, P=0.000007) and dose-dependently ameliorated acute knee hyperalgesia (Fig. 3.8c); with the highest dose of xylopic acid producing acute knee anti-hyperalgesia of 236.5±19.9% (Fig. 3.8d). Morphine (1-10 mg kg^{-1}) used as a positive control was effective at all the three doses producing a significant and dose-dependent acute knee anti-hyperalgesia ($F_{3,28}$=11.55, P= 0.00004) (Fig. 3.8e); with the highest dose producing primary anti-hyperalgesia of 165.3±14.34% (Fig. 3.8f). The potency of drugs were in the order morphine > xylopic acid > extract (Fig. 3.9).

3.4.5.2   *Chronic skeletal hyperalgesia*

Chronic skeletal hyperalgesia was measured in the ipsilateral and contralateral paws on the eighth day after injection of a mixture containing 3% kaolin and 3% carrageenan. A decrease in paw withdrawal reflex of the ipsilateral paw and the contralateral (the inserted graph) paw to a mechanical source of stimulus was seen across all treatment groups on the test day indicating secondary skeletal hyperalgesia. This decrease in paw withdrawal reflexes remained significant for the control group throughout the experiment. Chronic skeletal hyperalgesia was significantly and dose-dependently attenuated by the extract (30-300 mg kg^{-1}) (ipsi: $F_{3,28}$=13.96, P= 0.000009, contra*: $F_{3,28}$=7.9, P=0.0006) in the ipsilateral and contralateral paws (Fig. 3.10a). Maximum anti-hyperalgesia was observed at the highest dose of the extract administration (Fig. 3.10b).

Xylopic acid administration produced a significant and dose-dependent (ipsi: $F_{3,28}$=11.43, P=0.00005, contra: $F_{3,28}$=11.75, P=0.00004) increase in paw withdrawal latency in the ipsilateral and contralateral paws (Fig. 3.10c). Maximum possible chronic skeletal anti-hyperalgesia was achieved at the highest dose (Fig. 3.10d). Morphine (1-10 mg kg^{-1}) significantly and dose-dependently attenuated chronic skeletal hyperalgesia (ipsi: $F_{3,28}$=13.13, P= 0.00002, contra: $F_{3,28}$=7.52, P= 0.0008) in the ipsilateral and contralateral paws (Fig. 3.10e); with the highest dose of morphine

producing the maximum possible effect (Fig. 3.10f). The rank of potency in the acute and chronic musculoskeletal pain was in the order morphine > xylopic acid > extract (Fig. 3.11).
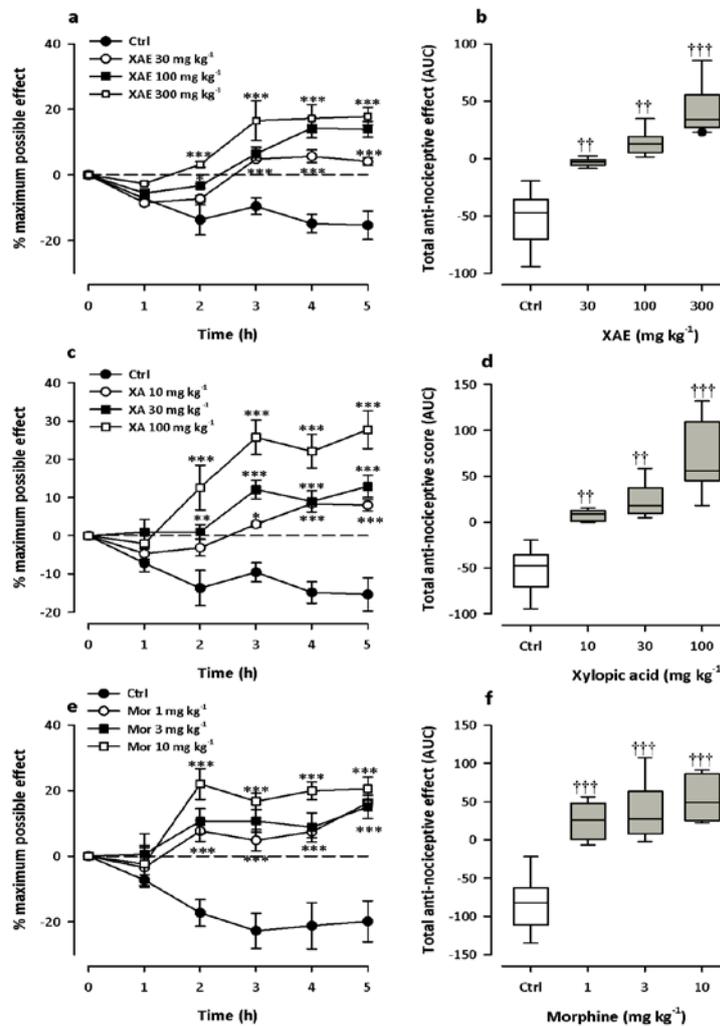
Figure 3.8 Effect of extract (30-300 mg kg-1 *p.o*), xylopic acid (10-100 mg kg-1 *p.o.*) and morphine (1-10 mg kg^{-1} i.p.) on the time course curve of paw withdrawal latency in the Randall-Selitto test (a, c and e) and the AUC (b, d and f) in acute knee pain. Data is presented as mean ± S.E.M. (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Symbol represents outlier. ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{†††}P<0.001$ $^{††}P<0.01$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
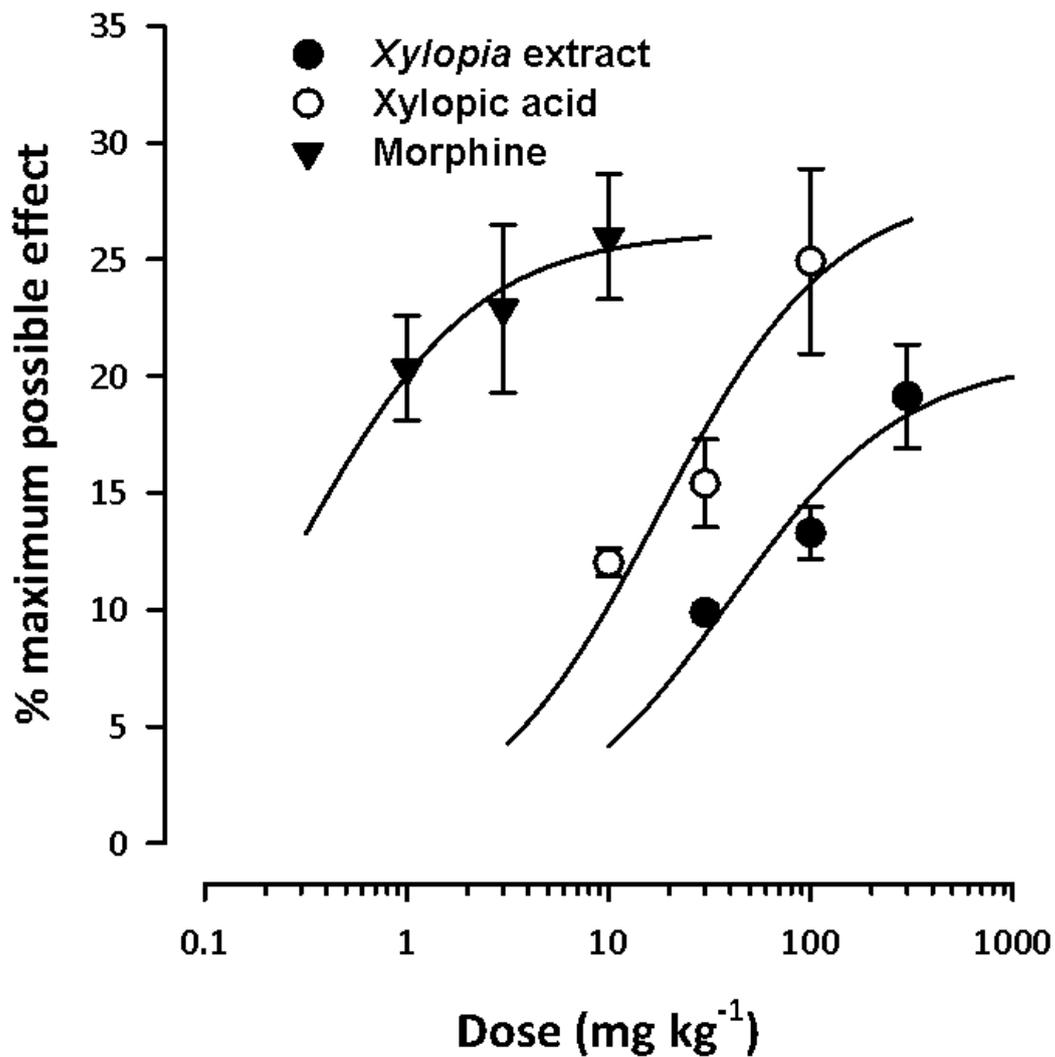
**Figure 3.9** Dose–response curves for the antinociceptive effect of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and morphine (1-10 mg kg^{-1}) in acute knee pain in rats. Each point is the mean ± S.E.M. of 8 animals.
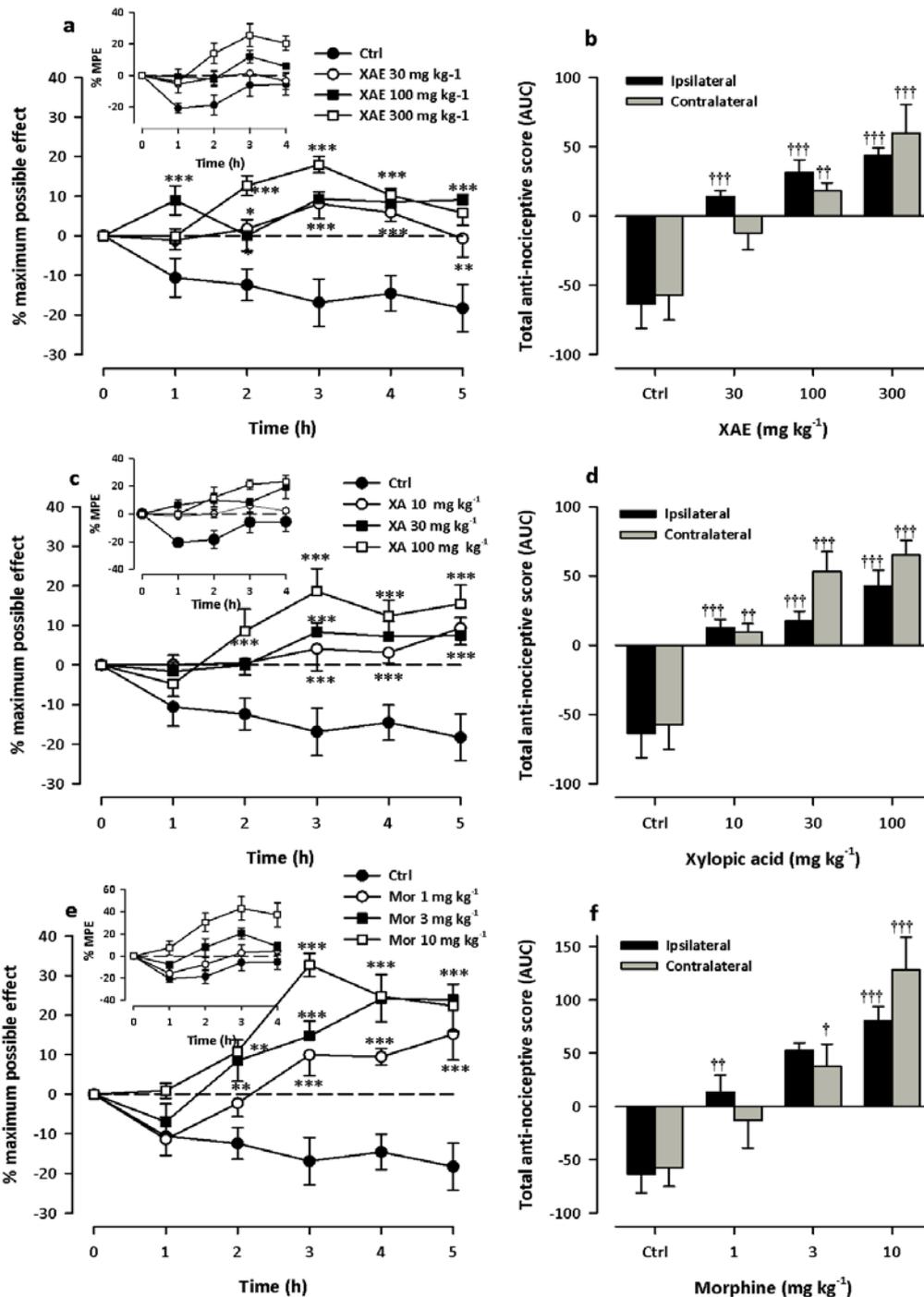
Figure 3.10 Effect of extract (30-300 mg kg^{-1} *p.o.*), xylopic acid (10-100 mg kg^{-1} *p.o.*) and morphine (1-10 mg kg^{-1} i.p.) on the time course curve of ipsilateral and contralateral paw (inserts) withdrawal latency in the Randall-Selitto test (a, c and e) and the AUC (b, d and f) in chronic knee pain. Data is presented as mean ± S.E.M. (n = 8); ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{†††}P<0.001$ $^{†}P<0.05$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
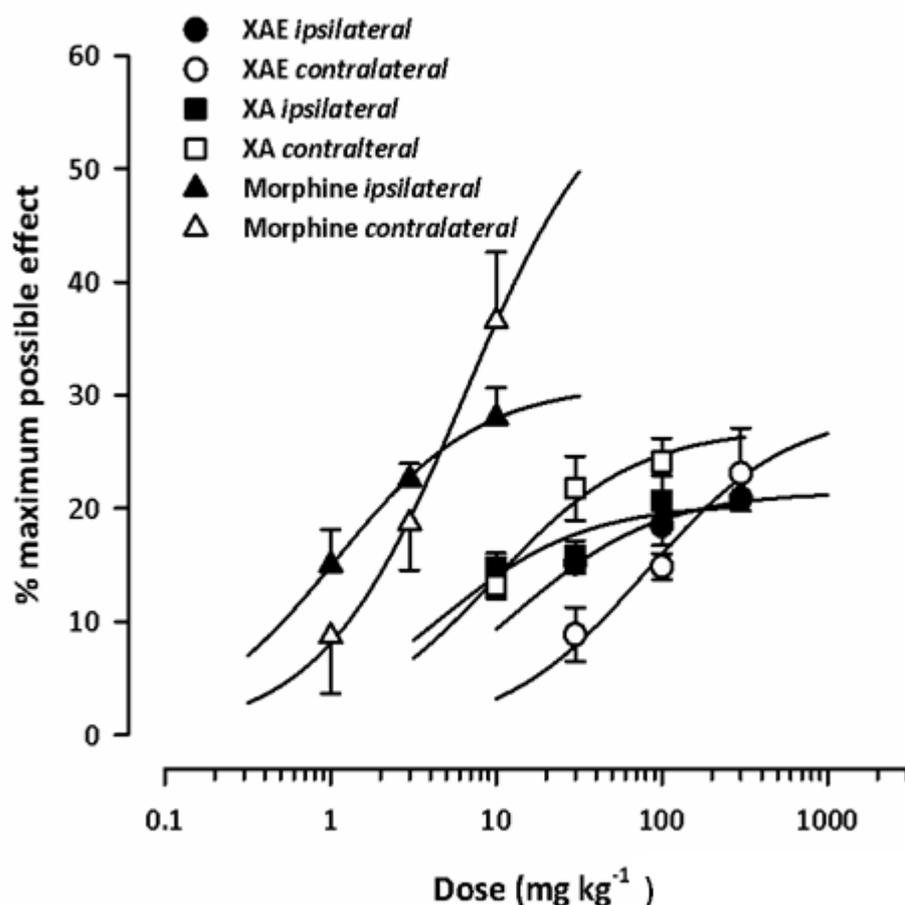
Figure 3.11 Dose–response curves for the antinociceptive effect of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and morphine (1-10 mg kg^{-1}) in chronic knee pain in rats. Each point is the mean ± S.E.M. of 8 animals.

3.4.6   Muscle hyperalgesia

The effects of the extract and xylopic acid in the acute and chronic muscle hyperalgesia are described below:

3.4.6.1   Acute muscle hyperalgesia

Acute muscle hyperalgesia induced with carrageenan was measured using grip strength assay. Muscle strength assessed twelve hours after the induction of muscle hyperalgesia after extract (30-300 mg kg^{-1}) administration significantly and dose-dependently increased ($F_{3,28}= 21.99$, $P=0.0000002$) (Fig.3.12a). The extract produced anti-hyperalgesic effect of 245.2±21.7% (Fig.3.12b) was by the highest dose of 300 mg kg^{-1}.

Xylopic acid (10-100 mg kg^{-1}) significantly and dose-dependently reduced acute muscle hyperalgesia ($F_{3,28}$=27.9., P=0.00000001, Fig.3.12c); producing possible maxima effect of 233.1±19.99% (Fig.3.12d) at the highest dose used.

Morphine (1-10 mg kg^{-1}) used as control was able to significantly and dose-dependently ameliorate acute muscle hyperalgesia ($F_{3,28}$=21.86, P=0.00000017, Fig. 3.12e). Morphine also produced acute muscle anti-hyperalgesic effect of 342.3±18.70% (Fig. 3.12f) at the maximum dose. The order of potency was morphine > xylopic acid > extract, Fig. 3.13.

3.4.6.2   *Chronic muscle pain*

Chronic muscle hyperalgesia was measured from both the ipsilateral and contralateral (insets) paws eight days after pain induction. The hyperalgesia induced in the contralateral paws was indicative of referred pain from muscle injury and central pain mechanism. The extract, xylopic acid and morphine were effective in attenuating hyperalgesia in the ipsilateral (ipsi) paw than the referred hyperalgesia in the contralateral (contra) paws. Chronic muscle hyperalgesia was significantly and dose-dependently attenuated by the extract (30-300 mg kg^{-1}) (ipsi: $F_{3,28}$=8.37, P=0.00039427, contra: $F_{3,28}$=18.25, P= 0.00000093, Fig. 3.14a) in the ipsilateral and contralateral paws. The maximum anti-hyperalgesic effect was achieved at the highest dose of the extract administration in both paws hyperalgesia measurement (Fig. 3.14b).

Xylopic acid attenuated chronic muscle hyperalgesia in both ipsilateral and contralateral paws significantly (ipsi: $F_{3,28}$=15.42, P=0.000004, contra: $F_{3,28}$=14.99, P= 0.000005, Fig. 3.14c) in a dose-dependent manner attaining maximum possible effect at the highest dose used, Fig. 3.14d.

Morphine (1-10 mg kg^{-1}) significantly and dose-dependently attenuated chronic muscle hyperalgesia (ipsi: $F_{3,28}$=5.2, P= 0.0056, contra: $F_{3,28}$=11.41, P= 0.00004610, Fig. 3.14e) in the ipsilateral and contralateral paws. Morphine exerted a maximum anti-hyperalgesic effect in both paws at the highest dose used (Fig. 3.14f). The order of potency was in the order morphine > xylopic acid > extract (Fig. 3.15).
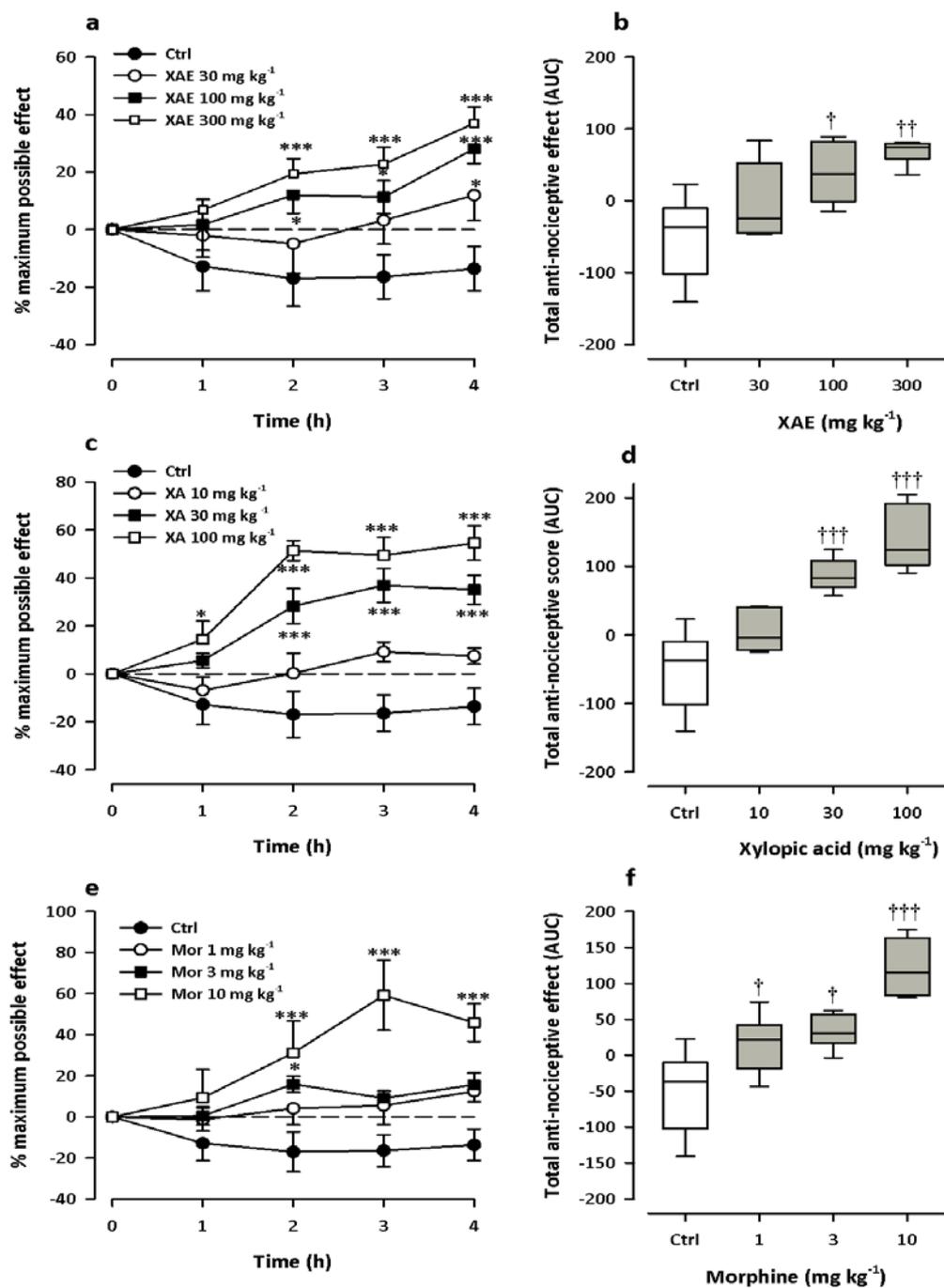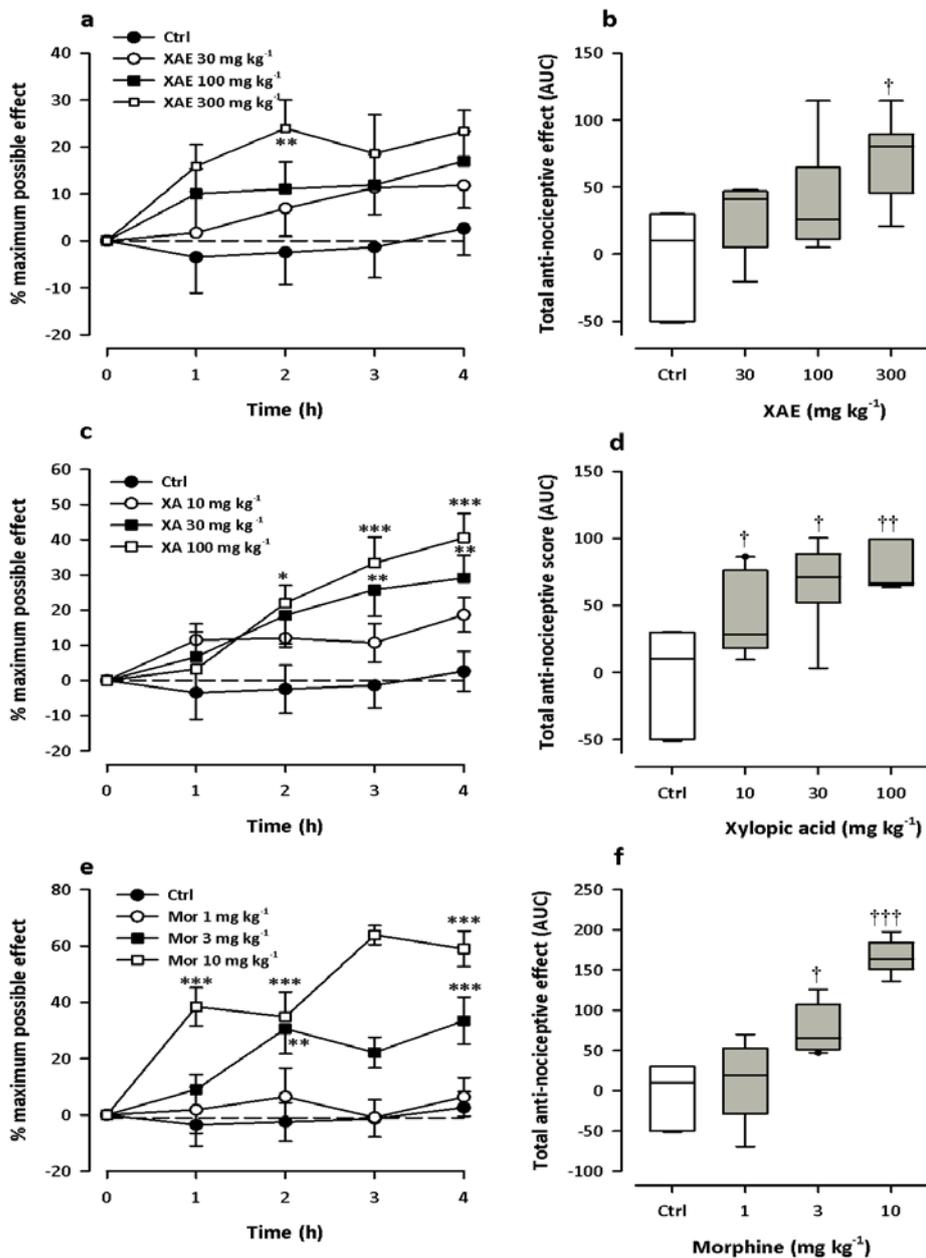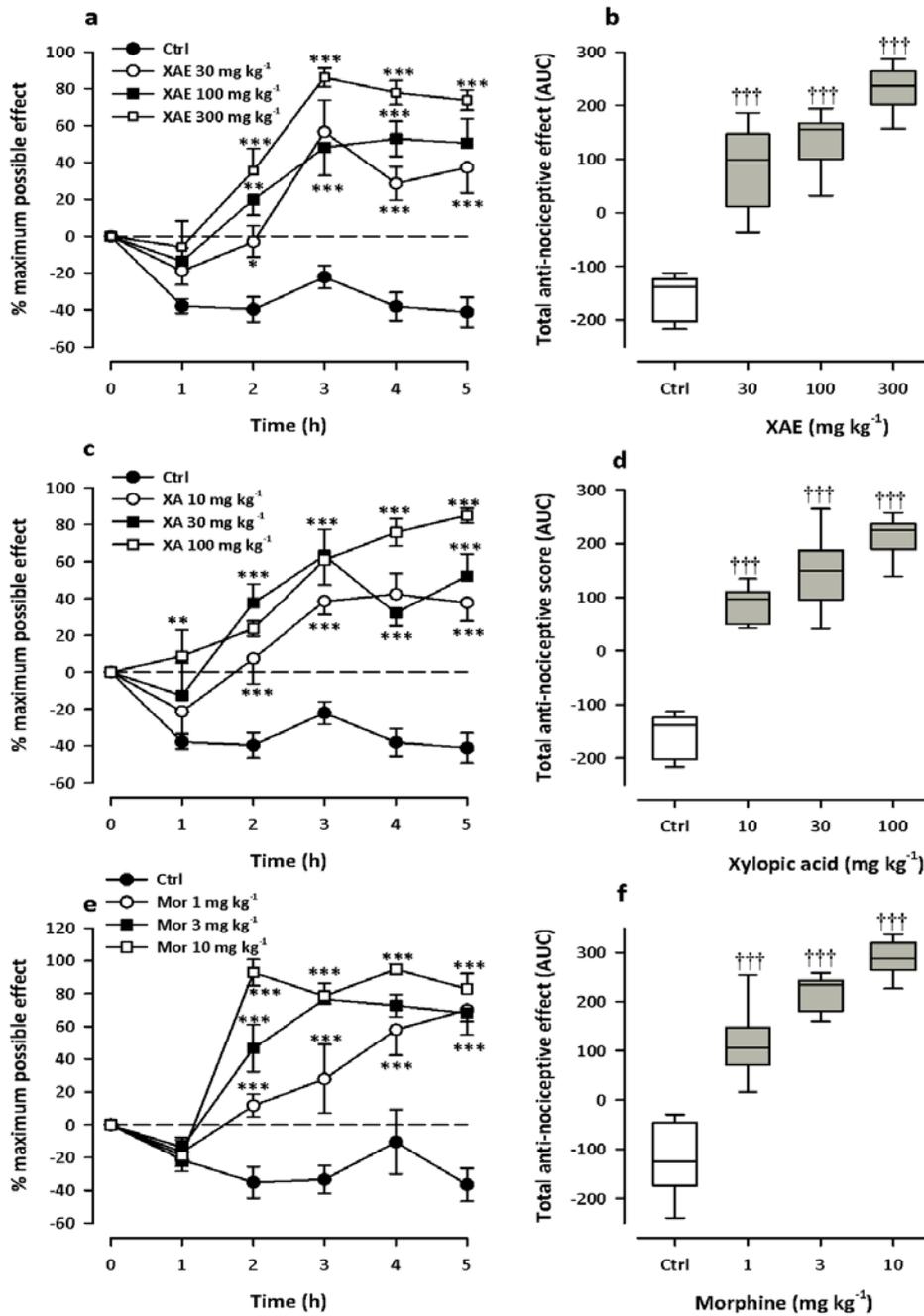
Figure 3.12 Effect of extract (30-300 mg kg^{-1} *p.o.*), xylopic acid (10-100 mg kg^{-1} *p.o.*) and morphine (1-10 mg kg^{-1} i.p.) on the time course curve of grip strength test (a, c and e) and the AUC (b, d and f) in acute muscle pain. Data is presented as mean ± S.E.M. (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ****P* < 0.001; ** *P* < 0.01; **P* < 0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{†††}P<0.001$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).

