ASSESSMENT OF POST- CASTRATION PAIN IN RABBITS TREATED WITH THE ETHANOL EXTRACT OF *CHENOPODIUM AMBROSIOIDES* LINN

BY

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DEPARTMENT OF VETERINARY SURGERY AND RADIOLOGY,
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OCTOBER, 2018

Declaration

I declare that the work in this dissertation titled "Assessment of Post-Castration Pain in Rabbits Treated with the Ethanol Extract of *Chenopodium ambrosioides* Linn" has been performed by me in the Department of Veterinary Surgery and Radiology, Ahmadu Bello University, Zaria, under the supervision of Dr E. O. Abidoye and Dr S. T. Fadason. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

LATEEFAT OPEYEMI OLADIMEJI _____ _____

Signature Date

Certification

This dissertation titled "ASSESSMENT OF POST-CASTRATION PAIN IN RABBITS TREATED WITH THE ETHANOL EXTRACT OF *CHENOPODIUM AMBROSIOIDES* LINN" by Lateefat Opeyemi OLADIMEJI, meets the regulations governing the award of the degree of Masters of Science of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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Dedication

This dissertation is dedicated to Almighty Allah, my mother (Hajia Khadijat Oladimeji), my husband (Dr Abdulrazaq Omowumi) and my beautiful daughters (Haleemat, Maryam, Khashiat) and son (Abdul Rahmaan).

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Abstract

This study was carried out to assess post-castration pain and distress using blood glucose concentration; oxidative stress parameters, algometer, and rabbit grimace scale in rabbits treated with the ethanol extract of *Chenopodium ambrosioides* (*C. ambrosioides*). Fifteen (15) sexually matured and clinically healthy bucks with the mean weight of about 2.5 ± 0.7 kg were used for the study. The animals were allowed to acclimatize to the management conditions for two weeks. The research was carried out in two phases: Phase 1 comprised three groups, A, B and C that contained 3 rabbits each, and were treated orally with normal saline, meloxicam tablet (0.1 mg/kg), and *C. ambrosioides* extract (500 mg/kg), respectively after castration. The phase 2 contained groups D and E with 3 animals each, and were treated topically with Fastum gel® (Ketoprofen 2.5 %) and *C. ambrosioides* (applied liberally) extract after castration. Two milliliter (2 ml) of blood sample was collected from each buck to assay for Malondialdehyde (MDA) concentration, Superoxide Dismutase (SOD), and Glutathione peroxidase (GPx) activities, 1 hr, 2 hr, and 3 hr post treatment, following the evaluation of blood glucose concentration. Post-castration pain assessment was done using the Rabbit Grimace scale at 1 hr post treatment for twenty minutes followed by post-castration pain evaluation using algometer around the surgical wound. The pain evaluation using algometer was also repeated on the day one of surgery at 1hr post treatment. Fifteen minutes after the treatments on day zero of surgery, the blood glucose concentration (mg/dL) of the normal saline treatment group (267.67 ± 18.77 mg/dL) was significantly higher ($p \leq 0.05$) than all other treatment groups. At two hours after treatment, the plasma MDA concentration (µmol/L) of the normal saline treatment group (44696 ± 19071 µmol/L) was significantly higher ($p \leq 0.05$) than all other treatment groups. The plasma SOD activity of oral *C. ambrosioides* (22 ± 5.7 mg/mL) treatment was higher than the oral meloxicam (14 ± 6 mg/mL) and normal saline (18 ± 8.4 mg/mL) treatments, while that of topical *C. ambrosioides* (4.8 ± 1.1 mg/mL) treatment group was higher than that of topical Ketoprofen (2.2 ± 1 mg/mL) treatment, though the difference was not significant. The force required in eliciting pain after 1 hour in topical *C. ambrosioides* (16.03 ± 2.52N) treatment group was significantly higher than the topical Ketoprofen (10.27 ± 0.17 N) treatment group. While the oral *C. ambrosioides* (13.47 ± 1.32

N) treatment was higher than the oral meloxicam (12.9 ± 1.42 N) and normal saline (9.87 ± 0.35 N) treatment groups though, the differences were not significant. The overall grimace pain score for the oral meloxicam (0.51 ± 0-0.9), oral *C. ambrosioides* (0.71 ± 0.1-1.4), topical *C. ambrosioides* (0.55 ± 0.3-1.2) extracts treatment groups was not different from that of the pre-surgical grimace pain score (0.36 ± 0-1), while that of the topical Ketoprofen score (0.8 ± 0.4-1.7) and oral *C. ambrosioides* (0.71 ± 0.1-1.4) extracts were not different from the normal saline (0.92 ± 0.5-1.6) treatment group. *Chenopodium ambrosioides* had better antioxidative effects when administered orally, while the topical application of *C. ambrosioides* extracts produced faster analgesic effects compared to the oral administration at the dosage used. This study has demonstrated that *C. ambrosioides* could be used to reduce post-castration pain and distress in rabbits as evaluated using oxidative stress parameters, algometer and rabbit grimace scale.

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Lists of Abbreviation

Ketoprofen (KP)

Non steroidal- anti inflammatory drugs (NSAIDs)

Chenopodium ambrosioides (C. ambrosioides)

Superoxide Dismutase (SOD)

Malondialdehyde (MDA)

Glutathione peroxidase (GPx)

Facial action units (FAUs)

Nicotinamide adenine dinucleotide phosphate (NADPH)

Very low density lipoprotein (VLDL cholesterol)

Day 1: day of Surgery

Day 2: second day of surgery

BK: Bradykinin

NO: Nitric oxide

SP: Substance P

MPO: Myeloperoxidase

ADA: Adenosine-deaminase

PGE2: Prostaglandin E2

TNF-α: Tumour necrotic factor alpha

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Orchidectomy or castration as is commonly called is the surgical removal of the testicles, epididymis and a portion of the *Vas deferens* (Murray, 2006). Indications for castration in food animals are different from that of small animals and they can be either elective or therapeutic. The indications for elective orchidectomy in food animals include reduction of inappropriate behaviour such as, aggression and unwanted sexual behaviour, prevention of some genetic or hereditary diseases and prevention of reproductive tract diseases (Murray, 2006; Lennox, 2008). The therapeutic indications for castration are testicular diseases such as infections and neoplasia, correction of an inguinal hernia, true cryptorchidism and trauma (Lennox, 2008). Castration has been known to have several advantages including reduction of aggression (towards other animals), improvement of handler safety, reduction of sexual behaviour and meat quality improvement (Okafor *et al.*, 2015). The three different techniques described for orchidectomy in rabbits are the traditional scrotal approach using a closed or open technique, the pre-scrotal approach and the abdominal approach which is possible because rabbits are functional cryptorchids (Capello and Lennox, 2006; Murray, 2006; Lennox, 2008). One major advantage of castration in rabbit as food animal is that it results in improved quality of rabbit meat in terms of its juiciness, tenderness and the flavour (Lebas *et al.*, 2000).

The pre-scrotal approach is the best technique for proper anatomical closure of the inguinal canals following orchidectomy in rabbits. Due to the position of the penis, manipulation of the delicate skin of the hemiscrotal sacs, including shaving and scrubbing, is completely avoided. In this technique as described by Capello and Lenox (2006), a 1.5–2 cm incision is made on the midline just cranial to the base of the hemiscrotal sacs. Blunt dissection of the subcutaneous tissue and inguinal fascia reveal the vaginal processes, where they lie just before entering the abdomen through the inguinal canal. The vaginal process is bluntly dissected from the surrounding soft tissues and isolated, and 3-0 or 4-0 suture material passed around it and tied loosely to act as a stay suture. The vaginal process is incised to access the testicle and spermatic cord, which are gently grasped and exteriorized through the incision. The ligament between the hemiscrotal sac and the tail of the epididymis is gently dissected, and the spermatic cord containing blood vessels and nerves is clamped, ligated and removed. The preplaced stay suture is tied, which will close the vaginal process. Inguinal fascia and subcutaneous tissue are sutured (Capello and Lennox, 2006).

Different types of castration are usually performed without anaesthesia or post-operative analgesia (Hewson *et al.*, 2007; Lumb, 2007), but their use could reduce the degree of pain experienced following the procedure (Rault *et al*, 2011). To minimize the pain experienced by castrated animals, different anesthetics and analgesics may be administered. Lidocaine can be administered either locally into the neck of the scrotum, thereby affecting the cord or testicles (Thüer *et al.*, 2007), or epidurally (Meyer *et al.*, 2007). Lidocaine is the most common local anaesthetic tested and has been found to reduce pain-related behaviours during surgery in both

swine and cattle. However, it seems to have no pain-relieving effects post-surgery (Rault *et al*, 2011).

Chenopodium ambrosioides L. is a medicinal plant belonging to the Chenopodiaceae family that is native to Central and South America which has been used as a traditional herbal remedy in tropical and subtropical regions (da Silva *et al.,* 2014). The common names are Mexican tea, Worm seed, Mexican gooseamerican, Gooxfoot (African Pharmacopoeia, 1985). It is popularly known as *Epazote* (meanning strong odour), and in Brazil it is called "*mastruz*" (Sousa *et al.*, 2012). In Nigeria, the Yoruba people call it '*ewe imi*' (meaning: leaf of excreta) or "*Arunpale*", the Ikulus in Kaduna State call it '*anyinyung'* (meaning smelly), while Hausas call it *kafi kashi wari* (meaning: your ordour is worse than excreta) (Amole and Izegbu, 2005; Lohdip and Aguiyi, 2013).

This plant is traditionally and widely used for its anti-parasitic, anti-inflammatory and antibiotic properties, the efficacy of which has been scientifically proven (Sousa *et al.,* 2012). It has antidiabetic properties and has been traditionally used in the treatment of dysmenorrhoea, uterine haemorrhage, and fibroids (Sundaram *et al.,* 2010). Alitonou *et al* (2012) reported that the leaves are used for the treatment of abscesses, epilepsy, vomit, cutaneous dermatoses, rheumatisms, articulars and neuralgias intercostales.

In West Africa, it is used traditionally for the treatment of sores, external swellings, pains, headache, coughs, tuberculosis, fever, and haemorrhoids. The plant was also reported to possess insecticidal, digestive, antimalarial, antihelminthic, sedative properties and it has been used for the treatment of snakebite (Lohdip *et al.*, 2013). In Brazil the leaves of *C. ambrosioides* has been blended with milk for traditional use (Matos *et al.,* 2004). It is useful as an analgesic

(Amole and Yusuf, 2002) and as a vermifuge to expel round-worms and hookworms (African Pharmacopoeia, 1985).

Domestication of rabbits started in 116 to 27 BC where they were kept in the leporaria (Lebas *et al.,* 1997). They were hunted as game animals for laurices (foetuses or newborn rabbits) (Lebas *et al.,* 1997). Importance of rabbits as food animal was first witnessed during World War II when serious efforts were made to meet the shortage of animal protein. They are especially useful for this purpose due to their advantageous features which include lack of direct competition with humans for food, most importantly for countries with no cereal surpluses contrary to what is seen in poultry production. Rabbits have short generation interval, high feed conversion efficiency, ability to rebreed shortly after parturition; each doe is capable of reproducing 4 times a year, an average of 8 kits per kindling, rapid growth rate and genetic diversity (Lebas *et al.,* 1997; Addass *et al.,* 2010; Khan *et al.,* 2014; Ugosor *et al.,* 2016).

In Nigeria, various breeds of rabbits exist but the most popular breeds are New Zealand White and California White which is due to the white fur and their good growth characteristics (Baruwa, 2014). Other breeds include Nigeria Idigenous breeds, Flemish Giant, California Red, Chinchilla, Champagne d' Argent, Crosses, Angora, Loap, Blue Beveren, Simonire, Himalayan, New Zealand, Polish Rex, Silver Martens and Ducth (Ugosor *et al.,* 2016).

Sexual maturity in rabbits is defined as the moment at which the daily spermatozoa production ceases to increase; this is observed at 32 weeks in New Zealand White rabbits (Campos *et al.,* 2014). Puberty occurs earlier in smaller breeds than in larger breeds of rabbits between 4-6 months. Puberty and sexual maturity in rabbits are of different phase as the former precedes the appearance of sperm in the ejaculate while the latter not only depends on age of the rabbits (125-

150 days) but on other factors such as breed, lineage, food and environmental factors such as photoperiod, temperature and season of the year (Campos *et al.,* 2014). The growth of rabbit's testes follows a sigmoid curve, increasing rapidly during puberty. Although the rabbit is pubertal in 4 months, the testes are not in the scrotum yet, the descent is observed only at six months of age (Fraser, 1988).

1.2 Statement of Research Problem

Post-castration pain in farm animals has often been neglected or at best undertreated in the past (Hewson *et al.,* 2007; Lumb, 2007). The reasons reported include lack of awareness that the procedure is painful, recognition of pain in farm animals (due to their natural instinct and ability to mask pain), inappropriate drug types (dearth of analgesics formulated solely for food animals), fear of drug residues in meat and meat products, cost of treatments, inappropriate route of administration, and sometimes unavailability of these drugs.

1.3 Justification of the Study

Pain and suffering in animals used in agriculture is of increasing concern to consumers of livestock products (Earley and Crowe, 2002). Although the pain of castration in cattle has been well documented (Fisher *et al.,* 1996, Coetzee, 2011), the procedure is commonly performed without analgesic or anaesthetic intervention. Considerable research on pain alleviation in castrated calves has been published (Fisher *et al.*, 1996, Earley and Crowe, 2002). However, the practicality and cost-effectiveness of these pain management strategies are a major limitation to their implementation (Petherick, 2005).

Castration has been shown to elicit physiological stress, inflammatory reactions (indicated by acute phase proteins), pain-associated behaviour, suppression of immune function, and a reduction in performance to varying degrees (Molony *et al.,* 1995; Fisher *et al.,* 1996 and 1997; Ahmed and Ahmed, 2011). Post-castration pain is one of the most important welfare concerns in pain control for routine management procedures in farm animals (Bayvel, 2004). There are different pharmacological agents used in post castration pain management in cattle and other ruminant including NSAIDs like meloxicam, ketoprofen, carprofen, flunixin meglumine and tolfenamic acid (Hudson *et al.,* 2008). Okafor *et al* (2015) reported the multimodal approach to surgical pain management to be the best option. Piroxicam and ascorbic acid combination is very effective in the management of post-surgical pain and control of behavioural signs of pain (Okafor *et al.,* 2015).

However, the uptake and use of analgesic therapies for husbandry procedures in commercially produced livestock largely depends on their cost, ease of administration and time to effectiveness (Mellor and Stafford, 1999). In addition, the requirement for veterinary or expert supervision and registration of pharmaceuticals affect the uptake of analgesic treatments on-farm (Mellor *et al.,* 2008). Other reasons for their limited uses for pain relief include delivery of anaesthesia and analgesia being too time consuming, willingness of the general public to pay for the extra costs, challenges in assessing pain in livestocks, considerations for residues in food producing animals, lack of long acting, cost-effective analgesic treatments for farm animals, and uncertainty on withdrawal periods for opioids and dissociative anaesthetics in farm animals (Cornish *et al.,* 2016). This also included the side effects of some of the analgesics used like NSAIDs causing gastric ulceration (Stanton and Bright, 1989; Neiger, 2003; Lascelles, *et al.,* 2005), nephrotoxicity (Bostrom *et al.,* 2002; Crandell *et al.,* 2004) and delayed bone healing (Ochi *et*

al., 2011), hence there is continual search for alternative agents that are more potent with little or no side effects. Phytotherapeautical agents are relatively cheaper and without fear of drug residue in the meat product. There is paucity of information on the therapeutic use of *Chenopodium ambrosioides* in animal subject, hence this research becomes important.

1.4 Aim

To evaluate the potentials of using *C. ambrosioides* Linn as an analgesic post orchidectomy in rabbits.

1.5 Objectives

The specific objectives are:

1. To evaluate the ameliorative effect of *C. ambrosioides* on blood glucose concentration due to the castration-induced stress and pain in rabbits
2. To evaluate the ameliorative effect of *C. ambrosioides* on castration-induced oxidative stress in rabbits
3. To evaluate anti-nociceptive property of oral and topical *C. ambrosioides* ethanol extract and Ketoprofen using algometer on castrated rabbits
4. To evaluate anti-nociceptive property of oral and topical *C. ambrosioides* ethanol extract and Ketoprofen using Rabbit Grimace Scale on castrated rabbits

1.6 Research Questions

1. Does ethanol extract of *C. ambrosioides* have ameliorative effect on blood glucose concentration changes due to the castration-induced stress and pain in rabbits?
2. Does ethanol extract of *C. ambrosioides* have ameliorative effect on castration-induced oxidative stress in rabbits?
3. Does oral and topical ethanol extract of *C. ambrosioides* and Ketoprofen gel possess anti-nociceptive property using algometer on castrated rabbits?
4. Does oral and topical ethanol extract of *C. ambrosioides*; and Ketoprofen gel possess anti-nociceptive property using rabbit grimace scale on castrated rabbits?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Castration

Castration is any procedure that removes the testicles surgically, damaging them irreparably or causing them to atrophy by stricture of the blood supply (Mohammed and James, 2013). It has been described as the commonest surgical procedure in both large and small animals (Fadason *et al.,* 2007; Remi-Adewunmi and Gyang, 2010). The three major methods used in castrating animals are physical, chemical and hormonal methods (Rajkumar, 2013).

The physical methods involve surgical removal of testicles, application of rubber ring at the base of the scrotum, and bloodless castration by using burdizzo clamps (Stilwell *et al.,* 2008).

Chemical castration involves injecting a sclerosing or toxic agents into testicular parenchyma to cause irreversible damage and functional loss (Fordyce *et al.,* 1989, Fadason, 2001). Various agents, all of which have one complication or the other, have been used for chemical castration in animals through intra-testicular injection. They include ferric chloride, BCG (Das *et al.,* 1982), glycerol (Immegart and Threlfall, 2000), Chlorhexidine and cotton seed oil (Fadason, 2001), formalin (Ijaz *et al.,* 2000; Bakir *et al.,* 2002), Purit® and cetrimide (Mohammed and James, 2013).

In hormonal techniques an injection of immuno-contraceptive is given to induce antibody production against gonadotropin releasing factor leading to decreased production of endogenous hormone (Fisher *et al.*, 1996).

Castration of male livestock is a routine husbandry practice performed by farmers of many countries, but this practice can be perceived as objectionable by the general public (Carter *et al.*, 2011). Data on the exact prevalence of castration is lacking, but in swine, it was reported that about 100% and 80% of male piglets are castrated in the United States and European Union, respectively, while in cattle, about 88% of beef cattle in the U.S. are castrated (Rault *et al.*, 2011). However, castration is a painful procedure that is a concern for both producers and animal welfare groups (Okafor *et al.*, 2015). In 2006, the American Veterinary Medical Associations (AVMA) listed castration among the procedure that elicit both acute and chronic pain (AVMA, 2006).

2.1.1 Methods of castration

There are several different methods of castration. Stafford and Mellor, (2005) classify the methods of castration as physical, chemical, or hormonal while Carter *et al.* (2011) classified castration methods into two categories: surgical or bloodless. Further subdivisions can be made, but castration is primarily achieved by removing the testicles surgically, damaging them irreparably, or causing them to atrophy by stricture of the blood supply (Currah *et al.*, 2009). The common methods of castration in food animals include: (1) orchidectomy, which is the removal of the testicles after making an incision into the scrotum; (2) band or ring castration, which is constricting the blood supply to the testicles and scrotum; and (3) Burdizzo castration, which

involves crushing of the spermatic cord (Okafor *et al.*, 2015). Depending on the species; a number of different castration methods are available. Swine, because of their anatomy, are castrated using the surgical castration technique, characterized by removal of the testes following scrotal incision. In sheep and cattle, other methods are also commonly used such as crushing the blood and nerve supply using clamps, rings or latex bands (Rault *et al*, 2011).

2.1.1.1 Surgical castration: This involves excision of the testes by splitting or removing the lower third of the scrotum and removing the testes by severing the spermatic cord in a manner that minimizes bleeding, usually with an emasculator, Henderson castrating tool, or knife (Carter *et al.*, 2011). According to Coetzee *et al.* (2010), surgical castration using scalpel is the most frequently used method of castration in farm animals, while the other methods of surgical castration that are less often used include; Newberry knife surgical castration, use of conventional knife to incise the scrotum, use of Henderson castration tool or surgical ligation to remove the testicles. Scalpel or other sharp knife either emasculators, surgical clamps or a dull knife were also reportedly used (Coetzee *et al.*, 2010).

Newberry knife surgical castration: This instrument is majorly used for scrotal incision. Newberry knife is the safest method of making two vertical incisions, one on each side of the scrotum, for testicle removal (Huston, 2015). This tool can be used on larger calves. “It’s a combination of knife and pliers, designed to split the side of the scrotum on both sides simultaneously, this approach allows access to the testicle very rapidly, exposing a lot of the testicle and spermatic cord (Huston, 2015). The advantage of this is speed. The opening created, provides good drainage which is important on larger calves (Huston, 2015). The disadvantage is that, to do a good job the operator needs to be very familiar with the tool and there’s a bit of a

learning curve to get to where you can use it properly. It needs to be sharp so the procedure can be done quickly and effectively without tearing the wall of the scrotum and causing undue pain (Huston, 2015).

Henderson Castration Tool (HCT): The HCT uses a twisting action to effectively severe and seal the severed spermatic cord, which reduces blood loss and the risk of haemorrhage via the severed spermatic cord (West, 2006). This also helps to minimize post-castration swelling, infection and pain (West, 2006). The instrument was originally designed for bulls but has now been redesigned to be used in stallions. It is to be used with a typical 14.4 volt variable speed drill with a 3-8 inch chunk.

Procedure for Henderson Castration Tool (HCT): According to West, (2006) general anaesthesia would be carried out on the animal following a standard pre-surgical examinations and preparation. Strip of skin of 2-3 cm over the surgical area on the scrotum would be removed; this is to reduce the likelihood of the instrument sagging a piece of skin when it is applied. The testes would be exteriorized through the opening and after removal of the fascia. The pliers of the HCT would be clamped onto the spermatic cord and the drill would be slowly rotated in a clockwise direction, maintaining slight tension on the cord without pulling on it. The cord would be shortening initially as it twists, and then lengthens as it fatigues and nears severance. After about 15-25 turns, the cord would be severed leaving a tightly coiled and sealed end of spermatic cord. The procedure is repeated for the other testicle and surgical site is stitched or left open.

Advantages and disadvantages of HCT: Some of the advantages of HCT techniques reported by West, (2006) include: fast to use; minimizes the risk of haemorrhage; post-operation swelling and pain is minimal and can be easily aborted using NSAIDs pre and post-operation; reduces

oedema, evisceration, septic funniculitis, hydrocoele, peritonitis and penile damage associated with misapplication of emasculator. While the disadvantage of HCT were due to the fact that: it is not recommended for a case of cryptorchidism; dread drill batteries can be problematic; if the clamp is not secure in the drill, it can't be used; there is risk of infection; spermatic cord can be torn if too much tension is applied on the cord; bleeding from large scrotal vessels as seen in older animals is a potential complication of any castration; cross–clamping the skin prior to removing it reduces bleeding.

Knife castration: This is the most common method of castration (Heather, 2015). Technique and sanitation are important, but using a sharp knife to remove a small testicle, pulling on the spermatic cord and scraping those blood vessels with the knife (to reduce bleeding) create minimal stress (Heather, 2015). This method is quick and sanitary, and the calves heal quickly. All cattle producers have sharp knife as a pocket instrument, so this doesn't require extra equipment. In young calves this is the preferred method (Heather, 2015).

2.1.1.2 Bloodless castration: bloodless castration is a type of physical method of castration and it involves use of methods that irreparably damage the testicles by interruption of the blood supply using burdizzo, rubber ring or latex band that ultimately results in testicular atrophy (Carter *et al.,* 2011; Okafor *et al.,* 2015). When using an emasculatome, the scrotum remains intact, while the spermatic cord of each testicle (within the scrotum) is placed in the jaws of the tool and crushed. The resulting damage causes a loss of blood flow to the testes and eventual testicular atrophy within the scrotum (Carter *et al.,* 2011).

Non-surgical castration (or bloodless castration) can be performed using an elastrator band or an emasculatome, also known as a Burdizzo clamp (Heather, 2015; Huston 2015). Some of the

benefit of bloodless castration include less blood loss, less risk of infection due to wet or dirty environments, and relative ease of performance (Heather, 2015; Huston 2015). A major disadvantage is the risk of tetanus, an often-fatal disease caused by the bacterium *Clostridium tetani*. When non-surgical methods are used, especially in older animals, it is recommended that tetanus vaccination be given at least 10-14 days prior to the procedure. Ideally, two vaccines should be given 6-8 weeks apart prior to castration for maximum protection (Heather, 2015; Huston 2015).