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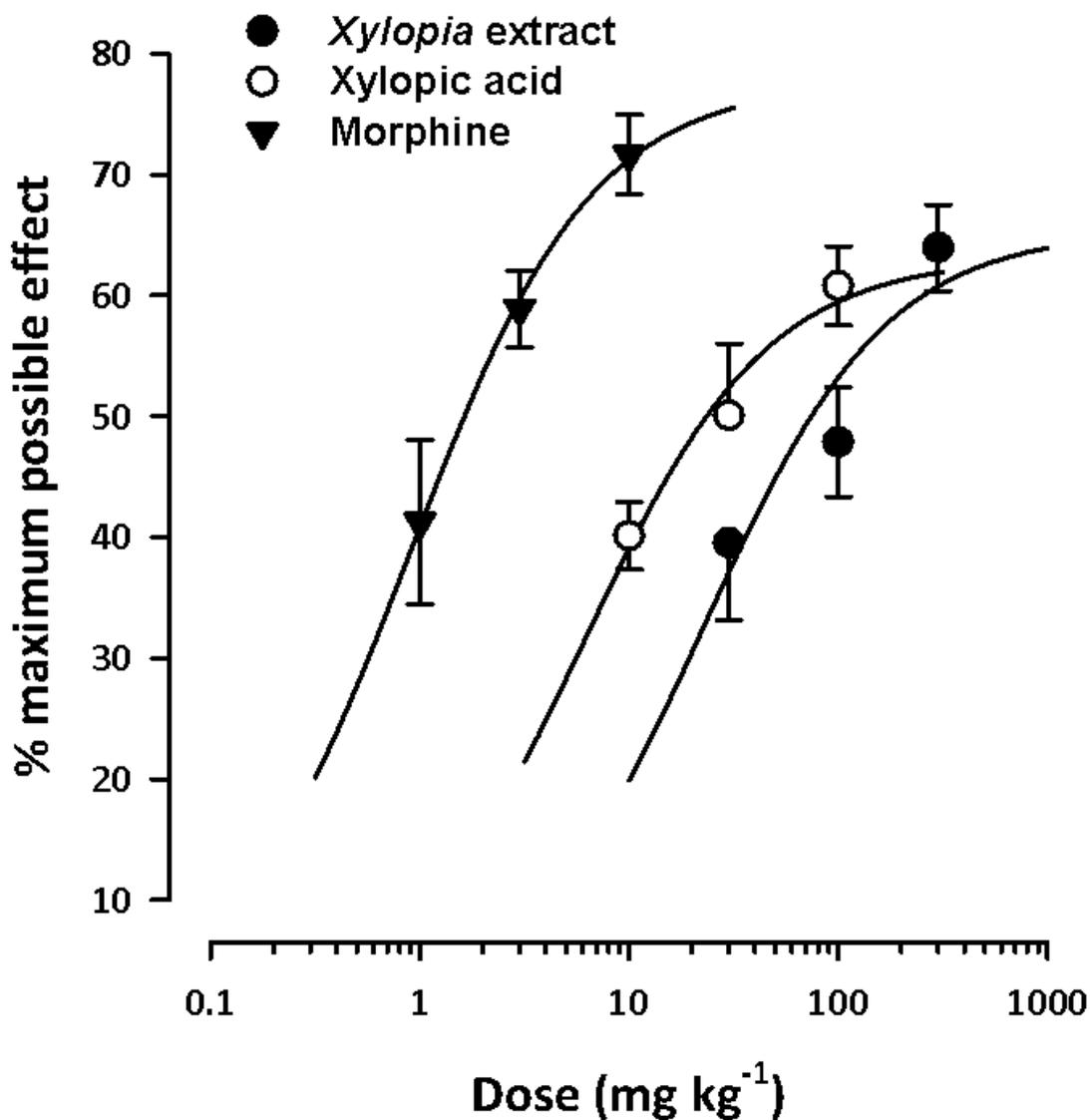
Figure 3.13 Dose–response curves for the antinociceptive effect of extract (30-300 mg kg⁻¹), xylopic acid (10-100 mg kg⁻¹) and morphine (1-10 mg kg⁻¹) in the acute muscle pain in rats. Each point is the mean ± S.E.M. of 8 animals.
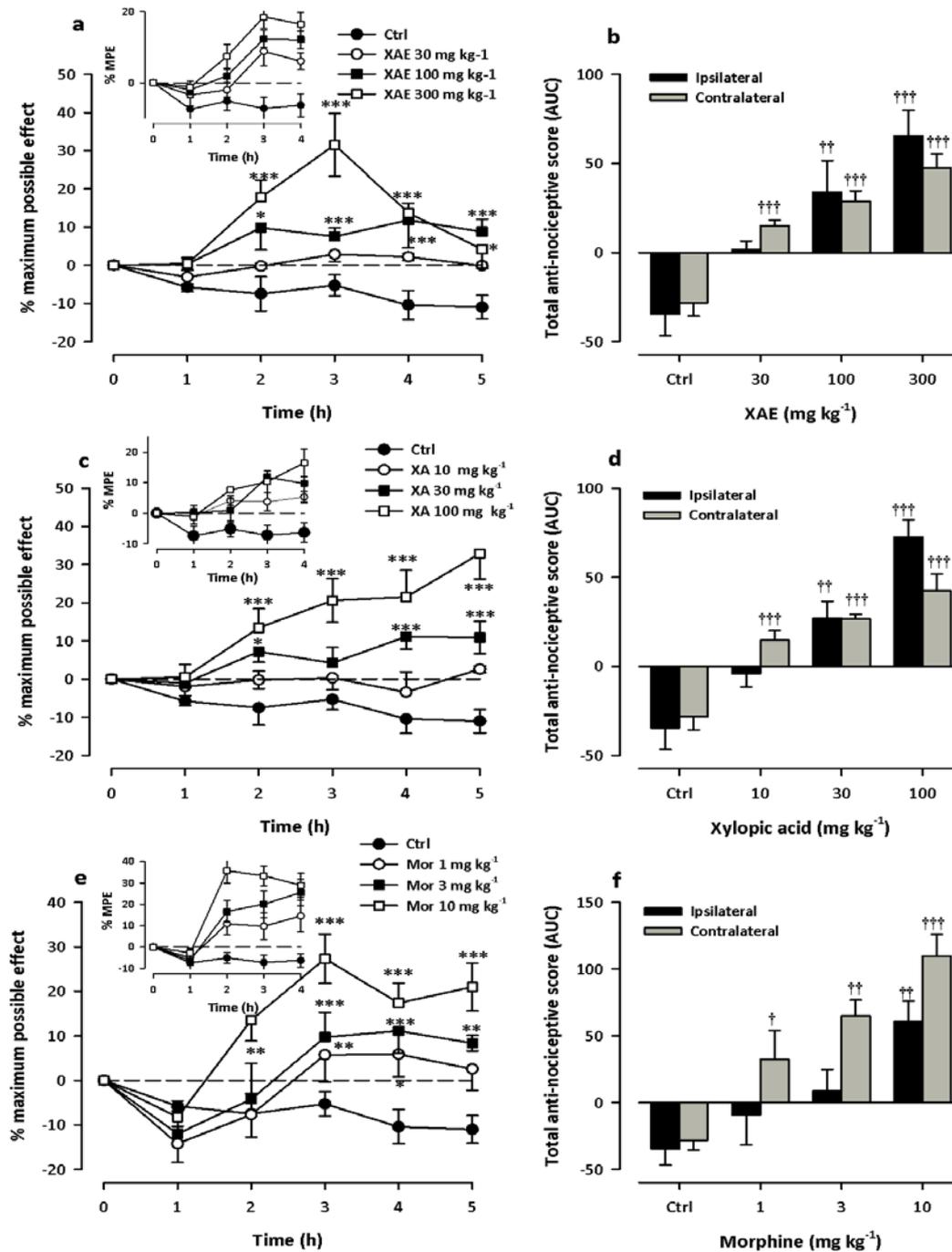
Figure 3.14 Effect of extract (30-300 mg kg^{-1} *p.o.*), xylopic acid (10-100 mg kg^{-1} *p.o.*) and morphine (1-10 mg kg^{-1} i.p.) on the time course curve of ipsilateral paw and contralateral (insets) paw withdrawal latency in the Randall-Selitto test (a, c and e) and the AUC (b, d and f) in chronic muscle pain. Data is presented as mean ± S.E.M. (n = 8); ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{†††}P<0.001$, $^{††}P<0.01$, $^{†}P<0.05$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
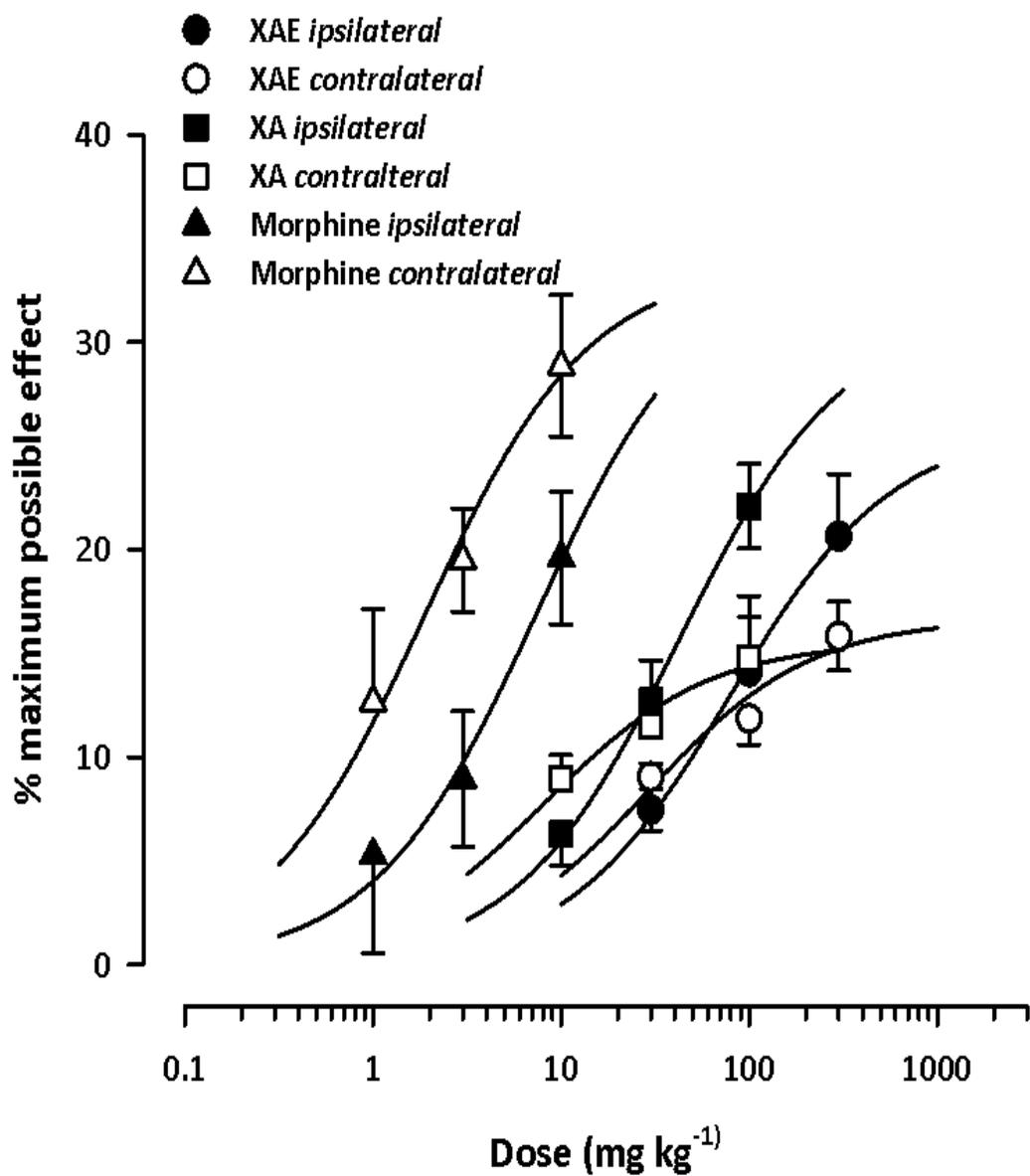
Figure 3.15 Dose–response curves for the antinociceptive effect of extract (30-300 mg kg⁻¹), xylopic acid (10-100 mg kg⁻¹) and morphine (1-10 mg kg⁻¹) in the chronic muscle pain tests in rats. Each point is the mean ± S.E.M. of 8 animals.

3.4.7 Tolerance Studies

Morphine (3 mg kg^{-1}, i.p.) significantly attenuated the basal nociceptive response in both phases (Fig. 3.16c) of the formalin test in animals chronically-treated with vehicle for eight days. However, the same dose of morphine administered on day 9 in animals chronically-treated with 6 mg kg^{-1}, i.p. morphine failed to show such effect indicating the development of tolerance in first phase (Fig. 3.17a) and second phase (Fig. 3.17b) of the formalin test.

The extract (300 mg kg^{-1}, *p.o.*) and xylopic acid (100 mg kg^{-1}, *p.o.*) exhibited anti-nociceptive activity in mice chronically-treated with the extract (600 mg kg^{-1}, *p.o.*) and xylopic acid (200 mg kg^{-1}, *p.o.*) respectively or vehicle for 8 days (Fig. 3.16a and b respectively), indicating failure of tolerance development to the anti-nociceptive effects of the extract and xylopic acid in the first phase (Fig. 3.17a) and second phase (Fig. 3.17b) of the formalin test. The extract (300 mg kg^{-1}, *p.o.*) and xylopic acid (100 mg kg^{-1}, *p.o.*) still demonstrated anti-nociceptive activity in mice chronically-treated with morphine (6 mg kg^{-1} i.p.) for eight days, indicating the absence of cross-tolerance development with morphine in the formalin test (Fig. 3.17).

3.4.8 Effect of extract and xylopic acid on motor coordination on the Rota-rod

Effect on motor coordination was measured as duration of stay on the rotating drum of the rota-rod. The results obtained indicated that there were no significant differences between xylopic acid-treated ($F_{3,28}$=0.39, P=0.7635; table 2) groups compared to the control group. ANOVA did not reveal any significant effect of the extract on the time spent on the rota-rod ($F_{3,28}$=2.44, P=0.0855; table 2). However, Bonferroni's *post hoc* analysis revealed significant effects (P<0.01, table 3.4) for the extract at the dose of 300 mg kg-1, when compared to vehicle treated controls.
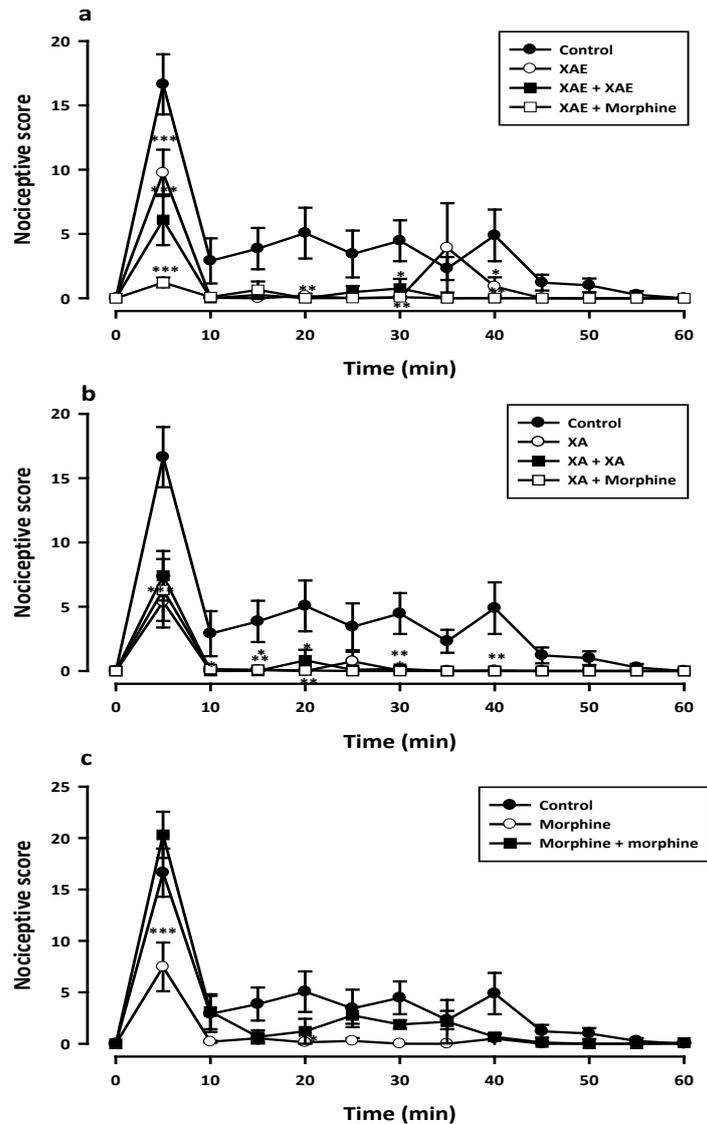
Figure 3.16 Effect of (a) extract (300 mg kg^{-1} p.o.), (b) xylopic acid (100 mg kg^{-1} *p.o.*) and (c) morphine (3 mg kg^{-1} i.p.) challenge on the time course effect of the total nociceptive score of formalin-induced licking of mice chronically treated with vehicle, extract (600 mg kg^{-1} *p.o.*), xylopic acid (200 mg kg^{-1} *p.o.*) or morphine (6 mg kg^{-1} i.p.) for 9 days. Each point represents the mean ± S.E.M. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (Two-way ANOVA followed by Bonferroni's *post hoc* test).
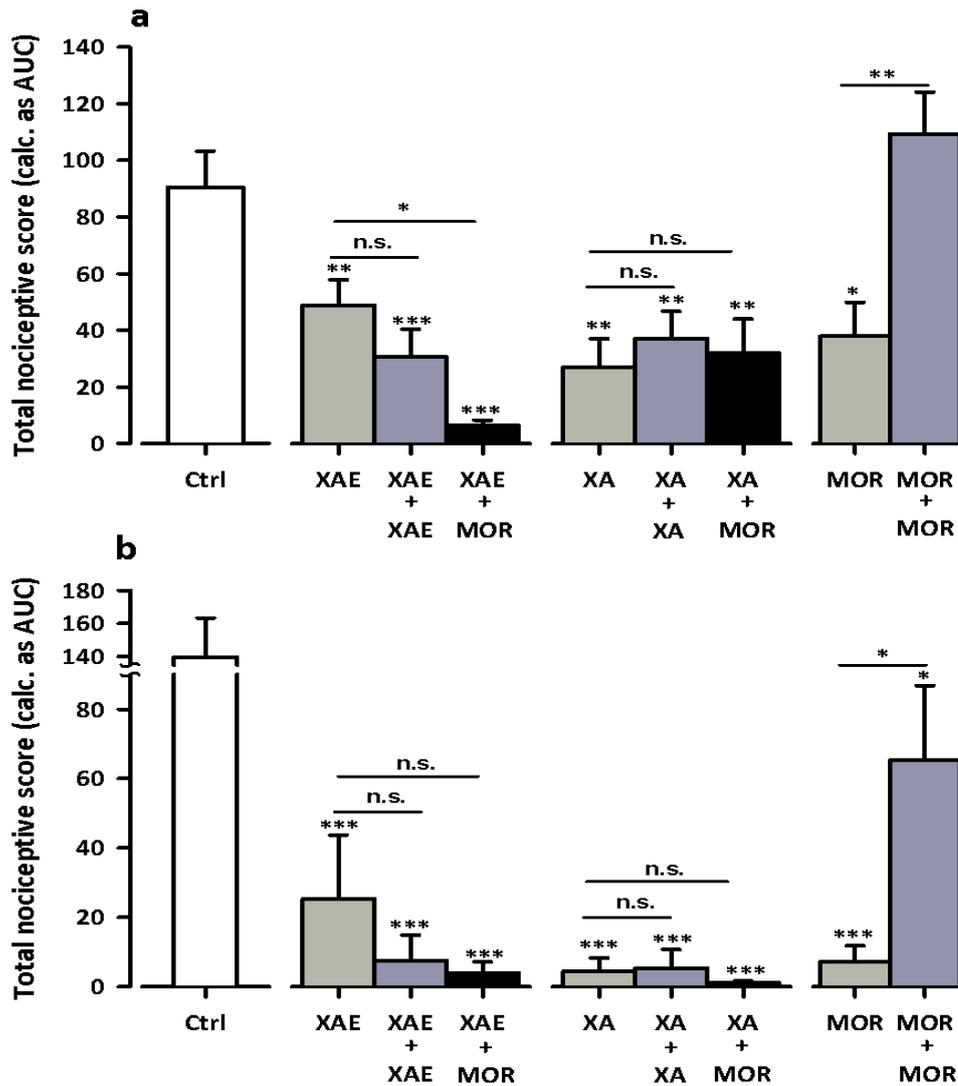
Figure 3.17 Effect of extract (300 mg kg^{-1} *p.o.*), xylopic acid (100 mg kg^{-1} *p.o.*) and morphine (3 mg kg^{-1} i.p.) challenge on mice chronically treated with vehicle, extract (600 mg kg^{-1} *p.o.*) and xylopic acid (200 mg kg^{-1} *p.o.*) or morphine (6 mg kg^{-1} i.p.) for 9 days on the total nociceptive score of formalin-induced licking test in mice AUC for (a) phase 1 and (b) phase 2 of formalin-induced pain respectively. Each column represent the mean ± S.E.M. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (one-way ANOVA followed by Newman-Keuls' *post hoc*).

3.4.9   Test for catalepsy and Grip strength assay

Post grip strength measured in the animals after the extract and xylopic acid administration did not differ from the baseline measurements (Table 3.2). Similar trends were observed for the extract and xylopic acid on the time spent on the bars (Table 3.3). The time spent on the bar by morphine treated mice however increased due to catalepsy (Table 3.3). The catalepsy induced in morphine treated mice affected grip strength measurement significantly (Table 3.2).

Table 3.2 Effect of XAE the (30-300 mg kg^{-1}), XA (10-100 mg kg^{-1}) and morphine (40 mg kg^{-1}) on grip strength measurement before (B) and after (A) XAE, XA and morphine administration.

Dose (mg/kg)	Control		XAE		XA		Morphine	
	B	A	B	A	B	A	B	A
0	30.9±3.3	30.6±2.1	-	-	-	-	-	-
10	-	-	-	-	25.9±2.1	25.8±1.8	-	-
30	-	-	30.9±1.4	31.2±3.1	30.4±1.5	31.2±2.5	-	-
40	-	-	-	-	-	-	29.1±1	39.8±7*
100	-	-	31.7±2.9	31.3±2.8	29.7±2.5	31.4±3.2	-	-
300	-	-	32.1±5.0	32.3±3.3	-	-	-	-

All data are expressed as a mean ± SEM. *$P < 0.05$ represents significant difference from respective pre-treated group animals of the same drug (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).

Table 3.3 Effect of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and morphine (40 mg kg^{-1}) on the total time (in seconds) spent on the bar in the Bar test of catalepsy before (B) and after (A) extract, xylopic acid and morphine administration.

Dose mg/kg	Control		extract		xylopic acid		Morphine	
	B	A	B	A	B	A	B	A
0	10.4±1.6	9.2±1.6	-	-	-	-	-	-
10	-	-	-	-	7.1±0.5	6.8±0.7	-	-
30	-	-	8.1±1.0	8.4±2.0	7.0±0.8	5.7±0.7	-	-
40	-	-	-	-	-	-	6.3±0.4	113.8±34***
100	-	-	6.4±0.7	6.0±0.5	6.8±0.7	6.1±1	-	-
300	-	-	7.7±0.7	7.9±2.5	-	-	-	-

All data are expressed as a mean ± SEM. ***$P < 0.001$ represents significant difference from respective pre-treated group animals of the same drug (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).

Table 3.4 Effect of extract and xylopic acid on motor coordination measured as time spent on the rota-rod.

Dose (mg kg^{-1})	Time spent on rod (s)			
	Vehicle	XAE		XA
	0h[#]	1h	2h	3h
0	120.0±0.0	116.0±2.1	118.6±0.9	118.0±2.0
30	120.0±0.0	118.4±1.2	112.2±3.7	115.4±1.2
100	120.0±0.0	110.6±5.3	113.6±4.4	116.8±1.6
300	120.0±0.0	105.6±7.0	102.6±5.9**	116.8±1.8
10	120.0±0.0	117.6±1.9	116.0±2.1	114.4±3.9
30	120.0±0.0	116.4±2.2	116.4±1.5	117.0±2.0
100	120.0±0.0	116.6±1.4	118.0±2.0	118.0±2.0

Data are expressed as mean ± SEM (n=8). **$P<0.01$ compared to vehicle-treated control group (Two-way ANOVA followed by Bonferroni's test). [#] Hours after treatment

3.5   DISCUSSION

A battery of tests was used to evaluate the antinociceptive properties of the extract and xylopic acid. The extract and xylopic acid were effective in all the pain models used. The models employed different stimuli types, intensity and sources in the induction of pain in order to provide a complete analgesic profile of the extract and xylopic acid. Acute, chronic, persistent and colic pain models were employed in order to evaluate centrally and peripherally mediated effects of the extract and xylopic acid. The acetic acid writhing test is useful in investigating visceral antinociceptive activity of drugs. Acetic acid injection causes the local release of pro-inflammatory substances, including prostaglandins, substance P, bradykinin, and/or cytokines (Raja *et al.*, 1988) resulting in the activation and sensitization of peripheral nociceptive afferent neurons. The antinociception of the extract and xylopic acid in this test may be due to possible inhibition of the synthesis and/or release of pro-inflammatory substances peripherally similar to diclofenac used as control (Panthong *et al.*, 2007). Both extract and xylopic acid inhibited prostaglandin E_2 (EP) and bradykinin (B 1 and/or B 2) receptors/ nociceptive pathways. Morphine exhibited similar effects and this agrees with other reports (Ferreira and Nakamura, 1979; Smith *et al.*, 1982; Stein *et al.*, 1988; Levine and Taiwo, 1989; Stein *et al.*, 1989; Parsons and Herz, 1990). These effects (inhibition of prostaglandin and bradykinin receptors/ nociceptive pathways) also contributed to the observed antinociceptive effects of the extract and xylopic acid in this model.

Furthermore, the antinociceptive effects of extract and xylopic acid observed in this test may have occurred spinally through the inhibition of pro-inflammatory mediators – mediated central sensitization in the spinal cord. Central sensitisation causes altered responsiveness of dorsal horn neurons leading to the expansion of the receptive fields of the neurons (Devor and Wall, 1978; Woolf and Fitzgerald, 1983). This eventually manifests as hypernociception (an increased   sensitivity to noxious stimuli) after the injection of acetic acid.

The acetic acid-induced writhing test therefore provided scientific evidence to the use of the fruits of *X. aethiopica* in management of colic pain, a form of visceral pain. In order to evaluate the antinociceptive effect of the extract and xylopic acid on other

forms of pain such as those arising from sensory nerves of which the fruits are reported to be used for, the formalin test was performed.

The formalin test is a tonic model of continuous pain resulting from formalin-induced tissue injury. Both extract and xylopic acid inhibited the neurogenic and inflammatory phases of the mouse formalin test. Morphine, an opioid analgesic used as control blocked both phases of the formalin test and this agrees with literature (Trongsakul *et al.*, 2003). The formalin test is a useful model, particularly for the screening of novel compounds, since the nociception produced in this test involves inflammatory, neurogenic, and central mechanisms (Ellis *et al.*, 1998). The formalin test was further used to evaluate the antinociceptive properties of the extract and xylopic acid because it is considered the most predictive of acute pain and is believed to be a more valid model for clinical pain (Dubuisson and Dennis, 1977; Tjolsen *et al.*, 1992). The possible mechanism(s) of analgesia produced by the extract and xylopic acid can be elucidated in formalin test (Hunskaar and Hole, 1987; Yun-Kyoung Yim, 1999; Tang *et al.*, 2007). The first phase which is transient is caused by the direct effect of formalin on transient receptor potential ankyrin subtype 1 receptors (TRPA 1) and transient receptor potential vanniloid receptors (TRPV 1) which are cation channels on sensory C-fibres (Mcnamara *et al.*, 2007). The second prolonged phase is associated with the combination of an inflammatory reaction in the peripheral tissue causing a release of nociceptive mediators such as serotonin, histamine, bradykinin and prostaglandins which subsequently cause sensitization of the central neurons leading to changes in central processing of pain (Da Rocha *et al.*, 2011; Santa-Cecilia *et al.*, 2011). The inflammatory pain has also been associated with higher brain centres like the anterior cingulate cortex (ACC). Inhibition of ACC decreases inflammatory nociceptive response of the prolonged, tonic, inflammatory portion of the formalin test manifested as reduction in paw licking/biting behaviour (Donahue *et al.*, 2001). The extract and xylopic acid like morphine acted peripherally by possibly inhibiting the activation of TRPA 1 receptors and centrally which may involve inhibition of the nociceptive effects of serotonin, adrenaline, nor adrenaline, prostaglandins, bradykinin, acetylcholine and adenosine to produce antinociception in the formalin test. The formalin test confirmed the traditional use of the fruits of *X. aethiopica* in the treatment of both neurogenic and inflammatory pain.

To further confirm the effect of the extract and xylopic acid on inflammatory pain the carrageenan- induced mechanical hyperalgesia and acute and chronic carrageenan/kaolin- induced musculosketal pain experiments were performed. Although these two experiments employ the same inflammation inducing agent, the pathophysiology underlining these two models vary and it has been demonstrated that the mechanisms of pain and hyperalgesia induced by injecting inflammatory agents into different tissues like muscle, joint and skin are different and may represent different pathophysiological processes (Radhakrishnan *et al.*, 2003). The effect of the extract and xylopic acid on mechanical hyperalgesia was evaluated in the Randall–Selitto assay. Acute inflammatory pain assay was conducted employing the Randall–Selitto assay, a test for quantification of thresholds of rat hind paw withdrawal reflex to nociceptive pressure stimulation. The extract and xylopic acid were effective in this model. The hyperalgesia in the carrageenan-induced acute pain model is due to the activation of slowly-adapting mechanoreceptors with decreased thresholds, which are predominantly C-fibers located in cutaneous and subcutaneous structures that would have required greater stimulus intensities for activation (Birder and Perl, 1994; Lewin and Moshourab, 2004). The extract and xylopic acid together with the control drugs; morphine and diclofenac produced anti-hyperalgesic effect in this model by possibly raising the thresholds of various mechanoreceptors and mechano transduction pathways which were lowered by the carrageenan-induced inflammation and/or antagonizing the pain mediating agents responsible for lowering the pain threshold (Park *et al.*, 2008).

The antinociceptive effects of the extract and xylopic acid in the musculoskeletal pain experiments were similar to the effects observed in the Randall–Selitto assay. Injection of 3% carrageenan into the muscle and a mixture of (3% carrageenan: 3% kaolin) into the knee provide a relevant model for assessing painful clinical conditions that are aggravated by mechanical pressure at the site of injury such as arthritis. Pain arising from musculoskeletal disorders is a major clinical problem globally and difficult to treat (Afable and Ettinger, 1993). The effectiveness of the extract and xylopic acid in this model give an indication that they may be useful in patients with musculoskeletal disorders such as arthritis and fibromyalgia (Skyba *et al.*, 2005). Histologically, there is an acute inflammatory response with neutrophilic infiltration for the first week. This converts to a macrophage-dominated chronic inflammation by 1 week that lasts through 8 weeks. Thus, carrageenan can be used as a model of acute inflammation of muscle or

joint for the first 24 h and as a model of chronic inflammatory hyperalgesia after one week (Radhakrishnan *et al.*, 2003). Acute musculoskeletal pain resulted from the activation of group III (Aδ-fiber) and group IV (C-fiber) polymodal muscle nociceptors. These nociceptors are sensitized by released pain mediators such as neuropeptides (substance P), citrulline and prostaglandins from the nerve endings eventually leads to peripheral sensitization and hyperalgesia in the ipsilateral limb (Schaible *et al.*, 1987; Mense and Simons, 2001; Sluka, 2002). Biochemically and histopathologically, the first 3 hours of the acute phase of knee joint inflammation is characterised by increased levels of cyclooxygenase enzyme-2 (COX-2 produces prostaglandin E_2) mRNA and inducible nitric oxide synthase (iNOS produces proinflammatory chemical NO) mRNA in the synovial fluid. This result in the synthesis and release of inflammatory mediators such as histamine, 5-HT, bradykinin and prostaglandins at 4 hours causing mild haemorrhage, oedema and minimal cell infiltrates of polymorphonuclear granulocytes (Di Rosa and Willoughby, 1971). More neutrophils are seen in the ipsilateral knee at 8 hours and at 24 hours, the acute inflammation is severe, accompanied by fibrinous exudate in the knee joint. Increased levels of the excitatory amino acid, glutamate, and nitric oxide metabolites as well as other cytokines such as nerve growth factor (NGF) and tumor necrosis factor-α (TNF- α) have been observed in the in the ipsilateral synovium of the acute phase (Manni and Aloe, 1998; Bement *et al.*, 2003).