Elastrator band application: An elastrator band can be applied by using an instrument that places a very tight rubber band around the neck of the scrotum (Heather, 2015; Huston 2015). Other methods of applying the band use a drill or specifically designed tool to tighten the rubber. It is important to ensure that both testicles are pulled down and encompassed below the band. The blood supply to the scrotum is cut off and the scrotal tissue and testicles will fall off in approximately 2-3 weeks, sometimes longer (Heather, 2015; Huston 2015). This procedure, while preferred by many when used in older bulls due to less bleeding, has the greatest risk of tetanus and is best when used in animals younger than a month of age. Another disadvantage to this method is the possibility of missing a testicle, resulting in a stag (Heather, 2015; Huston 2015). Breakage of the bands can be a problem if not properly applied or if old bands are used, requiring the scrotum to be cut off or reapplication of the band (Heather, 2015; Huston 2015).

Burdizzo castration: An emasculatome, or Burdizzo, resembles a large clamp which will shut off blood supply to the testicle resulting in its atrophy and resorption when properly applied (Heather, 2015; Huston 2015). With the calf in standing restraint and tailed to reduce kicking and movement, the neck of the scrotum is held in one hand and the opposite spermatic cord is pushed

to the side. The emasculatome is then applied to the cord and closed tightly about 2 inches above the testicle (Heather, 2015; Huston 2015). The emasculatome should be left in place for approximately 15-30 seconds. Each cord should be crushed separately in a staggered manner. The major disadvantage to this method is failure to completely clamp the spermatic cord, resulting in a stag. It is important that the emasculatome is in good condition in order to get a good crush of the blood and nerve supply to the testicle (Heather, 2015; Huston 2015).

2.1.2 Complications of different methods of castration

Pain is one of the commonest complications associated with castration (Coetzee *et al.*, 2010). Generally, surgical castration elicits the most acute pain and least chronic pain. Conversely, banding normally causes the least acute pain (measured by cortisol response), but is associated with the longest duration of chronic pain (Carter *et al.*, 2011). According to the American Veterinary Medical Association, calves castrated by elastic band have been observed to exhibit a pain response to palpation of the scrotal area after castration up to 4 weeks longer than calves castrated by emasculatome or surgery. Persistent wounds have also been observed in calves castrated by elastic bands, even after the scrotum has sloughed off. Castration by emasculatome is an appealing approach because it seems to cause less chronic pain than elastic banding and less acute pain than surgical castration. However, Swiss researchers reported that calves castrated by emasculatome when younger than 12 to 16 weeks may retain functional testes. It is not known whether the testes remained functional because the spermatic cord was too small at that age to fully crush or that regeneration occurred. Additionally, castration failure due to tool operator error has been reported more often for the emasculatome method than for other methods. The greatest advantage of the emasculatome over other methods is that, because the spermatic cords

are crushed within the scrotum, no open wound is exposed to the environment, protecting the injury from flies, mud, and infectious agents (Carter *et al.*, 2011). Immunocastration requires multiple injections and displays variable efficacy (Amatayakul-Chantler *et al.*, 2013; Norman and Collop, 2014).

2.1.3 The indications of castration

Indications for castration in rabbits are different from that of other livestocks and they can either be elective or therapeutic. The indications for elective orchidectomy in rabbits include population control, reduction of inappropriate behaviour such as urine marking, aggression and unwanted sexual behaviour and prevention of reproductive tract diseases (Murray, 2006; Lennox, 2008), while the therapeutic indications for castration are testicular diseases such as infections and neoplasia, correction of an inguinal hernia, true cryptorchidism and trauma (Lennox, 2008).

The three different techniques described for orchidectomy in rabbits are the traditional scrotal approach using a closed or open technique, the pre-scrotal approach and the abdominal approach which is possible because rabbit is a functional cryptorchid (Capello and Lennox, 2006; Murray, 2006; Lennox, 2008). One major advantage of castration in rabbit as food animal is that it results in improved quality of rabbit meat in terms of its juiciness, tenderness and the flavour (Lebas *et al.*, 2000).

Castration of male cattle is common practice throughout many parts of the world. The castration of male calves is an important management practice routinely performed in beef cattle (Earley and Crowe, 2002; Coetzee, 2011) to prevent unwanted breeding, facilitate fattening (Puig *et al.*,

2011) and improve meat quality (Coetzee, 2013). It also reduces aggression and mounting behaviours that cause injury and stress to other cattle (Earley and Crowe, 2002).

Although castration does inflict pain on the animal; which could result in slow growth rate and poorer feed efficiency, the benefits of castration, as viewed by the North American cattle industry, outweigh the negative aspects (Currah *et al.,* 2009). Castration prevents unwanted pregnancies and results in a lower level of testosterone being present in the animal, which decreases aggression in male animals and makes them less dangerous and easier to handle. The number of carcasses with a high muscle pH ("dark cutter") is also decreased and overall carcass quality is improved through increased tenderness and palatability of the meat (Seideman *et al.*, 1982).

It is argued that castration of lambs facilitates their management (Robertson 1965; Hosie *et al*. 1996). After castration of male lambs, all lambs may be housed or grazed together, irrespective of their sex, without the risk of indiscriminate breeding. Castration is thought to reduce the development of secondary sexual behaviours, including aggression and mounting. This assumption has been quantitatively assessed in two and three week old pigs in which aggression, mounting and attempts at mounting, were reduced by both surgical and immuno-castration (Cronin *et al*. 2003; Price *et al*. 2003).

2.1.4 The short scrotum method of castration in lambs

Standard rubber ring castration involves removal of the scrotum and testicles by the application of a tight rubber ring, which occludes the blood supply to these tissues by causing them to become, in turn, ischaemic, necrotic and eventually to be shed along with the rubber ring (Wood

et al. 1991). The short scrotum method of castration involves removal of the scrotum with a tight rubber ring as described above while leaving both the testicles close to or in the inguinal canal, as in cryptorchids *in situ* (anonymous 2). Short scrotum castration of lambs has been used by some farmers, particularly in New Zealand (Lester *et al*. 1991). It is proposed that removal of the scrotum prohibits thermoregulation of the testicles at less than body temperature and thus the proximity of the testicles to the heat of the body wall renders the process of spermatogenesis ineffective (anonymous 2). Research has demonstrated that the standard tight rubber ring castration procedure is associated with considerable acute and chronic pain in lambs and as such represents a significant welfare issue (Mellor and Murray, 1989; Kent *et al.*, 1997).

2.1.5 Castration in swine

Castration of male piglets is a common practice in many countries and the vast majority of male piglets in the United States are castrated. Castration is performed to avoid boar taint in the meat of sexually mature male pigs and to reduce aggression toward other pigs and caretakers. Boar taint is an accumulation of compounds, such as skatole and androstenone, in the meat of intact males that cause an unpleasant smell and taste that is released when pork is heated. About 75% of consumers find meat from boars objectionable in comparison with meat from castrated males (barrows) (Banon *et al.*, 2004).

2.1.5.1 Surgical Castration: Surgical castration of piglets is carried out prior to weaning, most commonly within the first three days of life. Piglets may be restrained for castration in a variety of ways including suspension by the hind legs using a castration stand or another stockperson, placement in a V-trough, or being held with a hand or between an individual's legs (Rault *et al.*,

2011). After the piglet is secure, either two vertical cuts or one horizontal cut is made to the skin of the scrotum, and the testes are removed by cutting the spermatic cord with a scalpel or pulling until the cord tears (Rault *et al.,* 2011). Castration is typically performed without anaesthesia or analgesia (Hewson *et al.,* 2007; Lumb, 2007; Hansson *et al.,* 2011; Rault *et al.,* 2011).

2.1.5.2: Immunocastration: Immunocastration involves the injection of a protein compound that works like an immunization to induce antibody production against gonadotropin releasing hormone (GnRH). The process requires two injections: the first 8 to 11 weeks prior to slaughter and the second 4 weeks prior to slaughter. Immunocastration results in decreased production of gonadal steroids (testosterone and androstenone) with an associated increase in metabolism of skatole (Quiniou *et al.,* 2012). Accordingly, there is a reduction in boar taint. Immunocastration is a temporary form of castration. Improvest® (an FDA-approved veterinary prescription product in the United States) is currently in use for immunocastration in pigs. This product is also labeled as Improvac™, a global brand that is approved for use in more than 60 other countries. Immunocastration is effective at reducing sexual and aggressive behaviours in male pigs e.g. mounting (Fabrega *et al.,* 2010; Rydhmer *et al.,* 2010; Brewster and Nevel, 2013) and knocking and fighting during the period the pig is immunocastrated (Brewster and Nevel, 2013).

2.1.6 Rabbit husbandry

Rabbit husbandry has been described to be suitable for all ages, gender, and class of people across the world (Lebas *et al.,* 1997). Rabbit meat is highly nutritious with low fat (9 %), low sodium and calories (8 %) and low cholesterol levels. It also has a high protein level of about 20.8 %, 28 % phosphorus, 13 % iron, 16 % zinc, 14 % riboflavin, 6 % thiamin, 35 % B12 and 48

% niacin and its consumption is of no known cultural and religious bias (Ume *et al.,* 2016). Rabbits, like horses, have the ability to digest large amount of fibrous feed as most non ruminant species cannot due to the presence of caecal microbes, with highest feed conversion ratios at 4:1 (Ugosor *et al.,* 2016). Rabbits are especially desirable to keep in a society with diminishing land size, as they require less space and therefore can be kept at the backyard (Lebas *et al.,* 1997; Ugosor *et al.,* 2016). Rabbit meat production has been described as suitable for a country with a continuous and rapidly increasing population and in a developing economy like Nigeria, rabbit meat production is important and more interesting than poultry production due to the low cost of production (Lebas *et al.,* 1997). All these features make rabbit farming not only suitable but also affordable investment by the poorest families (Baruwa, 2014). Rabbit skin is used in making carpet or rugs, jackets, and head gears. Rabbit manure contains high nitrogen and phosphorous, making it as useful as that of poultry in improving soil fertility (Ume *et al.,* 2016).

2.1.6.1 Sexual maturity in rabbits: The small breeds of rabbit (e.g. polish) mature at about 4 months of age, the medium size such as Chinchilla, New Zealand White mature at about 6-7 months of age and heavy breeds (e.g. Flemish giant) mature at about 9-12 months of age (Proverbs and Quintyne, 1992). Rabbits are pubertal when their testicles become androgenically active and accessory glands begin to produce fructose and citric acid and the animal assumes a characteristically male behaviour. In this context, in rabbits, sperm appear closer to the end of puberty than to the onset of puberty (Campos *et al.,* 2014). Skinner, (1967) observed from his research on the time-relationship between the androgenic and gametogenic function of the rabbit testis during puberty, that typical male characters are first seen on day 60 in male rabbits, while attempt to mount (bucking) was seen on day 70 (Skinner, 1967). At 126 days sperm motility was

as high as in adult bucks. Fraser, (1988), determined the male reproductive status from testes position (abdominal or scrotal) and weight, which are indicators of spermatogenetic activity, and concluded that in general, males with scrotal testes or with testes weights of 3-4 g are fertile (Fraser, 1988).

2.1.6.2 Reproduction in rabbits: Male rabbits are the basis of reproductive success because farm profitability depends upon the buck. Male fertility is an interesting trait in rabbit breeding because together with the doe it determines breeding success. Moreover, due to the fertilizing capacity of semen, the male can influence fertility, conception and the productivity of the does (Cherfaoui *et al.,* 2013). Rabbit Productivity is generally expressed as a number of kits produced per rabbit doe per year, but sometimes includes the litter size and number of litters per doe and per year (Lebas, 2005).

Although does are fertile 24 hours following kidding since they are induced ovulators and can be rebred at this time but a short rebreeding intervals after kidding may not allow for adequate recovery of the body reserve of the does, and this may lead to reduced fertility, decreased milk production, reduced growth rate, gestation length, recaptivity, stillbirth, litter size, decreased litter weight at weaning and an increase in kit mortality (Oguike and Okocha, 2008; Saha *et al.,* 2013). Moreover, it is considered inhumane and therefore not allowed by animal welfare group (Saha *et al.,* 2013). Weaning of litter at an early stage, artificial insemination, changing the nursing method or lighting schedule or duration (a short term light supplementation is beneficial), climatic condition is all factors influencing reproduction in rabbits (Lebas, 2005; Gerencsér *et al.,* 2011). Following parturition, rebreeding is generally done between 11 - 21days post-partum (Oguike and Okocha, 2008; Saha *et al.,* 2013).

Heat stress, as well as poor forage quality, has resulted in serious challenges to efficient reproductive and productive performance in most livestock production, rabbit inclusive in the tropic. Reproductive traits in rabbits were shown to be highly susceptible to detrimental effects of ambient temperature above the normal room temperature of 25 ^{0}C, which alter male fertility with the subsequent economic loss (Lebas, 2005; Abdulrashid and Juniper, 2016). Good husbandry practices are important in males as it ensures improved reproductive potential, which consequently result in increased rate of conception for sustainable production (Castellini *et al.,* 2010). Nutritional modifications of male rabbit feed have been shown to help avoid the negative effects of extreme temperature, with respect to seminal characteristics, sperm production rate and testicular sperm reserves (Cherfaoui *et al.,* 2013). The optimization of reproductive performance is one of the main facts that assure high productivity on rabbit farms. This requires that the management practices take into account the physiology and behaviour of the animals since environmental, managerial and sanitary aspects interfere with fertility and can impair it (Castellini *et al.,* 2010).

2.1.7 Castration techniques in rabbits

Rabbit's penis is located caudal to the testicles, which lie cranial to the penis in two separate hemiscrotal sacs. The inguinal canal remains open throughout life, and the testicles are free to move from the hemiscrotal sacs to the abdominal cavity. The testicles are oval shaped. The position of the testicles depends on many factors including body position, body temperature, breeding activity, gastrointestinal tract filling, and the amount of abdominal fat (Lennox, 2008). The epididymis is clearly visible at the caudal pole of the testicle, but not well developed. There is fat surrounding the testicles. The glans of the penis is not well developed, it is point shaped

and covered by a prepuce (Murray, 2006). In rabbits, 3 techniques can be used for orchidectomy and these include prescrotal, scrotal and abdominal surgical approach (Lennox, 2008).

2.1.7.1. Prescrotal approach: A single ventral midline incision located cranial to the scrotum is made to expose the vaginal tunics one at a time. Once the tunic is incised, the spermatic cord may be grasped, and with gentle traction the testicle and epididymis exposed. Once the ligament between the epididymis and the scrotum is broken down, the spermatic cord may then be doubled ligated along with the blood vessels and transected. It is important to double check to make sure that there is no haemmorhage before the stalk is allowed to retract into the body cavity, at which point any haemorrhage that may occur from the spermatic vessels will need to be approached through an abdominal approach (Murray, 2006). In order to prevent postoperative herniation, the vaginal tunic should be ligated as far cranially as possible. The skin is then closed with subcuticular sutures.

2.1.7.2 Scrotal surgical approach: For this technique, it can be open or closed castration and both approaches have been well described in rabbits (Murray, 2006; Lennox, 2008). The major disadvantage of the scrotal approach is challenging surgical preparation, as it is difficult to completely shave off the hair and adequately prepare the site for sterile surgery. Irritation can occur due to the adequate surgical scrubbing of the scrotum. Two separate skin incisions are made over the left and right testes respectively to open the vaginal process (open technique), which allows direct visualization and ligation of the spermatic cord and vessels, but necessitates the closure of the vaginal process at the end of the procedure. In the closed technique, the vaginal tunic is closed and spermatic cord and vessels simultaneously ligated.

2.1.7.3 Abdominal approach: The rabbit under general anaesthesia is placed in dorsal recumbency. The caudal area of the abdominal surface is surgically prepared routinely. Laparotomy is performed on the caudal midline 4-5 cm cranially to the hemiscrotal sacs. The urinary bladder is exposed, exteriorized and reflected caudally. The deferens is gently retracted, and testicles are exteriorized. Dissection of the tail of the epididymis from the caudal pole of the everted hemiscrotal sac and ligation of the spermatic cord is performed through the celiotomy access. Suturing of the abdominal wall and the overlying soft tissues are performed routinely (Murray, 2006; Lennox, 2008).

2.1.8 Management of pain due to castration in livestock

Castration has been shown to elicit physiological stress, inflammatory reactions (as indicated by acute phase proteins), pain-associated behaviour, suppression of immune function, and a reduction in performance (Molony *et al.*, 1995; Fisher *et al.*, 1996 and 1997; Ahmed and Ahmed, 2011; Olaifa and Akpan, 2017) to varying degrees. The degree of pain experienced by the animal depends on the method used and the animal's age. In swine, castration is usually conducted within the first few days of life. In cattle and sheep, the age and methods of castration are more variable depending on rearing systems such as intensive or extensive conditions (Rault *et al*, 2011).

Usually, castration is performed without anaesthesia or post-operative analgesia, but their use could reduce the degree of pain experienced (Rault *et al*, 2011; McCarthy, 2017). To minimize the pain experienced by castrated animals, different anaesthetics and analgesics may be administered. Analgesic pharmacological agents available for use in livestock include local

anaesthetic agents, NSAIDs, opioids and α-2 agonists (McCarthy, 2017). Lidocaine can be administered either locally into the neck of the scrotum, thereby affecting the cord or testicles (Thüer *et al.,* 2007), or epidurally (Meyer *et al.*, 2007). Lidocaine is the most common local anaesthetic tested and has been found to reduce pain-related behaviours during surgery in both swine and cattle. However, it seems to have no pain-relieving effects post-surgery (Rault *et al*, 2011). The use of analgesics such as opioids or non-steroidal anti-inflammatory drugs to tackle chronic or long-lasting pain has not been thoroughly investigated (Rault *et al*, 2011). Other methods that have been described for management of post-castration pain include use of topical anaesthetic gel containing lignocaine and bupivacaine applied immediately post-surgical castration (McCarthy, 2017) and the use of cryo-anaesthesia which has been shown to reduce the peri -operative pain (Lomax *et al.,* 2017). cryo-anaesthesia is a form of local anaesthesia which involves disruption of local sensory nerve function through reduction of tissue temperature via rapid evaporation of volatile liquid (vapocoolant) from the skin surface to which they are applied (Collado-Mesa *et al.*, 2015; Griffith *et al.*, 2016).

2.2 Pain

The International Association for the Study of Pain (IASP) defines pain as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Zaki, 2013). All animals possess the neuro-anatomic and neuro-pharmacologic components necessary for the transduction, transmission, and perception of noxious stimuli. Therefore the systems to consider in pain evaluation include neurological, cardiovascular, respiratory, skeletal, digestive, urinary and endocrine systems (Scharmann, 2012; Zaki, 2013). Behavioural parameters influencing pain scores are temperament, vocalization,

posture, locomotion and other behavioural changes. Also, threshold and response to pain vary according to species, breed, health status and age (Bufalari *et al.,* 2007). Although considerable progress has been made on pain in animals over the past 20 years, the recognition of pain in food animals undergoing managemental procedures, such as branding, dehorning and castration, remains difficult and frequently suboptimal, but the assessment of the degree of discomfort is even more difficult and problematic (Dalla Costa *et al.,* 2014).

2.2.1 Types of pain

Acute pain has been defined as pain that exists during the expected time of inflammation and healing after injury (up to 3 months), and chronic pain is defined as that which exists beyond the expected duration associated with acute pain. Therapy should be focused on the underlying cause of pain, (nociceptive, inflammatory, or pathological) rather than on arbitrary labels based on duration (Woolf, 2010).

Nociceptive pain occurs when peripheral neural receptors are activated by noxious stimuli (e.g., surgical incisions, trauma, heat, or cold) (Woolf, 2010; Mark *et al.,* 2015). Inflammatory pain results gradually from activation of the immune system in response to injury or infection, and pathological pain, also called maladaptive pain, occurs when pain is amplified and sustained by molecular, cellular, and microanatomic changes, collectively termed peripheral and central hypersensitization (Woolf, 2010; Mark *et al.,* 2015). Pathological pain is characterized by hyperalgesia (exaggerated response to noxious stimulus), allodynia (painful response to nonnoxious stimuli, such as touch or pressure), expansion of the painful field beyond its original

boundaries, and pain protracted beyond the expected time of inflammation and (Woolf, 2010; Mark *et al.*, 2015).

2.2.2 Analgesic therapies for painful husbandry procedures

Pain management is central to veterinary practice, not adjunctive. This is because of the pledge to 'the relief of animal pain and suffering' and to enhance the veterinarian-client-patient relationship (Mark *et al.,* 2015). A commitment to pain management identifies a practice as one that is committed to compassionate care; optimum recovery from illness, injury, or surgery; and enhanced quality of life (Mark *et al.*, 2015).

All types of tissue injury can be generators of pain and the pain response is unique to each individual and involves two components (Mark *et al.,* 2015):

(1) The sensory component is nociception, which is the neural processing of noxious stimuli and

(2) The affective component is pain perception, which is the unpleasant sensory and emotional experience associated with either actual or potential tissue damage.

Pain is the endpoint of nociceptive input and can only occur in a conscious animal; however, there is also the involvement of autonomic pathways and deeper centers of the brain involved with emotion and memory. Hence pain is a multi-dimensional experience; it is not just what you feel but also how it makes you feel (Reid *et al.* 2013).

Different forms of pain relief protocol for livestock husbandry are well documented in many literatures (Sutherland *et al.*, 2011; Coetzee, 2013; Stafford and Mellor, 2015). Pain can be

reduced using various pharmacological agents known as anaesthetics or analgesics. The analgesic drugs commonly used in cattle include local anaesthetics, NSAIDs, opioids and α-2 agonists (Schwartzkopf-Genswein *et al.,* 2012). However, a multi-modal approach to pain management involving combination of local anaesthetics and NSAID provides superior analgesia (Hudson *et al.,* 2008; McCarthy, 2017).

2.2.3. Recognition and assessment of pain

The Patient's Behaviour is the Key. Because animals are nonverbal and cannot self-report the presence of pain, the burden of pain assumption, recognition, and assessment lies with veterinary professionals. It is now accepted that the most accurate method for evaluating pain in animals is not by physiological parameters but by observations of behaviour. Pain assessment should be a routine component of every physical examination, and a pain score is considered the ''fourth vital sign,'' after temperature, pulse, and respiration (Hellyer, *et al.,* 2007; American Animal Hospital Association-American Veterinary Medical Association Preventive Healthcare Guidelines Task Force, 2011).

Routine pain control is considered one of the most important welfare priorities in livestock management (Bayvel, 2004). The activity of a surgical patient is seriously reduced (Wright-Williams *et al.,* 2007). This is due to pain which can be estimated (Hunton *et al.,* 2005). There are 3 basic means of assessing post-surgical pain in livestock: measurements of general body functioning like food and water intake and live-weight gain; physiological responses like plasma cortisol concentrations; and behaviours such as vocalizations (Weary *et al.,* 2006). Evaluation of behavioural signs has proven to be reliable indicators of pain (Morton and Griffiths, 1985;

Marchant-Forde *et al.*, 2009). In goats this include vocalization, teeth grinding, rapid and shallow breathing (Okafor *et al.*, 2015), standing and lying repeatedly, tail wagging, occasionally bleating, displaying neck extension, dorsal lip curling, kicking, rolling, and hyperventilation (National Academy of Sciences, 2009; Okafor *et al.*, 2015).

2.2.4 Pain scoring tools

Currently there is no gold standard for assessing pain in animals generally but the guidelines by Task Force strongly recommends utilizing pain-scoring tools both for acute and chronic pain. It should be noted that those tools have varying degrees of validation as acute and chronic pain scales are not interchangeable (Mark *et al.*, 2015). The use of pain scoring tools can decrease subjectivity and bias by observers, resulting in more effective pain management, which ultimately leads to better patient care (Mark *et al.*, 2015). Numerical rating scale (NRS) is used to rate pain intensity and assess pain distress and the degree of subjective suffering, caused by the pain on a numbered scale, such as the 0 to 10 Numeric Pain Intensity Scale (McCaffery and Pasero, 1999).

2.2.5 Pharmacological management of pain

Effective pain management generally involves a balanced or multimodal strategy using several classes of pain-modifying medications (Mark *et al.*, 2015). The rationale behind this approach is that it addresses targeting multiple sites in pain pathways, potentially allowing lower doses of each drug and minimizing the potential for side effects associated with any single drug (Mark *et*

al., 2015). The choice of medication should be based on anticipated pain levels and individual patient needs (Mark *et al.,* 2015).