The inflammation converts to a chronic state characterised by macrophage infiltration with a few scattered mast cells. The pain associated with the chronic inflammation involves central sensitization caused by glutamate, substance P, calcitonin gene-related peptide and prostanoids on dorsal horn neurons and supraspinal structures Chronic musculoskeletal hyperalgesia is a result of sensitization of deep-tissue nociceptors, followed by temporal summation, wind-up and central sensitization by glutamate, substance P, calcitonin gene-related peptide and prostanoids (synaptic plasticity and efficacy) leading to wide spread deep tissue hyperalgesia and expanded areas of referred pain (bilateral spread of pain) (Radhakrishnan *et al.*, 2003; Skyba *et al.*, 2005). In addition, descending inhibitory control of pain mediated via the descending inhibitory pain control system in the rostral ventral medulla (RVM), glutamate exitotoxity, increased descending facilitation of pain and destruction of K^+/Cl^- pump of the neurons (this causes accumulation of Cl^- ions intracellularly via stimulation of NMDA situated on GABA receptors and further GABA activation results in Cl^- ions

efflux leading to paradoxical GABA mediated excitation of neurons) contribute to the hyperalgesia observed in chronic musculoskeletal pain. Clinically, it has been observed that the descending inhibitory pain control system in the rostral ventral medulla is impaired in people with chronic musculoskeletal pain (Herrero and Cervero, 1996; Yang *et al.*, 1996; Urban *et al.*, 1999; Sluka *et al.*, 2001). Therefore chronic musculoskeletal pain (contralateral hyperalgesia) induced in the deep tissues is independent of primary afferent drive or input from the site of injury once it has been developed (Coderre and Melzack, 1985; Sluka *et al.*, 2001). It is therefore plausible to state that the extract and xylopic acid acted peripherally by acting against the released pro-inflammatory mediators such as neutrophils and centrally by suppressing the effect of infiltrated macrophages as well as facilitating the inhibitory activities of the RVM to inhibit acute and chronic pain in this model similar to morphine used as control (Skyba *et al.*, 2005).

The paw withdrawal response in the Hargreaves (plantar) thermal hyperalgesia test is mediated peripherally. The hyperalgesia observed in the Hargreaves (plantar) thermal test can therefore be inhibited by peripherally acting drugs (especially drugs that inhibit COX enzymes or the effect of the inflammatory mediators) (Dirig *et al.*, 1997; Lavich *et al.*, 2005). The tail-flick response is believed to be a spinally mediated reflex (Chapman *et al.*, 1985) but the mechanism of response could also involve higher neural structures (Jensen and Yaksh, 1986). The hyperalgesia observed in the Hargreaves (tail) thermal test can therefore be inhibited by drugs that act spinally and supra spinally. Employing the tail and paw withdrawal tests allow for the detection of both peripherally, spinally and supra spinally acting drugs. The extract, xylopic acid as well as morphine (Lavich *et al.*, 2005) acted peripherally, spinally and supra spinally to inhibit thermal hyperalgesia.

The development of tolerance that necessitates dose escalation regardless of disease progression, greatly limit the effectiveness and usage of some important analgesics such as the opioids (Tang *et al.*, 2006; Chen *et al.*, 2008). The extract and XE were chronically administered to animals to determine whether tolerance will develop to the analgesic effects of the extract and xylopic acid respectively and also if morphine tolerance could cross-generalize to the analgesic effects of the extract and xylopic acid. The results suggest that, unlike morphine, the extract and xylopic acid do not induce tolerance to their analgesic effects after chronic administration in the formalin test. Also

morphine tolerance does not cross-generalize to the effects of the extract and xylopic acid. These are significant findings and imply that the extract and xylopic acid can be used to treat pain in opioid tolerant individuals.

In order to eliminate the possibility that decreased motor function is confounding the results obtained in the nociceptive test models, motor performance of the mice was evaluated using the rota-rod. Some of the models employed such as the abdominal writhing response and grip strength measurement may be suppressed by drugs like muscle relaxants, catalepsy-inducing agents and adrenergic agonists which can lead to the misinterpretation of results (Pietrovski *et al.*, 2006). Muscle rigidity (cataleptic state) did not confound the antinociceptive effect of the extract and xylopic acid in the grip strength assay. Again, xylopic acid did not compromise motor coordination hence the measurement of their antinociceptive activity in models such as acetic acid-induced writhing test was not a false positive due to impairment in motor activity of the animals. The extract at the highest dose tested (i.e. 300 mg kg^{-1}) exerted some effect on motor coordination.

3.6   CONCLUSION

The extract and xylopic acid acts both centrally and peripherally to inhibit acute and chronic pain of neurogenic and inflammatory origin without the development of tolerance to their antinociceptive effects.

Chapter 4

EFFECT OF EXTRACT AND XYLOPIC ACID ON NEUROPATHIC PAIN

4.1 INTRODUCTION

The formalin test can be used as a predictor of agents that may be effective in neuropathic pain (Ellis *et al.*, 1998; Vissers *et al.*, 2006). The extract and xylopic acid as noted earlier exhibited antinociception in this (formalin) test. The extract and xylopic acid therefore may be effective in neuropathic pain. To further evaluate this claim, the analgesic properties of the extract and xylopic acid were evaluated in chemotherapy-induced neuropathic pain.

Neuropathic pain is defined as a form of chronic pain that results from damage or abnormal function of the central or peripheral nervous system (Reyes-Gibby *et al.*, 2009). Patients with neuropathic pain frequently report of sensory abnormalities including burning sensations, exaggerated responses to noxious stimuli (hyperalgesia), pain sensations resulting from innocuous stimuli (allodynia), an exaggerated response to touch such as to bed sheets (hyperesthesia), and spontaneous pain episodes (dysesthesia) (Schim, 2009). Neuropathic pain can also alter the patient's quality of life by interfering with emotional well-being. Cancer-related pain is a significant clinical problem and the two main sources of cancer-related pain are that from the malignancy itself and from the treatments utilized to alleviate the cancer (e.g. surgery, radiation and chemotherapy). Approximately one-third to half of all patients receiving anticancer treatment suffer from pain, and greater than two-thirds in the more advanced disease states (Lynch *et al.*, 2005). Although there has been an increase in the survival rate of cancer patients transforming the formerly lethal diseases into a chronic disease state, peripheral neuropathy and subsequent neuropathic pain related to chemotherapeutic treatment has been the dose limiting and greatest set back. This type of pain is often resistant to standard analgesics and severe enough for patients to skip chemotherapy treatment (Lynch *et al.*, 2005). To date no one drug or drug class is considered to be a 'safe and effective analgesic' in the treatment of chemotherapy-induced pain. Clearly the need exists for new pharmacotherapeutics for chemotherapy-induced neuropathic pain, either as stand-alone drugs or as supplements to already existing medications. Therefore, using doses of vincristine close to that used clinically, an animal model of

vincristine-induced neuropathic pain was used to evaluate the effect of the extract and xylopic acid in neuropathic pain (Aley *et al.*, 1996; Thibault *et al.*, 2008).

Vincristine-induced painful neuropathy in rats was chosen to evaluate the effect of the extract and xylopic acid in neuropathic pain due to the common features this protocol shares with vincristine-induced neuropathic pain in humans, specifically:

(i) Experimentally and clinically observed manifestation of pain occur at similar doses,

(ii) At doses above those required to alter nociception, motor function is also affected,

(iii) Both rapid onset and delayed onset forms of painful neuropathy are observed (Sandler *et al.*, 1969; Casey *et al.*, 1973; Ashburn and Lipman, 1993; Aley *et al.*, 1996; Thibault *et al.*, 2008).

4.2 MATERIALS AND METHODS

4.2.1 Animals

Sprague-Dawley rats (100–150 g) were used in this experiment. The animals were provided with the necessary conditions as described previously under section 3.2.1.

4.2.2 Drugs and chemicals

The following drugs were used: Pregabalin, (Lyrica®), was purchased from Pfizer Pharmaceuticals, Arzneimittelwerk Godecke, Freiburg, Germany; vincristine sulphate from Celon laboratory Ltd, Gajularamaram, India.

4.2.3 Vincristine-induced neuropathic pain

Vincristine sulphate was dissolved in saline and stored as a stock concentration of 1 mg 10 ml^{-1} at 4 °C. The animals received i.p. injection of vincristine at a final concentration of 0.1 mg kg^{-1} day^{-1} in two cycles of five consecutive 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 (Aley *et al.*, 1996). On day 15 baseline nociception 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 afterwards were treated with the extract (30-300 mg kg^{-1} *p.o.*), xylopic acid (10-100 mg kg^{-1} p.o.), pregabalin (10-100 mg kg^{-1} *p.o.*) or saline.

Three sets of experiments namely cold allodynia, Randall-Selitto mechanical hyperalgesia and Von Frey tactile, intermediate and mechanical hyperalgesia were then performed in order to evaluate the effects of the extract, xylopic acid and pregabalin on vincristine-induced painful neuropathy.

4.2.3.1 Assessment of tactile allodynia and mechanical hyperalgesia

To evaluate the effect of the extract, xylopic acid and pregabalin on mechanical allodynia/hyperalgesia, animals were placed in a restrainer and confined. 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 from a normally innocuous stimulus) because normal rats never withdraw from this stimulus (Flatters and Bennett, 2004; Siau *et al.*, 2006). Intermediate and mechanical hyperalgesia were assessed with Von Frey filaments of bending forces 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 (Flatters and Bennett, 2004; Siau *et al.*, 2006).

In ascending order of force, each filament was applied to the mid-plantar area (avoiding the base of the tori) of each hind paw five times, with each application held for 5 s. Withdrawal responses to the Von Frey filaments from both hind paws were counted and then expressed as an overall percentage response, e.g. if a rat withdrew to 6 out of the total 10 Von Frey applications, this was recorded as 60% overall response to that Von Frey filament (Flatters and Bennett, 2004; Siau *et al.*, 2006).

4.2.3.2 Cold allodynia

Cold allodynia was assessed by immersion of the rat's hind paw into a water bath containing cold water (4.5 °C) and latency to paw withdrawal was measured using a digital timer (Lynch *et al.*, 2005). Only one hind paw was tested during each immersion

at a time, with the maximum cut-off time limited to 20 s. For each animal, two recordings were made for each hind paw, and the data were reported as the mean of both hind paw values.

4.2.3.3 *Mechanical hyperalgesia in the Randall-Selitto test*

Mechanical nociception was measured with the IITC Life Science Model 2888 (Woodland Hills, CA, USA). Briefly, the rat's hind paw was placed into the pressure applicator, and a steadily increasing pressure stimulus (maximum cut-off of 250 g) was applied to the dorsal surface of the paw until withdrawal or vocalization occurred, at which weight the nociceptive threshold value was recorded. For each animal, two recordings were made for each hind paw, and the data were reported as the mean of both hind paw values (Stohr *et al.*, 2006; Woode *et al.*, 2009).

4.3 STATISTICAL ANALYSIS

Data were analysed as described under section 3.3

4.4 RESULTS

4.4.1 Assessment of vincristine-induced cytotoxicity

Vincristine treated animals showed overt signs of toxicity which manifested as loss in body weight and death of some animals (Fig 4.1). The initial mean weight of the animals was 105.53±3.25 which gradually reduced to a mean weight of 93.31±3.88. The percentage loss in body weight was 11.58%. Seven vincristine treated animals died: one animal died each on day six, eight and ten and four animals died on day nine.
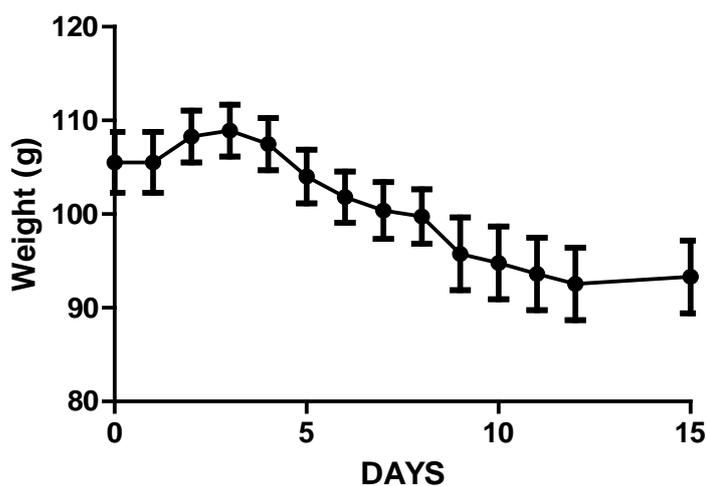
Figure 4.1 Daily weights and survival curve for groups of rats (n = 100) receiving vincristine (0.1 mg kg^{-1}). The x-axis (time) indicates days of vincristine treatment. The left y-axis illustrates the mean daily weights (g) ± S.E.M.

4.4.2 Measurement of Tactile allodynia using Von Frey filaments of 4 g

Intraperitoneal injection of vincristine for two 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 increase response to tactile allodynia compared to the treated animals. Extract (30-300 mg kg^{-1}) and xylopic acid (10-100 mg kg^{-1}) decreased the response to paw withdrawal ($F_{3,28}$=5.12, P=0.006: $F_{3,28}$=3.72, P=0.0229 respectively, Fig. 4.2a and c) producing tactile anti-allodynia of 61.3±11.4% and 65.8±23.8% respectively at the highest doses used (Fig. 4.2b and d).

The anticonvulsant, pregabalin (10-100 mg kg^{-1}) significantly and dose-dependently inhibited tactile allodynia ($F_{3,28}$=5.92, P=0.0029) (Fig. 4.2e):with the highest dose of pregabalin, 100 mg kg^{-1} producing an anti-allodynic effect of 62.4±8.93% (Fig.4.2f).

Xylopic acid was more potent than pregabalin and the extract. Pregabalin was also more potent than the extract (Fig.4.3).

4.4.3 Measurement of intermediate hyperalgesia using Von Frey 8 g filament

Von Frey filaments of 8 g was used to assess the effect of the extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and pregabalin (10-00 mg kg^{-1}) on mechanical hypernociception intermediate to tactile allodynia and mechanical hyperalgesia. The extract, xylopic acid and pregabalin produced significant anti-hyperalgesia (extract: $F_{3,28}$=2.91, P=0.0517, xylopic acid: $F_{3,28}$=3.61, P=0.0255, pregabalin: $F_{3,28}$=2.40, P=0.0889 Fig. 4.4a, c and e) respectively in this test. The highest doses of extract, xylopic acid and pregabalin produced possible maximum anti-hyperalgesic effects (Fig. 4.4b, d and f).

Xylopic acid was more potent and efficacious than pregabalin and the extract. Pregabalin was also more potent and efficacious than the extract (Fig.4.5).
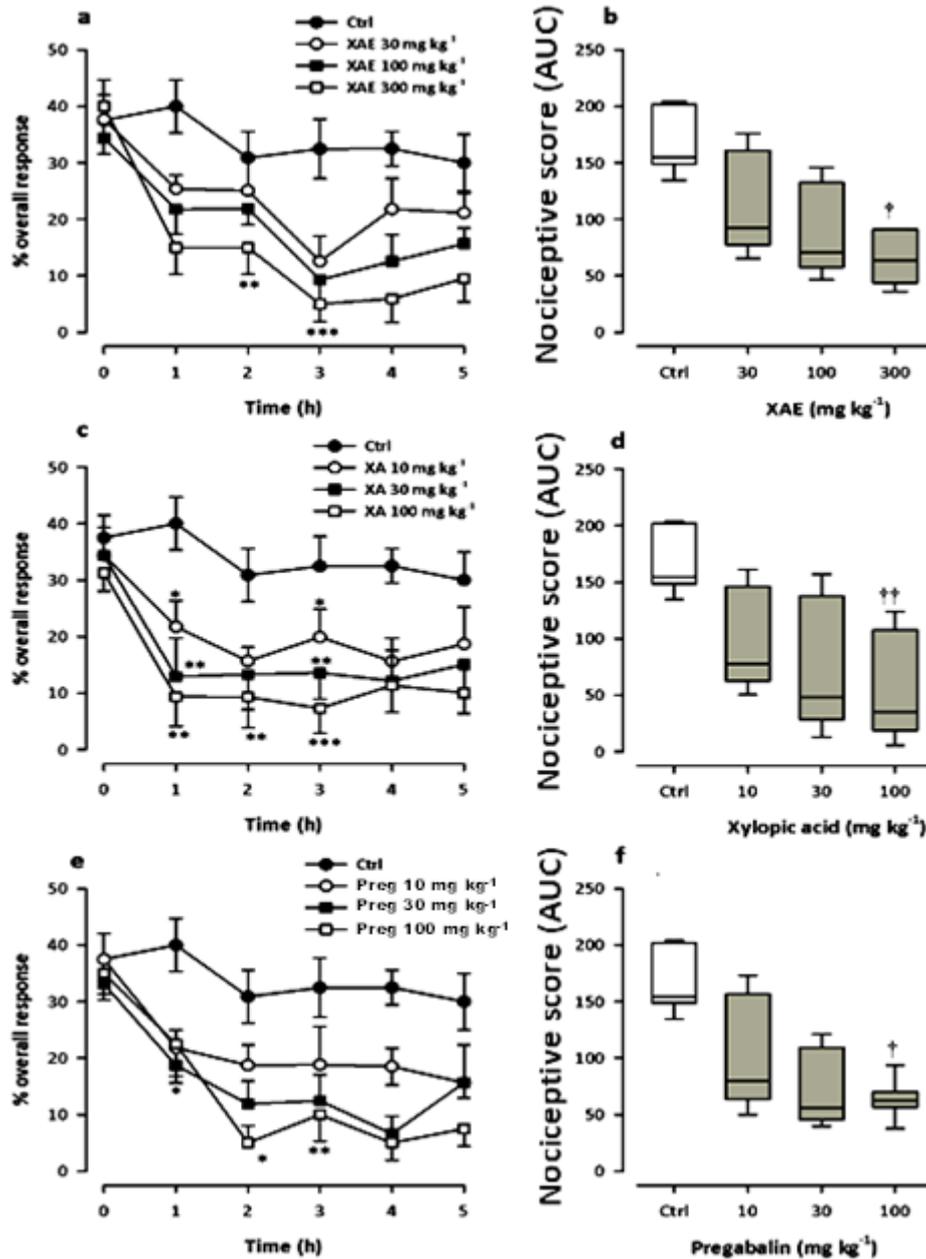
Figure 4.2 Effect of (a) extract (30-300 mg kg^{-1} *p.o.*), (c) xylopic acid (10-100 mg kg^{-1} *p.o.*) and (e) pregabalin (10-100 mg kg^{-1} *p.o.*) on the time course of vincristine-induced neuropathic pain (tactile allodynia, 4 g Von Frey filament) in rats. Each point represents Mean ± S.E.M (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{†††}P<0.001$ $^{††}P<0.01$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
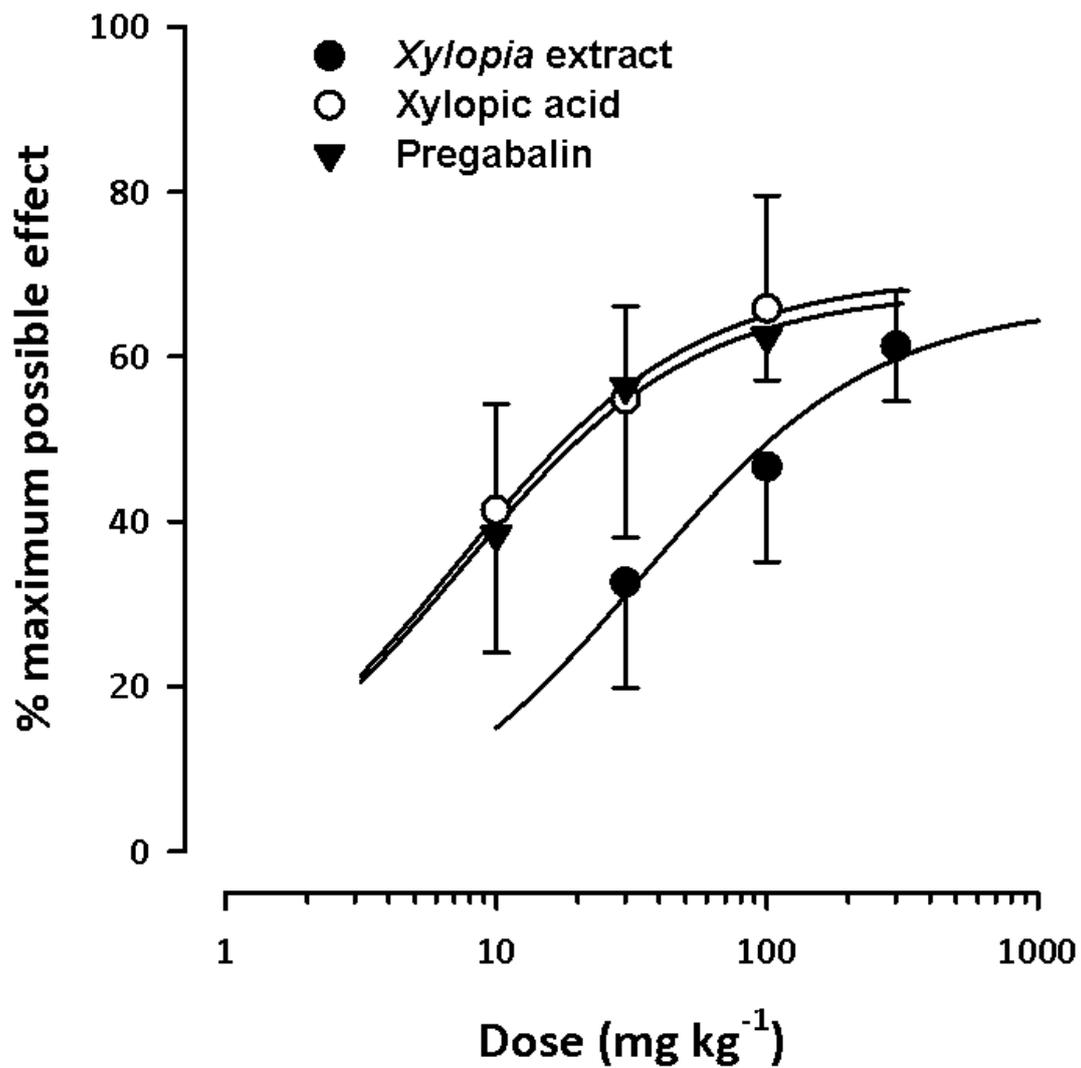
Figure 4.3 Dose–response curves for the tactile anti-allodynic effect of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and pregabalin (10-100 mg kg^{-1}) in vincristine-induced neuropathic pain. Each point is the mean ± S.E.M. of 8 animals.
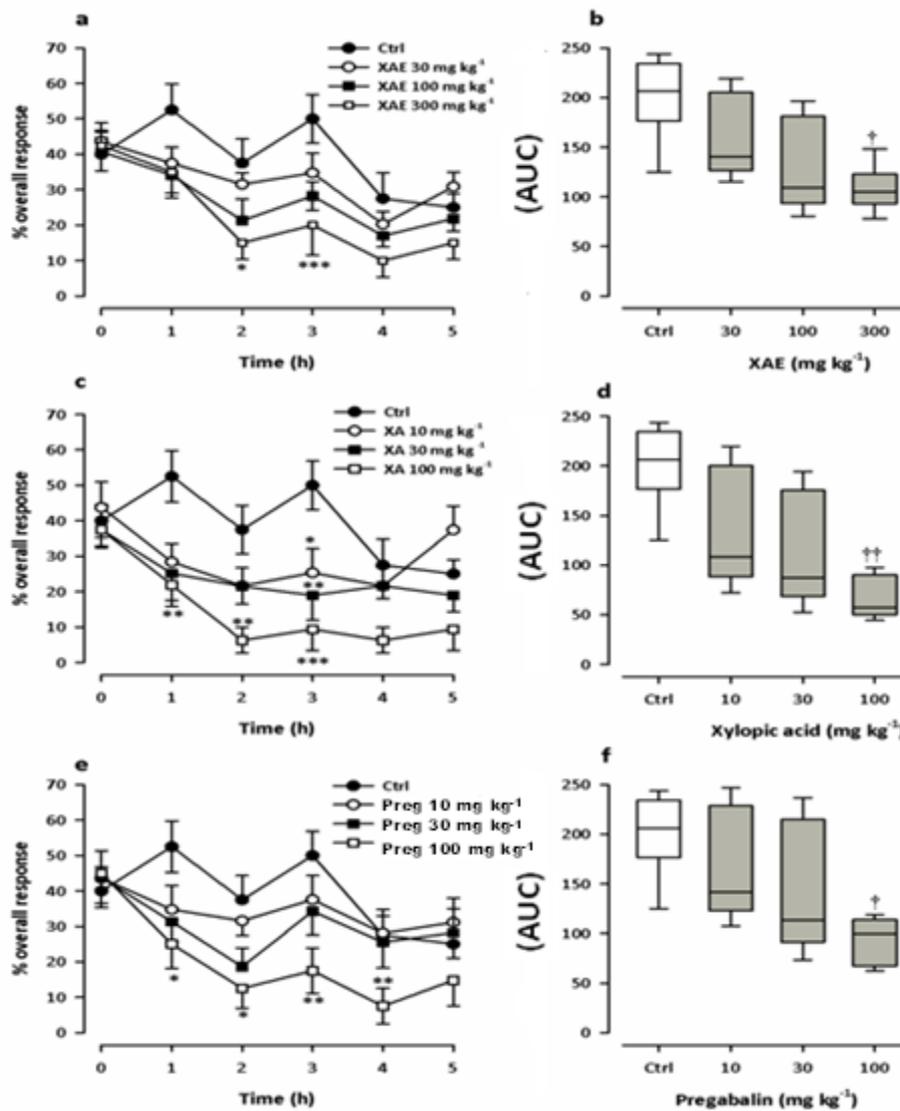
Figure 4.4 Effect of extract (a) (30-300 mg kg^{-1} p.*o*.), (c) xylopic acid (10-100 mg kg^{-1} p.*o*.) and (e) pregabalin (10-100 mg kg^{-1} p.*o*.) on the time course of vincristine-induced neuropathic pain (8 g Von Frey filament) in rats and the AUC (b, d and f). Each point represents Mean ± S.E.M (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ****P* < 0.001; ** *P* < 0.01; **P* < 0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{†††}P$<0.001 $^{††}P$<0.01 compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).

Figure 4.5 Dose–response curves for the antinociceptive effects of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and pregabalin (10-100 mg kg^{-1}) in vincristine-induced neuropathic pain (8 g Von Frey filament). Each point is the mean ± S.E.M. of 8 animals.

4.4.4 Measurement of Mechanical hyperalgesia using Von Frey 15 g filament

Baseline mechanical hyperalgesia taken on day 15 using Von Frey 15 g filament revealed that both hind paws exhibited marked static mechanical hyperalgesia. The extract (30–300 mg kg^{-1}, *p.o.*) produced a significant ($F_{3,28}$ =3.61, *P*=0.0255) and dose-dependent inhibition of static mechanical hyperalgesia (Fig. 4.6a).The highest dose of the extract decreased the overall response to mechanical hyperalgesia by 48.3 ± 32.5% (Fig. 4.6b).

Significant ($F_{3,28}$= 11.76, *P*=0.00003) and dose-dependent mechanical hyperalgesia was produced after xylopic acid (10–100 mg kg^{-1}, *p.o.*) administration (Fig. 4.6c): with the highest dose decreasing the overall mechanical hyperalgesia by 63.4 ± 25.5% (Fig. 4.6d).

The administration of pregabalin (10–100 mg kg^{-1}) significantly ($F_{3,28}$ = 10.87, *P* =0.00006) and dose-dependently inhibited mechanical hyperalgesia (Fig. 4.6e) and a maximum anti-hyperalgesia of 72 ± 25.1% (Fig. 4.6f) was achieved at the highest dose administered.

Pregabalin was more efficacious than xylopic acid and extract. Xylopic acid was also more efficacious than the extract (Fig.4.7).
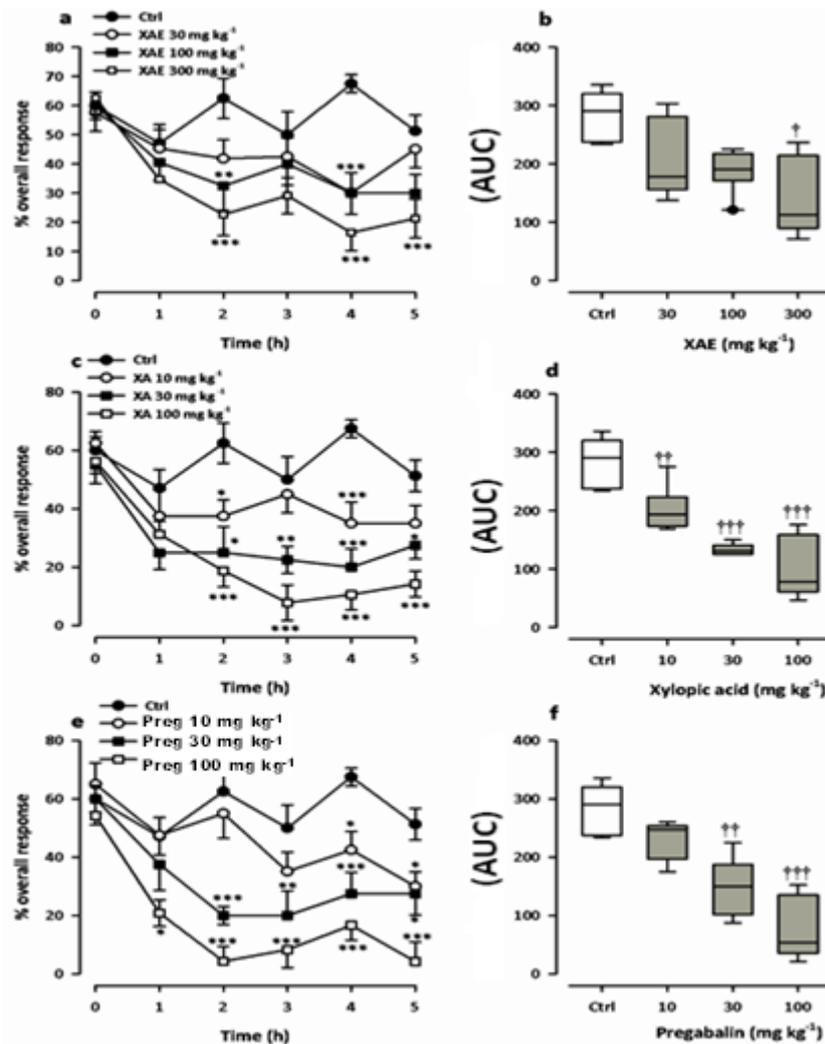
Figure 4.6 Effect of (a) extract (30-300 mg kg^{-1} p.o.), (c) xylopic acid (10-100 mg kg^{-1} p.o.) and (e) pregabalin (10-100 mg kg^{-1} p.o.) on the time course of vincristine-induced neuropathic pain (15 g Von Frey filament) in rats and the AUC (b, d and f). Each point represents Mean ± S.E.M (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Symbol represents outlier. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*); $^{†}P \leq 0.05$, $^{††}P \leq 0.01$, $^{††}P \leq 0.001$ (one-way ANOVA followed by Newman-Keuls' *post hoc*).
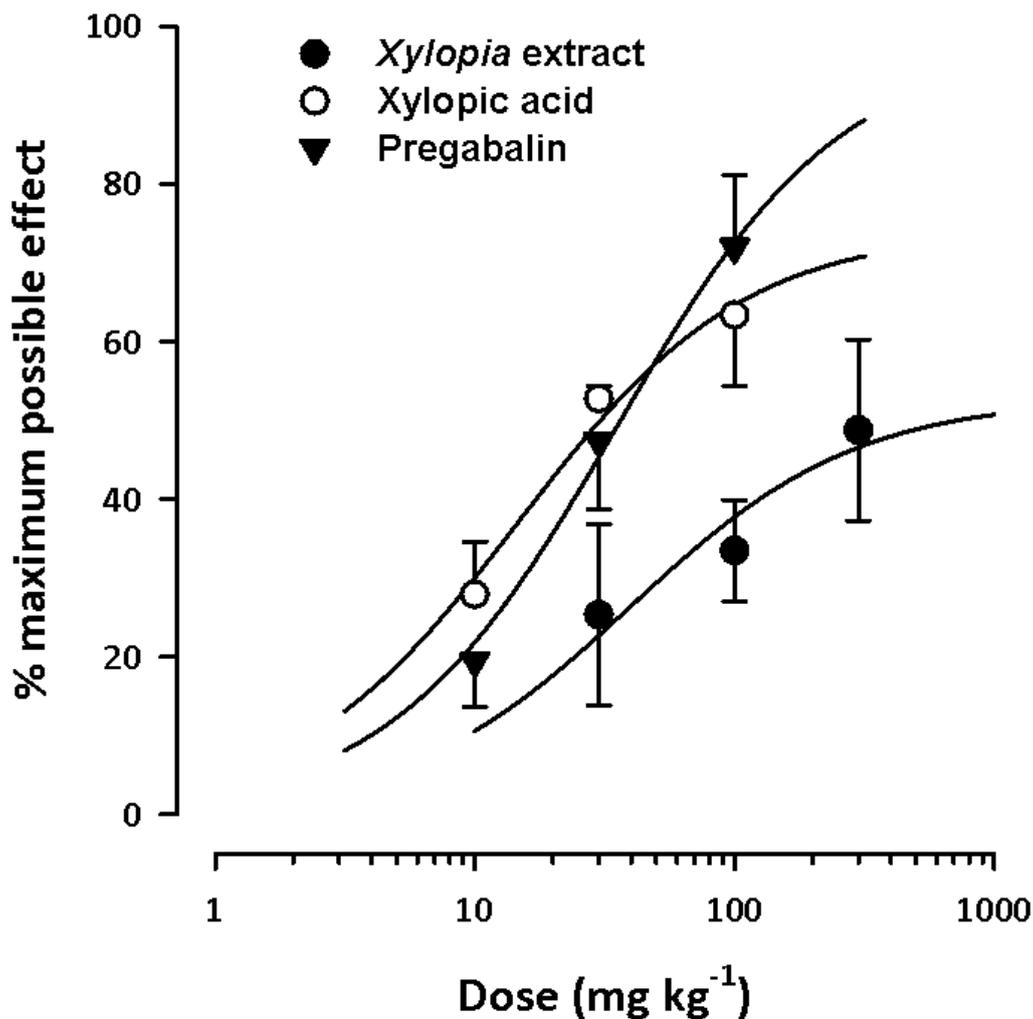
Figure 4.7 Dose–response curves for the anti-hyperalgesic effect of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and pregabalin (10-100 mg kg^{-1}) in vincristine-induced neuropathic pain. Each point is the mean ± S.E.M. of 8 animals.

4.4.5 *Mechanical hyperalgesia in the Randall-Selitto test*

Base line mechanical hyperalgesia taken on day 15 in the Randall-Selitto test revealed that both hind paws exhibited marked mechanical hyperalgesia. A change in hyperalgesic state was calculated as a percentage of the maximum possible effect. The extract (30–300 mg kg^{-1}, *p.o.*) produced a significant ($F_{3,28} = 14.34$, *P*=0.000007) and dose-dependent inhibition of mechanical hyperalgesia (Fig. 4.8a). The maximum possible effect was produced by the highest dose (Fig. 4.8b). Xylopic acid (10–100 mg kg^{-1}, *p.o.*) also produced a significant ($F_{3,28} = 14.85$, *P*=0.000006) and dose-dependent inhibition of mechanical hyperalgesia (Fig. 4.58) at all the three doses used. The highest

dose administered produces the maximum anti-hyperalgesic effect (Fig. 4.8d). The administration of pregabalin (10–100 mg kg^{-1}) significantly ($F_{3,28}$= 9.64, P=0.0002) and dose-dependently attenuated mechanical hyperalgesia (Fig. 4.8e); the maximum mechanical anti-hyperalgesic effect was achieved by the highest dose (Fig. 4.8f). Xylopic acid was more efficacious than pregabalin and the extract. Pregabalin was also more efficacious than the extract (Fig.4.9).

4.4.6 Cold allodynia

Base line cold allodynia was measured from both hind paws on day 15 using cold water at a temperature of 4.5 °C. The extract (30–300 mg kg^{-1}, *p.o.*) produced a significant ($F_{3,28}$ = 18.74, P=0.000002) and dose-dependent inhibition of cold allodynia (Fig.4.10a) which was demonstrated as increased latency to paw withdrawal. The highest dose of extract increased the latency to paw withdrawal to cold allodynia by $110.6 \pm 5.13\%$ (Fig. 4.10b).

Xylopic acid (10–100 mg kg^{-1}, *p.o.*) also produced a significant ($F_{3,28}$=15.61, P=0.000003) and dose-dependent inhibition of cold allodynia (Fig. 4.10c). The time of paw withdraw after xylopic acid administration was increased by $91.8 \pm 5.6\%$ (Fig. 4.10d) which occurred at the highest dose. The administration of pregabalin (10–100 mg kg^{-1}) inhibited cold allodynia ($F_{3,28}$=16.70, P=0.000002:Fig. 4.10e); the highest dose produced cold anti-allodynic effect of $111.4 \pm 5.19\%$ (Fig. 4.10f).

Pregabalin was more efficacious than extract. The extract was also more efficacious than xylopic acid (Fig.4.11).
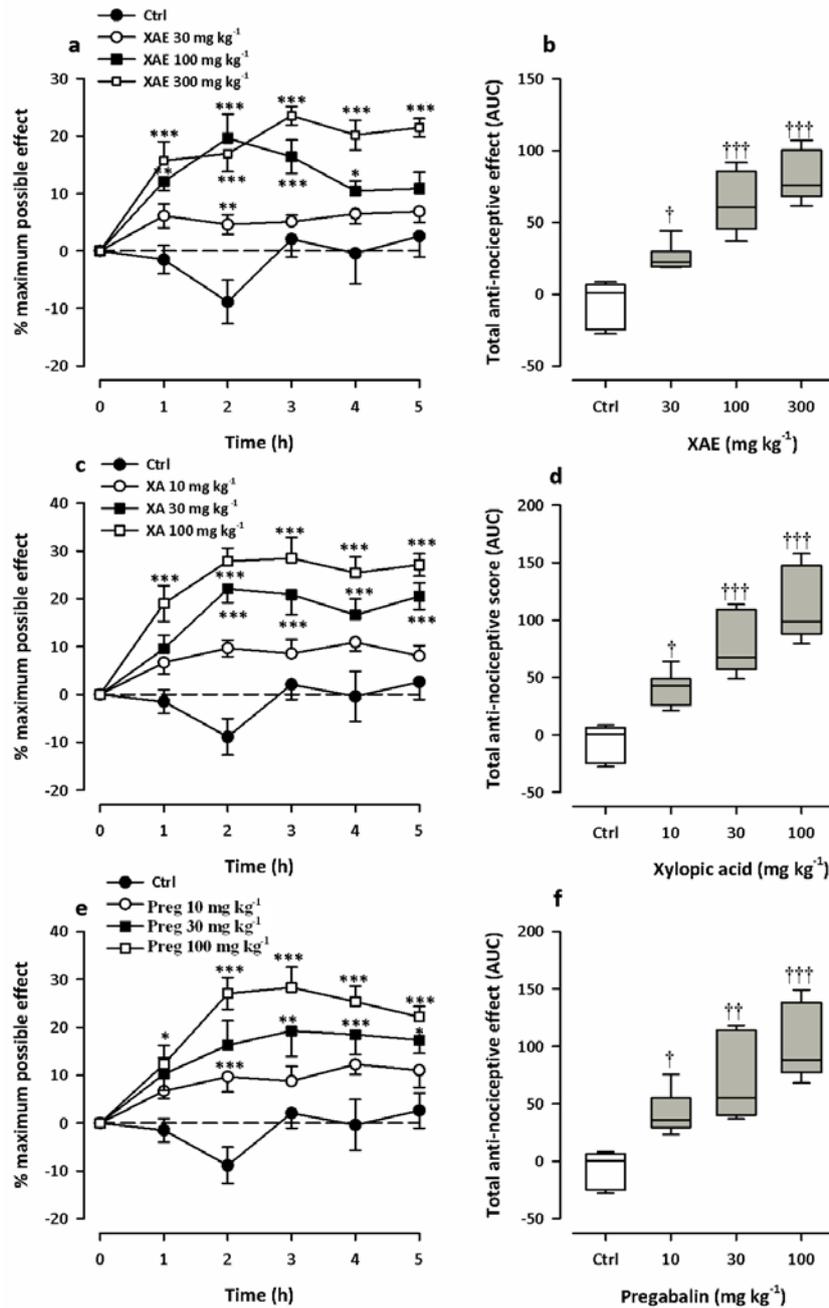
Figure 4.8 Effect of (a) extract (30 - 300 mg kg^{-1} *p.o*), (c) xylopic acid (10 - 100 mg kg^{-1} *p.o*) and (e) pregabalin (10-100 mg kg^{-1} *p.o.*) on the time course curve of vincristine-induced mechanical hyperalgesia in rat using the Randall- Selitto model and the AUC (b. d and f). Data is presented as mean ± S.E.M. (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test); $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
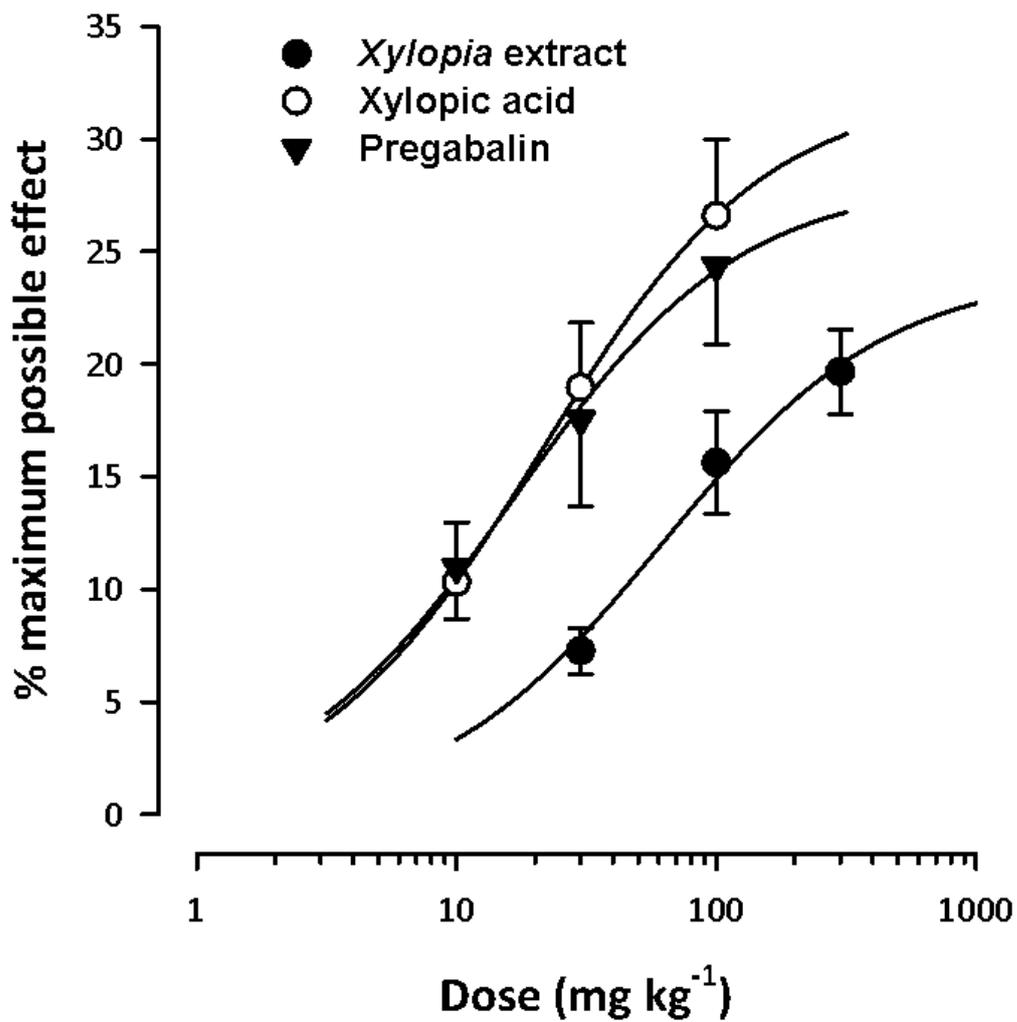
Figure 4.9 Dose–response curves for the anti-hyperalgesic effect of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and pregabalin (10-100 mg kg^{-1}) in vincristine-induced neuropathic pain (Randall- Selitto). Each point is the mean ± S.E.M. of 8 animals.
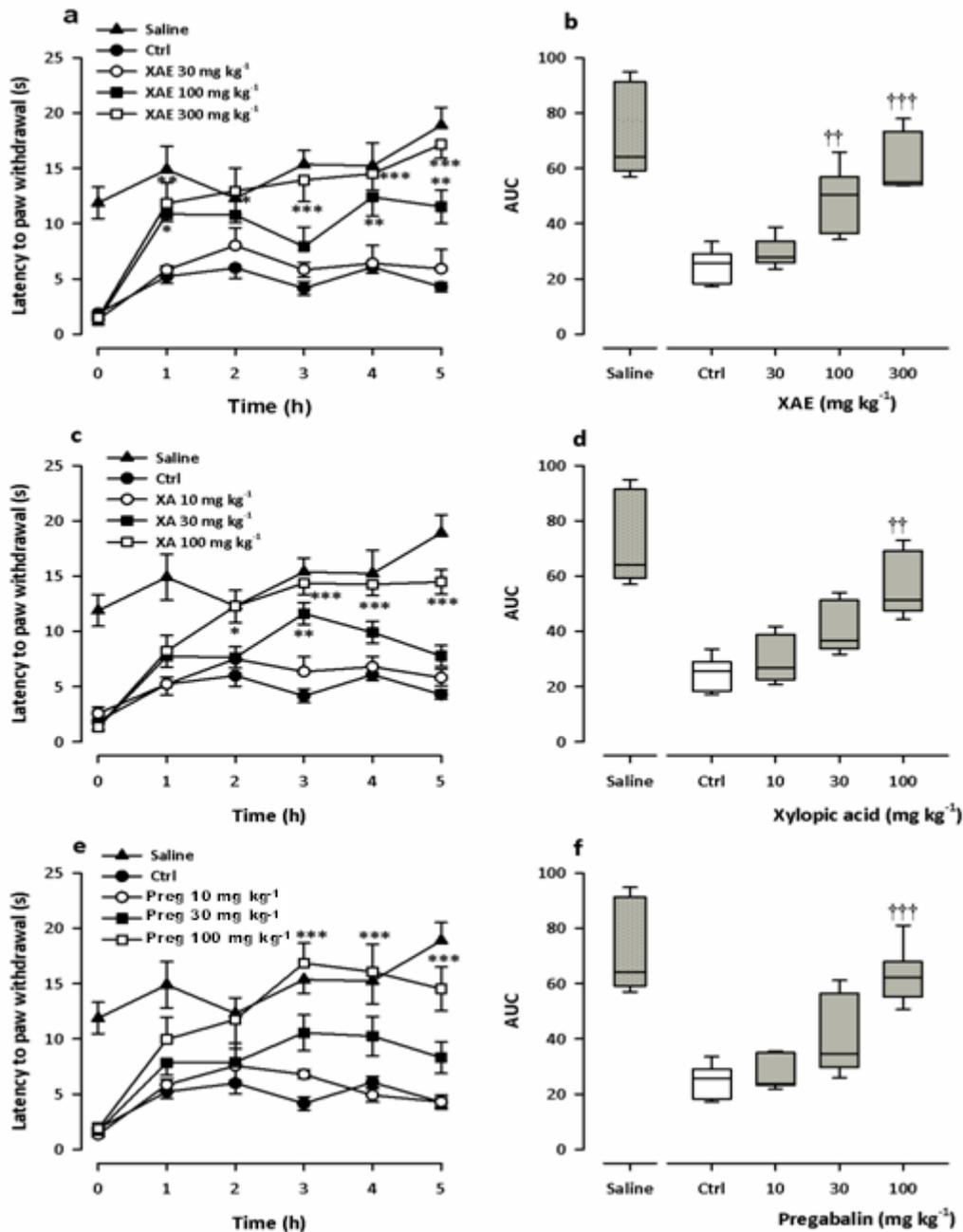
Figure 4.10 Effect of (a) extract (30 - 300 mg kg^{-1} *p.o*), (c) xylopic acid (10 - 100 mg kg^{-1} *p.o.*) and (e) pregabalin (10-100 mg kg^{-1} *p.o.*) on the time course curve of vincristine-induced cold allodynia and the AUC (b, d and f). Data is presented as mean $\pm$ S.E.M. (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test); $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
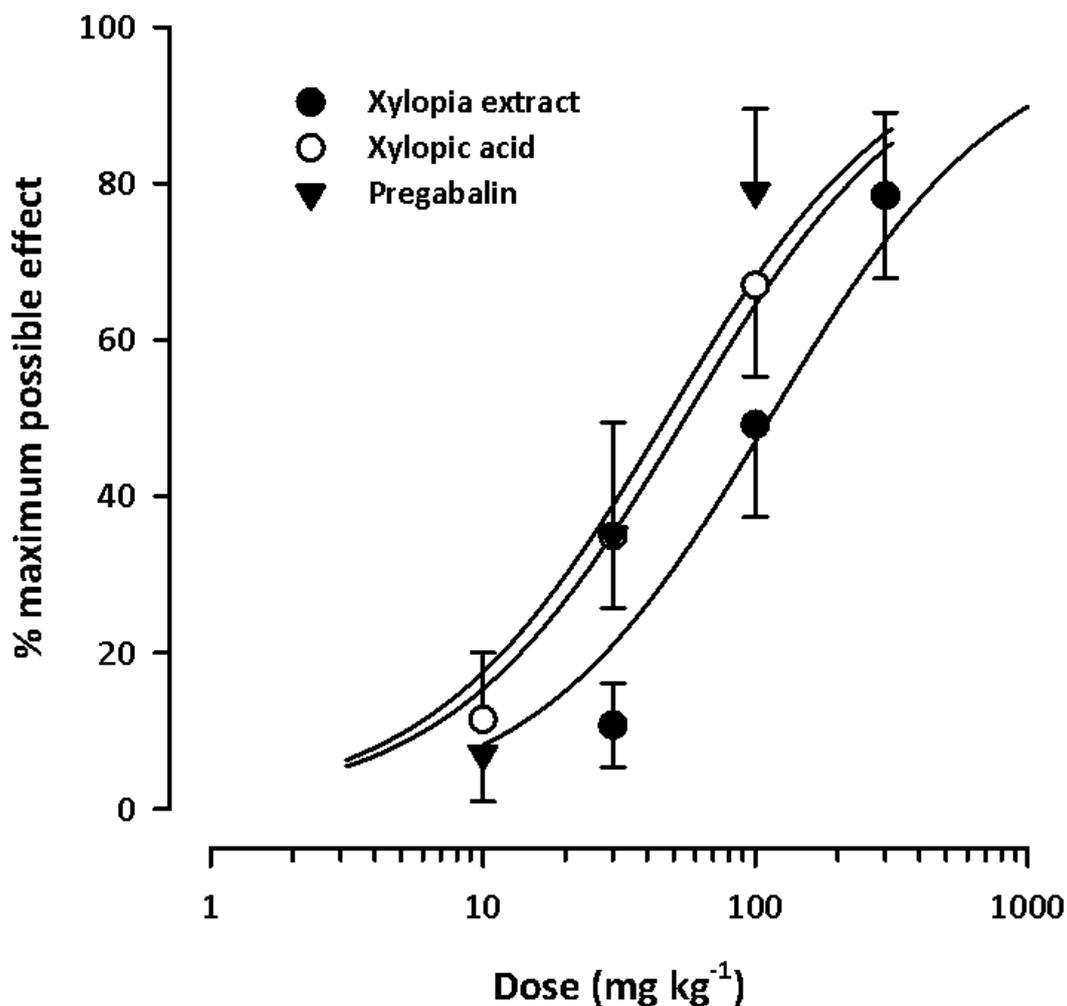
Figure 4.11 Dose–response curves for the cold anti-allodynic effect of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and pregabalin (10-100 mg kg^{-1}) in vincristine-induced neuropathic pain. Each point is the mean ± S.E.M. of 8 animals.

4.5 DISCUSSION

The extract, xylopic acid and pregabalin ameliorated vincristine-induced tactile and cold allodynia, and mechanical hyperalgesia which constitute the major and common symptoms experienced by patients with chemotherapy-induced neuropathy. Systemic treatment with vincristine damages Schwann cells and dorsal root ganglion (DRG) neurons of the peripheral nervous system leading to degeneration of myelinated and unmyelinated fibres. Vincristine-induced neuropathic pain involves peripheral (IL-6-

janus kinase (Jak) - transcription-3 (STAT3) pathway) and central (TNF-α - p38 MAP kinase pathway) mechanisms (Siau *et al.*, 2006; Kiguchi *et al.*, 2009; Authier *et al.*, 2003). The extract, xylopic acid and pregabalin may have exerted antinociceptive properties in this study through the inhibition of calcium channels on sensory nerves. It is generally admitted that small diameter C- and Aδ-sensory fibres are mainly involved in the response to cold and intense mechanical stimuli whereas large Aβ-fibres (low threshold fibres) response to tactile stimuli. Pregabalin an antagonist on α_2-δ_1 subunit of voltage dependent calcium channels is reported to be effective both experimentally and clinically in managing neuropathic pain (Schim, 2009; Kumar *et al.*, 2010). Also, Kaurenoic acid (present in the extract) and xylopic acid have been reported to posses calcium channel blocking effects (Somova *et al.*, 2001). Increased expression and activity of α_2-δ_1 Ca^{2+} channels (a subunit construct of N-type voltage-dependent Ca^{2+} channel) have also been observed in neuropathic pain states (Mao *et al.*, 1995; Yajima *et al.*, 2005). Furthermore the changes in the PNS and CNS occurring after vincristine treatment lead to spontaneous activity of C- and Aβ-fibres that result in spontaneous pain and abnormal sensations both peripherally and centrally. Inhibition of calcium channels prevent neuronal excitability and other cellular enzymatic cascade reactions that lead to pain sensation (Schim, 2009; Kumar *et al.*, 2010). It is therefore possible that the extract and xylopic acid may have blocked pain in this model by inhibiting calcium channels similar to pregabalin leading to inhibition of pain stimuli propagation in the affected unmyelinated and myelinated C-, Aδ- and Aβ-fibres.

4.6   CONCLUSION

The extract and xylopic acid exhibited analgesic properties in vincristine-induced neuropathic pain by ameliorating tactile and cold allodynia as well as mechanical hyperalgesia which constitute common symptoms seen in patients undergoing chemotherapy treatment.

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Chapter 5

MECHANISM OF ANTINOCICEPTION OF FRUIT EXTRACT OF *XYLOPIA AETHIOPICA* AND XYLOPIC ACID

</div>

5.1   INTRODUCTION

In the previous chapters the antinociceptive effect of the extract and xylopic acid were evaluated in several chemical, radiant and mechanical behavioural models of pain. In the first phase of the formalin test, substance P and bradykinin were involved in the nociceptors sensitisation while histamine, serotonin, prostaglandins, nitric oxide, bradykinin, TNF α, IL-6, noradrenalin, Ach and other pro-inflammatory pain mediators were involved in the late phase of the formalin test as well as the other models of pain used (Dalbo *et al.*, 2006; Lu *et al.*, 2007).

Again in the spinal cord the nociceptive information coming from gut, skin and other organs is submitted to a modulation by a great variety of transmitters that may filter and modulate the transmission of nociceptive impulses to the brain (Fürst, 1999; Millan, 2002). These modulating substances are able to act as pro- (descending facilitation) or antinociceptive (descending inhibition), depending on diverse factors, such as the type and intensity of the stimulation, the central region activated, receptor type, and others (Millan, 2002). The neurons projected by the central areas responsible for the control of the perception of pain (descending facilitation and descending inhibition) contain several transmitters, including noradrenalin, serotonin (5-HT), acetylcholine, γ-hydroxy-butyric acid (GABA), nitric oxide (NO), glutamate, dopamine and others (Fürst, 1999; Millan, 2002). In this context, the participation of the L-arginine/ nitric oxide pathway, opioidergic, adenosinergic, adrenergic, serotoninergic, muscarinic receptors or pathways in the antinociceptive effect of the extract and xylopic acid were investigated.

5.2   MATERIALS AND METHODS

5.2.1   Drugs and chemicals

Morphine hydrochloride was obtained from Phyto-Riker, Accra, Ghana: carrageenan, naloxone and N^G-Nitro-l-arginine methyl ester (L-NAME) from Sigma-Aldrich Inc., St. Louis, MO, USA**:** glibenclamide, Daonil$^®$ from Sanofi-Aventis, Guildford, UK: formalin and theophylline were purchased from BDH, Poole, England: Ondansetron from GlaxoSmithKline, Uxbridge, UK: Atropine sulphate was purchased from E. Merck AG-Darmstadt, Germany: yohimbine was obtained from Walter Ritter GmbH + Co. KG, Germany, L-glutamtic acid, prostaglandin -E_2, capsaicin and bradykinin acetate from Sigma-Aldrich Inc., St. Louis, MO, USA; adrenaline HCl from Wuhan Grand Pharm, Hubei, China; ketamine HCl from Brotex Medica, Trittau, Germany; propranolol from Ernest Chemist Ltd, Tema, Ghana and captopril from Teva UK Ltd, Eastbourne, UK. Tissue culture media, Geneticin, Fetal bovine serum (Invitrogen, Carlsbad, CA); [^{3}H]diprenorphine (specific radioactivity of 29 Ci/mmol from Perkin-Elmer Life Sciences, Boston, MA) and Ecolume scintillation fluid (ICN, Aurora, OH). All other biochemicals used in the binding studies were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

5.2.2   Animals

Male ICR mice (20-25 g) and Sprague Dawley rats (200-250 g) were used in this study. The animals were provided with the necessary conditions as described previously under section 3.2.1

5.2.3   Assessment of the mechanism of antinociception of extract and xylopic acid in the formalin test

The mechanism of analgesic action of the extract and xylopic acid was investigated using various antagonists in the formalin test. The receptor pathways investigated were the adenosinergic, opioid, adrenergic, serotoninergic, nitric oxide, ATP sensitive K^+ channels, and muscarinic receptors/pathways. The doses of antagonist, agonist and

other drugs were selected on the basis of previous literature data and in pilot experiments in the laboratory (Woode *et al.*, 2009; Woode and Abotsi, 2011).

5.2.3.1 Participation of the adenosinergic system

To investigate the role played by the adenosinergic systems in the anti-nociception caused by extract and xylopic acid, mice were pre-treated with theophylline (5 mg kg^{-1}, i.p., a non selective adenosine receptor antagonist). After 15 min the mice received extract (100 mg kg^{-1}, *p.o.*) or xylopic acid (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle. The nociceptive response to the intraplantar injection of formalin was recorded 60 min after administration of the extract and xylopic acid (100 mg kg^{-1}, *p.o.*) or vehicle and 30 min after morphine administration. The formalin test was then performed as described under section 3.2.4

5.2.3.2   Involvement of the ATP sensitive K$^+$ channels

Mice were pre-treated with glibenclamide (an ATP-sensitive K$^+$ channel inhibitor, 8 mg kg^{-1}, *p.o.*), or vehicle and after 30 min received extract (100 mg kg^{-1}, *p.o.*), xylopic acid (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle and the nociceptive responses to formalin were recorded as described earlier under section 3.2.4

5.2.3.3   Participation of the nitric oxide system

To verify the participation of nitric oxide/cyclic GMP pathway in the anti-nociceptive action caused by the extract or xylopic acid, mice were pre-treated with N^G-L-nitro-arginine methyl ester (L-NAME, a NO synthase inhibitor; 10 mg kg^{-1}, i.p.) or vehicle 30 min before extract (100 mg kg^{-1}, *p.o.*), xylopic acid (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle administration and the nociceptive responses to formalin were recorded as described earlier under section 3.2.4.

5.2.3.4 Involvement of serotoninergic system

In order to determine the contribution of $5HT_3$—receptors/pathway in the antinociceptive effects of xylopic acid and extract, mice were pre-treated with ondansetron ($5HT_3$ inhibitors; 0.5 mg kg^{-1} i.p.) or saline 30 min before extract (100 mg kg^{-1}, *p.o.*), xylopic acid (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle administration and the nociceptive responses to formalin were recorded as described earlier under section 3.2.4.

5.2.3.5 Participation of the muscarinic system

Mice were pre-treated with atropine (non-selective muscarinic antagonist, 5 mg kg^{-1} i.p.) 30 min before extract (100 mg kg^{-1}, *p.o.*), xylopic acid (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle administration and the nociceptive responses to formalin were recorded as described earlier under section 3.2.4.

5.2.3.6 Involvement of the adrenergic system

To assess the possible involvement of the α_2 receptor/system, mice were pre-treated with yohimbine (an α_2 receptor antagonist 3 mg kg^{-1} i.p.) 30 min before extract (100 mg kg^{-1}, *p.o.*), xylopic acid (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle administration and the nociceptive responses to formalin were recorded as described earlier under section 3.2.4.

5.2.3.7 Assessment of the opioid pathway

Assessment of the opioid receptors or pathway involvement was done by pre-treating mice intraperitoneally with naloxone (2 mg kg^{-1}, a non selective opioid receptor antagonist). After 15 min the animals received extract (100 mg kg^{-1}, *p.o.*), xylopic acid (30 mg kg^{-1}, *p.o.*), morphine (3 mg kg^{-1}, i.p.) or vehicle. The nociceptive response to intraplantar injection of formalin was recorded as described earlier under section 3.2.4.

5.2.4 Radioligand binding assay

Xylopic acid was examined for its μ-opioid receptor (MOR) binding in a [³H]diprenorphine ([³H] DPN) competition binding assay using membranes derived from C6μ cells (Divin *et al.*, 2009).

Cell Culture

C6 glioma cells stably transfected with the u-opioid receptor (C6μ) were maintained at 37 °C under 95% O_2/5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum (FBS) and 500 ug/mL Geneticin® (Invitrogen) to select for the μ-opioid receptor-expressing cells. Cells were grown in 10 cm^2 dishes to ~90% confluency on the day of the assay.

Membrane Preparation

C6μ cells were washed twice with ice cold phosphate-buffered saline (0.9% NaCl, 0.61 mM Na_2HPO_4 and 0.38 mM KH_2PO_4, pH 7.4), detached from the plate by incubation in warm harvesting buffer [20 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), pH 7.4, 150 mM NaCl and 0.68 mM EDTA) for 5 minutes, dispersed by agitation and pelleted by centrifugation at 1600 rpm for 3 min. The resulting cell pellet was resuspended in ice cold 50 mM Tris buffer, pH 7.4 and homogenized with a Tissue Tearor (Biospec Products Inc., Bartlesville, OK) for 20 s. The homogenate was centrifuged at 25000 *g* at 4 °C for 20 min. The resulting membrane pellet was resuspended in 50 mM Tris buffer, pH 7.4 and centrifuged as above. The final pellet was resuspended in 50 mM Tris, pH 7.4 with a Dounce homogenizer, and the homogenate was separated into 0.5 ml aliquots and stored at -80°C until use. Protein concentration was measured by the Bradford protein assay using bovine serum albumin as standard (Bradford, 1976).

Competition binding assay

For competition binding assays, cell membranes (15 ug protein) were incubated at room temperature (25 ° C) for 1 h with shaking with 0.2 nM [³H]diprenorphine ([³H]DPN) and increasing concentrations of unlabeled ligand [xylopic acid (0.1nM - 1mM) or ([D-Ala², N-MePhe⁴, Gly-ol]-enkephalin), a synthetic opioid peptide with high μ-opioid receptor specificity [DAMGO (0.001nM - 10μM)] in 50 mM Tris-HCl, pH 7.4.

Nonspecific binding was determined in the presence of 10 μM naloxone. The assay was stopped by rapid filtration through glass GF/C filters (Whatman, Clifton, NJ) using a Brandel harvester (MLR-24, Gaithersburg, MD), rinsing three times with ice-cold 50 mM Tris buffer, pH 7.4. Filters were dried at 50 °C for 20 min. Dried filters were saturated with EcoLume liquid scintillation mixture (MP Biomedicals, Solon, OH), heat sealed in polyethylene bags, and radioactivity was counted in a Wallac 1450 MicroBeta Liquid Scintillation and Luminescence Counter (Perkin Elmer, Boston, MA).