2.2.5.1 Opioids: Opioids are the most effective drug class for managing acute pain and can play a role in managing chronic pain. Opioids should be used as a routine preoperative medicant, preferentially in combination with a tranquilizer/sedative (e.g., acepromazine, midazolam, diazepam, or a-2 adrenergic agonist such as dexmedetomidine) when the patient's condition warrants their use (Sawyer *et al.,* 1991; Grimm *et al.,* 2000). Full agonists elicit greater and more predictable analgesia than partial agonists or agonists. In dogs, the antagonist/ agonist butorphanol, in particular, appears to have limited somatic analgesia and very short duration of visceral analgesia (Sawyer, *et al.,* 1991; Grimm, *et al.,* 2000).

Opioids exhibit analgesic effects by binding to spinal and supraspinal μ, κ and δ receptors and consequently decreasing propagation of the nociceptive signal by activating receptor linked potassium channels and inhibiting voltage-gated sodium channels. Administration of opioids can result in side effects including respiratory depression, decreased gastrointestinal motility, increased appetite, sedation, euphoria and nausea. To reduce these side effects, partial or mixed receptor opioids have been developed (Coetzee, 2011). Butorphanol is the most commonly used opioid in cattle (Anderson and Muir, 2005). Opioids may act synergistically with α-2 agonists and therefore these compounds are often administered in combination (Coetzee, 2011).

Treatment with xylazine (0.05 mg/kg), ketamine (0.1 mg/kg) and butorphenol (0.025 mg/kg) administered IM has been shown to result in a reduced chute exit velocity and reduced electrodermal activity following concurrent castration and dehorning of calves, likely due to the sedative effects of this drug combination (Baldridge *et al.,* 2011). In this study, treatment with

xylazine, ketamine and butorphanol only reduced serum cortisol for less than 1 h following the procedures, which is related to the short-lived effects of all the drugs in that combination treatment. There was no effect of this combination treatment alone on weight gain (Baldridge *et al.,* 2011). Similarly, a combination of butorphanol (0.07 mg/kg) and xylazine (0.02 mg/kg) administered to weanling bulls at the time of castration did not affect indicators of stress and did not improve performance, as measured through analysis of chute exit velocity, cortisol, haptoglobin, weight gain, feed intake, mortality and morbidity (Faulkner *et al.,* 1992). The apparent lack of analgesia was suggested possibly due to the dose rate or timing of administration (90s before castration) (Faulkner *et al.,* 1992).

2.2.5.2 Tramadol: Tramadol in dogs has a very short half-life (1.7 hr) and negligible amounts of the opioid M1 metabolite are produced (KuKanich and Papich, 2004; McMillan *et al.,* 2008; Giorgi *et al.,* 2009; KuKanich and Papich, 2011). Pharmacodynamic studies demonstrate the anaesthesia-sparing and pain-modifying effect of parenteral tramadol in dogs (Itami *et al.,* 2011; Kongara *et al.,* 2012; Neves *et al.,* 2012; Itami *et al.,* 2013; Teixeira *et al.,* 2013). In contrast to dogs, cats do produce the l-agonist M1 metabolite. A pain-modifying effect has been demonstrated in both a thermal threshold and clinical surgical model (Brondani *et al.,* 2009; Pypendop *et al.,* 2009).

2.2.5.3 Nonsteroidal Anti-Inflammatory Drugs: Non-steroidal anti-inflammatory drugs reduce inflammation by inhibiting the enzyme cyclo-oxygenase (COX) which synthesizes prostaglandins and other inflammatory mediators (Hudson *et al.,* 2008). The analgesic effect of NSAIDs is mediated by their anti-inflammatory action, reducing nociceptor sensitisation within injured tissue (Stafford and Mellor, 2015). Various NSAIDs with different pharmacokinetic and

pharmacodynamic properties are available for use in cattle (Anderson and Muir, 2005). In addition to peripheral analgesic effects, some NSAIDs also have various central analgesic effects, including inhibition of neurotransmitter synthesis, increasing nerve membrane potentials and reducing synaptic output (Stafford and Mellor, 2015). NSAIDs commonly used in cattle include meloxicam, ketoprofen, carprofen, flunixin meglumine and tolfenamic acid (Hudson *et al.,* 2008) while Piroxicam and ascorbic acid combination was used as reported by Okafor *et al.,* (2015). Unlike LA, NSAIDs act systemically and can be administered orally, IV or intramuscularly, IM (Stafford and Mellor, 2015).

Much work on the use NSAIDs for the relief of pain caused by dehorning and surgical castration has been reported with their efficacy (Stock *et al.,* 2013). Ketoprofen (3 mL) administered IV 15 to 20 min prior to scoop dehorning of 3 to 4-month-old dairy calves has been shown to have a minimal effect on the initial cortisol response however reduces the later phase of the cortisol response, suggesting that it may ameliorate inflammatory pain (McMeekan *et al.,* 1998). Similarly, meloxicam (0.5 mg/kg BW) administered IV to 16 to 20-week-old dairy calves immediately prior to scoop dehorning was demonstrated to have no effect on the cortisol response in the acute post-operative period. However, it has effect on longer term physiological, behavioural and performance indicative of analgesia (Coetzee *et al.,* 2012).

Oral administration of meloxicam has been recognised as having certain advantages over injectable administration, due to its differing pharmacokinetic profile. Although oral meloxicam has a greater 'time-to-peak-concentration' than meloxicam administered IV (Coetzee *et al.,* 2009), its mean plasma half-life is also greater, indicating that oral administration of the drug may result in longer lasting analgesia (Coetzee *et al.,* 2009). It also removes the risk of injection

site reactions as well as needle related injuries (Olson *et al.,* 2016), and requires less operator skill (Small *et al.,* 2014). More so, oral administration allows the drug to be delivered via feed thereby eliminating the requirement for restraint (Olson *et al.,* 2016). Oral meloxicam (1 mg/kg) administered to 4 to 5-month-old Holstein calves approximately 2 h prior to surgical and band castration has been shown to significantly reduce physiological and behavioural responses to procedural pain (Olson *et al.,* 2016). Similarly, yearling beef bulls exhibited a reduced acute inflammatory response following surgical castration with concurrent treatment of meloxicam bolus (1.1 mg/kg 71) administered directly into the rumen via esophageal intubation (Roberts *et al.,* 2015). Although NSAIDs administered orally can be effective, there are concerns about slow absorption rate (Small *et al.,* 2014). Drugs delivered by oral trans-mucosal absorption offer some advantages over drugs delivered orally, including abundant blood supply and improved bioavailability due to avoidance of degradation in the gastrointestinal tract and hepatic first-pass metabolism (Habib *et al.,* 2011). A buccal meloxicam (BM) gel has been developed for use in calves and lambs undergoing surgical husbandry procedures. When administered into the buccal cavity (0.5 mg/kg) immediately prior to surgical castration and tail docking in lambs, BM reduced pain-related behaviours in the first 8 h following the procedures (Small *et al.,* 2014).

The majority of conditions that cause pain have an inflammatory component hence; Non-steroidal anti-inflammatory drugs (NSAIDs) are a mainstay for management of chronic pain as well as for perioperative use (KuKanich *et al.,* 2012). NSAIDs should be used for their central and peripheral effects in both dogs and cats after consideration of risk factors, though there is no indication that any one of the veterinary approved NSAIDs is associated with any greater or lesser incidence or prevalence of adverse events (AEs) (KuKanich *et al.,* 2012). Canine and

feline veterinary-approved NSAIDs have demonstrated acceptable safety profiles, which is in contrast to non-approved NSAIDs such as aspirin, ibuprofen, naproxen, and meloxicam for human use (Innes *et al.,* 2010; Khan and McLean, 2012; Monteiro-Steagall *et al.,* 2013).

Although the overall incidence and prevalence of NSAID-related toxicity are unknown, it does appear to be very low relative to the number of doses administered (Monteiro-Steagall, *et al.,* 2013). This may include:

Gastrointestinal (GI) toxicity; Of the AEs associated with NSAIDs, gastrointestinal (GI) toxicity is the most common. The GI clinical signs associated with NSAID toxicity in dogs include vomiting, diarrhea, and inappetence while in cats inappetence appears to be the most common anaesthetics emergency (Stanton and Bright, 1989; Neiger, 2003; Lascelles *et al.,* 2005). The leading risk factors for NSAID-associated GI perforations are incorrect dosing, concurrent use with other NSAIDs or corticosteroids, and continued use despite GI signs or anorexia (Lascelles *et al.,* 2005; Monteiro-Steagall *et al.,* 2013).

Nephrotoxicity; Studies has shown administration of NSAIDs before anaesthesia in healthy dogs may lead to changes in renal parameters. Hence, the importance of maintaining a normotensive state during anaesthesia is considered paramount when utilizing preoperative NSAIDs (Bostrom *et al.,* 2002; Crandell *et al.,* 2004). Similar studies have not been conducted in cats undergoing anaesthesia, but one feline study revealed no alteration in glomerular filtration rate measured by iohexol clearance after 5 days of oral meloxicam (Goodman *et al.,* 2009).

Idiosyncratic hepatocellular necrosis; Idiosyncratic hepatocellular necrosis has been reported with various NSAIDs but remains exceedingly rare, only 1.4 cases/10, 000 dogs (0.052%),

usually occurring between 2 and 4 weeks after starting treatment. Preexisting elevated liver enzymes are not a risk factor (KuKanich *et al.*, 2012). Idiosyncratic hepatocellular necrosis is not a true toxicosis but rather an intrinsic, heritable reaction to the molecule being administered (Monteiro-Steagall *et al.*, 2013).

Delayed bone healing; Highly COX-2-selective NSAIDs have caused delayed bone healing in rabbit and rodent models, and one study in dogs demonstrated delayed healing of experimental tibial osteotomies following long-term NSAID use (Ochi *et al.*, 2011) and normal tissue healing is rapidly restored once the NSAID is withdrawn (Gerstenfeld *et al.*, 2007).

Piroxicam is a nonsteroidal anti-inflammatory drug (NSAID) of the oxicam class, which is non-selective. It undergoes enterohepatic circulation and is primarily used to treat arthritis and other musculoskeletal conditions (Agrawal and Gupta, 2010). Piroxicam exhibits anti-inflammatory (Hedner *et al.*, 2004), analgesic (Izdes *et al.*, 2003), and antipyretic activities in animal models. The mechanism of action of piroxicam, like any other NSAID, is not completely understood but may be related to prostaglandin synthetase inhibition (Abdallah *et al.*, 2013).

Piroxicam has been widely used to treat podagrous and rheumatoid arthritis, gonarthrosis, osteoarthritis, backaches, neuralgia, mialgia and other diseases accompanied by pain syndrome or an inflammatory process (Kormosh *et al*. 2011). It is primarily used to treat arthritis and other musculoskeletal conditions (Agrawal and Gupta 2010). It exhibits anti-inflammatory, anti-rheumatoid arthritis (Hedner *et al*. 2004), analgesic (Izdes *et al*. 2003) and antipyretic activities in animal models. The mechanism of action of piroxicam, like any NSAID, is not completely understood, but may be related to prostaglandin synthetase inhibition (Abdallah *et al*. 2011).

In goat, Okafor *et al.,* (2015) reported the multimodal approach to surgical pain management to be the best option of managing post castration pain. A combined administration of piroxicam and ascorbic acid is very effective in the management of postsurgical pain in goats (Okafor *et al.,* 2015).

2.2.5.4 Local Anesthetics (LAs): This is the only class of drug that renders complete analgesia. LAs are reported to be antimicrobial, immunomodulating, reduce postoperative maladaptive pain states and do not appear to delay tissue healing (Waite *et al.,* 2010). LAs can be administered either directly at a simple incision site or at a specific nerve to provide analgesia to a large region (or area). LAs are considered safe, with anaesthetic emergency generally limited to very high doses or inadvertent IV administration (DiBaise, 2007).

Local anaesthetics diminish sensation by blocking nerve depolarization and conduction via inhibition of sodium channels (Anderson and Muir, 2005). A popular local anaesthetics used in cattle is lignocaine. It is cheap and has limited toxic effects (Edmondson, 2014). It has an onset of action of 1 to 2 min and lasts for about 60 to 120 min. Other local anaesthetics agents include mepivacaine, procaine and bupivacaine which have different pharmacodynamics. Among these, Bupivacaine has a longer time for the onset and duration of action at 5 to 10 min and 4 to 12 h respecively (Stafford and Mellor, 2015).

Some of the techniques used for the administration of local anaesthesia in livestock include topical application, local blocks, ring blocks, selected peripheral nerve blocks and regional blocks (Anderson and Muir, 2005), depending on the procedure to be carried out.

Different topical agents have been used in livestock for surgical castration and other procedures with various outcomes. Earley and Crowe (2002) reported that lignocaine (2%) injected into each testicle (6 mL) and subcutaneously (SC) along each incision line of the scrotum (3 mL) 20 min prior to surgical castration significantly reduced the cortisol response of 5.5-month-old dairy calves to the procedure, whereas, Webster *et al.,* (2013) reported that injection of lignocaine (2%) into each testicle (6 mL) and SC around the spermatic cords (6 mL) 20 min prior to surgical castration was not sufficient to cause a significant reduction in the cortisol response of 2 to 3-month-old dairy calves. A topical anaesthetic gel containing lignocaine and bupivacaine applied immediately following dehorning and surgical castration of calves has been shown to reduce wound sensitivity for up to 5 and 24 h respectively (Espinoza *et al.,* 2013, Lomax and Windsor, 2014, Espinoza *et al.,* 2015). This similarly reduces pain-related behaviour in calves for the first 4 h following surgical castration (Lomax and Windsor, 2014). Topical anaesthetic gel is easy and quick to administer (Lomax and Windsor, 2014) but it is not effective for peri-operative pain relief. Topical application of local anaesthetics has been suggested to extend the duration of anaesthesia in comparison to injectable ones due to a slower absorption rate (Brofeldt *et al.,* 1989, Lomax *et al.,* 2013, Lomax and Windsor, 2014).

To manage peri-operative pain during minor surgical procedures, cryo-anaesthesia is preferable (Lomax *et al.,* 2017). This is a form of local anaesthesia which involves disruption of local sensory nerve function through reduction of tissue temperature (Griffith *et al.,* 2016). Topical vapocoolants can be used to reduce tissue temperature through the rapid evaporation of volatile liquid from the skin surface to which they are applied (Collado-Mesa *et al.,* 2015). This has been

used in beef calves to reduce the peri-operative pain response to ear tagging and ear notching (Lomax *et al.,* 2017).

*2.2.5.5 Alpha (*α) *-2 Adrenergic Agonists:* Alpha (α)-2 adrenergic receptors are located with opioid receptors. Thus, the two drug classes used together are highly synergistic for sedation and analgesia. Alpha (α)-2 Agonists have a versatile dosing profile (Mark *et al.,* 2015). That allows low and even micro doses in combination with opioids to be clinically useful and minimizes the cardiovascular effects. Clinicians should be mindful that cardiovascular side effects occur even with very low doses of α-2 adrenergic agonists that lower doses will have a shorter duration of effect, and that analgesic effects have a shorter duration than the sedative effects (Mark *et al.,* 2015).

Alpha (α)-2 agonists activate α-2-adrenoreceptors in the central and peripheral ANS producing analgesic and sedative effects by negatively affecting sympathetic activity and the release of noradrenaline (Hudson *et al.,* 2008). Side effects include decreased cardiac output, reduced respiratory rate, relaxation of muscles and depressed gastrointestinal motility. Xylazine is the most commonly used α-2 agonist in cattle (Coetzee, 2011). It is administered in combination with ketoprofen at the dosage of 0.2 mg/kg and 3 mg/kg respectively to reduce struggling behaviour during hot-iron disbudding of calves and reduce heart rate following the procedure (Caray *et al.,* 2015). Administration of xylazine alone was not sufficient to produce such analgesia during the hot-iron disbudding (Stilwell *et al.,* 2010).

2.2.5.6 Ketamine: Ketamine exerts a pain-modifying effect via its N-methyl-Daspartate receptor antagonist actions. Subanesthetic ketamine constant rate infusion (CRI) in humans prevents pain and has antihyperalgesic, and antiallodynic effects (Richebe *et al.,* 2005; Bell *et al.,* 2006).

Studies appear to support a similar clinical effect in dogs, although ketamine's analgesic effect has not yet been studied in a feline surgical model (Slingsby and Waterman-Pearson, 2000; Wagner *et al.,* 2002; Sarrau *et al.,* 2007).

2.2.5.7 Systemic Lidocaine: There is strong evidence of the safety and beneficial effects of IV lidocaine on pain after abdominal surgery (although not for other surgeries eliciting somatic pain) in humans and possibly in horses, including both analgesia and return of bowel function (McCarthy, *et al.,* 2010). Some investigators discourage the use of IV lidocaine in cats due to negative cardiovascular effects, but successful use in clinical practice has been anecdotally reported (Pypendop and Ilkiw, 2005). Various formulations for a combination of morphine, lidocaine, and ketamine CRIs have been described in dogs (Muir *et al.,* 2003).

2.2.5.8 Gabapentin: Gabapentin is an anticonvulsant with analgesic properties that may be primarily derived by down-regulating calcium channels (Longmire *et al.,* 2006). Because of its efficacy and tolerability, gabapentin is widely used in humans with neuropathic and other maladaptive pain conditions (Solak *et al.,* 2007). Along with published clinical case reports in animals, the data suggest a strong rationale for using gabapentin in dogs and cats with similar conditions (Rusbridge *et al.,* 2010; Plessas *et al.,* 2012).

2.2.5.9 Amantadine: Amantadine exerts a pain-modifying effect as an N-methyl-Daspartate receptor antagonist and remains a drug of interest for chronic pain (but not specifically for Degenrative Joint Diseases) in humans (Fisher *et al.,* 2000). One study demonstrated utility as an adjunct to NSAIDs in dogs with refractory DJD, and there is one case report utilizing amantadine to treat neuropathic pain in a dog (Lascelles *et al.,* 2007; Madden *et al.,* 2014). Toxicity and

pharmacokinetic studies have been performed in humans and cats but not in dogs (Vernier *et al.*, 1969; Siao *et al.*, 2011).

2.2.5.10 Tricyclic Antidepressants (TCAs): As a class, TCAs are the most effective medications for selective neuropathic pain conditions in humans (Finnerup *et al.*, 2005). In dogs, there exists only a single case report where amitriptyline was used for neuropathic musculoskeletal pain (Cashmore, *et al.*, 2009).

2.2.5.11 Selective Serotonin (Norepinephrine) Reuptake Inhibitors [SS (N) RIs]: These compounds exert their effect by increasing serotonin with or without norepinephrine in the synaptic cleft. At least one SS (N) RI, duloxetine, has a chronic pain label indication in humans. In dogs, bioavailability is poor and clinical efficacy is lacking (Patel *et al.*, 2011).

2.2.5.12 Acetaminophen: Acetaminophen is contraindicated in cats. In dogs, several early studies revealed a pain-modifying effect in orthopedic surgery, and pharmacokinetic data have been reported (Mburu *et al.*, 1988; Mburu, 1991; KuKanich, 2010). The literature does not appear to indicate that acetaminophen has a proclivity towards hepatotoxicity in dogs (Mark *et al.*, 2015).

2.2.5.13 Bisphosphonates: Bisphosphonates are administered by IV infusion, this class of drug exerts antiosteoclast activity and can contribute to pain relief in dogs with bone cancer (Fan *et al.*, 2005).

2.2.5.14 Corticosteroids: Corticosteroids are not primarily analgesic drugs, but may exert a pain-modifying effect by reducing inflammation. Their utility as an analgesic therapy in dogs and cats has not been reported (Pelletier and Martel-Pelletier, 1989; Pelletier and Martel-Pelletier, 1991; Pelletier *et al.*, 1994; Pelletier *et al.*, 1995).

2.2.6 Non-pharmacological management of pain

Shanan, (2011) reported different approaches to non-pharmacological management of pain to include cold compression, weight optimization, and therapeutic exercise. However, these are commonly carried out in pet animals. Other treatment options gaining increasing acceptance include acupuncture, physical rehabilitation, myofascial trigger point therapy and therapeutic laser (Shanan, 2011). In addition, nonpharmacologic adjunctive treatment includes an appreciation of improved nursing care, gentle handling, caregiver involvement, improved home environment, and hospice care (Shanan, 2011).

2.2.6.1 Acupuncture for Pain applications in human medicine, especially pain management. There is a solid and still-growing body of evidence for the use of acupuncture for the treatment of pain in veterinary medicine to the extent *Control:* Acupuncture has been recognized by the National Institutes of Health since 1998 as having that it is now an accepted treatment modality for painful animals (Laim *et al.*, 2009; Gakiya *et al.*, 2011; Groppetti *et al.*, 2011; Petermann 2011; Cassu *et al.*, 2012).

2.2.6.2 Physical Rehabilitation: Combined modality therapy to decrease pain and restore function is now considered an essential approach for musculoskeletal injury and post-surgical recovery (Au *et al.*, 2010). In the treatment of chronic diseases such as DJD or conformational abnormalities, rehabilitation should be considered an important component of an overall long-term treatment strategy (Edge-Hughes, 2007). The foundation of rehabilitation is a therapeutic exercise that aims to restore musculoskeletal strength and function, endurance, proprioception, and the reduction of pain. Most commonly it involves exercise and manual therapy, including joint mobilizations, massage, and myofascial release (Mark *et al.*, 2015).

Energy-based modalities are also often employed, including neuromuscular electrical stimulation, transcutaneous electrical nerve stimulation, cryotherapy with and without compression, therapeutic ultrasound, therapeutic laser, and extracorporeal shockwave therapy (Johnson *et al.,* 1997; Rochkind, 2004; Mueller *et al.,* 2007; Mueller *et al.,* 2009; Rexing *et al.,* 2010).

2.2.6.3 Nutrition Management for Pain Management: In the overweight patient, the prime nutritional emphasis should be achieving a leaner body condition. Weight control diets fortified with omega-3 fatty acids have been shown to be effective at reducing signs associated with both canine and feline Degenrative Joint Diseases (Lascelles, *et al.,* 2010; Corbee, *et al.,* 2013; Rialland, *et al.,* 2013).

2.2.6.4 Thermal Modification: In acute injury, including surgical areas, cold compression has a demonstrable benefit in reducing pain, inflammation, and return to function (Drygas, *et al.,* 2011). In the case of chronic injury, heat can improve comfort and function through a variety of mechanisms (Nadler, *et al.,* 2004).

2.2.6.5 Environmental Modifications: There is strong evidence that the stress of hospitalization inhibits normal behaviours in animals, including eating, grooming, sleeping, and elimination (Griffin and Hume, 2006). Fear, anxiety, stress, and distress lead to hyperalgesia in both humans and animals (Benedetti *et al.,* 2006; Khasar *et al.,* 2008; Martenson *et al.,* 2009; Stella *et al.,* 2013). Strategies to mitigate hyperalgesia, therefore, include providing bedding, blankets, or clothing from home with familiar scents; allowing visitation of hospitalized pets; separating the dogs from the cats; placing cages so that animals do not see each other; using species specific

synthetic pheromones; and proper handling, especially during procedures (Pageat and Gaultier, 2003; Kronen *et al.,* 2006; Kim *et al.,* 2010; Stella *et al.,* 2011; Ellis *et al.,* 2013).

2.2.6.6 Gentle Handling Techniques: The importance of gentle handling of patients with either acute or chronic pain cannot be overstated, especially when working with arthritic dogs and cats. Conscious avoidance of careless handling will ensure the patient's comfort, allow for a more thorough and effective physical exam, and avoid exacerbating anxiety in the agitated, fearful, or aggressive patient (Mark *et al.,* 2015).

2.2.7 Challenges due to the use of anaesthesia and analgesia in cattle and other food animals

Alpha (α)-2 agonists and opioids are not well suited for use on-farm due to the cost associated with these drugs and their sedative effects (Anderson and Muir, 2005; Hudson *et al.,* 2008). Therefore, most research has focused on local anaesthetics and NSAIDs for the relief of castration and dehorning in calves (Coetzee, 2011; Stock *et al.,* 2013; Stafford and Mellor, 2015). These were reportedly the best practice (Mellor and Stafford, 1999; Hudson *et al.,* 2008). However, the uptake and use of analgesic therapies for husbandry procedures in commercially produced livestock largely depends on their cost, ease of administration and time to effectiveness (Mellor and Stafford, 1999). In addition, the requirement for veterinary or expert supervision and registration of pharmaceuticals affect the uptake of analgesic treatments on-farm (Mellor *et al.,* 2008). Other reasons for their limited uses for pain relief include delivery of anaesthesia and analgesia being too time consuming, willingness of the general public to pay for the extra costs, challenges in assessing pain in animals, considerations for residues in food producing animals,

lack of long acting, cost-effective analgesic treatments for farm animals, and uncertainty on withdrawal periods for opioids and dissociative anaesthetics in farm animals (Cornish *et al.,* 2016).