5.2.5 Glutamate-induced neurogenic pain

The procedure used was similar to that described earlier by Beirith, (2002). Before testing, the animals were placed individually in 1 of 20 transparent Perspex chambers (15×15×15 cm). Following a 1-h adaptation period in the chamber, ten groups of mice (males) were pre-treated with extract (30-300 mg kg^{-1}, *p.o.*), xylopic acid (10-100 mg kg^{-1}, *p.o.*) or ketamine (1-10 mg kg^{-1}, i.p.) or vehicle (normal saline, 10 ml kg^{-1}, i.p.) 30 min (i.p.) or 1 h (*p.o.*) before intraplantar injection of glutamate (10 μmol/paw, 20 μl) on the right hind paw. The nociceptive behaviour (biting/licking of the injected paw) of the animals was captured (15 min) for analysis by a camcorder (Everio^{TM}, model GZ-MG1300, JVC, Tokyo, Japan) placed directly opposite the mirror and attached to a computer. Tracking of the behaviour was done using the public domain software JWatcher^{TM}, Version 1.0 (University of California, LA, USA, and Macquarie University, Sidney, Australia, available at http://www.jwatcher.ucla.edu/) to obtain the frequency and duration of biting/licking per 5 min, starting immediately after glutamate injection. A nociceptive score for each time block was calculated by multiplying the frequency and time spent in biting/licking. Data were expressed as the mean ± SEM of scores between 0 and 15 min after glutamate injection.

5.2.5.1 Capsaicin-induced nociception

The test was performed as described by Sakurada, (1992). After an adaptation period of 1 h in the test chambers, seven groups of mice (males) were pre-treated with the extract, xylopic acid or vehicle similar to that described for the glutamate test above. Mice were returned individually into the testing chambers immediately after intraplantar administration of capsaicin (1.6 μg/paw, 20 μl dissolved in 0.5% ethanol). The ethanol

did not cause any detectable analgesic effect on its own. The amount of time spent licking the injected paw was determined for 5 min following capsaicin injection and was considered as a nociceptive behaviour (section 3.2.4).

5.2.5.2   Bradykinin-induced hyperalgesia

Similar to that described by Meotti *et al.,*(2006) and Wilhelm *et al.,*(2009), seven groups of rats were pre-treated with the extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and vehicle after baseline testing and one hour later received intraplantar injection of bradykinin (20 μl: 10 nmol/ paw). Animals were pre-treated with captopril, 5 mg kg^{-1} s.c. (an angiotensin-converting enzyme inhibitor) 1 h the experiments to prevent bradykinin degradation. Mechanical nociceptive thresholds were measured in the rat paw pressure test using an analgesimeter (IITC Life Science Model 2888, Woodland Hills, CA, USA) which is based on the Randall–Sellito test (Randall and Selitto, 1957) as described previously under section 3.2.5.

5.2.5.3   Prostaglandin E$_2$- induced hyperalgesia

Similar to that described by Claudino, (2006) and Wilhelm *et al.,*(2009), baseline nociceptive testing was performed and ten groups of mice afterwards were treated with the extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and morphine (1-10 mg kg^{-1}). One hour later the mice received intraplantar injection of prostaglandin E$_2$ (20 μl: 1 nmol/ paw). Nociceptive threshold was determined as in the Randall and Selitto method as described earlier under section 3.2.5.

5.2.5.4   Epinephrine-induced hyperalgesia

Ten groups of rats were treated with the extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and propranolol (1-10 µg/paw) after taking the baseline responses and one hour later received intraplantar injection of epinephrine (20 μl : 450 nmol/paw) (Sakurada *et al.*, 1993 ; Meotti *et al.*, 2006). Nociceptive threshold was taken using the Randall and Selitto method as described earlier under section 3.2.5.

5.3 STATISTICAL ANALYSIS

GraphPad Prism for Windows Version 4.03 (GraphPad Software, San Diego, CA, USA) was used for all data and statistical analyses. $P<0.05$ was considered statistically significant. In all nociceptive tests, a sample size of seven animals (n=7-8) were used. The time-course curves were subjected to two-way (treatment $\times$ time) repeated measures analysis of variance (ANOVA) with Bonferroni's *post hoc* test. Total nociceptive score for each treatment was calculated in arbitrary unit as the area under the curve (AUC). Differences in AUCs were analysed by ANOVA followed by Student-Newman-Keuls *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) equation:

$$Y = \frac{a+(b-a)}{\left(1+10^{(LogED_{50}-X)}\right)}$$

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$_{50}$s) of the curves were compared statistically using F test (Miller, 2003; Motulsky and Christopoulos, 2003).

In the binding studies, each data point represents the mean $\pm$ S.E.M. of duplicate wells of at least two experiments. Specific binding of [³H]DPN was determined by subtracting non-specific binding counts from all values, and data were expressed graphically as a percentage of the total [³H]DPN binding ([³H]DPN binding in the absence of unlabeled ligand) against increasing concentration of unlabeled ligand (xylopic acid or DAMGO). Binding data were fitted by non-linear regression using one- or two-site binding models, and when possible the affinity of xylopic acid or DAMGO binding to the μ-opioid receptor (*Ki* or IC$_{50}$) was determined from the resulting curve fit. The IC$_{50}$ was defined as the concentration of drug that inhibited the specific [³H]diprenorphine binding by 50%.

5.4 Results

5.4.1 Analysis of mechanism of action of extract and xylopic acid in the formalin test

Results presented in (Fig. 5.1-5.3) show the effect of naloxone, glibenclamide, L-NAME, yohimbine, atropine, theophylline and ondansetron on the antinociceptive effects of the extract (Fig. 5.1), xylopic acid (Fig. 5.2) and morphine (Fig. 5.3). Pre-treatment of mice with 2 mg kg^{-1} naloxone and 5mg kg^{-1} theophylline reversed both phase 1 and phase 2 antinociception of extract (300 mg kg^{-1}, *p.o.*, Fig. 5.4a), xylopic acid (100 mg kg^{-1}, *p.o.*, Fig. 5.4b) and morphine (3 mg kg^{-1}, i.p., Fig. 5.4c).

The antinociception of the extract (300 mg kg^{-1}, *p.o.*, Fig. 5.4a) and xylopic acid (100 mg kg^{-1}, *p.o.*, Fig. 5.4b) in the first phase of the formalin test were blocked by 10 mg kg^{-1} L-NAME and atropine (5 mg kg^{-1}). The same doses of L-NAME and atropine however blocked the antinociception of morphine (3 mg kg^{-1}, i.p., Fig. 5.4c) in both phases of the formalin test.

Pre-treatment of the animals with ondansetron (0.5 mg kg^{-1}) blocked the second phase of the extract-induced anti-nociception (Fig. 5.4a). Ondansetron also antagonized xylopic acid and morphine-induced anti-nociception in the first phase of the formalin test (Fig. 5.4b & c).

Also, yohimbine (3 mg kg^{-1}) reversed the neurogenic anti-nociception of xylopic acid (Fig. 5.4b) but had no effect on analgesic activity of the extract and morphine in both phases of formalin-induced pain (Fig. 5.4a and c).

Glibenclamide (8 mg kg^{-1}, i.p.) reversed the anti-nociception of morphine (Fig. 5.4c) in the first phase but failed to significantly block the anti-nociception of the extract and xylopic acid in both phases of the formalin test (Fig. 5.4a and b).
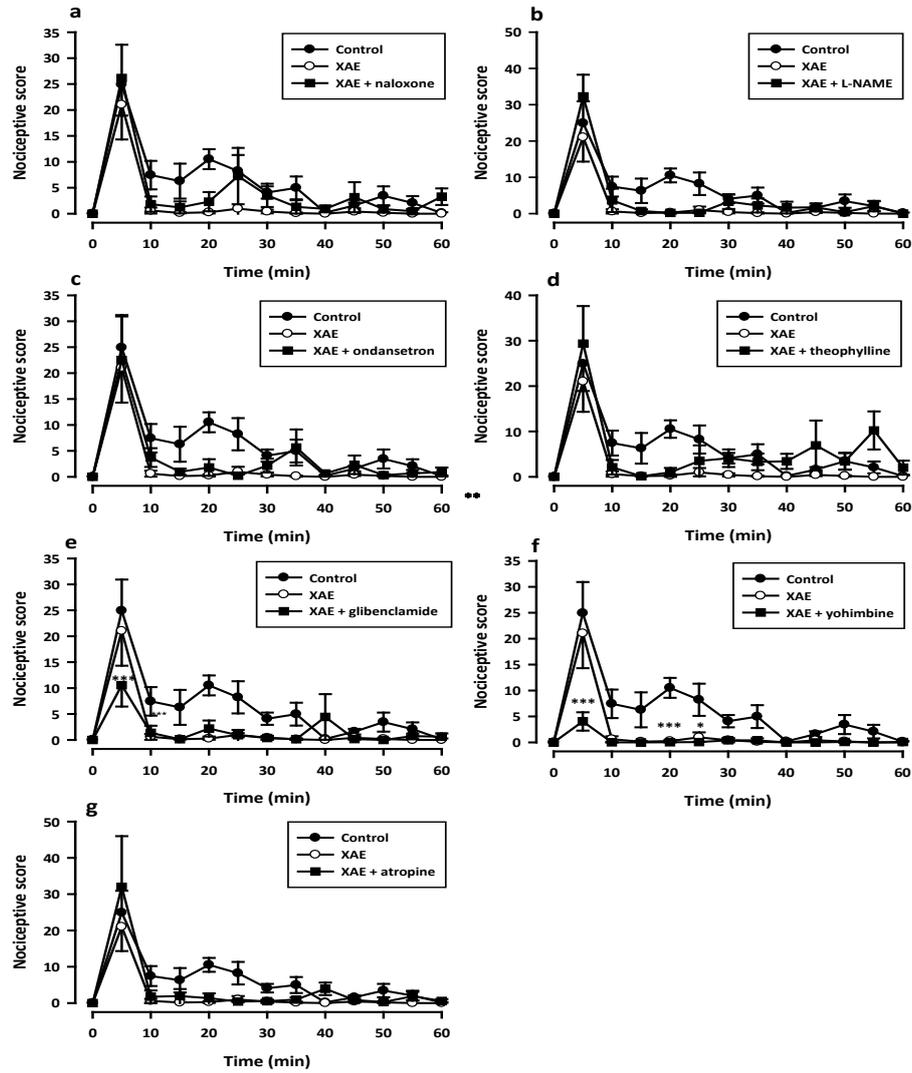
Figure 5.1 Effect of (a) naloxone (2 mg kg^{-1} i.p.), (b) L- NAME (10 kg^{-1} i.p.), (c) ondansetron (0.5 mg kg^{-1} i.p.), (d) theophylline (5 mg kg^{-1} i.p.), (e) glibenclamide (8 mg kg^{-1} i.p.), (f) yohimbine (3 mg kg^{-1} i.p.) and (g) atropine (5 mg kg^{-1} i.p) on the antinociceptive effect of the extract (300 mg kg^{-1} *p.o*) on the time course effect of the total nociceptive score of formalin-induced licking test. Each point represents the mean ± S.E.M. *$P \le 0.05$, ***$P \le 0.001$ compared to respective controls (Two-way ANOVA followed by Bonferroni's *post hoc* test).
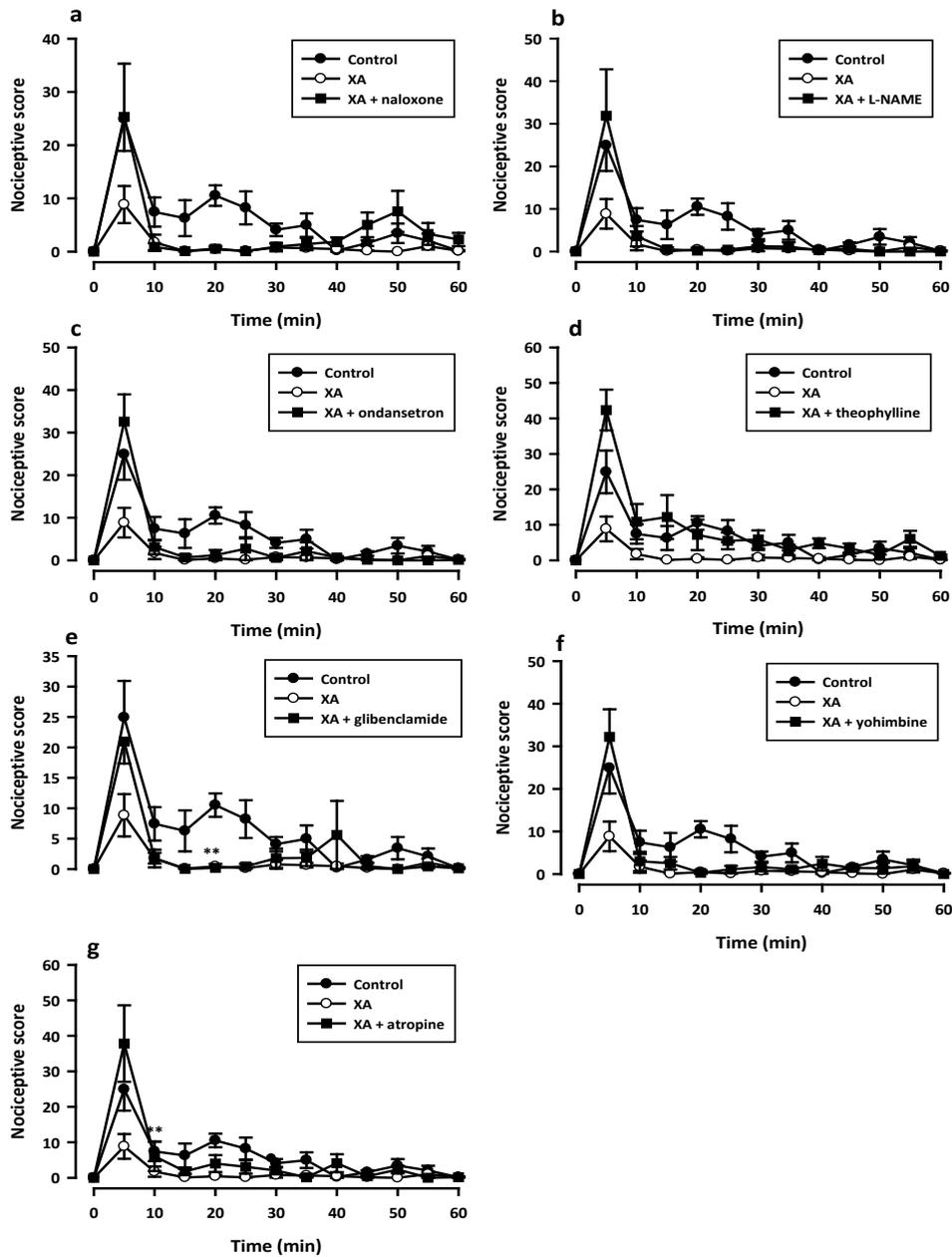
Figure 5.2 Effect of (a) naloxone (2 mg kg^{-1} i.p.), (b) L- NAME (10 kg^{-1} i.p.), (c) ondansetron (0.5 mg kg^{-1} i.p.), (d) theophylline (5 mg kg^{-1} i.p.), **(e)** glibenclamide (8 mg kg^{-1} i.p.), (f) yohimbine (3 mg kg^{-1} i.p.) and (g) atropine on the antinociceptive effect of xylopic acid (100 mg kg^{-1} *p.o.*) on the time course effect of the total nociceptive score of formalin-induced licking test. Each point represents the mean ± S.E.M. **$P \leq 0.01$ compared to respective controls (Two-way ANOVA followed by Bonferroni's *post hoc* test).

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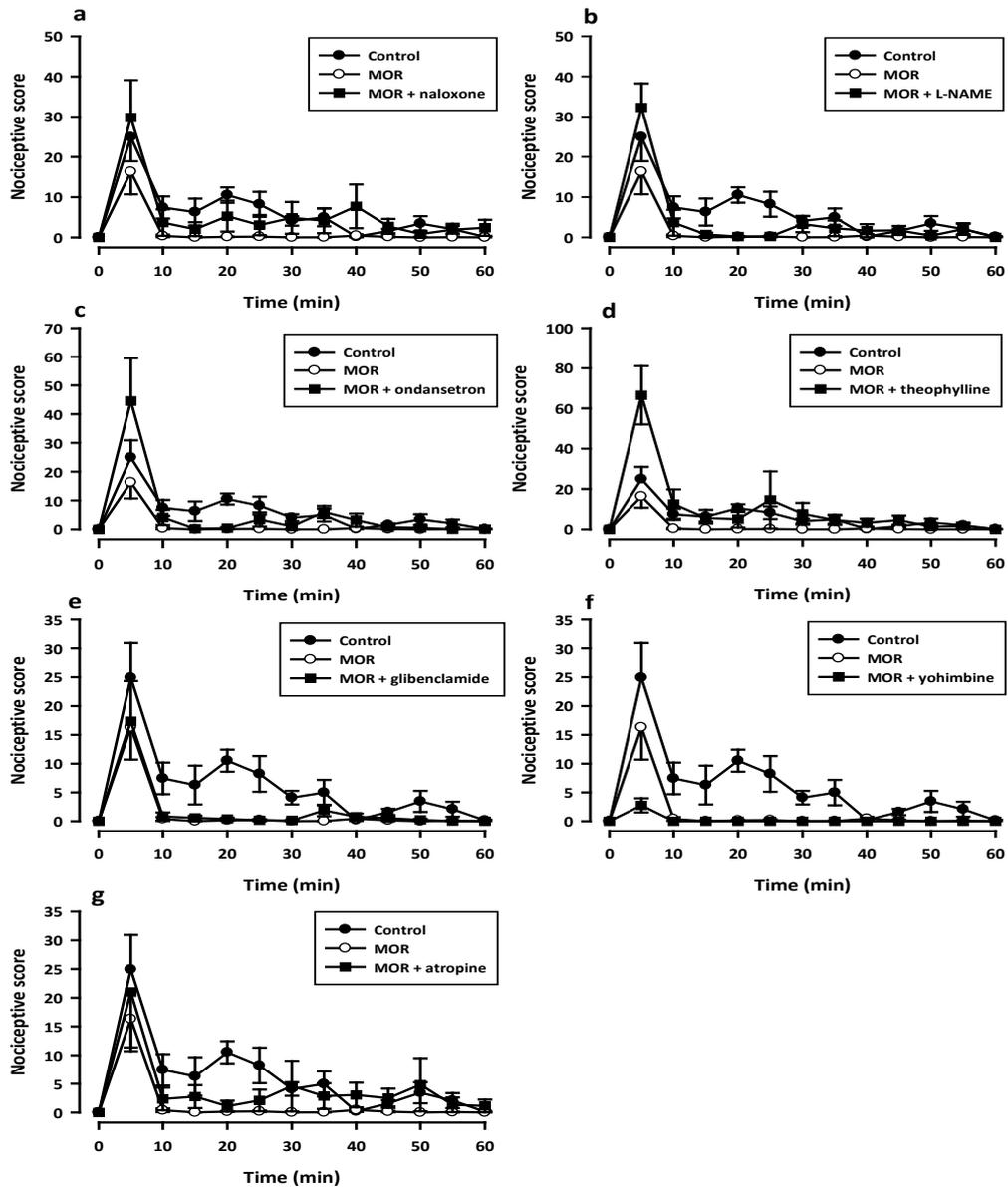
Figure 5.3 Effect of (a) naloxone (2 mg kg^{-1} i.p.), (b) L- NAME (10 kg^{-1} i.p.), (c) ondansetron (0.5 mg kg^{-1} i.p.), (d) theophylline (5 mg kg^{-1} i.p.), (e) glibenclamide (8 mg kg^{-1} i.p.), (f) yohimbine (3 mg kg^{-1} i.p.) and (g) atropine (5 mg kg^{-1} i.p.) on the antinociceptive effect of morphine (3 mg kg^{-1} i.p.) on the time course effect of the total nociceptive score of formalin-induced licking test. Each point represents the mean ± S.E.M.
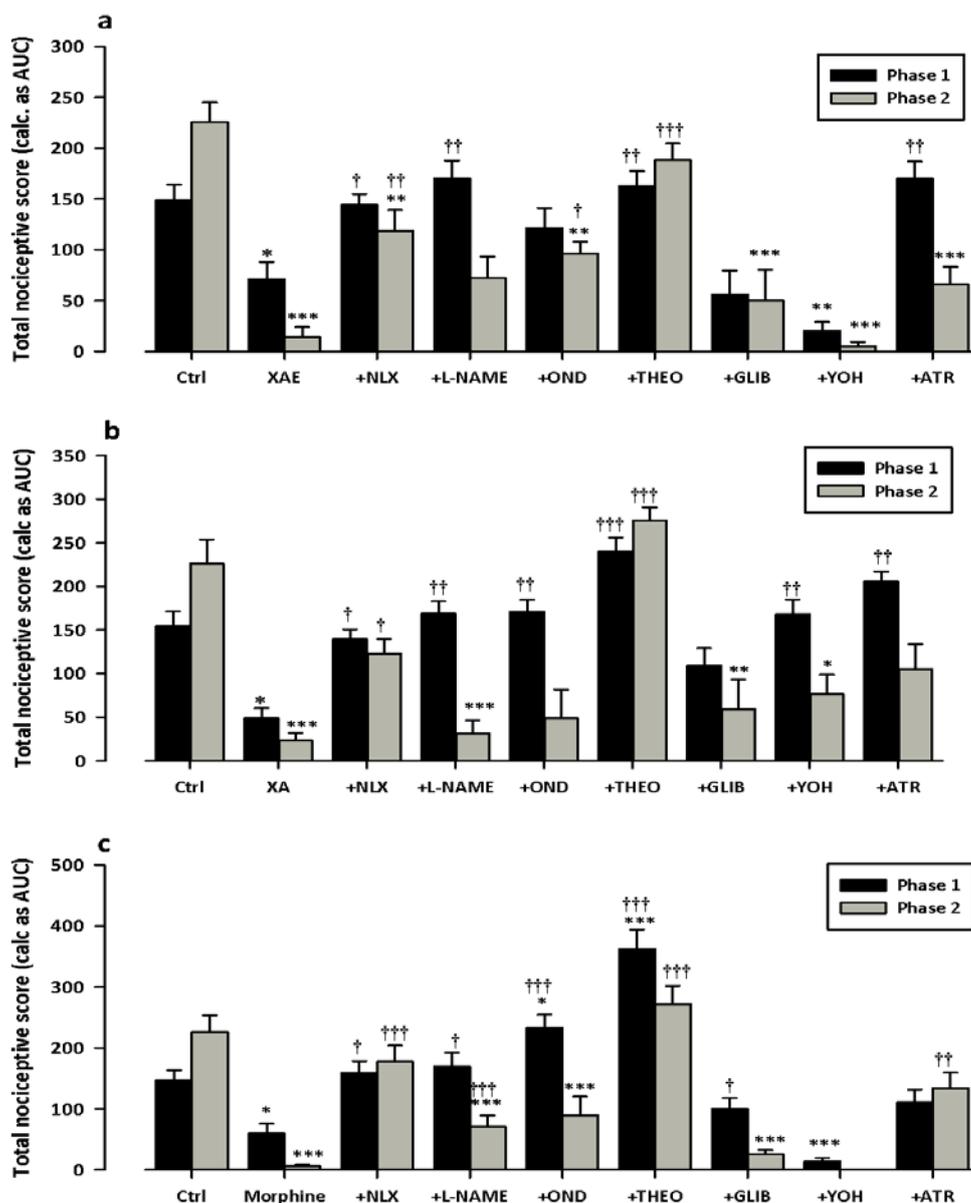
Figure 5.4 Effect of naloxone (2 mg kg^{-1} i.p.), L- NAME (10 mg kg^{-1} i.p.), ondansetron (0.5 mg kg^{-1} i.p.), theophylline (5 mg kg^{-1} i.p.), glibenclamide (8 mg kg^{-1} i.p.), yohimbine (3 mg kg^{-1} i.p.) and atropine (5 mg kg^{-1} i.p.) on the antinociceptive effect of (a) extract (300 mg kg^{-1}), (b) xylopic acid (100 mg kg^{-1}) and (c) morphine (3 mg kg^{-1}) for phase 1 and phase 2 of formalin-induced pain. Each column represents the mean ± S.E.M. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.00$, $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, $^{\dagger\dagger\dagger}P \leq 0.001$, compared to respective controls (one-way ANOVA followed by Newman-Keuls' *post hoc*).

5.4.2   *Radioligand binding assay*

Data for DAMGO was best fitted by a single binding site model. However, a comparison of the one-site/two-site model curve fits of the data for xylopic acid

revealed that the two-site model was better [$F_{1,12}$= 5.533, P= 0.0366; R^2 (one-site) = 0.74, R^2 (two-site) = 0.82]. Xylopic acid and DAMGO were able to competitively displace the binding of [^{3}H]DPN from the MOR (Fig 5.5). Table 5.1 shows the IC_{50} values of the inhibitions. Xylopic acid appears to bind to two different sites with IC_{50} values of 0.11 and 220.5 µM respectively. DAMGO showed very high affinity to the MOR with IC_{50} and K_i values of 0.0014 µM and 0.47 nM respectively. The affinity of DAMGO (based on the IC_{50} values) is about 100 times greater than xylopic acid at its high affinity binding site. There was also an apparent increase in [^{3}H]DPN binding that occurred at low concentrations of xylopic acid (Fig. 5.5a).

5.4.3   Glutamate-induced nociception

Nociceptive behaviour characterized by biting, flinching and licking after intraplantar administration of glutamate was inhibited by the extract, xylopic acid and ketamine during the 10 min observational period. Treatment of mice with the extract (30–300 mg kg^{-1}, *p.o.*, 60 min before) (Fig. 5.6a) produced a marked and dose-related inhibition of glutamate-induced neurogenic nociception ($F_{3,24}$=10.02, P=0.0002). Neurogenic antinociception of 69.60±2.1% was produced by the highest dose of extract (Fig. 5.6b).

Xylopic acid (10–100 mg kg^{-1}, *p.o.*, 60 min before) (Fig. 5.6c) also produced a marked and dose-related inhibition of glutamate-induced neurogenic nociception ($F_{3,24}$= 32.52, P=0.00000001): the highest dose causing a possible maximal antinociception of 88.9±0.31% (Fig. 5.6d).

Ketamine (1-10 mg kg^{-1}, i.p.) produced marked and dose-related inhibition of glutamate-induced neurogenic nociception ($F_{3,24}$= 24.31, P=0.0000002) (Fig. 5.6e). Ketamine reduced the nociception evoked by glutamate on neurogenic nociceptive behaviour by a maximum percentage effect of 77.4±1.20% (Fig. 5.6f).

The rank of potency was in the order ketamine > xylopic acid > extract as shown on Fig. 5.7.
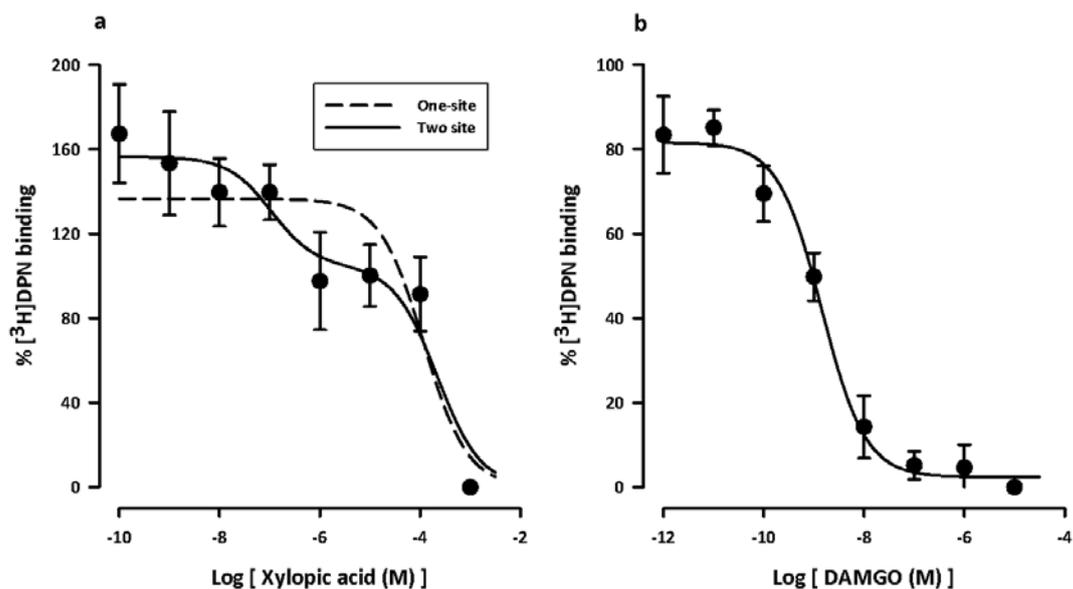
Figure 5.5 Competition binding curve of [^{3}H]Diprenorphine binding to the μ-opioid receptor in the presence of various concentrations of xylopic acid (a) or DAMGO (b). Each point represents the mean±S.E.M from two experiments. The one-site and two-site binding curves for xylopic acid were compared with the *F*-test.

Table 5.1 IC50 values from the competitive binding studies

Ligand	IC$_{50}$ (µM)	
	One-site binding	Two-site binding
Xylopic acid	261.4	0.1087, 220.5
DAMGO	0.001443	-
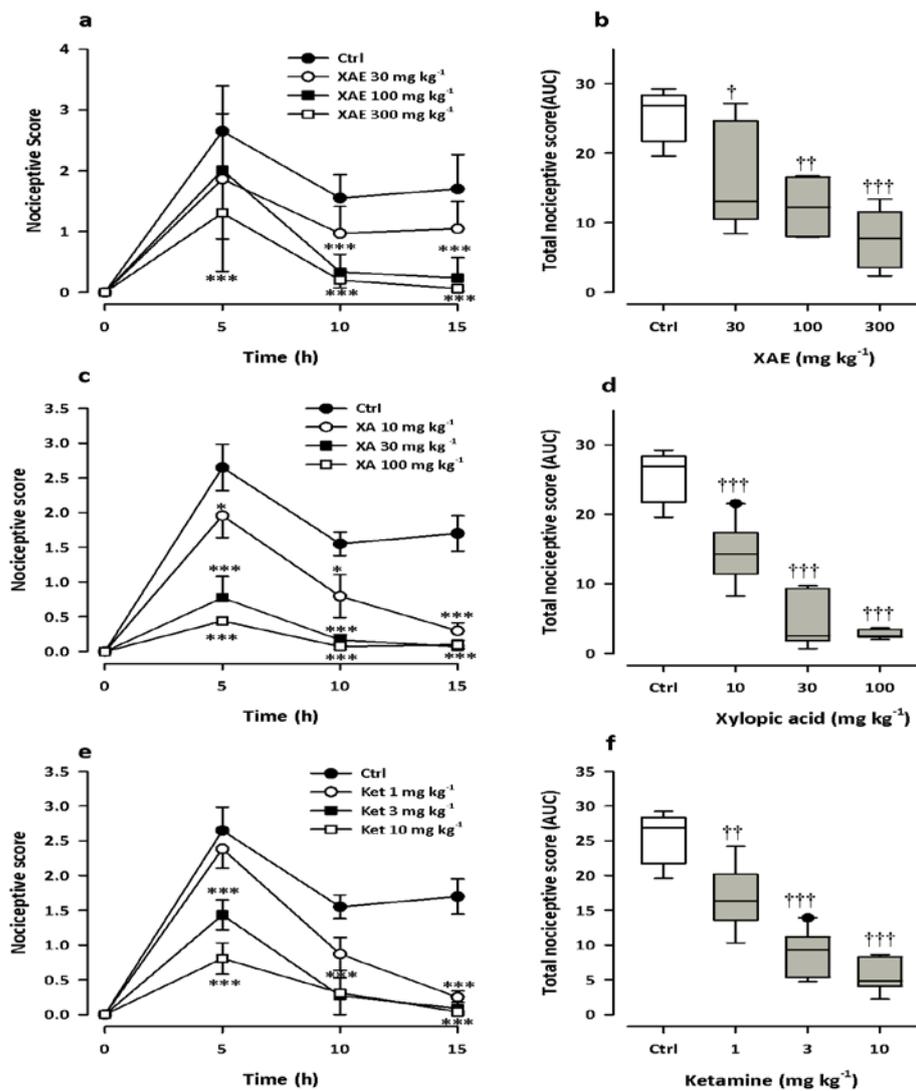
Figure 5.6 Effect of (a) extract (30 - 300 mg kg^{-1} *p.o*) (c) xylopic acid (10 - 100 mg kg^{-1} *p.o*) and (e) ketamine (1-10 mg kg^{-1}, i.p.) on the time course curve of glutamate-induced neurogenic pain and the AUC (b, d and f). Data is presented as mean ± S.E.M. (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles respectively. The median is shown as the horizontal line within the box. Symbol represents outlier. ***$P < 0.001$; compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test); $^{††}P < 0.01$, $^{†††}P < 0.001$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).

Figure 5.7 Dose–response curves for the antinociceptive activity of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and ketamine (1-10 mg kg^{-1}) in the glutamate-induced neurogenic pain assay in mice. Each point is the mean ± S.E.M. of 8 animals.

5.4.4 Capsaicin-induced neurogenic pain

Intraplantar injection of capsaicin into the paws of the animals produced nociceptive behaviour which was characterized by biting and licking of the injected paw. This started shortly after the administration of capsaicin and diminished in effect after 5 min. Treatment of the animals with extract and xylopic acid all produced marked antinociception in this test. The antinociception produced by extract (30–300 mg kg^{-1},

p.o.) was dose-related peaking at the highest dose used with a maximal antinociception of 86.0±0.23% (Fig. 5.8a). Xylopic acid (10–100 mg kg^{-1}, *p.o.*) also produced a marked and dose-related inhibition of capsaicin-induced nociception with the highest dose causing a maximal effect of 89.10±0.1% of the licking time (Fig. 5.8b). The order of potency was xylopic acid >extract (Fig.5.9).

5.4.5 Bradykinin-induced hyperalgesia

Hyperalgesia was induced in all the animals treated with bradykinin. A change in hyperalgesic state was calculated as a percentage of the maximum possible effect. Administration of extract (30–300 mg kg^{-1}, *p.o.*) significantly ($F_{3,24} = 8.85$, *P*=0.0004) and dose-dependently inhibited mechanical hyperalgesia (Fig. 5.10a). The highest dose of extract produced maximum possible effect at the highest dose used (Fig. 5.10b).

 The inhibition of mechanical hyperalgesia by xylopic acid (10–100 mg kg^{-1}, *p.o.*) was also significant ($F_{3,24}$=8.23, *P*=0.0006) and a dose-dependent effect was realized (Fig. 5.10c). A maximum possible mechanical anti-hyperalgesia induced by bradykinin was observed at the highest dose (Fig. 5.10d). Xylopic acid was more efficacious in this test than the extract as seen from Fig. 5.11.
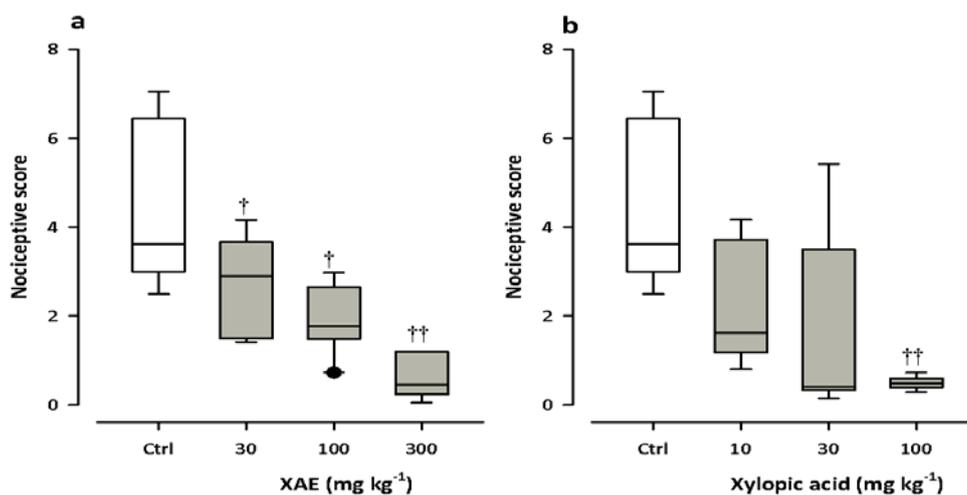
Figure 5.8 Effect of (a) extract (30 - 300 mg kg^{-1} *p.o*) and (b) xylopic acid (10 - 100 mg kg^{-1} *p.o*) on capsaicin-induced nociception. Data is presented as mean ± S.E.M. (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles respectively. Symbol represents outlier. The median is shown as the horizontal line within the box. $^{†}P < 0.05$, $^{††}P < 0.01$, compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
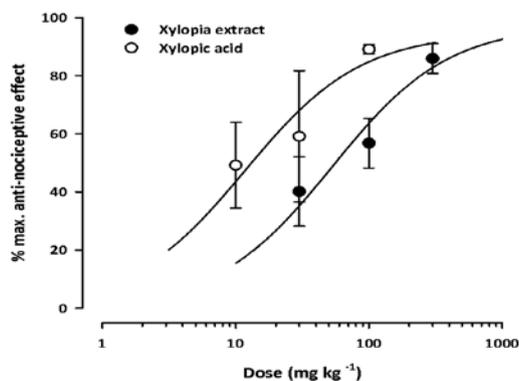
Figure 5.9 Dose–response curves for the antinociceptive activity of extract (30-300 mg kg^{-1}) and xylopic acid (10-100 mg kg^{-1}) in the capsaicin-induced neurogenic pain assay in mice. Each point is the mean ± S.E.M. of 8 animals.
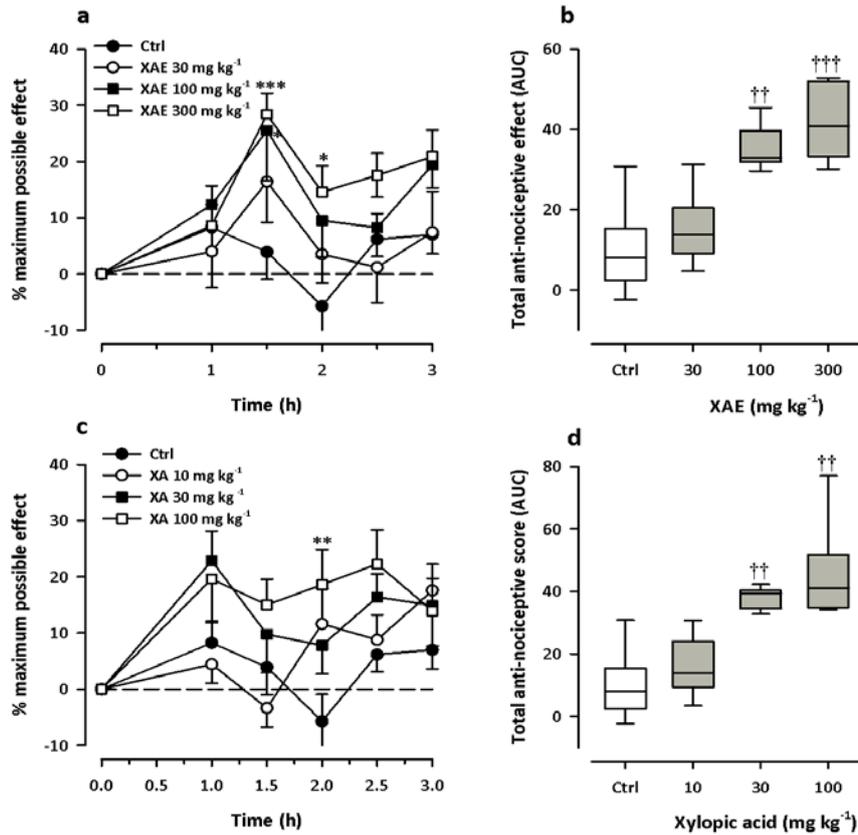
Figure 5.10 Effect of (a) extract (30 - 300 mg kg^{-1} *p.o*) and (c) xylopic acid (10 - 100 mg kg^{-1} *p.o*) on the time course curve of Bradykinin-induced mechanical hyperalgesia and the AUC (b and d). Data is presented as mean ± S.E.M. (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{†††}P<0.001$ $^{††}P<0.01$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
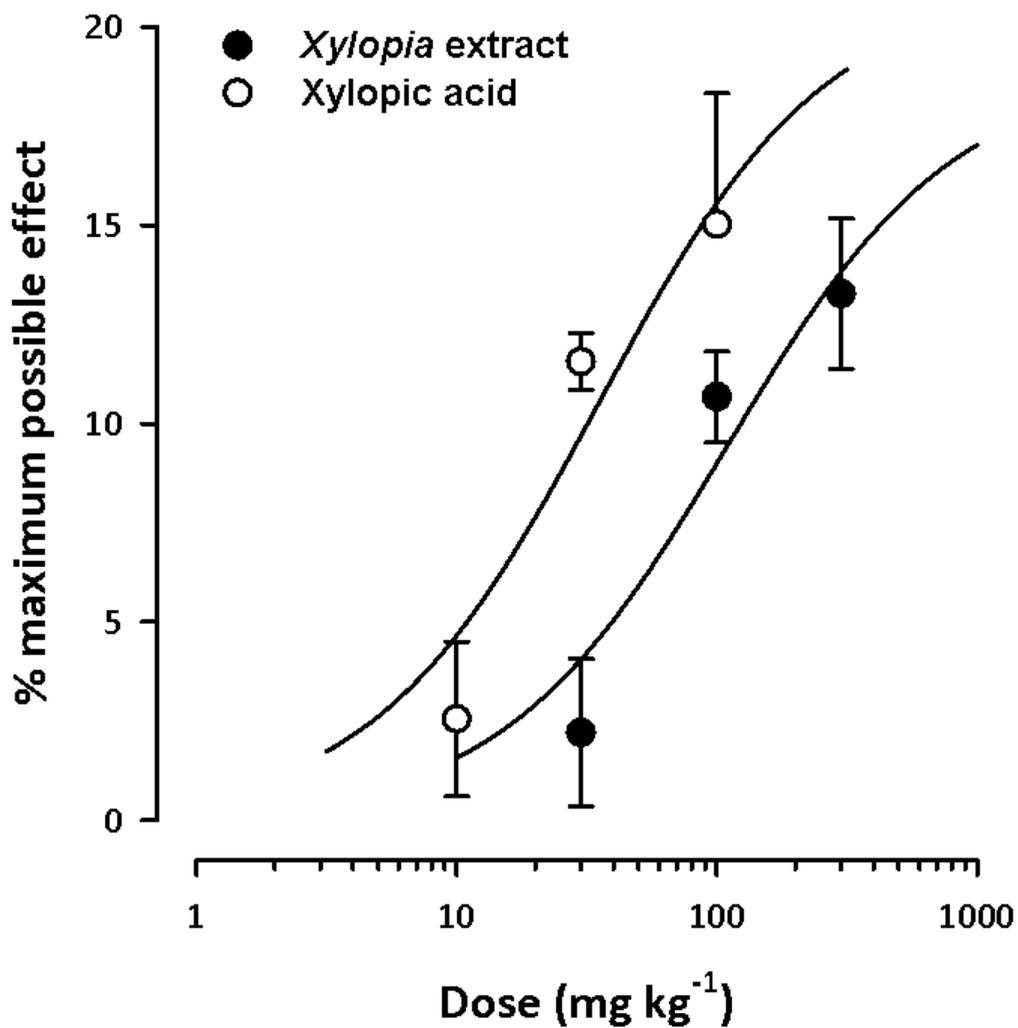
Figure 5.11 Dose–response curves for the antinociceptive activity of extract (30-300 mg kg^{-1}) and xylopic acid (10-100 mg kg^{-1}) in the bradykinin-induced hyperalgesia in rats. Each point is the mean ± S.E.M. of 8 animals.

5.4.6 Prostaglandin E₂-induced mechanical hyperalgesia

Intraplantar administration of the algogen, prostaglandin E_2 caused a painful state which was assessed using a mechanical source of stimulus. All the animals exhibited some degree of mechanical hyperalgesia after intraplantar prostaglandin E_2 injection which was attenuated by all the reference drugs used. The hyperalgesia produced was lowered after extract, xylopic acid and morphine treatments compared to control. The anti-hyperalgesia (extract: $F_{3,24} = 3.72$, $P=0.025$, xylopic acid: $F_{3,24} = 5.0$, $P=0.0078$, morphine: $F_{3,24} = 6.45$, $P =0.0023$) seen after the administration extract (30–300 mg kg^{-1}, *p.o.*), xylopic acid (10–100 mg kg^{-1}, *p.o.*) and morphine (1–10 mg kg^{-1}) (Fig. 5.12a, c and e) respectively increased dose-dependently and the maximum possible effect was attained at the highest doses used for all the drugs in (Fig. 5.12b, d and e). Xylopic acid exhibited the highest efficacy in this model. The extract was also more efficacious than the reference drug, morphine (Fig. 5.13).

5.4.7 Epinephrine-induced hyperalgesia

Mechanical stimulation of the hind paw of the rats after epinephrine injection revealed a state of hyperalgesia in all the animals which was seen as increase in paw withdrawal reflexes. Treatment of the animals with the test drugs extract (30–300 mg kg^{-1}, *p.o.*), xylopic acid (10–100 mg kg^{-1}, *p.o.*) and propranolol (1–10 µg/paw) resulted in a decrease ($F_{3,24}=4.97$, $P=0.0080$: $F_{3,24}=5.83$, $P=0.0038$: $F_{3,24}= 4.62$, $P=0.0109$ respectively) (Fig. 5.14a, c and e) paw withdrawal response to mechanical stimulation which was interpreted as anti-hyperalgesia produced by the various treatments. The possible maximum anti-hyperalgesia achieved at the highest doses of the various drug treatments (Fig. 5.14b, d and e) was in the order xylopic acid > extract > propranolol (Fig. 5.15).
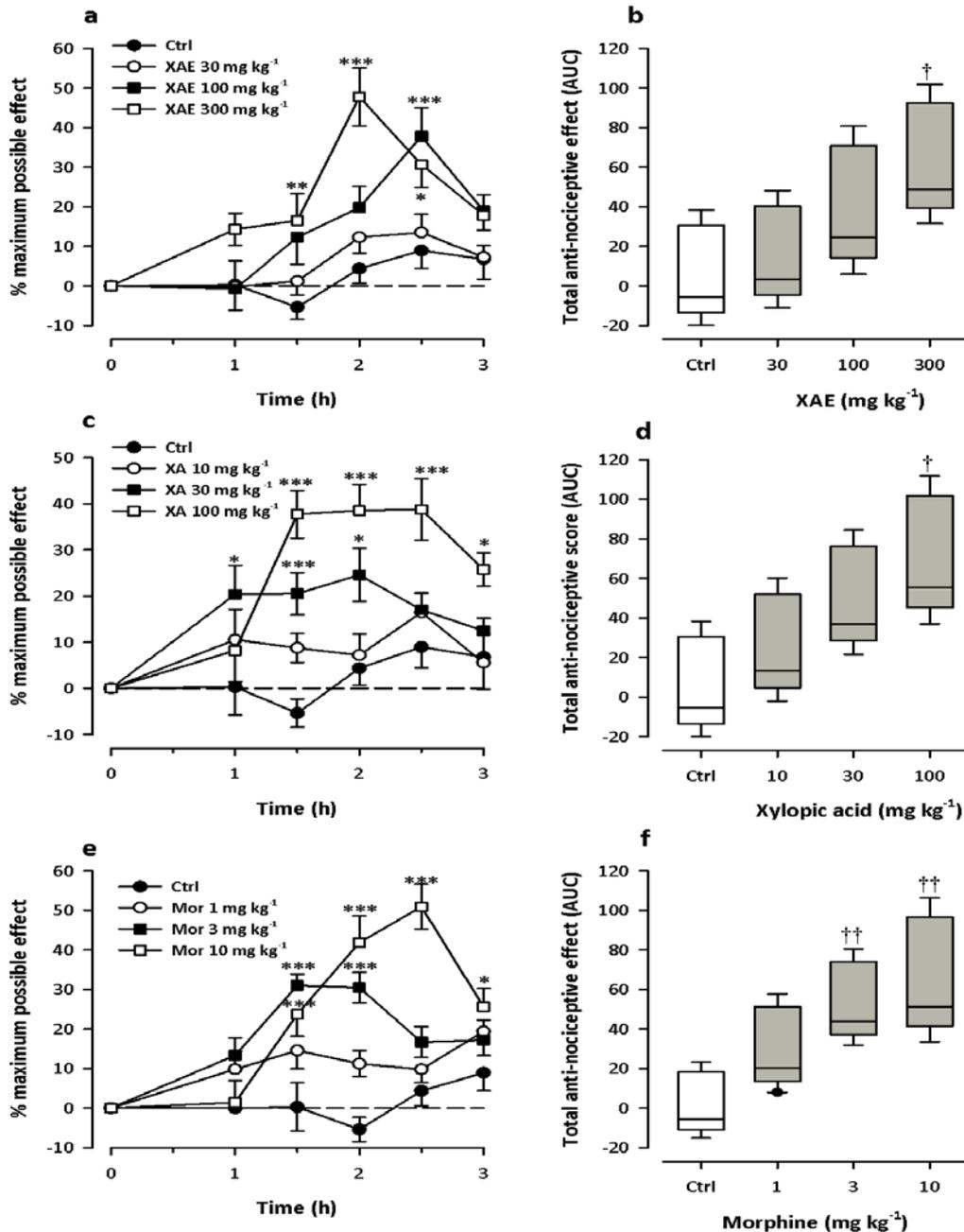
Figure 5.12 Effect of (a) extract (30 - 300 mg kg^{-1} *p.o*) (c) xylopic acid (10 - 100 mg kg^{-1} *p.o*) and (e) morphine (1-10 mg kg^{-1} i.p.) on the time course curve of Prostaglandin E$_2$-induced mechanical hyperalgesia and the AUC (b, d and f). Data is presented as mean ± S.E.M. (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Symbol represents outlier. ***P < 0.001; ** *P* < 0.01; *P < 0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test); $^{†}P$ < 0.05, $^{††}P$ < 0.01, compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
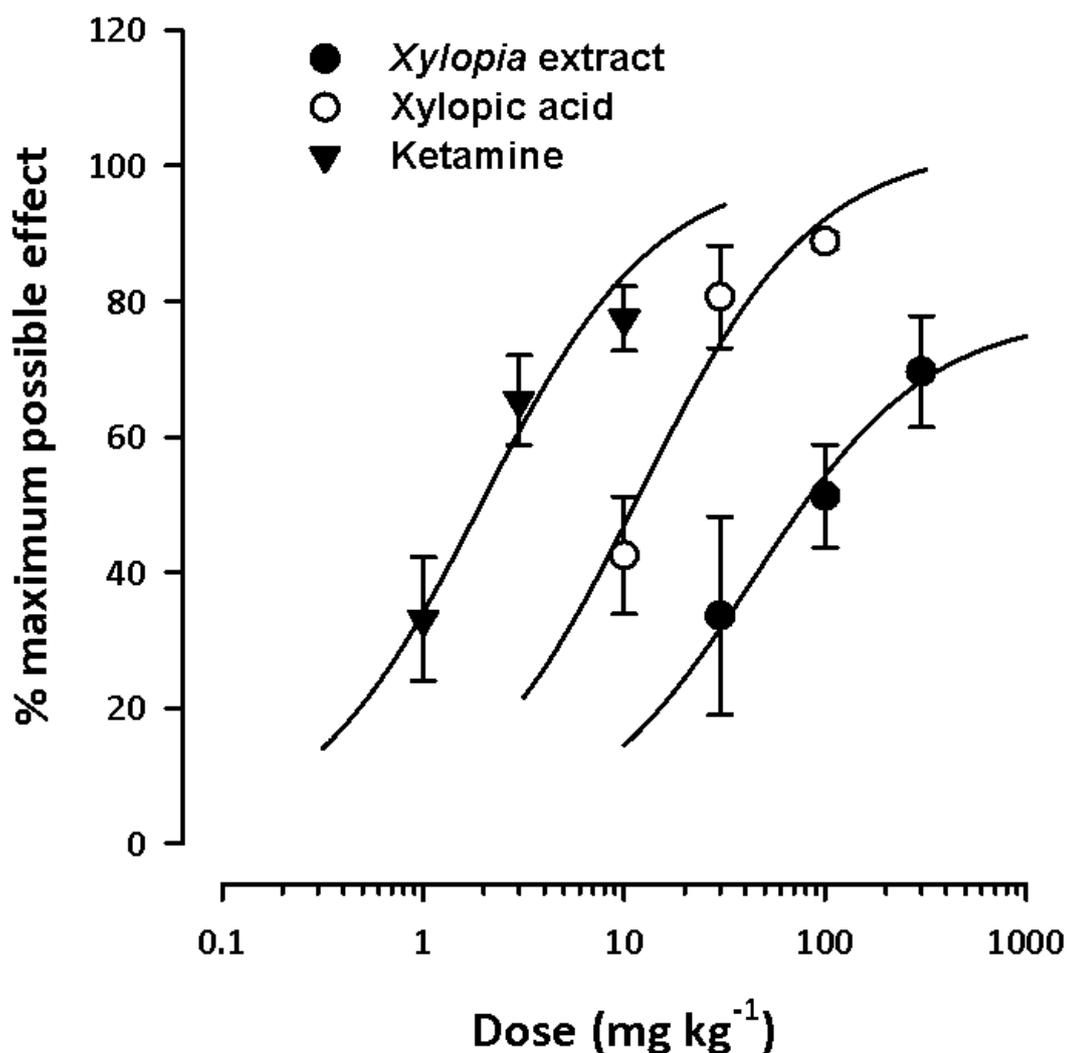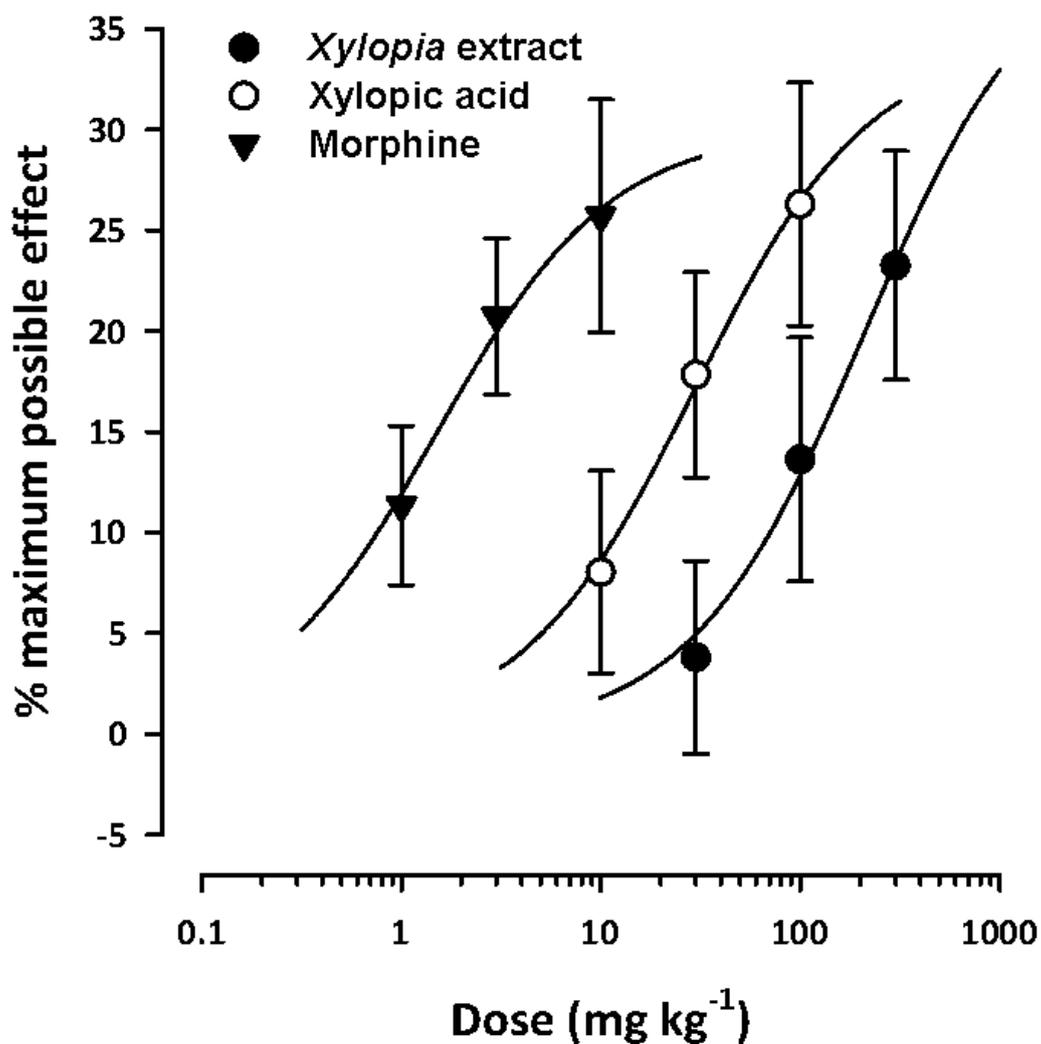
Figure 5.13 Dose–response curves for the antinociceptive activity of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and morphine (1-10 mg kg^{-1}) in the prostaglandin E$_2$-induced hyperalgesia in rats. Each point is the mean ± S.E.M. of 8 animals.
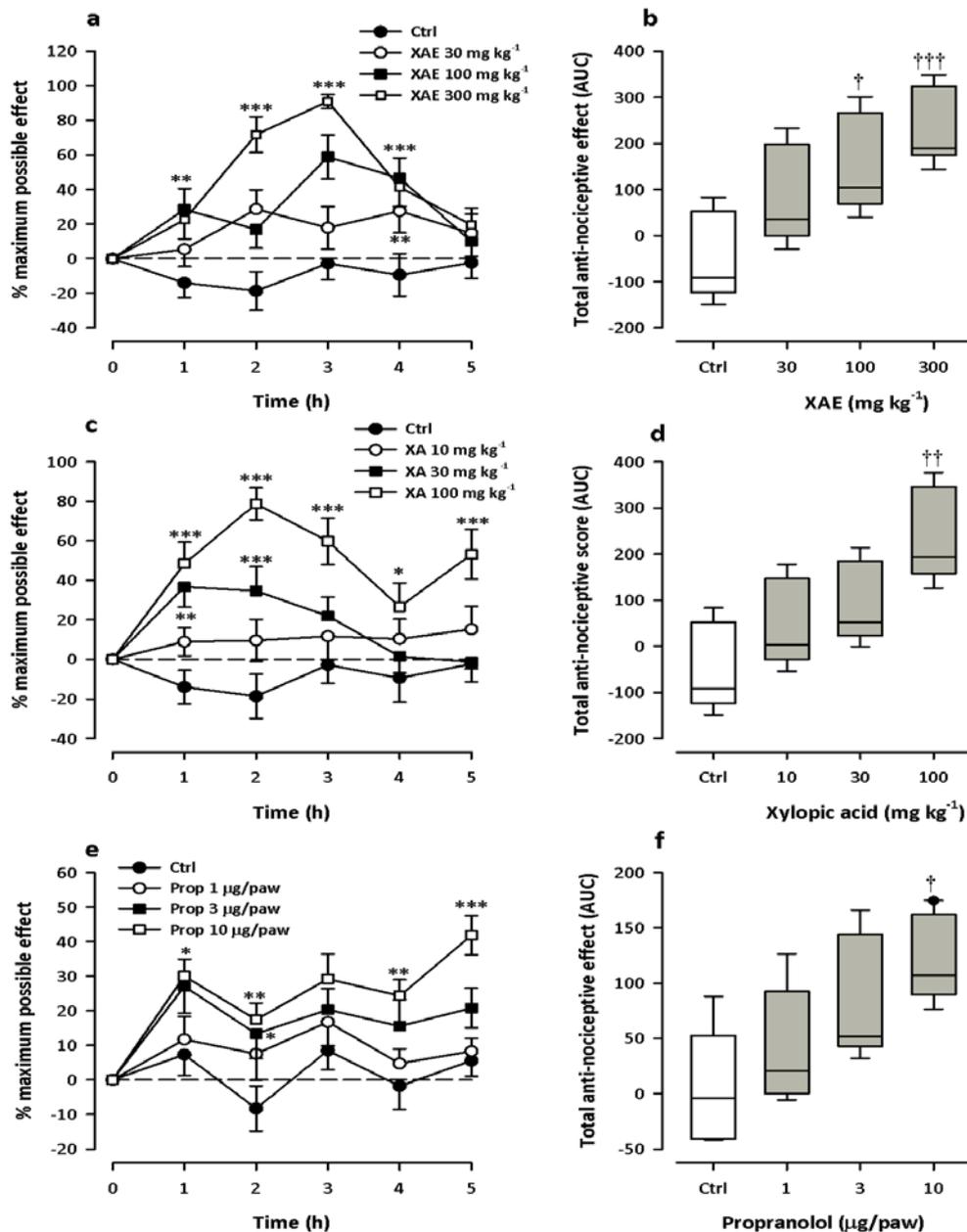
Figure 5.14 Effect of (a) extract (30 - 300 mg kg^{-1} *p.o*), (c) xylopic acid (10 - 100 mg kg^{-1} *p.o*) and (e) propranolol (1-10 µg/paw) on the time course curve of epinephrine-induced mechanical hyperalgesia and the AUC (b, d and f). Data is presented as mean ± S.E.M. (n = 8). The lower and upper margins of the boxes (b, d and f) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. Symbol represents outlier. ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test); $^{†}P < 0.05$, $^{††}P < 0.01$, $^{†††}P < 0.001$ compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls' *post hoc* test).
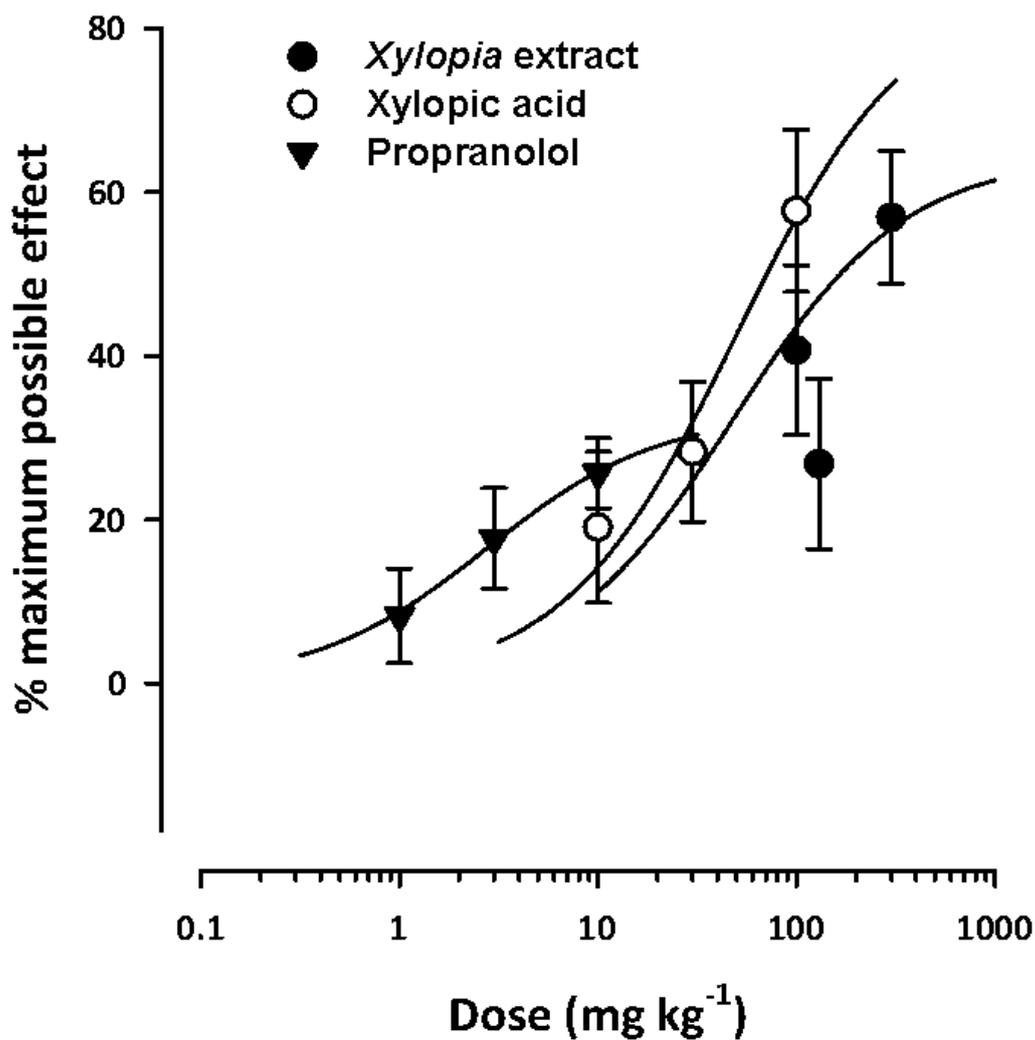
Figure 5.15 Dose–response curves for the antinociceptive effect of extract (30-300 mg kg^{-1}), xylopic acid (10-100 mg kg^{-1}) and propranolol (1-10 μg/paw) in the epinephrine-induced mechanical hyperalgesia in rats. Each point is the mean ± S.E.M. of 8 animals.