For example, in Northern Australian beef systems, the extra costs and time required to administer conventional forms of pain relief to large numbers of cattle is a major hindrance to the adoption of such forms of analgesia. More so, these cattle are unaccustomed to human handling and hence, longer restraint times or doubling handling could pose additional animal welfare issues (Petherick, 2005). Topical anaesthetic (TA) gel and buccal meloxicam (BM) gel are practical forms of analgesia for use in such extensive settings; therefore there is a need to investigate their efficacy for castration and dehorning of calves. However, a form of multi-modal analgesia involving the combination of TA and BM is most effective will likely provide superior analgesia than TA or BM administered alone.

2.2.8 Pain assessment in animals

Pain assessment, recognition, and treatment have gained a great momentum in veterinary practice throughout the world. For instance, pain has been listed to be the fourth vital sign especially for human patients (Mathews, 2000) and different new approaches of pain management are being discovered and the myths and misconceptions have been debunked (Mathews *et al.,* 2014). Pain recognition and assessment in animals require long term training, veterinary students should therefore be taught the skills required to identify, assess and implement pain management strategies for clinical patients and animals used for demonstration of invasive procedures (Mathews, 2000). For field veterinarians, continuing education at the

local or provincial level ranked as the most important source of knowledge for recognition and control of pain. Various researches done to evaluate the use of pain medication in animals showed that most surgical and post traumatic patients are not treated for pain in small animal practice where pain management is thought to have been more advanced than the food animal practice (Mathews, 2000; Mathews *et al.,* 2014). Mathews, (2000) reported that only 13% to 26% of cats and dogs undergoing routine ovariohysterectomy or castration in Canada, Australia, and Britain received analgesics, and from 50% to 70% received analgesics for non-neutering soft tissue procedures. Some of the reasons given for withholding pain medications include pain recognition difficulty, that analgesics 'mask' physiologic indicators (e.g., heart rate, respiratory rate) of patient deterioration (e.g., hypotension, hypoxia) and that analgesics are potentially toxic, so there is fear of the adverse reactions associated with drug administration.

In order to successfully treat pain, it must be recognized and its presence confirmed in a patient. When pain persists for a long time without being identified and properly treated, it can be detrimental to the overall healing process as well as to the general well-being of the animal, leading to a prolonged hospital stay and increases the potential for secondary problems such as immune suppression and secondary illness, in-appetence and loss of body condition (Mathews, 2000). The outward signs of pain in animals often involve changes from what is normal which is further complicated by understanding what the normal baseline response is (Bufalari *et al.,* 2007)

2.2.9 Postoperative pain assessment

Post-surgical pain is a kind of acute pain that is poorly understood and poorly treated, and management of this type of pain is not limited to use of analgesics but also avoidance of external

noxious stimuli such as rigorous activities. Acute pain in animals must be well controlled to avoid it leading to chronic pain as it happens in human (Mathews, 2000). Studies have shown that animals experience both acute and chronic pain and discomfort following castration (Dalla Costa *et al.,* 2014).

Incision-induced pain in animals undergoing the routine procedure (castration, tail docking, dehorning) or scientific research poses serious animal welfare concern (Sutherland *et al.*, 2010). The term "animal welfare" used to refer to an animal's quality of life means animals should be relatively free from negative states, such as pain, fear, and distress when they are used for scientific research or during routine husbandry (Fenwick *et al.*, 2014).

Minimizing pain is one of the most important responsibilities of veterinary professionals. To try to do this, pain should ideally be measured effectively and accurately, but this is difficult to do because animals cannot self-report (Mathews *et al.,* 2014). Self-reporting and discussion of the pain experience represent the "gold standard" in the diagnosis of a painful condition. The ability to self-report actually facilitates pain treatment in most human patients (Rocca, *et al.*, 2015). For animals, as for humans who cannot self-report (e.g., neonates, paediatric patients and those with cognitive impairment), other assessment tools must be put in place (National Research Council, 2009) for assessment of painful conditions. Research into pain assessment in animals is ongoing. Investigators have attempted to assess pain in animals by methods including analysis of physiological variables (heart rate, respiratory rate), measurement of hormones (cortisol, adrenaline) (Bufalari *et al.,* 2007), and use of subjective pain scales (visual analogue scores, numerical rating scales) (Mathews *et al.,* 2014), analysis of electrophysiological parameters (electroencephalograms, evoked potentials) and behavioural analysis (Molony and Kent, 1997).

The challenges with utilizing subjective measures to determine painful conditions are related to potential differences both between observers and among observers over time (Theurer *et al.,* 2013; Humm and Kellett-Gregory, 2016).

Digital pressure palpation of the painful area is commonly used to aid assessment of pain, but this can only indicate the presence of pain, not the degree of pain experienced by the animal. Pain is a subjective experience, but objective data relating to that pain are extremely valuable in quantifying the degree of pain and the response to treatment (Jolliffe *et al.,* 2011).

The digital pressure palpation that is normally used in the clinical practice to locate and assess pain is difficult to quantify and standardize because of the different degrees of pressure applied by the same or different examiners as well as the different response from the patients (Haussler *et al.,* 2008).

According to the U.S. Food and Drug Administration (FDA) Guidance Document 123 for the development of effectiveness data for nonsteroidal anti-inflammatory drugs (NSAIDs), a validated method of pain assessment must be used in order for a drug to be indicated for pain relief in the target species (Mohling *et al.,* 2014).

2.3. Blood Glucose Concentration

Some of the effects surrounding surgery such as the tissue damage, fasting, blood loss, effects of the medication (anaesthesia), and changes in temperature from the metabolic view, give rise to stress response that is characterised by increased secretion of pituitary hormone and activation of sympathetic nervous system (Barton, 1987; Desborough and Hall, 1993). These changes lead to

increased catabolic activity through increased release of cortisol and glucagon which are catabolic hormones. These lead to increased neoglucogenesis and hyperglycemia. The incidences of hyperglycemia in both diabetic and non-diabetic patients during the major surgeries such as cardiac and orthopaedic cases have been reported with the condition higher in non-diabetic patients (Sudhakaran and Surani, 2015). Therefore, the level of this stress response may be quantified by the incidence of hyperglycemia or increased blood glucose concentration (Maitra *et al.,* 2013). The metabolic perturbation appears to be proportional to the severity of the surgical trauma with plasma cortisol and blood glucose rising slightly during minor surgery such as castration, but significantly during major intra-abdominal and orthopaedic surgeries (Thorell *et al.,* 1993).

2.4 Oxidative Stress in Surgical Patients

Oxidative stress is defined as an imbalance between the production of oxidants and respective defense systems of an organism (Lovrić *et al.,* 2008). Oxidants include reactive oxygen species (ROS), reactive nitrogen species (RNS), sulfur-centered radicals (SCR) and others. Not all of these reactive species are arene-radicals, (molecules with one (or more) unpaired electrons), but in many cases, the reactive non-radical species will end up as radicals and damage the biomolecules by process of (one-electron) oxidation. The danger of this type of reaction is that the oxidation products formed are radicals themselves, which are in many cases able to propagate the reaction, leading to extensive damage (Lovrić *et al.,* 2008). Oxidants can be produced in numerous ways, such as by ionizing radiation, by chemical reactions, enzymatically, through redox-catalysis involving free transition of metal ions, or metal ions bound to enzymes. Important cellular sources of oxidative stress include the formation of reactive oxygen species by

incomplete reduction of oxygen in the respiratory chain of mitochondria and host defense systems, which include the 'oxidative burst' (respiratory burst; the rapid release of reactive oxygen species from different types of cells) mediated by Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase, producing superoxide radical, and myeloperoxidase, leading to the formation of hypochlorous acid (Lovrić *et al.,* 2008).

Oxidative status evaluation in farm animals is important for monitoring animal welfare to verify whether the animal can maintain a homeostatic condition in spite of stressful environmental stimuli directed at elevated meat productivity. Two different stressful situations, namely physiological or pathological, can be considered. In the case of physiological stress, animals are able to develop an adaptive response that is expressed by activating endogenous antioxidant mechanisms, which can compensate for the imbalance of oxidative status (Meineri *et al.*, 2017). Conversely, under conditions of pathological stress, adaptive response of the organism is inadequate and leads to excessive production of free radicals, which results in oxidative stress. Excessive generation and/or inadequate removal of free radicals results in destructive and irreversible cell damage. Oxidative stress can be measured directly, by detecting free radical production, or indirectly, by detecting antioxidant defenses of the organism. Cellular antioxidant defenses consist of a complex interacting network, and more than 40 molecules are involved in the oxido-reduction metabolism (Tsimikas, 2008).

Evaluation of the oxidative plasmatic status (the level of serum reactive oxygen metabolites – ROMs–and the level of serum antioxidants) may have several implications for veterinary interest. For instance, negative situations within the farm could be identified, thus suggesting the most appropriate interventions to create optimal conditions for the animals (Meineri *et al.*, 2017).

In fact, in farm animals, oxidative stress is involved in a number of pathological conditions, including those associated with animal production, reproduction, normal farm husbandry practices such as castration, dehorning and general welfare (Pastorelli *et al.* 2010; Meineri *et al.*, 2017). Evaluating plasma oxidative levels in animals would therefore be very useful in considering the health status of animals.

A first line body defense against oxidative stress produced by the generation of free radical and reactive oxygen species (ROSs) are antioxidants which can be defined as any substance which delays or inhibits oxidative damage to a target molecule. Antioxidants are classified into enzymatic antioxidant and non enzymatic. Enzymatic antioxidants are enzymes capable of removing, neutralizing, or scavenging free radicals and oxy-intermediates (Scandalio, 1993). Examples are glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase. While the examples of non-enzymatic include cysteine, hydroquinones, mannitol, vitamins C and E, flavonoids, some alkaloids, and β-carotene, bilirubin and glutathione (Scandalio, 1993; Fatin *et al.*, 2010). Three of the primary antioxidant enzymes contained in mammalian cells that are thought to be necessary for life in all oxygen metabolizing cells are superoxide dismutase (SOD), catalase, and a substrate specific peroxidase, glutathione peroxidase (GPx) (Weydert and Cullen, 2010).

Oxidative stress has been shown to occur following castration in the acinar epithelium of the rat ventral prostrate, as evidenced from a marked increase in 8-hydroxy-2-deoxyguanosine and 4-hydroxynonenal protein adducts in the regressing epithelium as shown by Tam *et al.*, (2003). Quantification of steady-state mRNA levels of 14 genes involved in the anabolism and catabolism of reactive oxygen species (ROS), showed that castration resulted in dramatic

increases of three ROS-generating NAD(P)H oxidases (N_{OXS}) including $N_{OX}1$, $gp91^{phox}$ (Nox2) and $N_{OX}4$; significant reductions of key ROS-detoxifying enzymes (superoxide dismutase (SOD) 2, glutathione peroxidase and increased malondialdehyde (MDA), which is involved in lipid peroxidation (Afolabi *et al.*, 2013).

2.4.1 Superoxide dismutase (SOD)

There are three SOD enzymes that are highly compartmentalized. Manganese-containing superoxide dismutase (MnSOD) is localized in the mitochondria; copper- and zinc-containing superoxide dismutase (CuZnSOD) is located in the cytoplasm and nucleus and extracellular SOD (ECSOD) is expressed extracellularly in some tissues (Weydert and Cullen, 2010). These three independent enzymes are involved naturally in hastening superoxide dismutation, the Cu- and Zn-containing superoxide dismutases (Cu,Zn SODs), the Fe or Mn SODs that are specific for or function with either of the two (Fe-SODs, Mn-SODs or Fe/Mn-SODs), and the SODs that use Nickel (Ni-SODs) (Miller, 2004).

SOD was first isolated from bovine blood as a green copper protein (Mann and Keilin, 1938) whose biological function was believed to be copper storage (Scandalio, 1993). Over the years, the enzyme has been variably referred to as erythrocuprein, indophenol oxidase, and tetrazolium oxidase. The catalytic function of the enzyme was discovered by McCord and Fridovich (1969). The enzyme is ubiquitous, being widely distributed among oxygen-consuming organisms, aerotolerant anaerobes, and some obligate anaerobes (Fridovich, 1986). All SODs, irrespective of source, are multimeric metalloproteins that are very efficient at scavenging the superoxide radical. The Cu/ZnSODs, as well as most prokaryotic MnSODs and FeSODs, are dimeric,

whereas the MnSODs from mitochondria and certain thermophilic bacteria are tetrameric. SODs catalyze a disproportionate reaction at a rate very near that of diffusion.

2.4.2. Malondialdehyde (MDA)

Malondialdehyde (MDA) is a highly reactive three carbondialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation and also during arachidonic acid metabolism for the synthesis of prostaglandins (Marbut *et al.,* 2009). MDA can combine with several functional groups of molecules including proteins, lipoproteins, RNA and DNA. The monitoring of MDA levels in biological materials can be used as an important indicator of lipid peroxidation in-vitro and in-vivo for various diseases. MDA is the most frequently used biomarker of oxidative stress in many health problems (Khoubnasabjafari *et al.,* 2015).

MDA has been found elevated in various acute and chronic diseases thought to be related to free radical damage (Tug *et al.,* 2005; Marbut *et al.,* 2009), determination of this biomarker has been widely applied as the most common approach for the assessment of lipoperoxidation in biological and medical sciences. MDA is also widely used in food sciences as an index of lipid oxidation and rancidity in foods and food products (Mateos *et al.,* 2005). Malondialdehyde is an aldehyde considered to be the most important marker for monitoring lipid peroxidation and oxidative damage induced by ROS (Marbut *et al.,* 2009). MDA act as a marker for oxidative stress and glutathione as a marker of antioxidant (Marbut *et al.,* 2009; Khoubnasabjafari *et al.,* 2015).

2.4.3 Formation of MDA

Malondialdehyde is a by-product of lipid peroxidation, which is a mechanism of cellular injury in both plants and animals. It is therefore used as an indicator of oxidative stress in cells and tissues. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, including MDA. MDA can be generated during cyclooxygenase (COX) catalysis in human platelets, formation of prostaglandin endo-peroxide (PGH2), catalyzed by thromboxane synthase and in liver cells by the breakdown of PGH2 (Jetawattana, 2005).

All of these activated oxygen species are extremely reactive and cytotoxic in all organisms. These highly reactive species can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in the plasmalemma or intracellular organelles (Khoubnasabjafari *et al.,* 2015). Peroxidation damage of the plasmalemma leads to leakage of cellular contents, rapid desiccation, and cell death. Intracellular membrane damage can affect respiratory activity in mitochondria, cause pigment breakdown, and cause loss of carbon-fixing ability in chloroplasts (Scandalio, 1993).

2.5 Pressure Algometry

Algometry is the mechanical noxious stimulation method for the quantification of pain or subsequent hyperalgesia (Kaka *et al.,* 2015). Algometers are devices that can be used to identify the pressure and/or force eliciting a pressure-pain threshold (Kinser *et al.,* 2009). The creation and the use of algometer originated from the digital palpation of a painful part of the body. For instance, digital palpation of the distal forelimbs of Tennessee Walking Horses to detect soring

which is illegally practiced in an effort to produce a highly exaggerated gait of the fore and hindlimbs (i.e. 'big lick') that has become a highly desired trait in some Tennessee Walking Horses (Haussler *et al.,* 2008). The digital palpation, which is not only considered to be the "gold standard" but also the most common clinical method for evaluating pain has the main disadvantages which include lack of quantitative assessment of its results and lack of repeatability (Więckiewicz *et al.,* 2015). The alternative remains the use of pressure algometry technique. Algometer has been in use for a long time (Ko *et al.,* 2016).

Pressure algometry provides quantitative information on nociception, through mechanical stimulation and the data can be retaken (Franco *et al.,* 2014). Pressure algometry has been used to investigate analgesic strategies for surgical castration (mechanical nociceptive threshold testing (MNTT) in cattle (*Bos indicus*) (Musk *et al.,* 2015), mechanical nociceptive thresholds (MNTs) within the axial skeleton and thoracic limb of normal horses (Haussler *et al.,* 2008), to quantify hyperalgesia associated with claw disorders in dairy cattle (Raundal *et al.,* 2014). The use of algometer has been clinically validated both in animals and human (Pelfort *et al.,* 2007; Więckiewicz *et al.,* 2015; Chantelau, 2016). It is useful in quantifying pain (pressure pain threshold) in soft tissue associated with trigger points. Its use in measuring pressure pain threshold of some parts of the body such as the head, neck, shoulder (Ko *et al.,* 2016), knee and foot pain (Pelfort *et al.,* 2007), has been validated. Algometer has been used to make diagnosis in patients, who have an atypical history of reflex sympathetic dystrophy (RSD) (Bryan *et al.,* 1991).

A pain device such as algometer, must meet some requirements to be useful experimentally and clinically and these involve measurability, controllability, and reproducibility of the stimulus; an

adequate range from threshold to ceiling in the clear perception of pain; minimal tissue damage; convenience; and simplicity (Więckiewicz *et al.,* 2015). Others are the availability of applying the stimulus at a body point where individual neuro-histological variations are at a minimum and a method of quantification of stimulus responses over time, the reliability and the capability of using the device "without the end result being influenced by the experimenter (Rainwater and McNeil, 1991).

There are various styles of algometer device designed in an effort to produce a unit that is easy to use while still allowing an adequate method of quantification. Two mechanical solutions to this problem emerged. One applied constant pressure to the skin and counted the number of seconds that elapsed before pain threshold or tolerance. The other applied ever increasing weight to the skin and determined how much pressure was needed to reach pain threshold or tolerance (Rainwater and McNeil, 1991).

The algometers used in various studies involve applying pressure with a probe over the testing points on the animal body. These devices consist of a probe with tip or disc, which is applied perpendicular to the skin in gradual increasing force to produce noxious stimulus (Więckiewicz *et al.*, 2015). Force applied to the tip of the probe is transmitted to a load cell and a voltage output is produced. The signal is transduced and amplified and the output is displayed in newtons or kilograms (Kaka *et al.,* 2015). Mathews *et al* (2014) reported that experimental animal models of pain show that the animal tries to escape from a noxious stimulus of about the same intensity that causes human subjects to first report pain. This corresponds to the algometer reading at which an animal subject reacts to pain when tested with algometer which is called Pain Detection Threshold (PDT). This is defined as the minimum amount of pressure that produces pain in the

subject (Ylinen, 2007). Pain Tolerance Threshold is defined as the highest level of pain elicited by applying pressure the subject can tolerate before struggling to withdraw from the noxious stimulus (Padmaja *et al.,* 2016). Reported pressure application rates using algometer have ranged from 0.05 to 20 N/s., ideally, compression should be performed slowly enough to allow the subject time to react when pain is felt.

2.6 Grimace Scale

Pain recognition and management have continued to be a serious challenge to veterinarians and animal handlers. In order for pain to be properly managed, it has to be identified and quantified. So far there has been more work on objective methods of pain assessment; they include grimace scale for different species of animals (horse, rabbit, and rat). The advantages of analyzing facial expressions as an alternative or complementary method of pain assessment have been emphasized (Göransson, 2016).

Facial expression analysis has only recently been validated as a tool to evaluate pain in animals and this has led to the development of species-specific grimace scales. The first grimace scale was introduced for mice in 2010 (Langford *et al.*, 2010; Leach *et al.*, 2012), there have been scales developed for rats (Sotocinal *et al.,* 2011), rabbits (Keating *et al.,* 2012), horses (Dalla Costa *et al.,* 2014), sheep (McLennan *et al.*, 2016), lambs (Guesgen *et al.*, 2016) and pigs (Viscardi *et al.*, 2017). Grimace scales require identification of specific facial action units (FAUs) that change when animals are in pain, such as ear position, orbital tightening, and nose bulge. Rabbit grimace scale (RbtGS) has five FAUs and this scale has demonstrated high inter-observer reliability among participants, which suggests that they are accurate and easy to use

(Viscardi *et al.*, 2017). Their potential clinical application and ability to permit rapid pen-side detection of pain is of special interest in the objective method of pain assessment and evaluation.

2.6.1 The facial action units

The RbtGS consists of 5 facial action units (FAUs) that are independently scored. These action units include orbital tightening, cheek flattening, nostril shape, whisker shape and position, ear shape and position. Each is scored on a 3-point scale, and then the sum of all 5 FAUs can be analyzed (Langford *et al.*, 2010; Keating *et al.*, 2012).

2.6.1.1 Orbital tightening: This is seen as the closing of the eyelid (narrowing of the orbital area). A wrinkle may be visible around the eye

2.6.1.2 Cheek flattening: Here cheeks have a sunken appearance and the face become more angular and less rounded.

2.6.1.3 Nostril shape: The nares (nostrils) are drawn vertically forming a 'V' rather than 'U' shape. The tip of the nose is moved down towards the chin

2.6.1.4 Whisker shape and position: The whiskers are pushed away from the face to 'stand on end'. Whiskers stiffen and lose their natural downward curve. The whiskers increasingly point in the same direction when 'obviously present', whiskers move downwards

2.6.1.5 Ear shape and position: Ears become more tightly folded/curled (more cylindrical) in shape. Ears rotate from facing towards the source of sound to facing towards the hindquarters. Ears may be held closer to the back or sides of the body (Keating *et al.*, 2012).

The intensity ratings: Intensity ratings are coded for each AU and are presented as follows according to Langford *et al.,* (2010); AU is not present = 0; AU moderately present = 1; AU severe/ obviously present = 2.

Calculating Rbtgs Scores according to analyzed (Langford *et al.,* 2010);

1. Average all AUs for each photograph to give RbtGS score. Most of these preliminary calculations are easily carried out using Excel
2. After unblinding, the data is sorted by subject (e.g., "rabbit") then by condition. Note that "no pain" photographs are almost always baseline photographs taken prior to noxious stimulation
3. The mean RbtGS score is then calculated for all "no pain" and all "pain" photographs for each subject and subtract the mean for the "no pain" photographs from the mean for the "pain" photographs to give a RbtGS difference score for each subject
4. Difference scores can then be averaged across subjects to give a mean difference score, which can be analyzed for significance using a one-sample Student's t-test compared to 0

2.7 Ethno-Veterinary Medicine

Matekaire and Bwakura, (2004) defined ethno-veterinary medicine as the scientific term for traditional animal health care that encompasses the knowledge, skills, methods, practices, and beliefs about animal health care found among the members of a community. The knowledge base differs not only from region to region but also among and within communities. According to Neils *et al* (2008) ethno-veterinary medicine/practice (EVM) is the sum total of all practice methods, supplementary materials and attempts of any kind in which for years have enabled man

to protect his animals from sickness, to alleviate or relieve suffering so as to bring about healing and to increase animal production within minimum cost for improved human protein intake. It is also referred to as the method of healing animals traditionally using different species of plant, especially the roots, stems, leaves and grasses.

Generally, Ethno-medicine is a sub-field of ethnobotany or medical anthropology that deals with the study of traditional medicines: not only those that have relevant written sources (e.g., traditional Chinese medicine, Ayurveda) but especially those for which knowledge and practices have been orally transmitted over the centuries. The practice of ethnoveterinary medicine occurs due to the strong bonds shared between animals and their owners, as far back as when domestication of animals started; for the purpose of maintaining their health either as a source of food, security, work or companionship (Neils *et al.,* 2008).

Advantages of ethnoveterinary practice include the fact that they are natural products, cheaper than orthodox medicine with few side effects, easily available, highly efficacious with lower chance of the development of resistance to the products, they promote biodiversity and conservation and are environmentally friendly (Fajimi and Taiwo, 2005; Shrivastava *et al.,* 2017). This traditional method of healing is in danger of extinction due to the dearth of proper written documentation from generation to generation in most communities (Matekaire and Bwakura, 2004; Alhaji and Babalobi, 2015), through visions or dreams and to others by inheritance (Neils *et al.,* 2008). The ethnoveterinary practice involves the use of acupuncture, chiropractic, homeopathy, flower essences, raw diets, nutraceuticals (the use of concentrated doses of vitamins, minerals, and enzymes to treat disease), Chinese medicine, and herbs (Alhaji

and Babalobi, 2015). This practice is either for therapeutic or prophylactic purposes in animal diseases or husbandry (Alhaji and Babalobi, 2015).

2.8 Chenopodium ambrosioides L

2.8.1 Common names

C. ambrosioides L. (Plate 2.1), which is also known as *Dysphania ambrosioides*, is an herbal medicinal plant belonging to the Chenopodiaceae family that is native from Central and South America has been used as a traditional medicine in tropical and subtropical regions (da Silva *et al.*, 2014).



Plate 2.1. ***Chenopodium ambrosioides***

The common names are Mexican tea, Worm seed, Mexican gooseamerican, Gooxfoot (African Pharmacopoeia, 1985). It is popularly known as "*mastruz*" in Brazil (Sousa *et al.,* 2012), "Epazote" (which means strong odour), in Nigeria, the Yoruba in Southwest call it 'ewe imi' or "Arunpale" (meaning: leaf of excreta), the Ikulus in Kaduna State call it 'anyinyung' (meaning smelly), Hausas call it "kafikashiwari" (meaning: your ordour is worse than excreta), "Kwima" in Swahili, "Ambarusya" in Arabic, (Amole and Izegbu, 2005; Lohdip and Aguiyi, 2013).

2.8.2 Origin and distribution

C. ambrosioides is an aromatic herb used by Mexicans as cuisine (Sundaram *et al.,* 2010), belonging to the family of Chenopodiaceae (Florian *et al.,* 2013). *Chenopodium ambrosioides* Linn is an odoriferous herb indigenous to Mexico, tropical regions of Central and South America, with broad geographic distribution in Brazil (Sousa *et al.,* 2012), Pakistan (Sousa *et al.,* 2012) and Mexico (Carrillo-Lòpez *et al.,* 2016), and to most of the world (Lohdip and Aguiyi, 2013). The plant originated from America, ruderal in Mediterranean Africa and the Sahel region (Burkill, 1985). It is widely distributed in West Africa especially in Nigeria, Senegal, Ghana, and Cameroon (Burkill, 1985; Friis and Gilbert, 2000).

2.8.3 Botanical description of *C. ambrosioides*

C. ambrosioides Linn is small, polymorphic plant with a branched stem that can either be annual or perennial depending on the environmental condition or the geography of the place it is grown. The leaves are oval and toothed, up to 4 cm in length and 1cm in width which grows in

long spikes or clusters along its stems (Lohdip and Aguiyi, 2013). *Chenopodium ambrosioides* L. (Chenopodiaceae) is a strongly aromatic herb, about 1.2 m high, sometimes with a woody base. It is a common weed of cultivated areas, often in seasonally wet sites, found at an altitude of 950-2500 m above sea level (Friis and Gilbert, 2000).

2.8.4 Phytochemical properties of *C. ambrosioides*

Some chemical constituents reported to be present in the plant are terpenes, saponins, trimethylamine, urease and vanillic-acid (Burkill, 1985). Also present in the plant are ascaridole, limonene and geraniol ferulic acid (Dembitsky *et al.,* 2008; Lohdip *et al.,* 2013). Palmitic acid, stigmasterol, and sitosterol have also been reported to be present in this plant material (Lohdip, 2012; Lohdip *et al.,* 2013)**.** In a study by Eguale and Giday, (2009), *C. ambroisiodes* was reported to contain polyphenols, saponins, and glycosides (oligosaccharids) among various phytochemical tests including polyphenols, cyanogenic glycosides, saponins, phytosteroides and withanoides, phenolic glycosides, glycosides (oligosaccharids), flavonoids, tannins, alkaloides, antraquinone glycosides

2.8.5 The pharmacological properties of *C. ambrosioides*

The pharmacological properties of *C. ambrosioides* include cytotoxic (Nibret and Wink, 2011; Borges *et al.,* 2012; Sousa *et al.,* 2012) antitumor (Nascimento *et al.,* 2006) anxiolytic, antipyretic (Bum *et al.,* 2011) analgesic, anti-inflammatory (Ibironke and Ajiboye, 2007), immunostimulatory (Rossi-Bergmann *et al.,* 1997; Cruz *et al.,* 2007) molluscocidal (Hmamouchi *et al.,* 2000), antiviral (Zanon *et al.,* 1999) antibacterial (Sousa *et al.,* 2012) antifungal (Kishore

et al., 1993; Prasad *et al.,* 2010; Sousa *et al.,* 2012) trypanocidal (Nibret and Wink, 2011; Borges *et al.,* 2012) antiplasmodial (Pollack *et al.,* 1990) antileishmanial (Franca *et al.,* 1996; Monzote *et al.,* 2007; Monzote *et al.,* 2011) antischistosomal (Kamel *et al.,* 2011) and anthelmintic (Kliks, 1985; Giove, 1996) activities. The primary essential oil in *C. ambrosioides* is the monoterpene ascaridole, which is most highly concentrated in the seeds (Okuyama *et al.,* 1993; Sagrero-Nieves and Bartley, 1995). Other components include aterpinene, p-cymene (Monzote *et al.,* 2011) limonene, transpinocarveol, ascaridoleglycol, aritasone, b-pinene, myrcene, phelandrene, alcanphor, a-terpineol (Sagrero-Nieves and Bartley, 1995) and other monoterpenes (Ahmed, 2000; Kiuchi *et al.,* 2002). The essential oil of *C. ambrosioides* is an irritant to the mucous membranes of the gastrointestinal tract and promotes lesions in the kidneys and liver. Presumably at high doses, the essential oil of *C. ambrosioides* has been reported to cause human fatalities, especially in children (Levy, 1914; Paget, 1926; Franca *et al.,* 1996; Montoya-Cabrera *et al.,* 1996; Zanon *et al.,* 1999). Furthermore, the exposure of human lymphocytes to *C. ambrosioides* extracts *in vitro* has been shown to increase the frequency of chromosomal aberrations and to reduce the mitotic index (Gadano *et al.,* 2002) while hepatocellular carcinomas were found in Egyptian toad (Bufo regularis) dosed with *C. ambrosioides* oil for 3 months (el-Mofty *et al.,* 1992). Although *C. ambrosioides* exhibits anti-parasitic properties in both humans (Kliks, 1985; Giove, 1996) and animals (Lans and Turner, 2011), its use as an antiparasitic agent has been limited by its toxicity, which is promoted primarily by ascaridole, however, infusions of the plant have been shown to retain its parasiticidal activity (MacDonald *et al.,* 2004).

2.8.6 Effects of *C. ambrosioides* L. extract on bone repair

Studies have proved the anthelmintic, anti-inflammatory, and analgesic properties of *C. ambrosioides* (Kiuchi *et al.,* 2002; Kumar *et al.,* 2007). These bioactive compounds present in *C. ambrosioides* generally exhibit anti-inflammatory activities, which indicate its use for preventing osteoporosis (Kiuchi *et al.,* 2002; Kumar *et al.,* 2007; Trivellato-Grassi *et al.,* 2013). *Chenopodium ambrosioides* enhances bone regeneration (Neto *et al.,* 2017). It significantly increases cortical area of the femur in the ovariectomised rats while the bone marrow of treated rats was preserved showing high cellularity (Soares *et al.,* 2015). Pereira *et al*. (2010) also reported that treatment with *mastruz* at higher doses was able to stimulate cell proliferation in the bone marrow of animals.

Soares *et al.,* (2015) reported that the bone marrow of the animals treated with "*mastruz*" showed higher number of native bone marrow cells, whereas the adipocyte numbers were lower indicating that a possible mechanism of action of "*mastruz*" is the activation of osteogenesis and suppression of adipogenesis, which would imply a higher number of osteoblasts, and consequently greater bone formation.

It was found that treatment with the "*mastruz*" extract did not change serum levels of calcium, phosphorus, and magnesium suggesting that the mechanism of action of this herbal medicine is probably at the cellular level, with osteoblast activation and osteoclast inhibition rather than in the metabolism of minerals. However, it has been reported that flavonoids extracted from medicinal plants may assist in osteoblast activation by binding to the estrogen receptors in these cells (Soares *et al.,* 2015). The hydroalcoholic extract of *mastruz* has effects on bone metabolism

by changing blood proteins and enzymes and preventing both bone loss and the substitution of bone marrow cells by adipocytes in ovariectomized rats (Soares *et al.,* 2015).

Calado *et al.,* (2015) reported that *C. ambrosioides* extract was effective in the treatment of osteoarthritis by reducing synovial inflammation and behavioural changes due to pain, the effect which may be related to the antagonistic effect of ascaridole on the N-methyl-D-aspartate (NMDA) receptor.

2.8.7 Antimicrobial properties of *C. ambrosioides*

Rios *et al*. (2017) reported that treatment with *C. ambrosioides* decreases bacterial growth by activation of phagocytes and ameliorates the general state of mice by reducing the systemic inflammatory response in sepsis.

2.8.8 Anti-inflammatory and analgesic properties of *C. ambrosioides*

A study by Lohdip and Aguiyi, (2013) revealed that *C. ambrosioides* possessed not only antipyretic but also hypothermic activity. The anti-inflammatory activity of hexadec-12-enoic acid isolated from *C. ambrosioides* may be due to prostaglandin synthesis inhibition (Lohdip and Aguiyi, 2013). The leaves extract has been reported to have anti-inflammatory, analgesic and anti-pyretic effects against egg albumin -induced paw oedema, acetic acid and hot plate-induced pain, and 20 % aqueous suspension of yeast-induced pyrexia (Lohdip and Aguiyi, 2013).

2.8.9 Anti-helminthic properties of *C. ambrosioides*

The oil of *C. ambrosioides* had been used for many years to treat parasite infections in humans and animals, in different parts of the world, before the emergence of more effective and less toxic modern anthelmintics (Githiori, 2004). Dry powder resulted in a 33- 36% reduction in mixed nematode parasite infections in sheep (Kato, 1997) while short-term administration of oil or freshly ground plant material of *C. ambroisiodes* was ineffective in reducing adult *H. contortus* populations in goats, although oil of *C. ambroisiodes* at concentration of 3.3 µl/ml induced 100% egg hatch inhibition (Ketzis *et al.,* 2002). The aqueous and hydroalcoholic extracts of *C. ambrosioides* produced complete egg hatch inhibition at concentrations lower than 1 mg/ml (Eguale and Giday, 2009). Longterm treatment of animals in a given farm may reduce hatchability of eggs excreted in faeces resulting in both reduced re- infection and lighter worm loads by decreasing pasture contamination (Eguale and Giday, 2009).

Ketzis *et al*. (2002) reported ineffectiveness of this plant extract against *H. contortus* infection in goats. Ascaridole, a monoterpene essential oil is reported to be the principal active component found in the extract of *C. ambrosioides,* responsible for the anthelmintic activity. The other components include isoascaridole, p-cymene, limonene and α-terpinene. The level of the different compounds varies depending on the part of the plant collected and age of the plant **(**Anonymous 1**)**.

2.8.10 The effects of *C. ambrosioides* on some haematological parameters

The crude extracts of *C. ambrosioides* generally increased the RBC and the crude leaf extract might be useful in improving Hb content, while crude stem and root if employed for such

purpose; might have to be supported with a Hb improving substance (Lohdip *et al.,* 2013). The white blood cells (WBC) in the mice after administration of the extracts of *C. ambrosiodes* was found to increase compared to those of the control group and that the crude extracts, especially at 500mg/kg could be useful in boosting the body defence against foreign attacks which could include envenomation (Lohdip *et al.,* 2013). Furthermore, Lohdip *et al*. (2013) reported that the platelets count for the crude extracts as compared to the control revealed only a slight increase with the leaf extract and a decrease with the stem extract. The decrease in bleeding time and clotting time, as well as in platelets count by the plant extracts presents the plant as a possible remedy for the treatment or management of bleeding. On the other hand, the increase in both bleeding and clotting times by leaf (500 mg/kg) implies that at this dosage the extract could be useful in restoring haemorrhagic activities when treating coagulation in snakebite patient.

2.8.11. Other traditional or medicinal uses of *C. ambrosioides*

This plant is traditionally and widely used for its anti-parasitic, anti-inflammatory and antibiotic properties which efficacy has been scientifically proven (Sousa *et al.,* 2012). It has digestive effects (Florian *et al.,* 2013), antidiabetic properties, traditionally used in the treatment of dysmenorrhoea, uterine haemorrhage, and fibroids. The essential oil of *C. ambrosioides* is known to inhibit the growth of yeast species (Chekem *et al.,* 2010), dermatophytes (Prassad *et al.,* 2008) and other filamentous fungi (Jardim *et al.,* 2008) and the hexane extract of *C. ambrosioides* is known to inhibit the growth of filamentous fungi (Jardim *et al.,* 2010). The oil from the plant is known as Baltimore oil with insecticidal activities (Shah *et al.,* 2014). Alitonou *et al.,* (2012) reported that the leaves are used for the treatment of abscesses, epilepsy, vomit, cutaneous dermatoses, and rheumatisms articulars and neuralgias intercostales.

In West Africa, it is used traditionally for the treatment of sores, external swellings, pains, headache, coughs, tuberculosis, fever, and haemorrhoids. The plant is also reported to possess insecticidal, digestive, antimalarial, antihelminthic, sedative properties and is used for the treatment of snakebite (Lohdip *et al.,* 2013). The most common traditional form of use as a medicinal plant in Brazil is leaves blended with milk (Matos *et al.*, 2004). The therapeutic uses of *C. ambrosioides* include anthelmintic, (Kliks, 1985; Giove, 1996) abortifacient, emmenagogue, (Conway and Slocumb, 1979) sedative, antipyretic, and antirheumatic applications. It has also been used for the treatment of digestive, respiratory, urogenital, vascular, and nervous disorders, as well as diabetes, hypercholesterolemia (De Feo and Senatore, 1993) and sickle cell anemia (Ameh *et al.,* 2012). It is useful as an analgesic (Amole and Yusuf, 2002) and as a vermifuge to expel round-worms and hookworms (African Pharmacopoeia, 1985). It is also useful as a purgative (Burkill, 1985). Long-term use of *C. ambrosioides* Linn has been associated with various pathologies in the lungs, stomach and kidney tubules (Kuete, 2014).

2.8.12 Toxicity of *C. ambrosioides* L

Chenopodium ambrosioides oil has been reported to be toxic because it is an irritant to the mucous membranes of the gastrointestinal tract and it promotes lesions in the kidneys and liver (da Silva *et al.,* 2014). Several human fatalities have been attributed to the use of high doses of *C. ambrosioides* oil (MacDonald *et al.,* 2004). Carvacrol, caryophyllene oxide, and ascaridole, which are the major compounds of this plant, have been shown to inhibit the mitochondrial electron transport chain (da Silva *et al.,* 2014). Carvacrol and caryophyllene oxide have been reported to affect mitochondrial electron transport by directly inhibiting the electron transferring

complex I, whereas the effects of ascaridole have been shown to depend strongly on the availability of redoxactive Fe^{2+} (Monzote *et al.*, 2009).

It has been suggested that the use of *C. ambrosioides* infusions in traditional medicine, especially as a vermifuge, is safer than the use of the essential oil (MacDonald *et al.*, 2004). Ascaridole has been shown to relax rat gastrointestinal tissue contracted by carbachol *in vitro*, but *C. ambrosioides* infusion at nematocidal concentrations was reported to have no effect at these contractions (MacDonald *et al.*, 2004). The acute and chronic administration of an aqueous extract of *C. ambrosioides* leaves resulted in mild toxic effects in the liver and kidneys, which were associated with changes in serum biochemistry and histological abnormalities (da Silva *et al.*, 2014). However, the observed effects are far less toxic than those that have been reported with use of the essential oil (De Pascual *et al.*, 1980; Montoya-Cabrera *et al.*, 1996). Similarly, the administration of a hydroalcoholic extract from *C. ambrosioides* leaves to mice for 15 days led to reductions in the blood levels of albumin, Very Low Density Lipoprotein (VLDL) cholesterol, and triglycerides (Pereira *et al.*, 2010) effects that might result from disturbances in hepatocyte function. In a study by da Silva *et al.*, (2014) lowered serum ALT activities in rats treated with 1.0 g/kg of *ambrosioides* leaves for 15 days was attributed to an initial liver damage, that was histologically observed as cytoplasmic vacuolation of hepatocytes and mild hepatic congestion.

Amole and Izegbu, (2005) reported the plant to induce weight loss along with some pathological features like congestion of the lungs, metaplastic changes in the mucosal surface of the stomach, and necrosis of the kidney tubules.

2.9 Meloxicam

Meloxicam [4-hydroxy-2-mcthyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3 carboxamide-1,1 -dioxide; UHAC 62 XX, Boehringer Ingelheim], is an enolic acid class of non steroidal anti-inflammatory drug (NSAID) (Turck *et al.,* 1996). It has a good tolerability, particularly in the gastrointestinal tract (Distel and Bluhmki, 1995), and is believed to be due to its preferential inhibition of inducible cyclooxygenase-2 (COX-2, involved in inflammatory processes) over COX-1, which has a physiological function (Engelhardt, 1994). Meloxicam could be administered as an intravenous or intramuscular solution as well as by oral capsule, tablet, or rectally as a suppository (Turck *et al.,* 1996).

2.9.1 Meloxicam's mechanism of action

Meloxicam inhibits the synthesis of PG E2 by inhibiting the constitutive cyclo-oxygenase effects (EMEA, 1999). It has anti-inflammatory, anti-pyretic and analgesic properties and inducible cyclooxygenase (EMEA, 1999). Compared to other NSAIDs tested meloxicam was shown to be a most selective inhibitor of inducible cyclooxygenase activity (EMEA, 1999). The 3 main metabolites (5-hdroxymethyl-, 5 Carboxy- and Oxalyl-metabolite) of meloxicam found in rats and humans showed negligible activity when tested for anti-inflammatory activity and cyclooxygenase inhibition (EMEA, 1999).

2.9.2 Absorption of meloxicam

The absorption of meloxicam has been investigated following administration of intramuscular solutions, oral capsules or tablets, and rectal suppositories (Turck *et al.,* 1996). The absolute

bioavailability (F) was 89% (Turck *et al.,* 1995) for oral capsules after a single 30 mg dose. Maximum meloxicam plasma concentrations (Cmax) were achieved after 5-6 h (fmax) when administered after breakfast (Turck *et al.,* 1995). During chronic therapy for osteoarthritis or rheumatoid arthritis it is generally more common to administer NSAIDs after a meal, thus the *tmaxjs* value of 5-6 h for meloxicam is probably the clinically more relevant value (Turck *et al.,* 1996). Absorption after intramuscular injection is faster than oral administration, with Cmax occurring after 1-1.5 h (Narjes *et al.,* 1996).

2.9.3 Distribution of meloxicam

Most NSAIDs are highly protein bound to albumin (Lin *et al.,* 1987) and meloxicam is no exception, being >99% bound (Turck *et al.,* 1995). The binding is consistent with the concentration range encountered in clinical practice (Turck *et al.,* 1996). This high protein binding results in a restricted volume of distribution (Vd) of 10-15 (Degner *et al.,* 1994), which is similar to that reported for other NSAIDs (Verbeeck *et al.,* 1983). Animal experiments suggest that meloxicam is predominantly distributed to highly perfused (albumin rich) compartments such as the blood, liver, kidney, etc. (Busch, 1994). Inflamed tissue is characterized by extravasation and probably, decreased pH values, compared with non-inflamed tissue (Turck *et al.,* 1996). Such conditions are ideally suited to 'trap' an NSAID from the circulation. Indeed, high concentrations of meloxicam in inflamed tissue have been observed in animal models (Busch and Engelhardt, 1990).

2.9.4 Metabolism of meloxicam

Meloxicam is almost exclusively eliminated by metabolic degradation. The drug undergoes roughly equal renal and faecal elimination, with ≤0.25% excreted unchanged in urine and 1.6% of the parent compound present in the faeces. The latter may also represent a small amount of unabsorbed drug (Schmid *et al.,* 1994).

2.9.5 Elimination of meloxicam

Oral meloxicam has a total clearance of 0.42-0.48 1/h and an elimination half-life of ~20 h (Turck *et al.,* 1995). Steady-state plasma concentrations are achieved within 3-5 days. In common with most other NSAIDs, a considerable variation between subjects (inter individual coefficient of variation 30%) has been observed. In comparison with other NSAIDs of the same class, meloxicam has a relatively short elimination half-life of ~20 h (Turck *et al.,* 1996).

2.9.6 Interactions of meloxicam with other drugs

The concomitant administration of food, as well as other agents, is known to affect the absorption, as well as elimination, of NSAIDs (Turck *et al.,* 1996). Thus several pharmaco kinetic interaction trials involving meloxicam have been undertaken which demonstrated the lack of clinically relevant interactions with high-fat food (Turck *et al.,* 1995), P-acetyldigoxin (Degner *et al.,* 1995), methotrexate (Hubner *et al.,* 1994), cimetidine, antacids, acetylsalicylic acid (Turck *et al.,* 1996) or furosemide (Muller *et al.,* 1995). Recent data also indicate that there is no relevant interaction with warfarin (Turck *et al.,* 1996).

2.10 Ketoprofen

Ketoprofen 2-(3-benzolphenyl) is a propionic acid class with anti-inflammatory, analgesic and antipyretic properties (Rençber *et al.,* 2009). It is used in the treatment of a variety of acute and chronic inflammatory diseases and including rheumatoid arthritis, osteoarthritis, ankylosing spondylitis as well as menstrual abdominal cramps (Rençber *et al.,* 2009). Ketoprofen's oral, dermal, rectal or intravenous formulations are available (Rençber *et al.,* 2009). The average ketoprofen elimination half-life is 2-4 hours (Rençber *et al.,* 2009). It has a simple metabolism, and a broad therapeutic window, and does not show accumulation in the body following multiple administrations. It is metabolized in the liver and excreted in the urine and to a lesser extent in the faeces (Rençber *et al.,* 2009).

The use of topical non-steroidal anti-inflammatory drugs (NSAIDs) in relieving pain in patients with acute and chronic musculoskeletal disorders, including osteoarthritis (OA), tendonitis, and muscle strains is well established (Haroutiunian *et al.,* 2010). Topical formulations of NSAIDs have analgesic effects similar to that of oral formulations but are associated with less systemic exposure and, therefore, with fewer serious adverse events (AEs) (Altman and Barkin, 2009). The favourable benefit/risk ratio of topical NSAIDs has been further confirmed by a Cochrane meta-analysis of 47 randomized studies (Massey *et al.,* 2010).

Ketoprofen (KP), a widely used NSAID, is effective and well-tolerated in the treatment of acute and chronic pain, of both rheumatic and traumatic origin, as well as postoperative pain (Sarzi-Puttini *et al.,* 2010). The clinical experience with this drug is extensive (Sarzi-Puttini *et al.,* 2010), and a number of different formulations are available - including capsules, suppositories, injectable solutions, sustained release formulations, and a topical gel (Veys, 1991).

KP is a NSAIDs which belongs to the group of substituted 2-phenyl propionic acids [2-(3-benzoylphenyl) - propionic acid] (Coaccioli, 2011). It was first synthesized in 1967 at Rhone-Poulenc Research Laboratories, Paris, and was approved for clinical use as an oral formulation in France and the United Kingdom in 1973 (Coaccioli, 2011). The main indication for KP 2.5% gel is the local treatment of musculoskeletal pain and inflammation in muscles and joints (contusions, distortions, strains, stiff neck, and lumbago) (Coaccioli, 2011). The topical release of the active molecule is locally effective, and because of the low systemic bioavailability is associated with fewer adverse events (AEs) than with systemic formulations (Haroutiunian *et al.*, 2010). Importantly, KP should only be used on the intact skin **(**as single or repeated application) to avoid any irritant action on mucosa and scarred skin (Coaccioli, 2011).

2.10.1 Pharmacokinetics and pharmacodynamics of topical ketoprofen

The pharmacokinetic profile of topical ketoprofen (tKP) following its application has been extensively studied *in vitro* and *in vivo*. In one of the first studies, tKP was applied to the entire surface of the knee once a day for three days in patients scheduled for surgical intervention. KP concentrations in the plasma, synovial fluid and tissue were determined by HPLC9 KP was detected in the plasma 2 hours after the administration and its concentration remained constant for 12 hours (Coaccioli, 2011). Local tissue concentrations at the site of application were about 100-fold higher than the plasma concentrations (Coaccioli, 2011).

KP levels in the knee joint were considered to be a result of a direct transcutaneous diffusion, rather than plasma diffusion. In a 10-day study in 10 healthy volunteers, the total amount of KP in the urine following daily application of tKP was 2.6% of the initial dose applied and the

apparent half-life of unchanged KP was 17.1 ± 9.1 hours, and no accumulation was reported (Flouvat *et al.,* 1989). Another study, conducted to determine the concentration of KP in the synovial fluid following topical application, showed that KP concentrations were comparable with those following intramuscular administration (Parier, 1994).