5.5 DISCUSSION

In order to identify some possible mediators involved in the anti-nociceptive activity of the extract and xylopic acid, an antagonism study was carried out in the formalin test. The anti-nociceptive effects of the extract and xylopic acid were assessed in the presence of various antagonists including naloxone, theophylline, L-NAME, glibenclamide, atropine, ondansetron and yohimbine. Systemic administration of the opioid receptor antagonist naloxone inhibited the anti-nociceptive effects of the extract and xylopic acid in both phases of the formalin test. This strongly suggests an opioidergic involvement in the actions of the extract and xylopic acid.

Pre-treatment of the mice with N^G-Nitro-L-arginine methyl ester (L-NAME; NO synthase inhibitor) reversed the anti-nociception of extract and xylopic acid in the neurogenic phase of the formalin test. This finding implicates the involvement of the NO/cGMP pathway. Nitric oxide plays a complex and diverse role in the modulation of nociceptive transmission in both the peripheral and central nervous system (Cury *et al.*, 2011). NO exerts a dual effect on nociception and there are suggestions that this may be due to the existence of different subsets of nociceptive primary sensory neurons in which NO plays opposing roles (Vivancos *et al.*, 2003; Cury *et al.*, 2011). ATP-sensitive K^+ channels seem not to be involved in the actions of the extract and xylopic acid since glibenclamide (ATP-sensitive K^+ channel blocker) did not significantly alter their anti-nociceptive effects. Potassium channels other than the ATP sensitive K^+ channels involvement may not entirely be excluded.

The anti-nociceptive effects of the extract and xylopic acid were reversed by pre-administration of theophylline implicating the involvement of adenosinergic pathway in their actions. Adenosine acts at several P1 receptors (A_1, A_{2A}, A_{2B}, and A_3) all of which are coupled to G proteins (Fredholm *et al.*, 2001). In the periphery, adenosine A_1 receptor activation produces pain suppression, while adenosine A_2 receptor activation produces pain enhancement (Sawynok, 1998). Within the spinal cord, adenosine A_1 receptor activation produces anti-nociception (Sawynok, 1998). Adenosine A_3 receptor activation produces pro-nociception in the CNS (Pedata *et al.*, 2010) and also peripherally—secondary to mast cell degranulation and release of histamine and 5-hydroxytryptamine (5-HT) that exert nociceptive actions at sensory nerve terminal (Sawynok, 1998). Since theophylline blocks adenosine A_1 and A_2 receptors, the anti-

nociceptive effects may be due to activation of A_1 receptors and/or an increment in endogenous adenosine either centrally or peripherally. The finding that both opioidergic and adenosinergic mechanisms are possibly involved in the anti-nociceptive effects of the extract and xylopic acid is not surprising since both opioid and adenosine receptor agonists are known to share some similar anti-nociceptive mechanisms (Aley *et al.*, 1995; Sawynok, 1998). The A_1 receptor has been proposed to exist as part of a multireceptor complex, in association with μ-opioid and $α_2$-adrenergic receptors on the basis of a demonstrated cross antagonism, cross tolerance and cross withdrawal between these systems (Aley and Levine, 1997; Sawynok, 1998) and activation of one of these receptors may affect the rest (Suh *et al.*, 1997).

Activation of muscarinic receptors induces anti-nociception in various pain paradigms including thermal, inflammatory and neuropathic pain (Wess *et al.*, 2003; Jones and Dunlop, 2007). M_1, M_2, M_3 and M_4 receptors are involved in mediating the analgesic effects of muscarinic agonists at the spinal and supraspinal level (Naguib and Yaksh, 1997; Ghelardini *et al.*, 2000; Honda *et al.*, 2000; Lograsso *et al.*, 2002; Wess *et al.*, 2003) while peripheral activation of M_2 receptors likely contributes to analgesia via reduced CGRP release (Bernardini *et al.*, 2002; Wess *et al.*, 2003). The anti-nociceptive effects of the extract and xylopic acid involve the muscarinic cholinergic pathway since it was antagonized by atropine. Atropine also blocked the analgesic effects of morphine. It is already well established that the analgesic effect of systemic morphine is mediated by a descending cholinergic pathway (Chiang and Zhuo, 1989) as well as spinal endogenous acetylcholine acting through muscarinic receptors (Chen and Pan, 2001; Radhakrishnan and Sluka, 2003; Honda *et al.*, 2004; Chen *et al.*, 2005).

Pre-treatment of mice with yohimbine (an $α_2$-adrenoceptor antagonist) also reversed the anti-nociception of xylopic acid in the first phase of the formalin test but affected neither the extract nor morphine. This implicates the involvement of $α_2$-adrenergic mechanisms in the anti-nociceptive activity of xylopic acid. Alpha-2-receptors are important in peripheral, spinal and supraspinal pain modulation. In the spinal cord, for instance, noradrenaline released from descending pathways suppresses pain by inhibitory action on $α_{2A}$-adrenoceptors on central terminals of primary afferent nociceptors (presynaptic inhibition) or by direct $α_2$-adrenergic action on pain-relay neurons (postsynaptic inhibition)(Pertovaara, 2006). Serotoninergic neurons also play a crucial role in the control of pain (Fields *et al.*, 1991) and the diversity of subtype

receptors for serotonin, makes this system able to exert either facilitatory or inhibitory function (Bardin *et al.*, 2000). Spinal 5-HT$_3$ receptors haven been shown to mediate anti-nociception, possibly via GABA release (Alhaider *et al.*, 1991; Millan, 2002). Pre-administration of ondansetron attenuated the anti-nociceptive of the extract and xylopic acid in the formalin test. This implicates 5-HT$_3$-serotoninergic involvement in the anti-nociceptive activity of the extract and xylopic acid.

The μ-receptor subtype (MOR) is generally thought to be responsible for most of the analgesic effects of the opioids. Since antagonism in the formalin test revealed the involvement of opioidergic pathways, further study was performed to determine whether xylopic acid actually binds to opioid receptors via radioligand competitive binding studies. Considering, the IC$_{50}$ values of xylopic acid in comparison with DAMGO, it appears that xylopic acid has some (albeit moderately weak) affinity for MOR. Unfortunately, it is not yet clear what the binding sites actually represent. It could signify binding to more than one of the proposed μ-opioid receptor subtypes (Goldberg *et al.*, 1998; Cadet, 2004) or it could represent both low and high affinity binding to the same μ-receptor (e.g. high affinity in the presence of G protein, low affinity in the absence). The involvement of other opioid receptor subtypes (i.e. δ or κ) in the analgesic actions of xylopic acid is also possible and this requires further investigation. Perhaps, the most interesting finding from the binding studies was the apparent increase in [^{3}H]DPN binding that occurred at very low concentrations of xylopic acid. It suggests that xylopic acid may be able to enhance the binding of orthosteric ligands, such as DPN. Xylopic acid may, therefore, be producing its analgesic effects by acting as an allosteric modulator of the μ-opioid receptor *in vivo* to potentiate the anti-nociceptive effects of endogenous μ-opioid receptor ligands. Further studies are necessary to confirm this observation.

In order to ascertain the involvement of the glutamatergic system in the mechanism of action of extract and xylopic acid, the glutamate test was carried out with ketamine as a reference drug. The extract, xylopic acid and ketamine attenuated the nociceptive behaviour induced by glutamate. It has been shown that the nociceptive response induced by glutamate appears to involve peripheral, spinal and supraspinal sites of action and is greatly mediated by both NMDA and non-NMDA receptors by a mechanism which largely depends on the activation of L-arginine-nitric oxide pathway (Beirith *et al.*, 2002). The anti-nociceptive activity of the extract and xylopic acid in the

glutamate test, therefore, probably involves an inhibition of the production/action of NO, or an interaction with the glutamatergic system. This is a significant finding because glutamate and its receptors, both ionotropic and metabotropic, are critical for nociceptive processing. Hyperfunction or dysfunction of glutamatergic neurotransmission is known to be a key mechanism of pain-related plastic changes in the central and peripheral nervous system (Neugebauer, 2007). NMDA receptor-mediated mechanisms are particularly important as NMDA-receptor antagonists have been shown to effectively alleviate pain-related behavior, reduce opioid-induced hyperalgesia and retard opioid tolerance development in both animal models as well as in clinical situations (Mao, 1999; Price *et al.*, 2000; Dupen *et al.*, 2007).

The extract and xylopic acid suppressed nocifensive responses in the capsaicin test. Capsaicin activates the vanilloid receptor, TRPV1, a ligand-gated non-selective cation channel present in primary sensory neurons (Caterina *et al.*, 1997). This leads to the release of neuropeptides, excitatory amino acids, nitric oxide, and pro-inflammatory mediators from the periphery, and transmits nociceptive information to the spinal cord or causes spinal sensitization through protein kinase A and C activation (Calixto *et al.*, 2005; Meotti *et al.*, 2006). The anti-nociceptive effect of the extract and xylopic acid may therefore involve an interaction with TRPV1 or the inhibition of production or action of some of these mediators. It has been shown that μ-opioid receptor activation can inhibit the activity of TRPV1 via $G_{i/o}$ proteins and the cAMP pathway (Endres-Becker *et al.*, 2007). Therefore, xylopic acid and the extract may have acted to inhibit capsaicin-induced nociception through the opioid pathway.

The extract and xylopic acid inhibit bradykinin-, prostaglandin E_2 -and epinephrine-induced hyperalgesia. Bradykinin, an important peripheral mediator of pain, elicits nociception or hyperalgesia by direct stimulation of the nociceptors Aδ and C-fibers. Several inflammatory and algogenic substances such as products derived from arachidonic acid pathways, cytokines and nitric oxide as well as neuropeptides such as calcitonin gene-related peptide and substance P may also be released by bradykinin (Vasko *et al.*, 1994; Calixto *et al.*, 2001). The mechanical hyperalgesia induced by bradykinin involves a B_2 receptor-mediated direct activation of protein kinase C and the indirect activation of the protein kinase A (Ferreira *et al.*, 2004; Meotti *et al.*, 2006). Epinephrine and prostaglandin E_2 also act through β and EP receptors respectively, to activate cAMP/PKA and PKC pathways leading to hyperalgesia (Khasar *et al.*, 1999 ;

Villarreal *et al.*, 2009). Xylopic acid and the extract may have acted directly or indirectly to inhibit B_2/β/EP receptors or PKA and/or PKC pathways.

5.6   CONCLUSION

The antinociception mechanism of the extract and xylopic acid was mediated through the adenosinergic, muscarinic, serotoninergic and opioidergic-NO-cGMP pathways. Xylopic acid additionally acted on the α_2- adrenergic pathway to produce antinociception. The extract and xylopic acid produced antinociception by inhibiting B_2/β/EP receptors or PKA and/or PKC pathways.

Chapter 6

ISOBOLOGRAPHIC ANALYSIS OF THE ANTINOCICEPTION OF XYLOPIC ACID, MORPHINE AND DICLOFENAC COMBINATIONS

6.1   INTRODUCTION

Combination of different analgesics is a common practice in Ghana and this strategy is aimed at achieving one or more therapeutic goals, such as facilitating patient compliance, improving efficacy without increasing adverse effects, or decreasing adverse effects without loss of efficacy (Tallarida, 2000). In Ghana a lot of combination drugs are in use including anti hypertensives such as Nilol (Nifedipine 20 mg : atenolol 50 mg), Caduet (Amlodipine besylate 5 mg : Artovastatin calcium 10 mg) and analgesics such as Efpac (Aspirin 150 mg : Paracetamol 250 mg : Caffeine 30 mg), APC (Aspirin 250 mg : Paracetamol 125 mg : Caffeine 25 mg), Parabru (Brufen 250 mg: Paracetamol 325 mg); Kwik Action (Ephedrine Hcl 10 mg : Paracetamol 500 mg : Caffeine 30 mg), Gebedol forte (Diclofenac 50 mg : Paracetamol 500 mg : Chlorzoxazone 250 mg) and Inflnil (Diclofenac 50 mg : Paracetamol 650 mg). Inappropriate combination of the constituents of a combination product may lead to antagonism or simple additive effect. Therefore strategic combination of the constituents is needed in order to achieve synergism. In order to determine the proportion of the constituents of combination products of xylopic acid/morphine and xylopic acid/diclofenac that produced synergism, isobolographic analysis was employed.

Isobolographic analysis provides a fundamental basis for assessing whether biological responses induced by mixtures of agents are greater, equal or smaller than would have been expected on the basis of the individual activities of the component agents (Tallarida, 2000). The concept of dose additivity, antagonism or supra-additivity aimed at the development of novel pain relief strategies involving different combinations of analgesics that target both central and peripheral pain pathways and to produce greater analgesia at reduced and more tolerable doses of individual drugs has become important in disease treatment. Synergism when observed in isobolographic analysis may also provide information on mechanism of drug action (Tallarida *et al.*, 1999). The principle

of independent joint action (the two drugs should produce overtly similar effects through different mechanism) is used as a guide for selection of drugs for isobolographic analysis and the realization of addditivity indicates that the same pathway is activated by the constituents of the combination (Miranda *et al.*, 2007). Xylopic acid acted on opioidergic, serotoninergic, adenosinergic and NO- cAMP pathway to exert its antinociception. There is no single safe and effective analgesic for the treatment of pain although some of the reasons for this is attributable to inappropriate or insufficient use of existing therapies (Bandolier, 2003; Mcmahon and Koltzenburg, 2006; Chen and Tang, 2011). There is a possibility of the co administration of xylopic acid and other drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. This may lead to potentiation or antagonism of the combination. Commonly used analgesics, opioids and NSAIDs come with severe side effects.

While opioids such as morphine are the most widely used and most effective drugs for the treatment of many pain syndromes, they have some well-known adverse effects, including sedation, respiratory depression, nausea and constipation. Furthermore, on long term administration, they can lose their efficacy through the development of tolerance and very high doses of the drug are necessary in these cases. A further important issue is that some chronic pain states, such as neuropathic pain, are not effectively treatable with opiates (Pelissier *et al.*, 2003). NSAIDs are mainstays in acute and chronic pain management and their beneficial actions have been linked to their ability to inhibit cyclooxygenases: constitutive COX-1 and inducible COX-2 among others. Long term uses of the NSAIDs come with gastric ulceration which limits their usefulness (Miranda *et al.*, 2007). Xylopic acid (15α-Hydroxy-*ent*-kaur-16-en-19-oic acid) belongs to diterpenoid class of compounds and a closely related diterpene (*ent*-kaur-16-en-19-oic acid) contained in the extract has been reported to be cytotoxic and embryotoxic. Kaurenoic acid at a concentration of 78 μM produced growth inhibition of leukemic cells by 95%, MCF-7 breast and HCT-8 colon cancer cells by 45% each. Furthermore, kaurenoic acid induced a dose-dependent hemolysis of mouse and human erythrocytes with an EC_{50} of 74.0 and 56.4 μM, respectively (Costa-Lotufo *et al.*, 2002). Xylopic acid however has been reported to be relatively safer in brine shrimp exhibiting a low toxicity with LC_{50} of 0.5 ng/ml. Hexane extract of *X. aethiopica* has

also been reported to poses low toxicity with LC_{50} of 0.3 ng/ml in brine shrimp (Somova *et al.*, 2001).

In the light of the above it is obvious that alternative strategies based on drug combinations need to be considered in order to solve the drawbacks of opioids and NSAIDs especially with agents with low toxicity profile such as xylopic acid.

6.2   MATERIALS AND METHODS

6.2.1   Animals

Male ICR mice (20-25 g) were used for this study. The animals were provided with the necessary conditions as described previously under section 3.2.1.

6.2.2   Drugs and chemicals

Diclofenac sodium was purchased from Troge Medical GmbH, Hamburg, Germany: morphine hydrochloride was obtained from Phyto-Riker, Accra, Ghana**:** formalin and acetic acid were purchased from BDH, Poole, England.

6.3   ISOBOLOGRAPHIC ANALYSIS OF XYLOPIC ACID/MORPHINE, XYLOPIC ACID/DICLOFENAC COMBINATIONS.

The method of isobolographic analysis was performed similarly as described (Pinardi *et al.*, 2005; Miranda *et al.*, 2007; Miranda *et al.*, 2002). The formalin test was chosen for this assay because of its ability to detect interaction of drugs both peripherally and centrally (Lapa Fda *et al.*, 2009). The acetic acid writhing assay was also employed due to its sensitivity to detect drugs of weak analgesic properties (Pietrovski *et al.*, 2006). The ED_{50}'s of xylopic acid, morphine and diclofenac were obtained from the formalin test and acetic acid writhing assay for isobolographic analysis. This was achieved by determining the dose–response curves for the administration of xylopic acid, morphine and diclofenac using eight animals (n=8) in the formalin and acetic acid-induced writhing test. A least-square linear regression analysis of the log dose–response curves

obtained from the two nociceptive tests allowed the calculation of the doses that produced 50% of antinociception when each drug was administered alone. ED_{50} was used in the formalin and acetic acid-induced writhing tests as the equieffective doses for isobolographic analysis since all the administered drugs attained maximum responses (full agonists on their receptors). Then dose response curves were also obtained and analyzed after the co-administration of xylopic acid with morphine or with diclofenac in fixed ratio (1:1) combinations based on the following fractions 1/2, 1/4, 1/ 8 of their respective ED_{50} for both formalin and writhing tests (Tallarida, 2000; Miranda *et al.*, 2009).

To obtain the experimental ED_{50} for xylopic acid/morphine co-administration for isobolographic analysis, animals were grouped into thirteen with each group consisting of eight mice and were treated as follows in the formalin test:

Group I	were treated with 0.15 mg kg^{-1} morphine i.p. (ED_{50} of morphine) and then received 10 µl of 5% formalin intraplantar 30 minutes afterwards for the phase 1 analysis.
Group II	were treated with 0.0004 mg kg^{-1} morphine i.p. (ED_{50} of morphine) and then received 10 µl of 5% formalin intraplantar 30 minutes afterwards for phase 2 analysis.
Group III	were treated with 13.56 mg kg^{-1} xylopic acid *p.o.* (ED_{50} of xylopic acid) and then received 10 µl of 5% formalin intraplantar 1 hour afterwards for phase 1 analysis.
Group IV	were treated with 7.61 mg kg^{-1} xylopic acid *p.o.* (ED_{50} of xylopic acid) and then received 10 µl of 5% formalin intraplantar 1 hour afterwards for phase 2 analysis.
Group V-VIII	mice were treated with [morphine + xylopic acid]: $(0.15 + 13.56)$ mg kg^{-1}, $(0.15 + 13.56)$ mg $kg^{-1}/2$, $(0.15 + 13.56)$ mg $kg^{-1}/4$, $(0.15 + 13.56)$ mg $kg^{-1}/8$ followed by administration of 10 µl of 5% formalin intraplantar for phase 1 analysis.
Group IX-XII	mice were treated with $(0.0004 + 7.61)$ mg kg^{-1}, $(0.0004 + 7.61)$ mg $kg^{-1}/2$, $(0.0004 + 7.61)$ mg $kg^{-1}/4$, $(0.0004 + 7.61)$ mg $kg^{-1}/8$

then received 10 µl of 5% formalin intraplantar 1 hour afterwards for phase 2 analysis.

Group XIII   mice were treated with vehicle (10 ml kg^{-1} of *p.o.*) and 60 min afterwards received 10 µl of 5% formalin intraplantar.

Isobologram (a cartesian plot of pairs of doses that, in combination, yield a specified level of effect) was then built by connecting the theoretical ED_{50} of morphine plotted on the abscissa with that of xylopic acid plotted on the ordinate to obtain the additivity line. For each drug mixture, the ED_{50} (experimental) and its associated 95% confidence intervals were determined by linear regression analysis of the log dose–response curve (and compared by a 't'-test to a theoretical additive ED_{50}) obtained from the calculation;

$Z_{add} = f (ED_{50})$ of morphine + (1-f) ED_{50} of xylopic acid

Where f is the fraction of the each component in the mixture and the variance (*Var*) of Z_{add} was calculated as:

Var $Z_{add} = f^2$ (Var ED_{50} of morphine) + $(1-f)^2$ Var ED_{50} of xylopic acid.

From these variances S.E.M.'s were calculated and resolved according to the ratio of the individual drugs in the combination. A supra-additive or synergistic effect is defined as the effect of a drug combination that is higher and statistically different (ED_{50} significantly lower) than the theoretically calculated equieffect of a drug combination in the same proportion. If the ED_{50}'s are not statistically different, the effect of the combination is additive and additivity means that each constituent contributes with its own potency to the total effect. The degree of interaction was calculated using fractional analysis by dividing the experimental ED_{50} (Z_{mix}) by the theoretical ED_{50} (Z_{add}). A value close to 1 was considered as additive interaction. Values lower than 1 are an indication of the magnitude of supra-additive or synergistic interactions ($Z_{mix}/Z_{add} < 1$), and values higher than 1 correspond to sub-additive or antagonistic interactions (Miranda *et al.*, 2007).

Isobolographic analysis of xylopic acid/diclofenac combination was performed similarly as described above.

6.4   STATISTICAL ANALYSIS

In all experiments, a sample size of eight animals (n=8) were used. All data are presented as mean ± S.E.M (n=8). The time-course curves were subjected to two-way (treatment × time) repeated measures analysis of variance (ANOVA) with Bonferroni's post hoc test. Total nociceptive score for each treatment was calculated in arbitrary unit as the area under the curve (AUC). To determine the percentage inhibition for each treatment, the following equation was used.

$$\% \ inhibition = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}} \right) \times 100$$

Differences in AUCs were analyzed using one-way ANOVA with drug treatment as a between- subjects factor. Further comparisons between vehicle- and drug-treated groups were performed using the Newman–Keuls 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:

$$Y = \frac{a + (b - a)}{\left(1 + 10^{(LogED_{50} - X)}\right)}$$

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$_{50}$s) of the curves were compared statistically using F test (Miller, 2003; Motulsky and Christopoulos, 2003). GraphPad Prism for Windows Version 4.03 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED$_{50}$ determinations. $P < 0.05$ was considered statistically significant.

Isobolographic calculations were performed with the program Pharm Tools Pro (version 1.27, the McCary Group Inc.). Results are presented as mean ± S.E.M. or as ED$_{50}$ values with 95% confidence limits (95% CL). The statistical analysis of the isobolograms were performed according to Tallarida (Tallarida, 2006) and the statistical

difference between experimental and theoretical values was assessed by the Student's 't' test for independent means, and the P values < 0.05 were considered significant.

6.5   RESULTS

6.5.1   Isobolographic analysis of xylopic acid and morphine

Xylopic acid, morphine and the fractions of xylopic acid and morphine combination inhibited both neurogenic and inflammatory pain in the formalin test (Fig. 6.1a). However the neurogenic inhibition of pain by both morphine and xylopic acid was higher than the inflammatory pain inhibition. Xylopic acid significantly inhibited neurogenic pain by $77.32\pm15.9\%$ for phase 1 and $79.9\pm15.90\%$ for phase 2 respectively (Fig. 6.1b). Morphine also inhibited neurogenic pain by $42.99\pm15.51\%$ for phase 1 and $34.4\pm17.50\%$ for phase 2 respectively (Fig. 6.1b). Morphine was however more potent than xylopic acid in both phases (Table 6.1).

Table 6.1 $ED_{50} \pm$ S.E.M. of morphine and xylopic acid for both phases of the formalin test.

Drugs	Phase 1 ED_{50} (mg kg^{-1})	Phase 2 ED_{50} (mg kg^{-1})
Morphine	0.15 ± 0.42	0.0004 ± 2.3
Xylopic acid	13.56 ± 4.5	7.61 ± 2.3

Values are expressed as mean $\pm$ S.E.M. (n=8).

$ED_{50}s \pm$ S.E.M. were obtained by least-square nonlinear regression as described in materials and methods.
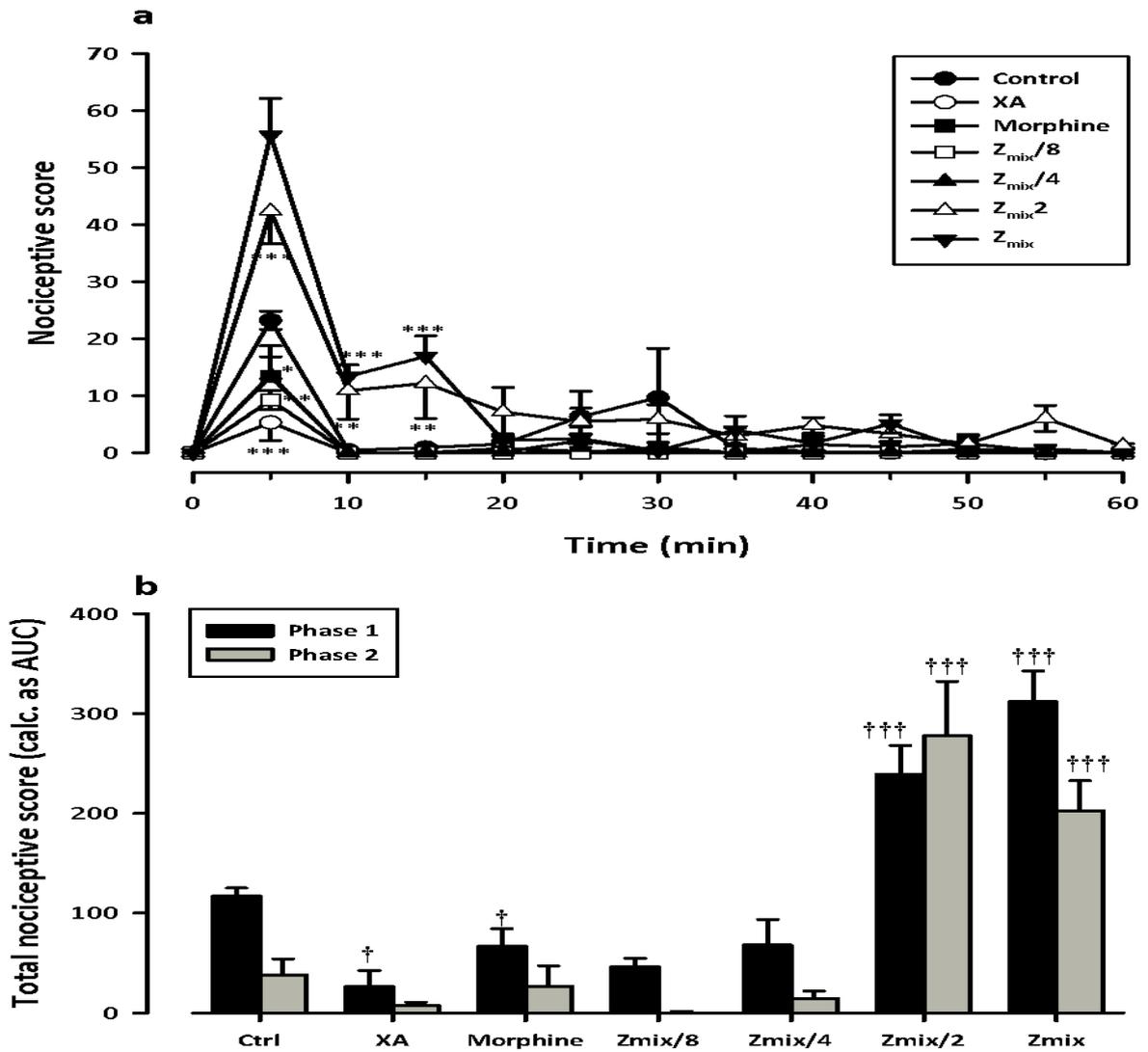
Figure 6.1 Effect of xylopic acid, morphine and fractions of their combination on (a) the time course curve of formalin-induced licking test. Each point represents the mean ± S.E.M, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (Two-way ANOVA followed by Bonferroni's *post hoc* test) and (b) the total nociceptive score of formalin-induced licking test in mice AUC for phase 1 and phase 2 of formalin-induced pain. Each column represent the mean ± S.E.M. [†]$P \leq$ 0.05 and [†††]$P \leq 0.001$, compared to respective controls (one-way ANOVA followed by Newman-Keuls *post hoc*).

6.5.2 *Isobologram of xylopic acid and morphine*

The experimental ED_{50} (Z_{mix}) obtained by non-linear regressional analysis for phase 1 (Fig.6.2a) was 2.58 ± 0.49 mg kg^{-1} and 2.31 ± 0.81 mg kg^{-1} for phase 2 (Fig. 6.2b) both indicating potentiation of the antinociceptive effect of the two drugs.

The degree of potentiation calculated as the interaction index by fractional analysis indicated synergism for phase 1 and phase 2 and graphically displayed as Z_{mix}'s lying below the line of additivity (Fig. 6.2 c and d respectively) of the isobologram.

The theoretical additive ED_{50} (Z_{add}) was computed as 6.86 ± 2.24 mg kg^{-1}for phase 1 (Fig. 6.2c) and 3.81 ± 2.5 mg kg^{-1} for phase 2 (Fig. 6.2d) and (Table 6.2).

Table 6.2 Theoretical and experimental ED50 $\pm$ S.E.M. of morphine and xylopic acid for both phases of the formalin test with their computed interaction indices.

Combinations	xylopic acid /morphine	xylopic acid /morphine
	phase 1	phase 2
Theoretical ED_{50} (mg/kg)	6.86 ± 2.24	3.81 ± 2.5
Experimental ED_{50} (mg/kg)	2.58 ± 0.49	2.31 ± 0.81
Interaction index	0.38	0.61
Drugs ratio	89.7:1	19025:1

Values are expressed as mean $\pm$ S.E.M. (n=8). The values were obtained from experiments described earlier.

ED_{50}s $\pm$ S.E.M. were obtained by least-square nonlinear regression as described in materials and methods.
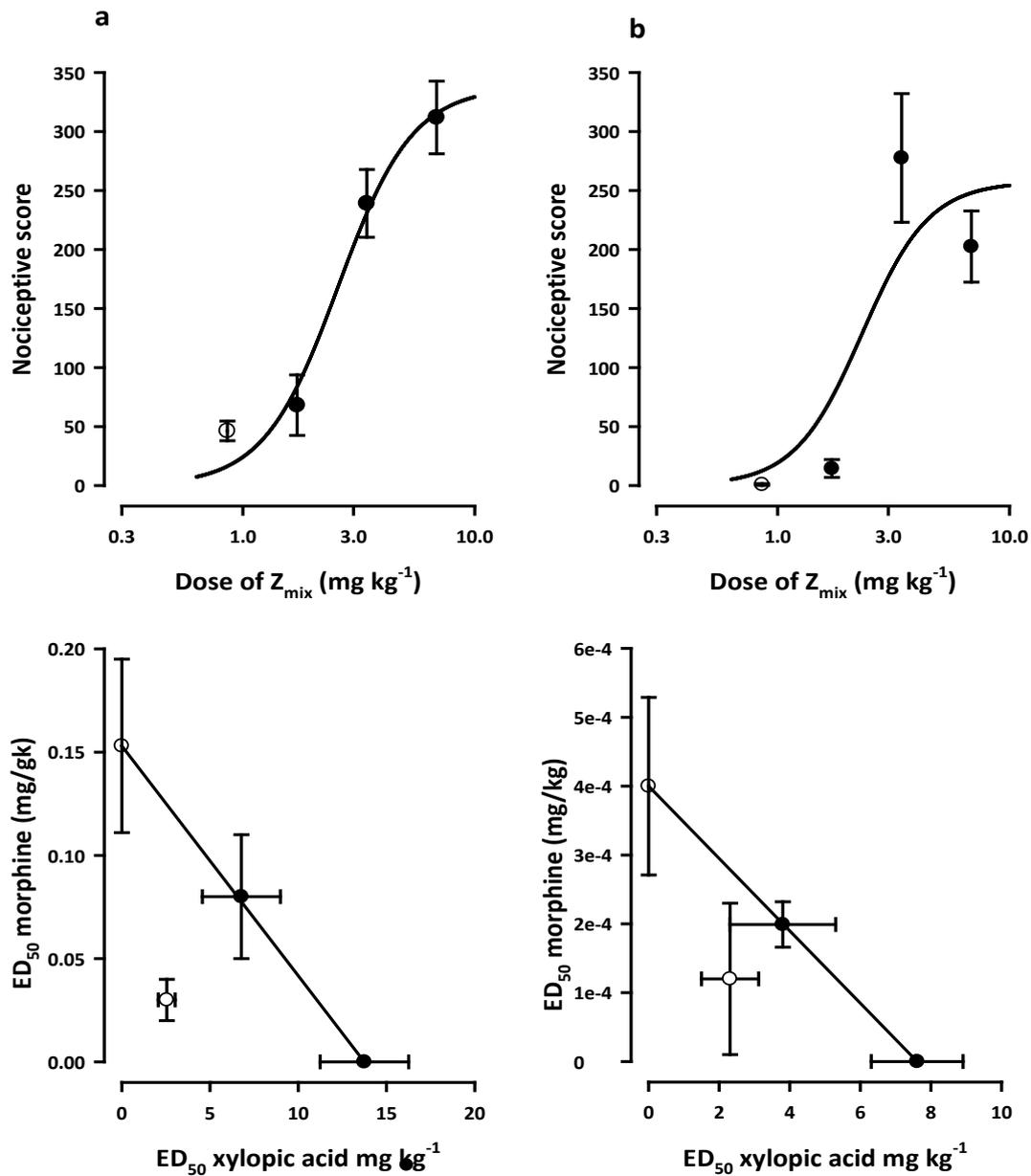
Figure 6.2 Dose response curves for xylopic acid and morphine and fractions of their combination for (a) phase 1 and (b) phase 2 of the formalin-induced licking test respectively. Isobologram for the combination of morphine and xylopic acid in (c) phase I and in (d) phase II of formalin test in mice. Filled circles (●) are the theoretical ED_{50}'s ± S.E.M. and open circles (o), the experimental ED_{50}'s ± S.E.M.

6.5.3   Isobolographic analysis of xylopic acid/diclofenac combination

6.5.3.1   Acetic acid-induced writhing assay

Xylopic acid and diclofenac inhibited visceral pain in the acetic acid-induced writhing test during the 30 min observational period. Xylopic acid (10 -100 mg kg^{-1}, *p.o.*, 60 min before) reduced the writhings induced by the acetic acid with the highest dose causing a reduction of 78.01 ± 0.26%. Diclofenac reduced the total number of writhes with the highest dose a percentage inhibition of 91.8 ± 0.27. The ED$_{50}$'s of xylopic acid and diclofenac were found to be 12.94 ± 1.24 mg kg^{-1} and 1.462 ± 0.27 mg kg^{-1} respectively (Table 6.3).

Table 6.3 ED$_{50}$ ± S.E.M. of diclofenac and xylopic acid in the acetic acid-induced writhing test.

Drugs	ED$_{50}$ (mg kg^{-1})
Xylopic acid	12.94 ±1.24
Diclofenac	1.462 ± 0.27

Values are expressed as mean ± S.E.M. (n=8).

ED$_{50}$s ± S.E.M. were obtained by least-square nonlinear regression as described in materials and methods.

6.5.4   Isobolographic analysis of xylopic acid/ diclofenac and their fractions

Xylopic acid, diclofenac and the fractions profoundly inhibited visceral pain in the acetic acid-induced writhing test during the 30 min observational period (Fig. 6.3a). Xylopic acid inhibited nociception in this test by 84.81±3.82% (Fig. 6.3b). Diclofenac also significantly inhibited visceral pain by 76.59±7.25% (Fig. 6.3b). The fractions also inhibited visceral pain dose dependently with $Z_{mix}/8$ inhibiting nociception by 50.94±12.54%, $Z_{mix}/4$ exhibiting antinociception of 69.43113±9.56, $Z_{mix}/2$ and Z_{mix} inhibiting nociception by 89.17±6.065% and 99.19±3.29% respectively Fig. 6.3b.
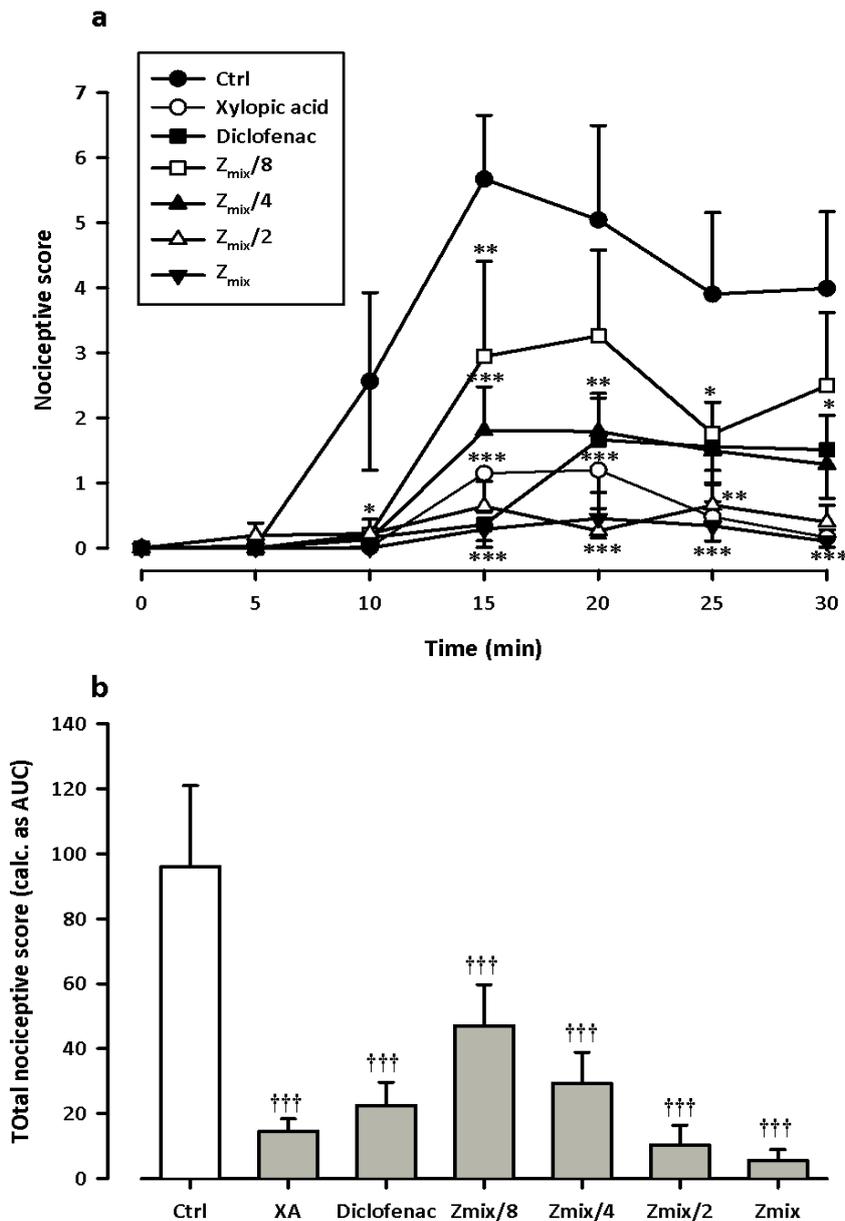
Figure 6.3 Effect of (a) xylopic acid, diclofenac and fractions on time course curve of acetic acid- induced writhing test in mice in 5 min blocks up to 30 min post acetic acid injection. Each point represents Mean ± S.E.M (n = 8). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared to respective controls (two-way repeated measures ANOVA followed by Bonferroni's post hoc) and (b) AUC (total response). Each column in b represents the mean ± S.E.M. $^{\dagger\dagger\dagger}P \leq 0.001$, (one-way ANOVA followed by Newman-Keuls *post hoc*).

6.5.5 *Isobologram of xylopic acid and diclofenac*

The experimental ED_{50} obtained after administration of xylopic acid and diclofenac and in fixed ratio of their combination in the acetic acid-induced writhing test was 1.944 ± 0.97 Fig. 6.4a.

The degree of potentiation calculated as the interaction index by fractional analysis indicated synergism for the combination and graphically displayed as Z_{mix}'s lying below the line of additivity (Fig. 6.4 c and d respectively) of the isobologram. The theoretical additive ED_{50} (Z_{add}) was computed as 6.86 ± 2.24 mg kg^{-1}for phase 1 (Fig. 6.4c) and 3.81 ± 2.5 mg kg^{-1} for phase 2 (Fig. 6.4d) and (Table 6.4).

Table 6.4 Theoretical and experimental $ED_{50} \pm$ S.E.M. of xylopic acid in the Acetic acid-induced writhing test and their computed interaction indices.

Z_{add} (mg kg^{-1})	Z_{mix} (mg kg^{-1})	Interaction index	Drug ratio
7.201 ± 0.403	1.944 ± 0.97	0.27	1 : 3.70

Values are expressed as mean $\pm$ S.E.M. (n=8). The values were obtained from experiments described earlier.

ED_{50}s $\pm$ S.E.M. were obtained by least-square nonlinear regression as described in materials and methods.
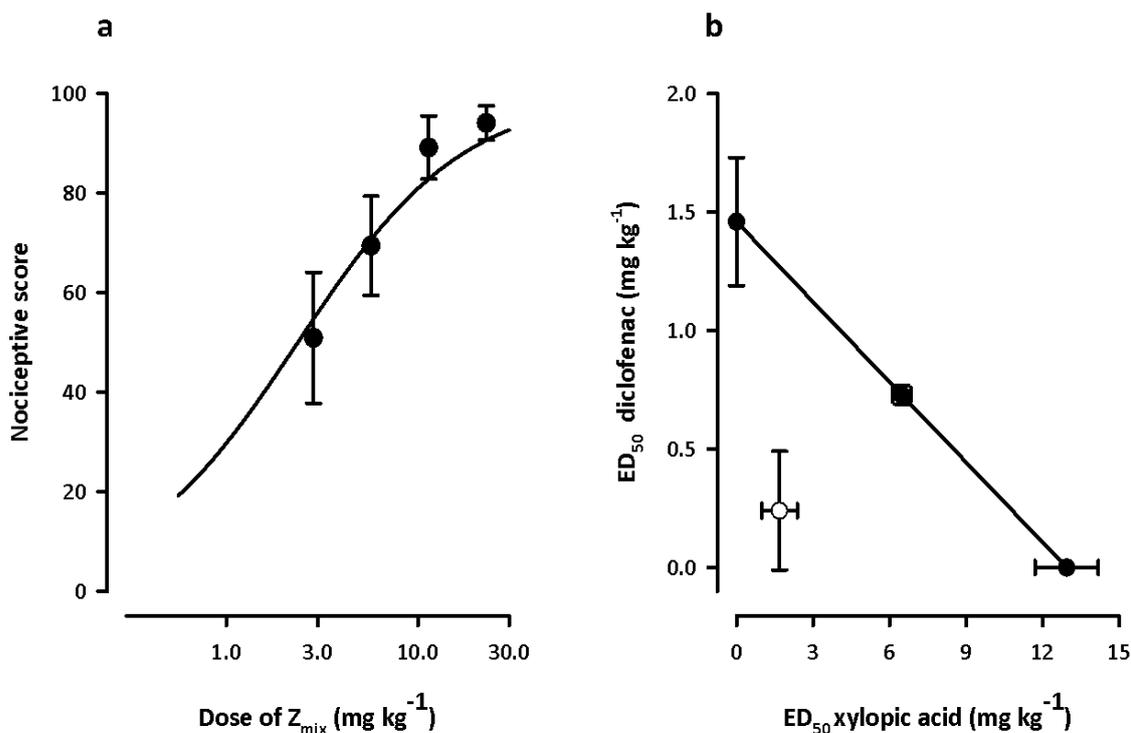
Figure 6.4 Dose response curve for xylopic acid, diclofenac and fractions of their combination in the acetic acid-induced writhing test and (b) Isobologram for the combination of diclofenac and xylopic acid. Filled circles (●) are the theoretical ED_{50}'s ± S.E.M. and open circles (o), the experimental ED_{50}'s ± S.E.M.

6.6   DISCUSSION

Co-administration of xylopic acid/morphine and xylopic acid/diclofenac produced antinociceptive effects in the formalin and acetic acid-induced writhing tests. The observed antinociception was greater than would be achieved for the administration of the individual drugs alone.

The formalin test is considered to be one of the standard animal models for screening analgesics and it is considered to provide more validity to animal model of nociception. The first phase (neurogenic phase) of paw licking/biting response is generated in the periphery, and it is considered to be a result of the direct stimulation of C-fibre nociceptors by the injected formalin (Dubuisson and Dennis, 1977). The second phase (inflammatory phase), which appears later, is considered to be due to the combination of an inflammatory reaction in the peripheral tissue and changes in central processing (Yun-Kyoung Yim, 1999). The first phase is sensitive to drugs that interact with the

opioid system as well as local anaesthetics such as lidocaine and the second phase is inhibited by non steroidal anti-inflammatory drugs and narcotics. Inhibition of the late phase is due to inhibition of inflammatory mediators such as serotonin, histamine, bradykinin and prostaglandins, which at least to some degree, can cause sensitization of the central nociceptive neurons (Santa-Cecilia *et al.*, 2011). Xylopic acid inhibited neurogenic pain in the formalin test by interacting with opioidergic- NO-cGMP, serotoninergic, adenosinergic and adrenergic system. Morphine inhibited neurogenic pain in this model by interacting with opioidergic-ATP-sensitive K^+ channels-NO-cGMP system. Xylopic acid and morphine co- administration produced synergistic antinociceptive effect in the first and second phase of formalin test. Based on the principle of independent joint action, co-administration of xylopic acid and morphine as a combination activated different pathways to produce synergistic effect in the first and second phase of the formalin test since additivity would at best be realized if only a single pathway was activated. Molecular interaction involving cAMP between xylopic acid and morphine though may have occurred considering the pathways xylopic acid and morphine activated to inhibit pain in both phases. Based on the elucidated mechanism of action of xylopic acid, it was possible that xylopic acid and morphine combination may activate G-protein coupled receptors and if they co-exist on neurons, they could share a common pool of G-protein and activation of one receptor may cause a redistribution of its G-protein, which will therefore lead to an increases in the sensitivity of the other receptor (Diaz-Reval *et al.*, 2004) for the compounds.

Xylopic acid /morphine combination also exhibited synergism in the second phase of the formalin test. The synergism of the combination in the inflammatory phase was however lower than the neurogenic phase. The common pathway shared by xylopic acid and morphine in inhibition of inflammatory pain was mediated through the opioidergic, adenosinergic and muscarinic pathways. Additionally, xylopic acid acted on adrenergic pathway while morphine acted on serotoninergic pathway. One of the reasons for the observed greater antinociception of xylopic acid/morphine combination in the neurogenic pain compared to the inflammatory pain may be that considering the individual antinociceptive mechanistic pathways of xylopic acid and morphine, more pathways were activated in the first phase than the second phase. The combination may therefore have activated more pathways in the first than the second phase. It is worth noting that the explicit mechanism(s) of the combination however need to be

elucidated. This study has made it obvious that lower doses of morphine and xylopic acid can be used to achieve greater analgesic effect at the same time minimizing their side effects.

Xylopic acid and diclofenac combination also produced synergism in the acetic acid-induced visceral pain and this is in agreement with the results obtained from the inflammatory phase of the formalin test. Diclofenac, an example of an NSAID is known to block pain through the inhibition of prostaglandin biosynthesis, modulations of endogenous opioids, serotoninergic and noradrenergic mechanisms, and the inhibition of NO/cGMP pathway (Fürst, 1999; Diaz-Reval *et al.*, 2004). Pain produced in this model was due to peripheral and central sensitization of nociceptors by inflammatory pain mediators.

The inhibition of pain in the acetic acid-induced writhing test was higher than that seen in the second phase of formalin test although both have similar pathophysiology. This may be due to the aptitude of xylopic acid/diclofenac combination to inhibit production of inflammatory pain mediators as well as inhibiting the neuronal sensitizing effect of prostaglandin if any is still produced providing an efficient and systematic pain inhibition compared to morphine/xylopic acid combination. xylopic acid has a low toxicity profile and further use of lower doses with morphine and diclofenac as seen from fractional analysis demonstrated that the extent of potentiation of xylopic acid/morphine and xylopic acid/diclofenac combinations were synergistic. This makes the combinations useful agents compared to the use of the drugs individually for the treatment of both neurogenic and inflammatory pain conditions.

6.7   CONCLUSION

Xylopic acid/morphine combination exhibited marked potentiation after isobolographic analysis of the combination in both phases of the formalin test. The degree of potentiation calculated as the interaction index revealed synergism in both phases of the formalin test. Similarly, xylopic acid/diclofenac combination exhibited synergism in the acetic acid-induced writhing test.

Chapter 7

GENERAL DISCUSSION AND CONCLUSION

The extract and xylopic acid exhibited both central and peripheral analgesic properties. Again this study has demonstrated that extract and XA are effective in acute and chronic pain conditions.

The fruit of *X. aethiopica* is used traditionally for the treatment of colic pain, rheumatism and neuralgia (Igwe *et al.*, 2003). The extract contains among other constituents kaurenoic and xylopic acid. Kaurenoic acid has been shown to possess anti-inflammatory and analgesic properties in animal models through the inhibition of NF-κB (Castrillo *et al.*, 2001). The extract and xylopic acid inhibited visceral pain in the acetic acid - induced writhing test. This provided scientific evidence to the traditional use of the fruit of *X. aethiopica* as an analgesic for the treatment of visceral pain such as colic pain. Similarly, the extract and xylopic acid blocked thermal pain in the Hargreaves hyperalgesia model. The results obtained from the Hargreaves tail-flick test indicated the involvement of central mechanisms in the anti-nociceptive effects of the extract and xylopic acid. The tail-flick response is believed to be a spinally mediated reflex (Chapman *et al.*, 1985) but the mechanism of response could also involve higher neural structures (Jensen and Yaksh, 1986). The activity of the extract and xylopic acid in this model shows they act, at least in part, by spinally mediated central mechanisms.

Treatment of pain of neurogenic and inflammatory origin has been practiced using the fruit of *X. aethiopica* (Igwe *et al.*, 2003). Results obtained from the formalin-induced acute persistent pain model showed that the extract and xylopic acid inhibited both neurogenic and inflammatory pain. The inflammatory phase of the formalin test, similar to the inflammation induced by carrageenan involves the release of proinflammatory pain mediators such as iNOS, COX-2 products, TNF- α, serotonin, histamine and prostaglandins. Proinflammatory pain mediators have also been implicated in development of musculoskeletal pain. It is therefore not surprising that the extract and xylopic acid were effective in musculoskeletal pain, Hargreaves paw withdrawal test and phase 2 of the formalin test. In fact, a significant number of kaurene diterpenes, including kaurenoic acid are known to inhibit iNOS, COX-2 and TNF- α (Castrillo *et*

al., 2001; Leung *et al.*, 2005; Park *et al.*, 2007; Sosa-Sequera *et al.*, 2010). Additionally, activation of the transcription factor NF-κB has been shown to be a key component in the expression of genes involved in the production of inflammatory mediators, which may exacerbate pain, hyperalgesia and nociception (Haddad; Baeuerle, 1998; Lee *et al.*, 2004). Therefore, the inhibition of activation or action of NF-κB can effectively control inflammation and associated inflammatory pain. Castrillo *et al* (2001) have shown that kaurene diterpenes inhibit NF-κB activation through a mechanism that involves an impairment of IKK activity as a result of the inhibition of NIK and the lack of a coordinate activation of p38 and/or ERK1 and ERK2. This mechanisms possibly explains, at least in part, the observed analgesic actions shown by xylopic acid, a kaurene diterpene, and the extract (which contains at least two kaurenes: kaurenoic acid and xylopic acid) against inflammatory pain. The extract and xylopic acid were effective in acute and chronic musculoskeletal pain models. Kaolin/carrageenan injected into the joint of rats leads to biochemical and histopathological changes in the knee joint. The acute phase lasts for 24 hours. The first 3 hours of the acute phase of knee joint inflammation is characterised by increased levels of cyclooxygenase enzyme-2 (COX-2 produces prostaglandin E_2) mRNA and inducible nitric oxide synthase (iNOS produces proinflammatory chemical NO) mRNA in the synovial fluid. This result in the synthesis and release of inflammatory mediators such as histamine, 5-HT, bradykinin and prostaglandins after 4 hours leading to mild haemorrhage, oedema and minimal cell infiltrates of polymorphonuclear granulocytes (Di Rosa and Willoughby, 1971). More neutrophils are seen in the ipsilateral knee at 8 hours and at 24 hours, the acute inflammation is severe, accompanied by fibrinous exudate in the knee joint. Increased levels of excitatory amino acids (glutamate and aspartate) and nitric oxide metabolites as well as other cytokines such as nerve growth factor (NGF) and tumor necrosis factor-α (TNF- α) have been observed in the in the ipsilateral synovium of the acute phase (Manni and Aloe, 1998; Bement *et al.*, 2003). The inflammation converts to a chronic state characterised by macrophage infiltration with a few scattered mast cells. The pain associated with the chronic inflammation involves central sensitization with spinal and supraspinal involvement associated with sensitisation of dorsal horn neurons by glutamate, substance P, calcitonin gene-related peptide and prostanoids. Impairment in ionic conductance of the neurons due to calcium and potassium channels disturbance have been observed. Supraspinal involvement was due to descending facilitation of pain control system as a result of impaired descending inhibitory pain control system. Acute

musculoskeletal pain is a major symptom of some disease states such as malaria and this will make the extract and xylopic acid good candidates for the treatment of malaria and its associated musculoskeletal pain due to reports of *X. aethiopica* being used to treat malaria traditionally (Tatsadjieu *et al.*, 2003). This in effect will reduce the cost and number of drugs used to treat malaria (Iwu *et al.*, 1992). The formalin test is a very useful test because not only can it detect drugs that inhibit neurogenic and/or inflammatory pain but can also predict drugs that may be effective in neuropathic and neurogenic painful conditions (Ellis *et al.*, 1998; Vissers *et al.*, 2006).

 The extract and xylopic acid exerted potent neurogenic pain inhibition in the vincristine-induced neuropathic, formalin, glutamate and capsaicin models of pain. The pain induced by vincristine has been associated with damages inflicted on schwann cells and dorsal root ganglion (DRG) neurons of the peripheral nervous system resulting in the release of inflammatory mediators and further recruitment of macrophages in the peripheral nervous system (PNS). The recruited macrophages may also release the inflammatory cytokine IL-6, which elicits neuro-inflammation and activates janus kinase (Jak) - transcription-3 (STAT3) pathway (Jak-STAT3 pathway), leading to neuropathic pain. CNS sensitization due to secondary signal transduction of pain from the PNS to the CNS may activate microglia and astrocytes to release proinflammatory cytokines such as TNF-α which in turn activates the p38 MAP kinase pathway to contribute to the development and maintenance of neuropathic pain in the CNS (Siau *et al.*, 2006; Kiguchi *et al.*, 2009; Authier *et al.*, 2003). Neuropathic pain, especially the cancer pain type is opioid resistant due to down regulation of mu-opioid receptors in dorsal spinal cord, mediated through the activation of NMDA receptors and protein kinase A (Mizoguchi *et al.*, 2009). It was therefore unlikely that the extract and xylopic acid acted on the opioidergic receptors/pathways to block pain in this model. Again, at neuropathic pain states, there is an increase expression and activity of voltage-dependent Na^+ channels and α_2-δ_1 Ca^{2+} channels (a subunit construct of N-type voltage-dependent Ca^{2+} channel) as well as increased expression of α_2 – adrenergic receptors (indicating the maintenance of neuropathic pain by the sympathetic system) on neuronal terminals.   This is supported by the fact that administration of NMDA receptor antagonists or protein kinase C inhibitors (Mao *et al.*, 1995; Yajima *et al.*, 2005) suppress the development of the hyperalgesia and allodynia in neuropathic pain states.

The fruit and seeds of *X. aethiopica* have been used in the management of cardiac palpitation in Cameroon (Sandberga *et al.*, 2005) as well as oedema (Somova *et al.*,

2001). Further research on the fruits and seeds revealed that extracts of fruits of *X. aethiopica* posses hypotensive, diuretic and naturetic properties (Somova *et al.*, 2001) and the hypotensive property of the fruits and extract of *X. aethiopica* was due to the calcium blocking effects of xylopic acid and kaurenoic acid. The diuretic and naturetic effects may account for the use of the fruit for managing oedema. Calcium channel antagonists such as pregabalin and gabapentin have proven beneficial in the management of neuropathic pain. Effectiveness of the extract and xylopic acid in the neuropathic pain model can be attributed to their inhibition of Ca^{2+} influx via calcium channel blockade and subsequently, inhibiting the release of excitatory neurotransmitters (e.g., glutamate, substance P) from the primary afferent nerve fibres which are sensitized during neuropathic pain states similar to pregabalin. Again, the extract and xylopic acid directly blocking the α_2 – adrenergic- protein kinase C and capsaicin –sensitive C-fibres- NMDA system further might have contributed to the resulted attenuation of the neuropathic pain.

Furthermore xylopic acid and extract may have inhibited pain in this model by possibly inhibiting COX-2 and TNF- α. Kaurene diterpenes, including kaurenoic acid are known to inhibit iNOS, COX-2 and TNF- α (Castrillo *et al.*, 2001; Leung *et al.*, 2005; Park *et al.*, 2007; Sosa-Sequera *et al.*, 2010) and this may have resulted in the inhibition of one of the essential pathways, p38 MAP kinase pathway in neuropathic pain generation.

Extract of *X. aethiopica* is reported to possess anticancer properties in rodents although the active constituent responsible for this effect has not been yet elucidated (Adaramoye *et al.*, 2010; Adaramoye *et al.*, 2011). This places the extract and xylopic acid in high priority of drug development consideration as it may not only be another source of anticancer agent but play the dual role of preventing or ameliorating neuropathic pain associated with anticancer therapy.

The antinociception of the extract and xylopic acid were mediated through the adrenergic, muscarinic, opioidergic-NO-cGMP, adenosinergic and serotoninergic pathways. The protein kinase A and Cε pathways may also be involved in the antinociception of the extract and xylopic acid. This finding was not surprising because the opioidergic, muscarinic, adenosinergic and adrenergic receptors are coupled to the PKA and PKC pathways. It is possible that aside the effect of the extract and xylopic

acid on these enzyme pathways, they may have exerted some effect on the receptors as well as demonstrated by the binding of xylopic acid on MOR receptors.

In spite of the observation that the extract and xylopic acid acts on the opioidergic as well as adenosinergic pathways to produce antinociception both peripherally and centrally, they do not induce tolerance to their antinociception. Tolerance has been a worrying side effect of opioids that has limited the use of opioids and the search for opioid agonists devoid of causing tolerance has been ongoing. The extract and xylopic acid can therefore be used in patients for the management of chronic pain without tolerance being produced to their antinociceptive properties. Again tolerance developed to the antinociceptive properties of opioids does not cross-generalize to the antinociception of the extract and xylopic acid. This implies that morphine can be substituted for the extract and xylopic acid in patients that have developed tolerance to the antinociception of morphine.

To further minimize the side effects of xylopic acid that may come with its use, minimize poly pharmacy and enhance the efficacy of xylopic acid, morphine and diclofenac, isobolographic analysis of xylopic acid/morphine and xylopic acid/diclofenac were performed. Using relatively very low doses of xylopic acid/morphine and xylopic acid/diclofenac combinations resulted in profound antinoception compared to the use of the individual drugs alone. The side effects of these drugs may have drastically been reduced due to smaller doses used.

CONCLUSION

This study has provided pharmacological evidence to support the use of the fruit of *X. aethoipica* as an analgesic. Furthermore it illustrated that the analgesic effect of the fruit was partly due to the presence of xylopic acid. The analgesic effect of the ethanolic extract of *X. aethoipica* was mediated by:

- Stimulation of opioid receptors-nitric oxide-cyclic GMP (NO/cGMP) pathway devoid of tolerance development to the analgesic effect of the extract.

- Stimulation of adenosinergic, muscarinic and $5HT_3$ pathways.

- Inhibition of EP, B1 and B2, TRPV1 (vanilloid), glutamate, adrenergic receptors- cAMP-protein kinase A and protein kinase Cε pathways.

The analgesic effect of xylopic acid was partially or wholly due to:

- Stimulation of opioid receptors-nitric oxide-cyclic GMP-pathway as well as causing an increment in the binding of endogenous opioids devoid of tolerance development to the analgesic effect of xylopic acid.

- Stimulation of adenosinergic, muscarinic, adrenergic and 5HT$_3$ pathways.

- Inhibition of EP, B1 and B2, TRPV1 (vanilloid), glutamate, adrenergic receptors- cAMP-protein kinase A and/or protein kinase Cε pathways.

Isobolographic analysis of xylopic acid/morphine and xylopic acid/diclofenac combination indicated synergism for both combinations.

7.1   RECOMMENDATIONS

This study has provided scientific evidence to the use of the fruits of *Xylopia aethiopica* and xylopic acid as analgesic in rodents. This cannot directly be extrapolated to human beings and therefore care should be taken when being used in human subjects. The following future works can be performed to improve upon the knowledge on the fruits and xylopic acid.

➢ Acute, sub acute and chronic toxicity profile of the extract and xylopic acid should be conducted to assess the safety of the extract and xylopic acid since - the fruits of *X. aethiopica* is used as a delicacy.

➢ In order to elucidate the direct inhibition of PKA and PKCε as well as possible inhibition of COX-1 and COX-2, western blotting or ELISA should be employed.

➢ Bradykinin 1 (B1) and B2 specific agonists induced nociception should be employed to explicitly determine the bradykinin receptor(s) the extract and xylopic acid acted on.

➢ The nociceptive pathways the extract and xylopic acid attenuated were associated with specific receptors. Binding studies should therefore be carried

out in order to elucidate the receptors and receptor subtypes the extract and xylopic acid acted upon.

➤ The extract should further be fractionated to isolate other compounds and kaurenoic acid derivatives and their antinociception determined. This will help fully characterize the extract both pharmacologically and phytochemically as any additional pure compounds would serve as marker compounds for the extract and this would enhance the finger print database on the extract.

➤ Isobolographic analysis of xylopic acid and other clinically used drugs for neuropathy should be performed so as to increase the efficacy of these drugs.

➤ This work should further be conducted in primates in order to extract relevant scientific data before use in man.

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APPENDIX

PREPARATION OF CARRAGEENAN SUSPENSION

A 2% carrageenan suspension was prepared by sprinkling small amounts of the powder (200 mg) evenly over the surface of 10 ml of 0.9% NaCl solution and left to soak between additions. It was then left for 2-3 hours before use.

PREPARATION OF PHOSPHATE BUFFER SALINE

One tablet of phosphate buffer saline was dissolved in 400 ml of distilled water to obtain   1 M sodium phosphate buffer, pH 7.4.

PREPARATION OF STOCK SOLUTION OF PROSTAGLANDIN E$_2$

A stock solution of 1 M prostaglandin-E$_2$ was prepared by disolving 1 mg of prostaglandin -E$_2$ in 1 ml of PBS and stored at -20 ° C. Aliqoute of 0.5 ml of this solution was taken and diluted to 23 ml to obtain a 1 nM/ 0.02 ml solution.

PREPARATION OF STOCK SOLUTION OF BRADYKININ

A stock solution of 1 M bradykinin was prepared by dissolving 0.5 mg of bradykinin in 5 ml of PBS and stored at -20 ° C. Aliqoute of 0.5 ml of this solution was taken and diluted to 23 ml to obtain a 1 nM/ 0.02 ml solution.

PREPARATION OF EMULSION OF EXTRACT AND XYLOPIC ACID.

Emulsion of the extract and xylopic acid were prepared by tritrating the acid or extract into fine powder. This was followed by the addition of 0.2 ml tween 80 and tritrated further. Distilled water was then added while tritrating till an emulsion was formed.