In line with other NSAIDs, the pharmacodynamic activity of KP relies on the inhibition of the metabolism of arachidonic acid. KP is one of the most powerful inhibitors of cyclooxygenase (COX) (Carabaza *et al.,* 1996) and the inhibition of prostaglandins synthesis gives it its anti-inflammatory, analgesic, and antipyretic effects (Haroutiunian *et al.,* 2010). In addition, KP is characterized by other pharmacological effects that may be relevant to its anti-inflammatory and analgesic activities (Coaccioli, 2011). It is a powerful inhibitor of bradykinin (an important chemical mediator of pain and inflammation) (Julou *et al.,* 1976); it stabilises the lysosomal membranes against the osmotic damage (Migne *et al.,* 1976) and it prevents the release of lysosomal enzymes that mediate the tissue destruction in the inflammatory reactions (Kantor, 1986).

2.10.2 Efficacy of topical ketoprofen (tKP)

The efficacy of tKP gel has been studied in a series of controlled trials versus placebo or other analgesic drugs such as diclofenac or etofenamate, in a broad range of patients, with acute and chronic painful conditions (sport lesions, low back pain, tendonitis, osteoarthritis of the knee and hand, soft tissue rheumatic pain) (Coaccioli, 2011)..

2.10.3 Safety and tolerability of topical ketoprofen

Overall KP 2.5% gel is well tolerated (Avouac and Teule, 1988). Results of meta-analyses, conducted in a wide range of patients, consistently show that local and systemic adverse events associated with the administration of tKP are rare and in most cases mild (Moore *et al.,* 1998; Mason *et al.,* 2004; Massey *et al.,* 2010). In a double-blind, placebo controlled study no adverse effects were observed over a 7- day period (Odaglia, 1987). These findings are consistent with those reported in other trials, in which the application of tKP was not associated with any local or systemic adverse effects (Matucci-Cerinic and Casini, 1988). Drug interactions are not to be expected when using the topical route, and no cases of over dosage have been reported (Coaccioli, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Location of the Research

The study was carried out in the Department of Veterinary Surgery and Radiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, North-Western Nigeria with geographical coordinates of 11^0 4'0'' North and 7^0 42'0'' East (A.B.U., 2000).

3.2. Matrials

3.2.1 Exprimental animals

A total of 15 sexually matured rabbits between 4 to 6 months of age were used for this study. They were acquired from the National Animal Production Research Institute (NAPRI), Shika. The mean weight of the bucks was 2.5 ± 0.7 kg. The animals were kept for two weeks before the surgery for pre-surgical acclimatization, during this period the bucks were treated for mange (diagnosed upon arrival), different degree of superficial injuries, worm infestation and coccidiosis as a precaution (Wright-Williams *et al.*, 2013). Three rabbits were housed per cell upon their arrival. The rabbits were made to be accustomed to daily routine activities of the animal handlers such as cleaning, feeding, weighing, video-taping, presence of observers and the video monitoring equipment (Keating *et al.*, 2012). The animals were fed with pelletised feed (vital feed®) twice daily and given water *ad libitum.*

3.2.2 Equipment and instruments

The equipment and instrument used were surgical packs, drapes, surgical tables, working table stands, surgical lamps, waste trolleys and pans, thermometer, micro haematocrit bottles and micro haematocrit reader, clear transparent rabbit cages, High definition cameras, basket, drug pipette, active glucometer, active glucose strip and pressure algometer.

Drugs/medications: The drugs and medications used were fastum gel (ketoprofen 2.5%), meloxicam tablets, chlorpromazine and ivermectin injection.

3.2.3 Consumables

The consumables used were surgical blades, surgical gloves, gauze, chlorhexidine, porvidone iodine, syringes and needles, cotton wool, sample bottles, razor blades, pelletized rabbit feed (vital feed®), washing soap and methylated spirit.

3.3 Methodology

Phase I

3.3.1 Plant material and extraction

The plant of *C. ambrosioides* was collected from Ago, Asa Local Government Area, Kwara State. The plant was identified at the Herbarium of the Department of Biological Sciences of Ahmadu Bello University, Zaria and a Voucher Specimen Number (1725) was obtained. The plant was cleaned and dried under shade. It was then reduced to powder with mortar and pestle

and kept in a cool, dry place until it was used for extraction for further study as described by Ior *et al.* (2012). Dried powder of the plant sample was extracted with 75% ethanol kept on rotary shaker for 24 h. Thereafter, it was filtered through Whatmann No 1 filter paper and centrifuged at 5000G for 15 min. The extract was stored at 4° C in air tight bottles as described by Prashanthi *et al.* (2012). Fifty grams (50g) of each extract was dissolved in 100ml of distilled water (500mg/ml) for use.

3.3.2 Determination of acute oral toxicity

The LD_{50} of the ethanol extract of the *C. ambrosioides* was determined according to Lorke (1983)'s method described by Imafidon *et al.* (2016). The procedure was conducted in two phases. In the first phase, nine wistar rats were divided into three groups of three rats per group. Each group of rats were administered different doses 10, 100 and 1000 mg of ethanol extract of *C. ambrosioides* per kg body weight of the rats orally using cannula. All the rats were kept under the same conditions and monitored for signs of toxicity and mortality for 24 h. In the second phase, a total of three wistar rats were used. The rats were divided into three groups of one rat each and were administered 1600, 2900 and 5000 mg/kg per os of the ethanol extract of *C. ambrosioides*. They were also observed for signs of toxicity and mortality for 24 h.

Phase II

3.4 Pre-surgical Evaluation and Surgical Procedure

3.4.1 Restraint/anaesthesia

The lower abdomen along with the scrotal area was shaved liberally and then scrubbed with a mild antiseptic (diluted chlorhexidine) solution and povidone iodine. Each rabbit was placed on a dorsal recumbency on the surgical table and the limbs were secured with ropes. A triangular draping using 3 surgical drapes that were secured with towel clamps. The castration was carried out on each rabbits following a standard surgical procedure under mild sedation with chlorpromazine (0.3 mg/kg).

3.4.2 Surgical technique

Prescrotal approach was used for castration in this study (Murray, 2006). For the prescrotal approach, a single ventral midline incision located cranial to the scrotum was made to expose the vaginal tunics one at a time. Once the tunic was incised, the spermatic cord was visualised and grasped, and with gentle traction, the testicle and epididymis were exposed. The spermatic cord was doubly ligated along with the blood vessels and transected (Murray, 2006). In order to prevent postoperative herniation, the vaginal tunic was ligated as far cranially as possible. The skin was then closed with subcuticular sutures.

3.4.3 Animal grouping

This comprised of 5 groups with 3 rabbits per group (Table 3.1). Rabbits in groups A, B and C were castrated (orchidectomised) and treated with normal saline, oral meloxicam tablet (0.1mg/Kg) and oral plant extract (500mg/Kg) respectively. Blood sample for glucometry was taken immediately after surgery and just before treatment, then at 15, 30 and 60 minutes after treatment. Blood sample for oxidative stress indices was collected 1hour, 2 hours and 3 hours following treatment. Grimace scale evaluation was done 1 hour post treatment for twenty minutes according to Langford *et al.* (2010); this was then followed with pain evaluation using algometer around the surgical wound. The pain evaluation with algometer was performed at 1 hr, 2 hr, 3 hr following treatment on the day of surgery (day 0) and repeated on day 1 post surgery. The rabbits in groups D and E were castrated (orchidectomised) and treated with Fastum gel® topically (Ketoprofen 2.5%) and the plant extract. They were then evaluated for pain as done in phase 1. The data were collected for evaluation.

Table 3.1: Summary table of the methodology

	Animal (N0.)	Group (N0.)	N0. of Animals in each subgroups	Treatment	Dosage (mg/Kg)	Route of administration
Phase 1	Wistar Rats (12)	Group 1 (9)	3	E.E. Ch	10	Oral
(Toxicity studies)			3	E.E. Ch	100	Oral
			3	E.E. Ch	1,000	Oral
		Group 2 (3)	1	E.E. Ch	1,600	Oral
			1	E.E. Ch	2,900	Oral
			1	E.E. Ch	5,000	Oral
Phase 2	Castrated bucks (15)	A (3)	-	Normal saline	Liberal	Oral
(Analgesic evaluation)		B (3)	-	Meloxicam tablet	0.1	Oral
		C (3)	-	E.E. Ch	500	Oral
		D (3)	-	Ketoprofen 2.5%	Liberal	Topical
		E (3)	-	E.E. Ch	Liberal	Topical

Key:

E.E. Ch: ethanol extract of *C. Ambrosioides*

N0. Number

3.5 Determination of Blood Glucose Concentration and the Oxidative Stress Makers

3.5.1 Determination of blood glucose concentration

The glucometer was switched on by pressing the power button. The test strip was inserted correctly into its slot. A drop of blood was added to the strip on the coloured spot containing the reagent (Anonymous 3). The blood spread down by capillary action. While the blood glucose concentration appeared at the average of about 5 seconds in mg/dl (Anonymous 3)

3.5.2 The assessment of plasma malondialdehyde (MDA)

3.5.2.1 Methods: The assessment of MDA was carried out according to Okhawa *et al.*, (1979) as described by Atawodi *et al.* (2011) using trichloroacetic acid (TCA) and thiobarbituric acid (TBA). Two milliliter (2ml) of thiobabitutric acid (TBA) was added to 2ml of 15% trichloroacetic (TCA) acid inside a test tube, and 100µl of plasma was added. The mixture was incubated at 80°C for 30 minutes in a water bath and allowed to cool for some time followed by centrifugation at 3000 rpm for 10 mins. A clear supernatant was collected and the absorbance was determined at 535 nm on a spectrophotometer.

3.5.2.2 Calculations: Molar extinction coefficient is 1.56×10^5 $cm^{-1}M^{-1}$

$$\text{MDA Concentration} = \frac{\text{Absorbance of sample}}{1.56 \times 10^5 cm^{-1}M^{-1}}$$

3.5.3 Determination of superoxide dismutase activity (SOD)

*3.5.3.1 Principle***:** The rate of autooxidation of epinephrine and the sensitivity of this autooxidation to inhibition by superoxide dismutase are "both augmented, as the pH is raised from 7.8 to 10.2. The autooxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving 0_2 and hence inhibitable by superoxide dismutase. This chain reaction accounted for progressively greater fraction of the total oxidation as the pH was raised. The ability of superoxide dismutase to inhibit the autooxidation of epinephrine at pH 10.2 has been used as the basis for this enzyme assay. The oxidation of epinephrine was followed in terms of the production of adenochrome, which exhibits an absorption maximum at 450nm (Fridovich, 1989).

*3.5.3.2 Reagents***:**

Sodium carbonate - bicarbonate buffer (0.05M, pH10.2). Prepared by dissolving 0.53g of sodium carbonate and 0.42g of sodium bicarbonate in 100ml distilled water. Ethylene diamine tetra acetic acid [EDTA]. Prepared by dissolving 4mg in 100ml of distilled water [4mg %]. Epinephrine. Prepared by dissolving 0.55 gm of epinephrine in 100ml of 0.1 N HCl. It is important to note that epinephrine is stable in acidic pH, hence its pH must be 2.0**.**

3.5.3.3 Methods*:* Superoxide dismutase (SOD) activity was determined by the method described by Fridovich (1989). 0.1 ml of supernatant was diluted in 0.9 ml of distilled water to make 1:10 dilution of serum. An aliquot mixture of 0.20 ml of the diluted serum was added to 2.5 ml of 0.05 M carbonate buffer. The reaction was started with the addition of 0.3 ml of 0.3 mM adrenaline. The reference mixture contains 2.5 ml of 0.05 M carbonate buffer. 0.3 m1 of 0.3 mM

adrenaline and 0.20 ml of distilled water. Absorbance was measured over 30 up to 150 s at 480 nm.

3.5.3.4 Calculations:

Increase in absorbance per minute = $\frac{(A2-A1)}{2.5}$

% inhibition = $\mathbf{100} - \frac{\textbf{increase in absorbance for sample}}{\textbf{increase in absorbance of blank}} \times \mathbf{100}$

A1 is absorbance at 30s and A2 is absorbance at 150s

1 unit of SOD activity is the quantity of SOD necessary to elicit 50 % inhibition of the oxidation of adrenaline to adrenochrome in one minute.

3.5.4 Assay of decreased glutathione peroxidase activity (GPx)

3.5.4.1 Reagents:

Precipitating solutions: It was prepared by dissolving 1.67 gm of glacial metaphosphoric acid and 0.2gm EDTA and 30gm of NaCI in 100ml of distilled water. Phosphate solution: It was prepared by dissolving 0.3M Na2HP04 (disodium hydrogen phosphate) in distilled water. Ellman's reagent: Prepared by dissolving 20 mg DTNB in 100ml of 1% sodium citrate. Sodium Citrate solution: 1% solution of Sodium Citrate in distilled water.

3.5.4.2 Methods: Reduced glutathione peroxidase (GPx) activity measurements were done according to ElIman (1959) as described by Rajagopalan *et al.* (2004). To 150 µl of plasma (in phosphate saline pH 7.4), 1.5 m1 of 10 % TCA was added and centrifuged at 1500 rpm for 5

min. 1 ml of supernatant was treated with 0.5 ml of ElIman's reagent and 3 ml of phosphate buffer (0.2 M, pH 8). The absorbance was read at 412 nm.

3.6 Pressure Algometry

The pain evaluation with algometer was performed at 1 hr, 2 hr, 3 hr following treatment on the day of surgery (day 0) and repeated on day 1 post surgery. Pain detection threshold was measured using pressure algometer 60 minutes post-surgery before treatment. This involved applying pressure with a probe over the testing points on the animal body, in this case, around the surgical wound. The device was applied perpendicular to the skin in gradual increasing force to produce noxious stimulus. Force applied to the tip of the probe was transmitted to a load cell and a voltage output was produced (Więckiewicz *et al.,* 2015).

3.7 Rabbit Grimace Scale

Following surgery, each rabbit was treated and kept in its cage. At 1 hour post-treatment, rabbits were video-taped in a clear transparent 6″x8″x6″ dimension cage for 20 minutes (Langford *et al.*, 2010) using 2 High definition video camera (Plate 3.1).

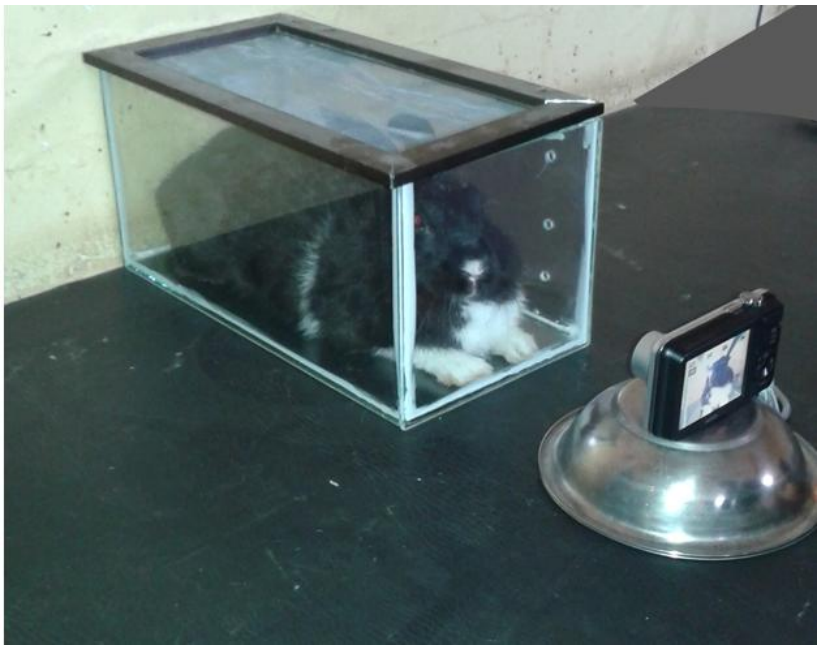


Plate 3.1Video recording of a rabbit in a clear transparent 6″x8″x6″ dimension cage

The cameras were placed at fixed distances from the front and back of the cage. This arrangement gave the highest probability of capturing the face of the rabbit during filming (Keating *et al.*, 2012).

The method described by Keating *et al.* (2012) for Rabbit Grimace Scale was used. From each video sequence of the rabbits' head and face, still images were extracted, enabling generation of a number of clear and high quality images of each rabbit before and during each treatment. Each image was cropped so that only the head and not the body of the rabbit were visible. This prevented the observers from being biased by the body of the animal, when attempting to score facial expressions as described by Langford *et al.* (2010). Images taken before and during each procedure were compared to identify changes in facial expressions (Appendix 4) associated with these procedures by the treatment-blind participants for the scoring of the images. This included animal handlers, nurse, clinicians, post graduate students and 600L clinical students in the Faculty of Veterinary Medicine, ABU Zaria.

3.8 Data Analysis.

All data were expressed as means ± SEM (standard error of mean). One way and two ways ANOVA were used to analyze different data as appropriate. Multiple pare-wise comparisons were performed by one-way ANOVA Kruskal-Walis, followed by post-test Dunn's. P-Values ($P \leq 0.05$) were considered as statistically significant. Statistical analyses were performed using the Graphpad prism 5.0 for sciences software (Graph Pad, 2000).

CHAPTER FOUR

4.0 RESULTS

4.1 Preliminary Phytochemical Studies

The preliminary phytochemical studies (qualitative analysis) on the leaves of *C. ambrosioides* ethanol extract revealed that the plant contained carbohydrates, glycoside, saponins, steroids, triterpenes, flavonoids, alkaloids and tannins (Table 4.1).

4.1.1 Determnation of acute oral toxicity

None of the rats showed any sign of toxicity or die after 24 hrs of administration at the dosages used.

Table 4.1 Phytochemical constituents of *C. ambrosioides*

S/N	Chemical Test	*C. ambrosioides*
1	Carbohydrates	
	Molisch's test	+
	Fehling's test	+
2	Cardiac glycoside	
	Kella-killiani test	+
	Kedde's test	+
3	Free Anthraquinones	
	Borntrager's test	-
	Modified Borntrager's test	-
4	Combined Anthracene	
	Modified Borntrager's test	-
5	Saponins	
	Frothing test	+
	Haemolysis test	-
6	Steroids and Triterpenes	
	Leiberman-Burchards test	+
	Salkowskis Test	+
7	Flavonoids	
	Shinoda test	+
	Ferric chloride test	+
	Sodium hydroxide test	+
8	Tannins	
	Lead sub-acetate test	+
	Ferric chloride test	+
	Bromine water	-
9.	Alkaloids	
	Mayer's test	-
	Dragendorff's test	+
	Wagners Test	-

Key: +: Present

- : Absent

4.2. Evaluation of the Ameliorative Effect of *C. ambrosioides* on Blood Glucose Concentration (mg/dL) due to the Castration-induced Pain in Rabbits

The blood glucose concentration (mg/dL) of the castrated rabbits treated with meloxicam and *C. ambrosioides* was presented in Figure 4.1, Appendix 1a; and 1b. The blood glucose concentration for rabbits treated with oral meloxicam and topical ketoprofen immediately after the surgery were 251.67 ± 18.00 and 259.67 ± 18.85 mg/dL, respectively while that of oral and topical administration of *C. ambrosioides* extracts to rabbits were 242.67 ± 1.86 and 262.33 ± 13.87 mg/dL, respectively. Rabbits treated with normal saline had the glucose concentration of 270 ± 14.84 mg/dL. The blood glucose concentration in rabbits treated with oral meloxicam and topical ketoprofen after 15 min of treatment were 147.33 ± 13.78 and 146.67 ± 10.09 mg/dL, respectively while that of oral and topical administration of *C. ambrosioides* extracts to rabbits were 137.67 ± 3.38 and 184.33 ± 31.87 mg/dL, respectively. Rabbits treated with normal saline had the glucose concentration of 267.67 ± 18.77 mg/dL. The blood glucose concentration in rabbits treated with oral meloxicam and topical ketoprofen after 30 min of treatment were 126.33 ± 3.18 and 131.67 ± 5.21 mg/dL, respectively while that of oral and topical administration of *C. ambrosioides* extracts were 131 ± 3.06 and 149.33 ± 23.22 mg/dL, respectively. Rabbits treated with normal saline had the glucose concentration of 156.67 ± 20.00 mg/dL. After 60 minutes of trearment, the plasma glucose concentration in the rabbits treated with oral meloxicam and topical ketoprofen were 120 ± 0.67 and 146.67 ± 2.03 mg/dL, respectively while the values of 120 ± 0.67 and 133 ± 16.62 mg/dL were observed for the oral and topical treatment with the *C. ambrosioides* extracts. Rabbits treated with normal saline had the glucose concentration of 108 ± 15.72 mg/dL (Figure 4.1).

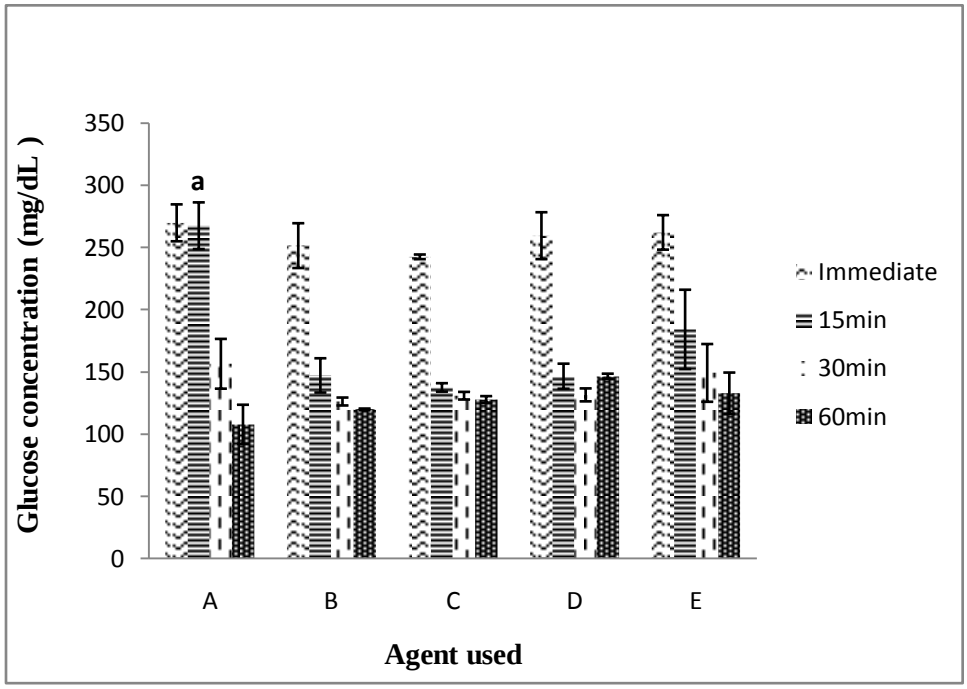


Figure 4.1. The mean and standard error of mean for blood glucose concentration (mg/dL) of the castrated rabbits treated with different agents.

[a]Means in the same column with different superscript alphabet was significantly different ($p \leq 0.05$).

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical)

4.3 Evaluation of the Ameliorative Effect of *C. ambrosioides* on Castration-induced Oxidative Stress in Rabbits

4.3.1 Effects of Meloxicam and *C. ambrosioides* on the castration-induced increase in plasma malondialdhyde (MDA) concentration

The plasma MDA concentration (µmol/L) of the castrated rabbits treated with meloxicam and *C. ambrosioides* was presented in Figure 4.2; Appendix 1c and 1d. The plasma MDA concentrations in the rabbits treated with oral meloxicam and topical ketoprofen at 1 hr after the surgery were 18381 ± 11236 and 3218 ± 340 µmol/L, respectively while the values of 8280 ± 551 and 3731 ± 419 µmol/L were observed for the oral and topical treatment with the *C. ambrosioides* extracts, respectively. Rabbits treated with normal saline had the MDA concentration of 5837 ± 2615 µmol/L. The plasma MDA concentrations in the rabbits treated with oral meloxicam and topical ketoprofen at 2 hrs after the surgery were 7332 ± 732 and 3044 ± 212 µmol/L, respectively while the values of 18505 ± 4170 and 3313 ± 155 µmol/L were observed for the oral and topical treatment with the *C. ambrosioides* extracts, respectively. Rabbits treated with normal saline had the MDA concentration of 44696 ± 19071 µmol/L. After 3hrs, the plasma MDA concentration in the rabbits treated with oral meloxicam and topical ketoprofen were 16711 ± 1866 and 4414 ± 1364 µmol/L, respectively while the values of 20128 ± 14686 and 3105 ± 95 µmol/L were observed for the oral and topical treatment with the *C. ambrosioides* extracts, respectively. Rabbits treated with normal saline had the plasma MDA concentration of 18323 ± 5959 µmol/L.

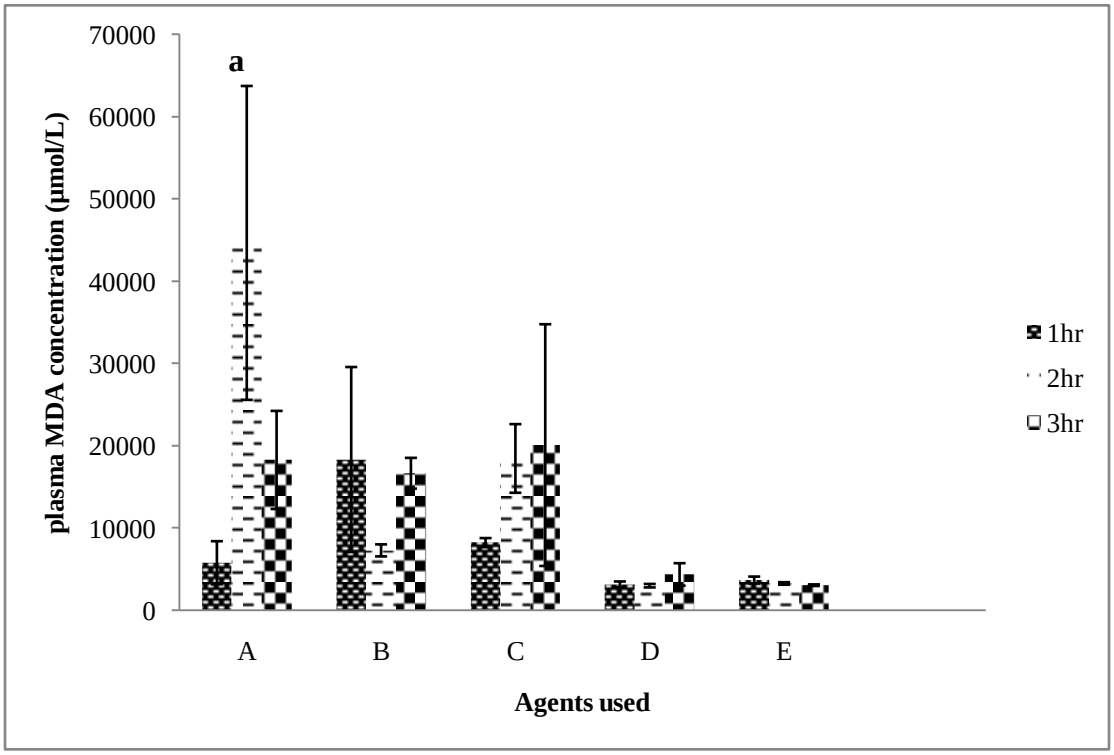


Figure 4.2. The mean and standard error of mean for plasma malondialdhyde (MDA) concentration (µmol/L) of the castrated rabbits treated with different agents.

[a]Means in the same column with different superscripts alphabets were significantly different ($p \leq 0.05$).

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); F: Baseline data before surgery

4.3.2 Effects of Meloxicam and *C. ambrosioides* on castration-induced decrease in plasma superoxide dismutase (SOD) activity

The plasma SOD activities (mg/mL) of the castrated rabbits treated with meloxicam and *C. ambrosioides* were presented in Figure 4.3; Appendix 1e and 1f. The plasma SOD activities in the rabbits treated with oral meloxicam and topical ketoprofen at 1 hr after the surgery were 13 ± 6.9 and 2.8 ± 1.1 mg/mL, respectively while the values of 19 ± 4.3 and 5.8 ± 0.39 mg/mL were observed for the oral and topical treatment with the *C. ambrosioides* extracts, respectively. Rabbits treated with normal saline had the SOD activity of 15 ± 7.3 mg/mL.

The plasma SOD activities in the rabbits treated with oral meloxicam and topical ketoprofen at 2 hrs after the surgery were 14 ± 6.0 and 2.2 ± 1.0 mg/mL respectively, while the values of 22 ± 5.7 and 4.8 ± 1.1 mg/mL were observed for the oral and topical treatment with the *C. ambrosioides* extracts, respectively. Rabbits treated with normal saline had the SOD concentration of 18 ± 8.4 mg/mL.

After 3hrs, the plasma SOD activities in the rabbits treated with oral meloxicam and topical ketoprofen were 22 ± 5.8 and 4.1 ± 0.78 mg/mL, respectively while the values of 8.9 ± 4.3 and 4.4 ± 1.3 mg/mL were observed for the oral and topical treatment with the *C. ambrosioides* extracts respectively. Rabbits treated with normal saline had the plasma SOD activity of 14 ± 5.3 mg/mL 3hrs after the surgery.

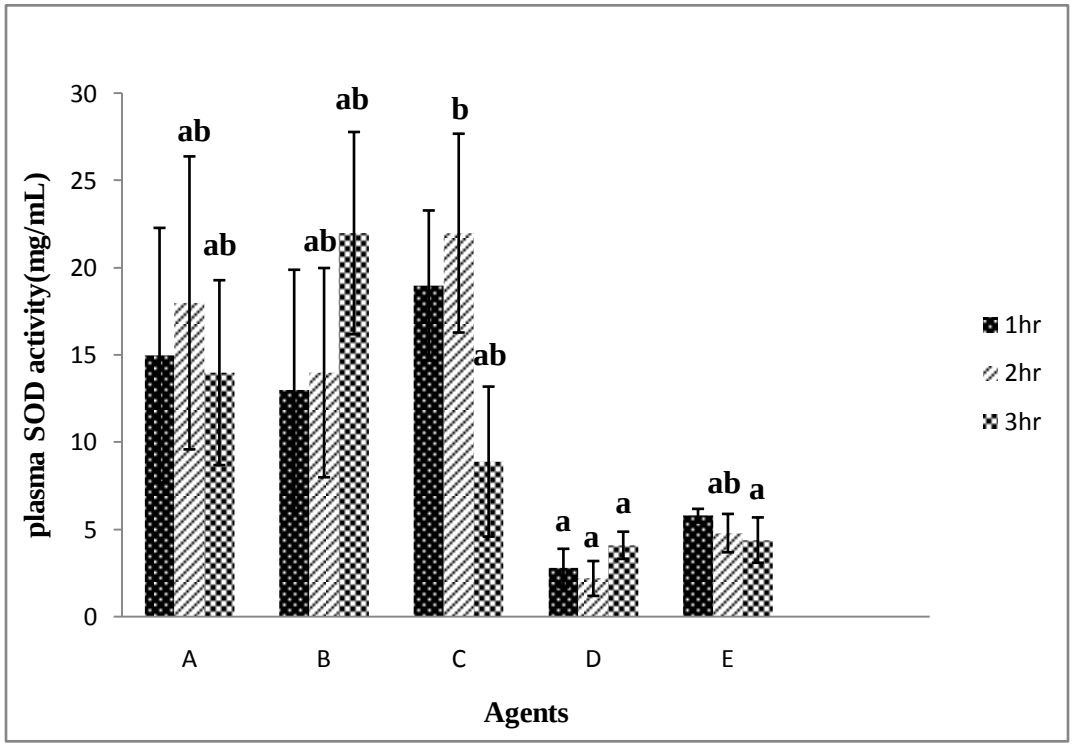


Figure 4.3. The mean and standard error of mean for plasma superoxide dismutase (SOD) activity (mg/mL) of the castrated rabbits treated with different agents.

[ab]Means in the same column with different superscripts alphabets were significantly different ($p \leq 0.05$).

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); F: Baseline data before surgery

4.3.3 Effects of Meloxicam and *C. ambrosioides* on the castration-induced decrease in plasma glutathione peroxidase (GPx) activity

The plasma GSH activities (Ug/ml) of the castrated rabbits treated with meloxicam and *C. ambrosioides* were presented in the Figure 4.4; Appendix 1g and 1h . The plasma GSH activities in the rabbits treated with oral meloxicam and topical ketoprofen at 1 hour after the surgery were 80 ± 34 and 63 ± 11 Ug/ml, respectively while the value of 48 ± 3.5 and 93 ± 49 Ug/ml were observed for the oral and topical treatment with the *C. ambrosioides* extracts, respectively. Rabbits treated with normal saline had the GSH activities of 86 ± 41 Ug/ml.

The plasma GSH activities in the rabbits treated with oral meloxicam and topical ketoprofen at 2 hrs after the surgery were 51 ± 0.5 and 85 ± 37 Ug/ml, respectively while the values of 96 ± 33 and 20 ± 2.7 Ug/ml were observed for the oral and topical treatment with the *C. ambrosioides* extracts, respectively. Rabbits treated with normal saline had the GSH activities of 138 ± 47 Ug/ml.

After 3hrs, the plasma GSH activities in the rabbits treated with oral meloxicam and topical ketoprofen were 92 ± 12 and 59 ± 20 Ug/ml, respectively while the value of 77 ± 42 and 100 ± 28 Ug/ml were observed for the oral and topical treatment with the *C. ambrosioides* extracts, respectively. Rabbits treated with normal saline had the plasma GSH activities of 121 ± 42 Ug/ml 3hrs.

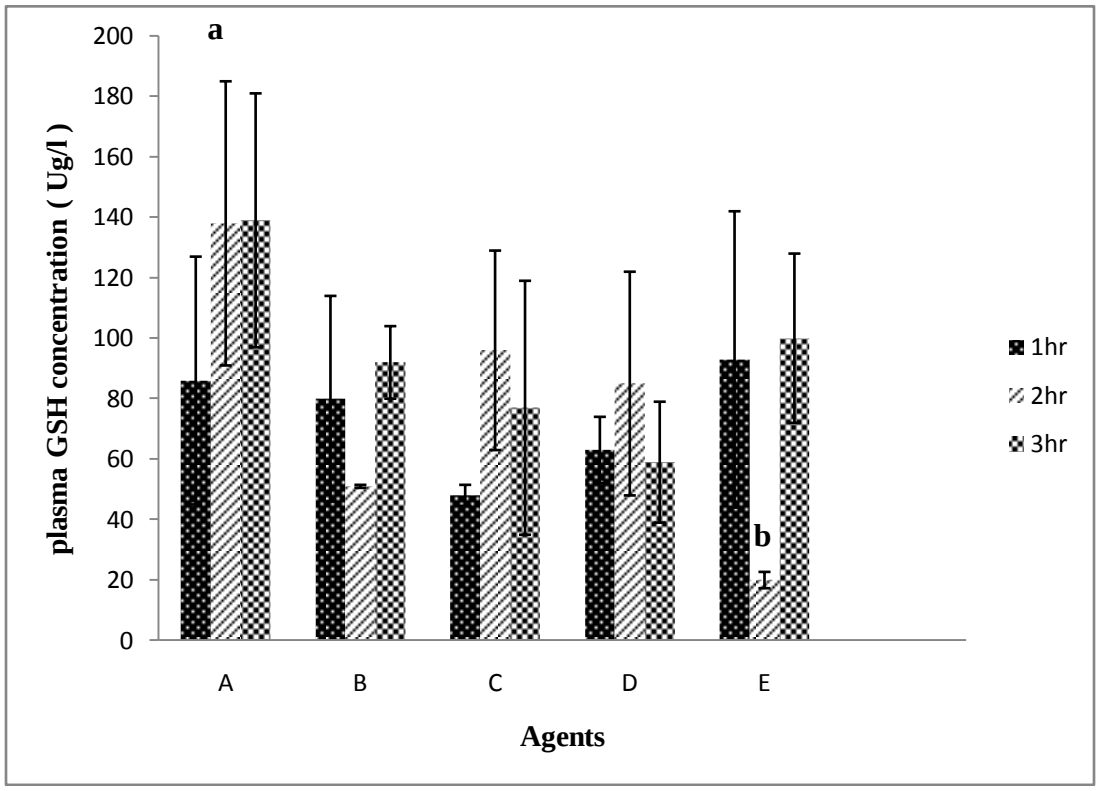


Figure 4.4. The mean and standard error of mean for plasma glutathione peroxidise (GPx) activity (Ug/ml) of the castrated rabbits treated with different agents.

[ab]Means in the same column with different superscripts alphabets were significantly different ($p \leq 0.05$).

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); F: Baseline data before surgery

4.4 Evaluation of Anti-nociceptive Property of Meloxicam, Ketoprofen, and *C. ambrosioides* Extract on Castrated Rabbits

4.4.1 Evaluation of algometer – induced pain in castrated rabbits treated with various agents at the day 1 (day of surgery).

One hour after the surgery, the minimum force required to elicit pain in rabbits treated with *C. ambrosioides* extracts oral and topically were 13.47 ± 1.32 and 16.03 ± 2.52 N, respectively while 12.9 ± 1.42 and 10.27 ± 0.17 N were recorded for oral meloxicam and topical ketoprofen medication use respectively (Table 4.2 and Appendix 2a). The force required to elicit pain in rabbits treated with normal saline after 1hr was 9.87 ± 0.35 N.

Two hours after the surgery, the minimum force required to elicit pain in rabbits treated with *C. ambrosioides* extracts oral and topically were 16.63 ± 1.45 and 14.17 ± 0.85 N, respectively while 18.73 ± 0.70 and 10.37 ± 0.12 N were recorded for oral meloxicam and topical ketoprofen medication use respectively. The force required to elicit pain in rabbits treated with normal saline after 2hr was 6.47 ± 1.50 N.

Three hours after the surgery, the minimum force required to elicit pain in rabbits treated with *C. ambrosioides* extracts oral and topically were 21.9 ± 0.81 and 16.53 ± 2.07 N, respectively while 18.4 ± 1.45 and 12.37 ± 0.39 N were recorded for oral meloxicam and topical ketoprofen medication use respectively. The force required to elicit pain in rabbits treated with normal saline after 3hrs was 6.17 ± 1.37 N.

Table 4.2 The mean and standard error of mean values of the force (N) required to elicit pain with the use of algometer in castrated rabbits treated with different agents on the day 1.

Time	A	B	C	D	E
1h	9.87 ± 0.35^{b}	12.9 ± 1.42^{bcd}	13.47 ± 1.32^{bcd}	10.27 ± 0.17^{bc}	16.03 ± 2.52^{ad}
2h	6.47 ± 1.50^{c}	18.73 ± 0.70^{a}	16.63 ± 1.45^{a}	10.37 ± 0.12^{bc}	14.17 ± 0.85^{ab}
3h	6.17 ± 1.37	18.4 ± 1.45^{ab}	21.9 ± 0.81^{b}	12.37 ± 0.39^{c}	16.53 ± 2.07^{ac}

^{abc}Means in the same row with different superscripts alphabets were significantly different ($p \leq 0.05$).

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical).

4.4.2 Evaluation of algometer – induced pain in castrated rabbits treated with various agents at day 2 (second day of surgery).

On the day 2 of surgery, the minimum force required to elicit pain in rabbits treated with *C. ambrosioides* extracts oral and topically was 9.6 ± 1.3 and 13 ± 4 N, respectively while 17 ± 3 and 8.7 ± 0.73 N were recorded for oral meloxicam and topical ketoprofen medication respectively at 1 hour after the administration of different agents (Table 4.3 and Appendix 2b). The force required to elicit pain in rabbits treated with normal saline after 1hr was 7.1 ± 0.22 N.

Two hours after the surgery, the minimum force required to elicit pain in rabbits treated with *C. ambrosioides* extracts oral and topically was 13 ± 1.8 and 15 ± 1.4 N, respectively while 18 ± 3.3 and 12 ± 2.6 N were recorded for oral meloxicam and topical ketoprofen medication, respectively. The force required to elicit pain in rabbits treated with normal saline after 2hrs was 4.8 ± 0.43 N.

Three hours after the surgery, the minimum force required to elicit pain in rabbits treated with *C. ambrosioides* extracts oral and topically was 18 ± 0.17 and 15 ± 2.8 N, respectively while 22 ± 1.4 and 17 ± 1.3 N were recorded for oral meloxicam and topical ketoprofen medication respectively. The force required to elicit pain in rabbits treated with normal saline after 3hrs was 4.5 ± 0.41 N.

Table 4.3 Mean and standard error of mean values of the force (N) required to elicit pain with the use of algometer in castrated rabbits treated with various agents on day 2.

Time	A	B	C	D	E
1h	7.1 ± 0.22^{b}	17 ± 3^{a}	9.6 ± 1.3^{b}	8.7 ± 0.73^{b}	13 ± 4^{ab}
2h	4.8 ± 0.43^{b}	18 ± 3.3^{a}	13 ± 1.8^{a}	12 ± 2.6^{ab}	15 ± 1.4^{a}
3h	4.5 ± 0.41^{b}	22 ± 1.4^{a}	18 ± 0.17^{ac}	17 ± 1.3^{ac}	15 ± 2.8^{c}

^{abc}Means in the same row with different superscripts alphabets were significantly different ($p \leq 0.05$).

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical).

4.5 Evaluation of Anti-nociceptive and Anti-inflammatory Properties of Oral and Topical *C. ambrosioides* Extract on Castrated Rabbits

4.5.1 Pain score values of various facial expression under different treatment groups of castrated rabbits using Rabbit Grimace Scale

The mean scores (minimum and maximum) values for various facial expression was presented in Table 4.4 and Appendix 3.1a and 3.1b, while the overall score values for individual groups was presented in Table 4.5 and Appendix 3.2a and 3.2b. It was observed that the pre-surgical pain scores for all the facial expressions were lower, while that of the normal saline treated group (A) was higher (Table 4.4).

Also, the overall mean pain scores for pre surgical values was 0.36, while 0.92, 0.51, 0.71, 0.8 and 0.55 were observed for normal saline, meloxicam, (oral), *C. ambrosioides* extracts (oral), ketoprofen (topical) and *C. ambrosioides* extracts (topical), respectively (Table 4.5).

Plate 4.1 - 4.6 presented below were for various facial expression observed from castrated rabbits pre-surgically and following treatment with normal saline (oral), meloxicam (oral), *C. ambrosioides* extracts (oral), ketoprofen (topical) and *C. ambrosioides* extracts (topical) respectively.

In this study, it was observed that the mean orbital pain score for the normal saline treated groups was significantly higher ($p \leq 0.05$) than the score obtained for pre-surgical values. The orbital pain scores for the oral meloxicam and *C. ambrosioides* extracts treatment groups, and the topical Ketoprofen and *C. ambrosioides* extracts treatment groups were however not different

from the pre-surgical scores. Though the pain score values observed for the normal saline treated groups were higher than pre-surgical and all other treatment groups (B, C, D, and E) for the cheek flattening, nostril shape and whisker positioning, the difference was statistically significant. The ear position pain score observed for the Ketoprofen topical treatment group was significantly higher than the pre-surgical values recorded suggesting that the *C. ambrosioides* extracts topical treatment was more potent and effective.

Table 4.4 The mean and standard error of mean (minimum and maximum) values for the pain scores recorded for various facial expression under different treatment groups using Rabbit Grimace Scale (n=15) after 1hr.

	pre	A	B	C	D	E
Orbital	0.1 ± 0.06 (0-0.2)	0.93 ± 0.19[b] (0.7-1.3)	0.23 ± 0.12 (0-0.4)	0.37 ± 0.22 (0.1-0.8)	0.57 ± 0.09 (0.4-0.7)	0.4 ± 0.06 (0.3-0.5)
Cheek	0.3 ± 0.12 (0.1-0.5)	0.83 ± 0.12 (0.6-1)	0.23 ± 0.09 (0.1-0.4)	0.73 ± 0.24 (0.4-1.2)	0.6 ± 0.06 (0.5-0.7)	0.37 ± 0.07 (0.3-0.5)
Nostril	0.5 ± 0.26 (0.1-1)	0.7 ± 0.1 (0.5-0.8)	0.63 ± 0.07 (0.5-0.7)	0.57 ± 0.23 (0.2-1)	0.5 ± 0.1 (0.4-0.7)	0.57 ± 0.09 (0.4-0.7)
Whisker	0.47 ± 0.03 (0.4-0.5)	0.83 ± 0.03 (0.8-0.9)	0.63 ± 0.09 (0.5-0.8)	0.63 ± 0.15 (0.4-0.9)	0.73 ± 0.18 (0.4-1)	0.6 ± 0.1 (0.4-0.7)
Ear	0.43 ± 0.13[a] (0.3-0.7)	1.3 ± 0.25 (0.8-1.6)	0.8 ± 0.06 (0.7-0.9)	1.2 ± 0.12 (1-1.4)	1.6 ± 0.06[b] (1.5-1.7)	0.8 ± 0.2 (0.6-1.2)
Over all mean pain scores	0.36 ± 0.07[a] (0-1)	0.92 ± 0.08[b] (0.5-16)	0.51 ± 0.07[ac] (0-0.9)	0.71 ± 0.11[ab] (0.1-1.4)	0.8 ± 0.12[bc] (0.4-1.7)	0.55 ± 0.06[ac] (0.3 -1.2)

[ab]Means in the same row with different superscripts alphabets were significantly different ($p \leq 0.05$).

KEY: Pre: pre surgical values; A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical).



Plate 4.1: Pre-surgical facial expression of rabbit with the eyelid wide opened and the normal ear shape and carriage.



Plate 4.2: Facial expressions of the rabbit treated with normal saline at 1hr post castration. The eyelid was narrowed while the ears were curled and held closer to the back (dorsum).



Plate 4.3: Facial expressions of the rabbit treated with oral meloxicam (0.1mg/Kg) at 1hr after castration with the opened eyelid, more rounded face and the nares forming "U" shape.



Plate 4.4: Facial expressions of the rabbit treated with oral administration of *C. ambrosioides* extracts (500mg/Kg) at 1 hr after castration. The eyelids were opened while face was rounded with "U" shaped nares.



Plate 4.5: Facial expressions of the rabbit treated with topical administration of Ketoprofen (topical) at 1hr after castration. The eyelids were partially closed, face was slightly angled while the ears were curled and held closer to the back (dorsum).



Plate 4.6: Facial expressions of the rabbit treated with topical administration of *C. ambrosioides* extracts (liberal) at 1hr after castration with partially closed eyelid, slightly angled face and curled ears that were held closer to the back (dorsum).

CHAPTER FIVE

5.0 DISCUSSION

The preliminary phytochemical studies (qualitative analysis) on the leaves of *C. ambrosioides* ethanol extract revealed that the plant contained carbohydrates, glycoside, saponins, steroids, triterpenes, flavonoids, alkaloids and tannins, which corroborated the findings of several researchers (Eguale and Giday, 2009; Hallala *et al.,* 2010). In a study by Eguale and Giday, (2009), *C. ambroisiodes* was reported to contain polyphenols, saponins, and glycosides (oligosaccharids) among various phytochemical tests including polyphenols, cyanogenic glycosides, saponins, phytosteroides and withanoides, phenolic glycosides, glycosides (oligosaccharids), flavonoids, tannins, alkaloides, antraquinone glycosides. Hallala *et al.* (2010) similarly reported *C. ambroisiodes* contained saponins, glycosides, flavonoids, tannins and alkaloids while evaluating its analgesic and antipyretic activities.

In this study, the blood glucose concentrations immediately after surgery were constant for all the treatment groups (A, B, C, D, and E). Fifteen minutes after the administration of various agents, the blood glucose concentrations of other treatment groups (B, C, D, and E) were transiently significantly lower ($p \leq 0.05$) than the normal saline treatment group. There was no significant difference between the blood glucose concentrations of the topical and oral treatment with *C. ambrosioides* compared with the topical and oral treatment with Ketoprofen and meloxicam, respectively. The blood glucose increase observed in the normal saline treatment group was indicative of the higher distress and pain perception in the normal saline treatment group as reported by several authors (Wing *et al*. 1985; Yamada *et al*. 1993; Uresin *et al*. 2004;

Sim *et al.,* 2012). It is widely accepted that pain and stressors (such as hypoxia, hypothermia, and sepsis) elevate blood glucose level (increase blood glucose) and worsen glycaemic control in patients with diabetes (Wing *et al*. 1985; Yamada *et al*. 1993; Uresin *et al*. 2004; Sim *et al.,* 2012). However, at 2 and 3hrs after treatment, there was no significant difference in the blood glucose concentration of all the treatment groups (A, B, C, D, and E).

In this study, the plasma MDA concentration was found to be significantly ($p \leq 0.05$) higher with the normal saline treated groups, when compared to baseline values obtained before the surgery and other treatment groups (B, C, D, and E). Hence the oral use of meloxicam and *C. ambrosioides* significantly reduced the MDA concentration. At 1hr after administration, the *C. ambrosioides* reduced the MDA concentration to the base line level after which it increased slightly, while the meloxicam administration reduced the plasma MDA to the baseline level at 2hr after which it also increased slightly. This implies that the antioxidant effect of *C. ambrosioides* was immediate while it took meloxicam an hour to take effect. The topical administration of Ketoprofen and *C. ambrosioides* also significantly lowered ($p \leq 0.05$) the plasma MDA concentration compared with the normal saline treated group but not different from that of the baseline values obtained before the surgery. An-qi *et al*. (2012) reported a similar occurrence, when they studied the effects of decomposition liquids from residue of *C. ambrosioides* on the antioxidant system in *Glycine max* (L) root tips. The antioxidant system of *Glycine max* (L) root tips were significantly inhibited by the decomposition liquids from the residue with the essential oil of *C. ambrosioides* due to its ability to decrease the contents of the MDA and increase activities of the SOD and catalase enzyme (CAT), which can reduce the oxidative damage to the soya bean root tip cells to a certain degree. Since malondialdehyde

(MDA) is the marker most frequently used to indicate the level of lipid peroxidation in the field of exercise and sports, reduction of plasma MDA by *C. ambrosioides* indicate that the plant reduces lipid peroxidation and free ionic radicals production (An-qi *et al.*, 2012).

The plasma SOD activity was higher in the oral treatment with the meloxicam and *C. ambrosioides* groups indicating ameliorative effects on oxidative stress. The rapid increase was observed with the oral *C. ambrosioides* treatment group, while in the oral meloxicam treatment group the increase was after 2hrs of treatment. The topical treatment with the Ketoprofen and *C. ambrosioides* further lowered the plasma SOD activity significantly ($p \leq 0.05$) compared to the baseline data observed, implying that the topical use of this agent increased oxidative stress. There is likely that the topical treatment with Ketoprofen and *C. ambrosioides* cause local reaction which lead to the observed lower plasma SOD activity. Boojar and Goodarzi (2007) reported the anti-oxidative enzymes in *C. ambrosioides* to play a key role in defence system against oxidative damages and in tolerance due to Cu contamination of the soil that it was cultivated on.

In this study, the GSH activities observed in the oral meloxicam treatment group, topical Ketoprofen treatment group, oral and topical *C. ambrosioides* extract treated groups were higher ($p \leq 0.05$) than the baseline values recorded pre-surgically with a slight variation. This suggests their anti-oxidant properties. The GSH activity values obtained in the normal saline treatment group were, however, higher at 2 and 3hr after treatment than all other treatment groups (B, C, D and E), though the differences were not statistically significant except between normal saline and *C. ambrosioides* topical treatment group at 2hrs post treatment. This showed that all the treatment groups including normal saline had antioxidant effects. The anti-oxidant effect of *C.*

ambrosioides is similar to that of oral meloxicam and topical ketoprofen treatments whereas, that of the topical treatment was not as effective as the oral treatment.

This study had shown that *C. ambrosioides* significantly reduces the plasma MDA and increased the plasma SOD and GSH indicative of its antioxidant properties. The antioxidative effects could be attributed to high flavonoids and terpenoids content as reported by several authors (Cruz *et al.,* 2007; Kinupp, 2007; Dembitsky *et al.,* 2008; Grassi, 2011; Degenhardt *et al*, 2016) who reported *Chenopodium* to be rich in flavonoids and terpenoids and having very diverse pharmacological activities, including antioxidant and chemopreventive effects against cancer, as well as antimicrobial, anti-inflammatory, and analgesic properties. Christian *et al.* (2016) also reported that flavonoids scavenge for oxygen-derived free radicals, reduce lipid peroxidation and inflammation. Whereas, Matos (2011) and Degenhardt *et al*. (2016) reported that the anti-inflammatory activity of *Chenopodium* is due mainly to ascaridole, which is one of the major components of the essential oil of the plant. More so, Trivellato-Grassi *et al.* (2013) reported *C. ambrosioides* to inhibit mediators Bradykinin (BK), Nitric oxide (NO), Substance P (SP), Prostaglandin E2 (PGE2), Tumour necrotic factor alpha (TNF-a) and enzymes Myeloperoxidase (MPO), Adenosine-deaminase (ADA) that were involved in the inflammatory and pain processes. However, Calado *et al.* (2015) demonstrated that treatment with *C. ambrosioides* promotes analgesic and anti-inflammatory effects, improving the clinical aspects of experimental osteoarthritis, due to the antagonistic action of ascaridole on the N-methyl-D-aspartate (NMDA) receptor. Furthermore, Thakur *et al*. (2011) reported the free radical scavenging properties of the plant to be due to tannin content. Tannins have a wide range of pharmacological activities including antimicrobial, wound healing, antioxidant and anti-inflammatory activities (Fakhim *et*

al., 2015; Christian *et al.,* 2016). Haslam, (1996) suggested that tannins act by virtue of their complexation, astringent, antioxidant and radical scavenging activities, and their ability to form complex with proteins. Similarly, sterols and polyphenols have been reported to reduce lipid peroxidation due to its free radical-scavenging and antioxidant activity (Thakur *et al.,* 2011). This suggests that the ability of *C. ambrosioides* to alleviate the oxidative stress caused by the surgical pain was due to its tannins, flavonoids and sterol contents as explained by several authors (Haslam, 1996; Thakur *et al.,* 2011; Fakhim *et al.,* 2015; Christian *et al.,* 2016).

At day 1, it was generally observed that the force (N) required in eliciting pain was generally lower with the normal saline treatment group. The force required in eliciting pain in the oral meloxicam treatment group was not different from that of the oral treatment with *C. ambrosioides* extracts at 1hr, 2h and 3hrs, whereas the force applied in both groups were significantly higher ($p \leq 0.05$) than that of normal saline treated groups at 2 and 3hrs. The force required in eliciting pain in the *C. ambrosioides* extracts topical treatment was significantly higher ($p \leq 0.05$) than that of the topical treatment with Ketoprofen at 1hr after treatment but, thereafter not different from the latter. Whereas the force required to eliciting pain in the *C. ambrosioides* extracts topical treatment was significantly higher ($p \leq 0.05$) than that of the normal saline at 1hr, 2hr, and 3hrs. This implies that *C. ambrosioides* extracts have higher analgesic effects at the concentration used and the analgesic effects were immediately, similar to what was observed with the rapid decrease and increase in the plasma MDA concentration and SOD activity, respectively as reported earlier in this study. The analgesic properties possessed by *C. ambrosioides* using the algometer was due to the flavonoids and tannins present in the leaf as described by Akpalo *et al.* (2015); Fakhim *et al.* (2015); Christian *et al.* (2016).

At day 2 of surgery, it was observed that the force required in eliciting pain was lower in the normal saline treatment groups compared to the previous day while for other treatment groups seems to be fairly constant. This showed an increased pain perception in the normal saline treatment groups. At 1hr after treatment on the day 2 of surgery, the force required in eliciting pain in oral meloxicam treated group was significantly higher ($p \leq 0.05$) than that of oral treatment with *C. ambrosioides* extracts but not significantly different thereafter. Whereas, the force required in eliciting pain in oral treatment with *C. ambrosioides* extracts and meloxicam was significantly higher than that of normal saline treatment group at 2 and 3hr after treatment. Though the force required in eliciting pain in the *C. ambrosioides* extracts topical treatment group was higher than that of the Ketoprofen topical treatment group, the difference was not significant. The force required in eliciting pain in the *C. ambrosioides* extracts topical treatment group was however significantly higher ($p \leq 0.05$) than that of normal saline at 2 and 3 hrs post treatment, while that of Ketoprofen treated group, was only significantly higher ($p \leq 0.05$) than that of the normal saline group at 3hrs post treatment. This explained the rapid effect of analgesia induced by the *C. ambrosioides* extracts on topical application. This was the reason why the plant is effective in folk medicine as a topical anti- inflammatory (Lorenzi and Matos, 2002). In Brazilian folk medicine, *C. ambrosioides* is crushed and served in the form of tea, infusions or poultices as a diuretic and an anthelmintic, and it is also used to treat wounds, respiratory problems, inflammatory and painful processes, bronchitis, tuberculosis and rheumatism (Kumar *et al.*, 2007). In this study, it was found that *C. ambrosioides* was very effective when administered topically. This agrees with what was reported by Trivellato-Grassi *et al*. (2013), who found that ethanol extract of *C. ambrosioides* exhibit the anti-inflammatory and anti-nociceptive effects by inhibition of mediators (such as bradykinin (BK), nitric oxide

(NO), substance P (SP), PGE2, and TNF-a) and enzymes (like myeloperoxidase (MPO) and adenosine deaminase (ADA) that were involved in the inflammatory and pain processes. These anti-inflammatory effects validate in part its popular use and demonstrate its efficacy, when it is used topically (Trivellato-Grassi *et al.*, 2013).

The overall Grimace pain score values for the oral meloxicam treatment group, oral and topical *C. ambrosioides* extracts treatment group were not different from that of the pre-surgical Grimace pain score, while that of the topical Ketoprofen score and oral *C. ambrosioides* extracts were not different from the score values for normal saline treatment group. This showed that the topical application of *C. ambrosioides* extracts produced rapid analgesic effects compared to the oral used. This Grimace pain score further confirmed what was observed earlier in this study, whereby the force required in eliciting pain was higher with the topical treatment of *C. ambrosioides* extracts than its oral use immediately after treatment. This was due to the advantage from the route of administration and is suggesting that *C. ambrosioides* extracts might be a better local analgesic agent.

Face grimace scale was recently validated for the pain assessment in rats, mice, and rabbits (Asgar, 2015). Similar to this study, Rabbit Grimace Scale (RbtGS) has been used by Keating *et al*. (2012) for the evaluation of EMLA cream in preventing pain during tattooing of Rabbits while Rat Grimace Scale (RGS) has been used by Dreancă *et al*. (2017) to find the most effective post-surgery (ovariectomy) analgesic protocol. The analgesic effect of *C. ambrosioides* was believed to be as a result of ascaridole components of its essential oil. Several authors had reported that monoterpene ascaridole is the main component of essential oil of *C. ambrosioides* which has sedative and anthelmintic effects (Olajide *et al.*, 1997; Efferth *et al.*, 2002; Cavalli *et*

al., 2004; Dembitsky *et al.,* 2008); anti-inflammatory and anti-nociceptive properties (Ibironke and Ajiboye, 2007); analgesic, sedative and antifungal effects (Olajide *et al.,* 1997). Trivellato-Grassi *et al*. (2013) reported that the ethanol extract of *C. ambrosioides* significantly reduce both phases of nociception (neurogenic and inflammatory). The ethanol extract of *C. ambrosioides* produces its anti-oedematogenic effect by inhibiting the synthesis of eicosanoids and cytokines, in which PGE2 and TNF- α were the main oedematogenic mediators (Saraiva *et al.,* 2011). Also, Trivellato-Grassi *et al*. (2013); Ibironke and Ajiboye (2007) reported that ethanol extract of *C. ambrosioides* effectively inhibited oedema induced by carrageenan. The effect was due to inhibition of mediators involved in the events such as histamine, BK, SP and prostaglandins (Trivellato-Grassi *et al.,* 2013). It also produced significant inhibition of the inflammatory response with respect to leucocyte migration in relation to neutrophils and mononuclear cells to the inflammatory site (Trivellato-Grassi *et al.,* 2013). Similarly, it inhibits the enzyme activity; NO levels; and reactive oxygen species including the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-), this contributed to limiting the infiltration of leucocytes and subsequent cell damage (Trivellato-Grassi *et al.,* 2013).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Chenopodium ambrosioides has analgesic effect post-castration as demonstrated by:

i. The blood glucose concentration at one hour post treatment with oral Meloxicam, oral *C. ambrosioides* extracts, topical Ketoprofen and topical *C. ambrosioides* extracts were 147.33 ± 13.78 mg/dL, 137.67 ± 3.38 mg/dL, 146.67 ± 10.09 mg/dL and 184.33 ± 31.87 mg/dL respectively, and they were significantly lower ($P< 0.05$) than the normal saline treated group (267.67 ± 18.77 mg/dL).

ii. Two hours after administration of Meloxicam and *C. ambrosioides* orally, plasma malondialdehyde concentration was signicantly ($p \leq 0.05$) reduced to 7332 ± 732 µmol/L and 18505 ± 4170 µmol/L, respectively, when compared with the control (44696 ± 19071 µmol/L).

iii. *C. ambrosioides* had better anti-oxidative properties when administered orally because after 2 hours the plasma SOD activity of oral *C. ambrosioides* (22 ± 5.7 Mg/mL) treatment was higher than the oral meloxicam (14 ± 6 Mg/mL) and normal saline (18 ± 8.4 Mg/mL) treatments while that of topical *C. ambrosioides* (4.8 ± 1.1 Mg/mL) treatment group was higher than that of topical Ketoprofen (2.2 ± 1 Mg/mL) treatment, though the different was not significant.

iv. *C. ambrosioides* had better anti-oxidative properties when administered orally because after 2 hours the GSH activity of oral *C. ambrosioides* (96 ± 33 Ug/ml) was higher than that of oral meloxicam (51 ± 0.5 Ug/ml) while that of topical Ketoprofen (85 ± 37 Ug/ml) was higher than that of topical *C. ambrosioides* (20 ± 2.7 Ug/ml) though, difference was not significant. However, the GSH activity of the normal saline (138 ± 47 Ug/ml) treatmet group was significantly higher ($p \leq 0.05$) than that of topical *C. ambrosioides* (20 ± 2.7 Ug/ml).

v. On day 1 of surgery *C. ambrosioides* was better as topical analgesic because the force required in eliciting pain after 1 hour in topical *C. ambrosioides* (16.03 ± 2.52 N) treatment group was significantly higher than the topical Ketoprofen (10.27 ± 0.17 N) treatment group. While the oral *C. ambrosioides* (13.47 ± 1.32 N) treatment was higher than the oral meloxicam (12.9 ± 1.42 N) and normal saline (9.87 ± 0.35 N) treatment groups though, the differences were not significant.

vi. On day 2 of surgery *C. ambrosioides* was also better as topical analgesics because the force required in eliciting pain after 1 hour in topical *C. ambrosioides* (13 ± 4 N) treatment group was higher than the topical Ketoprofen (8.7 ± 0.73 N) treatment group but the difference is not significant ($p > 0.05$). While the oral meloxicam (17 ± 3 N) treatment was significantly higher ($p \leq 0.05$) than the oral *C. ambrosioides* (9.6 ± 1.3 N) and normal saline (7.1 ± 0.22 N) treatment groups whereas, not different from the topical *C. ambrosioides* (13 ± 4 N) treatment.

vii. The mean orbital pain score for the normal saline (0.9 ± 0.7-1.3) treated groups was significantly higher ($p \leq 0.05$) than the score obtained for pre-surgical evaluation (0.1 ± 0-0.2) whereas, the orbital pain scores for the oral meloxicam (0.23 ± 0-0.4), oral *C.*

ambrosioides extracts (0.37 ± 0.1-0.8), topical Ketoprofen (0.57 ± 0.4-0.7) and topical *C. ambrosioides* (0.4 ± 0.3-0.5) extracts treatment groups were not different from the pre-surgical scores (0.1 ± 0-0.2).

viii. The topical application of *C. ambrosioides* extracts produced rapid analgesic effects and more effective compared to the oral administration at the dosage used because the overall Grimace pain score for the oral meloxicam (0.51 ± 0-0.9), oral *C. ambrosioides* (0.71 ± 0.1-1.4), topical *C. ambrosioides* (0.55 ± 0.3-1.2) extracts treatment groups was not different from that of the pre-surgical Grimace pain score (0.36 ± 0-1), while that of the topical Ketoprofen score (0.8 ± 0.4-1.7) and oral *C. ambrosioides* (0.71 ± 0.1-1.4) extracts were not different from the normal saline (0.92 ± 0.5-1.6) treatment group.

6.2 Recommendations

i. Further studies should be done on the analgesic effect of *C. ambrosioides* extract on clinical cases.

ii. Further studies should be carried out on the analgesic effect of *C. ambrosioides* using the ascaridole isolate as reported by Matos (2011), Calado *et al.,* (2015) and Degenhardt *et al*, (2016) that was responsible for the observed analgesic and anti-inflammatory activities.

iii. Improvement on this work and commercialization of the products from these plants for animal and human use especially in the developing countries is highly recommended.

6.3 Limitations

i. Willingness of the observers to read the pictures obtained for grimace scale.

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Appendices

Appendix 1a: Evaluation of the plasma glucose concentration (mg/dL) of castrated rabbits treated with different agents.

Time	A			B			C			D			E		
Immed	290	279	241	287	240	228	244	245	239	256	229	294	261	239	287
15 min	300	268	235	174	140	128	142	140	131	142	132	166	200	230	123
30 min	193	153	124	126	132	121	135	133	125	123	141	131	194	138	116
60 min	133	79	112	122	120	120	132	129	123	150	143	147	166	120	113

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); Immed: Immediately

Appendix 1b The mean and standard error of mean for plasma glucose concentration (mg/dL) of the castrated rabbits treated with different agents after surgery.

Time	A	B	C	D	E
Immediate	270 ± 14.84	251.67 ± 18.00	242.67 ± 1.86	259.67 ± 18.85	262.33 ± 13.87
15 min	267.67 ± 18.77[a]	147.33 ± 13.78	137.67 ± 3.38	146.67 ± 10.09	184.33 ± 31.87
30 min	156.67 ± 20.00	126.33 ± 3.18	131 ± 3.06	131.67 ± 5.21	149.33 ± 23.22
60 min	108 ± 15.72	120 ± 0.67	128 ± 2.65	146.67 ± 2.03	133 ± 16.62

[a]Means in the same row with different superscripts alphabets were significantly different ($p \leq 0.05$).

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical).

Appendix 1c: Evaluation of the plasma malondialdhyde concentration (µmol/L) of castrated rabbits treated with different agents.

GRP A			GRP B			GRP C			GRP D			GRP E			GRP F
1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr	Pre-surg.
3167.1	64254.5	22718.8	40240.5	8722.8	13501.4	7598.5	13414.9	3176	2595.5	2748.4	7141.4	2922.9	3207.8	3118.2	11653.7
11065.8	6559.1	6530.6	2937	6240.2	16664.8	7871.7	15328.7	7831.6	3292	2929	3091.9	3944.2	3618	3263.3	11065.8
3277.8	63274.5	25718.2	11966.2	7031.6	19966.6	9370	26771.2	49376.7	3767.7	3454.4	3007.6	4326.3	3111.8	2933.5	6306.1

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); F: Baseline data before surgery, GRP: group

Appendix 1d: The mean and standard error of mean for plasma malondialdhyde (MDA) concentration (µmol/L) of the castrated rabbits treated with different agents after surgery.

Time	A	B	C	D	E	F
1h	5837 ± 2615	18381 ± 11236	8280 ± 551	3218 ± 340	3731 ± 419	9675 ± 1693
2h	44696 ± 19071[a]	7332 ± 732	18505 ± 4170	3044 ± 212	3313 ± 155	9675 ± 1693
3h	18323 ± 5959	16711 ± 1866	20128 ± 14686	4414 ± 1364	3105 ± 95	9675 ± 1693

[a]Means in the same row with different superscripts alphabets were significantly different ($p \leq 0.05$).

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); F: Baseline data before surgery

Appendix 1e: Evaluation of the plasma superoxide dismutase (SOD) activity (Mg/mL) of castrated rabbits treated with different agents.

GRP A			GRP B			GRP C			GRP D			GRP E			GRP F		
1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr
11.58	34.97	4.37	26.23	26.23	32.79	22.3	11.15	0.87	1.31	1.69	2.63	5.63	5.82	5.45	36.07	36.07	36.07
28.85	7.87	22.3	3.28	8.31	13.11	24.48	24.04	10.05	2.07	4.13	4.51	6.57	5.82	5.82	28.85	28.85	28.85
4.32	12.3	16.7	8.52	8.31	19.23	10.49	30.6	15.74	4.88	0.75	5.26	5.26	2.63	1.88	8.26	8.26	8.26

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); F: Baseline data before surgery, GRP: group

Appendix 1f: The mean and standard error of mean for plasma superoxide dismutase (SOD) activity (Mg/mL) of the castrated rabbits treated with different agents after surgery.

Time	A	B	C	D	E	F
1h	15 ± 7.3	13 ± 6.9	19 ± 4.3	2.8 ± 1.1[a]	5.8 ± 0.39	24 ± 8.3[b]
2h	18 ± 8.4[ab]	14 ± 6[ab]	22 ± 5.7[b]	2.2 ± 1[a]	4.8 ± 1.1[ab]	24 ± 8.3[b]
3h	14 ± 5.3[ab]	22 ± 5.8[ab]	8.9 ± 4.3[ab]	4.1 ± 0.78[a]	4.4 ± 1.3[a]	24 ± 8.3[b]

[ab]Means in the same row with different superscripts alphabets were significantly different ($p \leq 0.05$).

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); F: Baseline data before surgery.

Appendix 1g: Evaluation of the plasma glutathione peroxidise (GPx) activity (Ug/ml) of castrated rabbits treated with different agents.

GRP A			GRP B			GRP C			GRP D			GRP E			GRP F		
1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr	1hr	2hr	3hr
17.72	199.02	190.02	137.56	50.08	75.12	54.63	75.12	25.37	85.37	44.55	75.12	43.58	16.91	137.56	69.76	69.76	69.76
81.46	45.85	44.55	19.35	51.71	85.37	43.58	52.03	46.18	51.71	159.35	19.35	190.24	17.72	115.77	81.46	81.46	81.46
159.02	169.23	127.55	83.41	50.41	115.77	44.55	159.35	159.02	52.03	50.41	83.41	45.85	25.37	46.18	51.38	51.38	51.38

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); F: Baseline data before surgery, GRP: group

Appendix 1h: The mean and standard error of mean for plasma glutathione peroxidise (GPx) activity (Ug/ml) of the castrated rabbits treated with different agents after surgery.

Time	A	B	C	D	E	F
1h	86 ± 41	80 ± 34	48 ± 3.5	63 ± 11	93 ± 49	68 ± 8.8
2h	138 ± 47[a]	51 ± 0.5	96 ± 33	85 ± 37	20 ± 2.7[b]	68 ± 8.8
3h	121 ± 42	92 ± 12	77 ± 42	59 ± 20	100 ± 28	68 ± 8.8

[ab]Means in the same row with different superscripts alphabets were significantly different ($p \leq 0.05$).

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); F: Baseline data before surgery.

Appendix 2a: The force (N) required to elicit pain with the use of algometer in castrated rabbits treated with different agents on the day 1 of surgery

TIME	A			B			C			D			E		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
1h	10	9.2	10.4	15.7	11.1	11.9	11	15.5	13.9	10.6	10.1	10.1	14.7	12.5	20.9
2h	9.3	4.2	5.9	20	17.6	18.6	19.2	16.5	14.2	10.2	10.3	10.6	15.3	12.5	14.7
3h	8.9	5	4.6	19.9	15.5	19.8	22.9	20.3	22.5	12.9	12.6	11.6	17.4	12.6	19.6

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); R1: Rabbit 1; R2: Rabbit 2; R3: Rabbit 3.

Appendix 2b: Evaluation of the force (N) required to elicit pain with the use of algometer in castrated rabbits treated with different agents on the day 2 of surgery

TIME	A			B			C			D			E		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
1h	7.4	6.7	7.3	14.5	13.6	22.9	11.4	7.1	10.2	7.5	8.5	10	12	7.3	21
2h	5.6	4.1	4.8	22	11.8	21.3	16.4	10.5	11.8	6.7	13.9	15	13.8	13.7	17.9
3h	5.3	4.4	3.9	23.6	19.4	23.8	17.5	17.8	18.1	14.7	17.8	19.1	13.3	10.9	20.3

KEY: A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical); R1: Rabbit 1; R2: Rabbit 2; R3: Rabbit 3.

Appendix 3. 1a The pain scored by ten observers for different facial expression using the Rabbit Grimace Scale.

Orbital																														
	Pre										A										B								
	a	B	c	d	e	f	g	h	i	j	a	b	c	d	E	f	g	h	i	j	a	b	c	d	E	f	g	h	i	j
R1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
R2	0	0	0	0	0	0	1	1	0	0	1	1	1	2	2	1	0	0	0	0	1	0	0	0	0	1	1	0	0	0
R3	0	0	0	0	0	0	0	1	0	0	2	2	2	1	2	2	0	0	1	1	1	0	1	0	1	0	0	1	0	0
Cheek																														
	Pre										A										B								
	a	B	C	D	e	f	g	h	i	j	a	b	c	d	E	f	g	h	i	j	a	b	c	d	E	f	g	h	i	j
R1	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	1	0	0	0	0	0	1	0	0
R2	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	2	1	0	0	0	0	0	0	1	0	0	0	0
R3	1	0	0	0	0	0	0	1	0	1	0	2	1	1	1	2	0	1	1	1	1	0	1	1	0	0	0	0	0	1
Nostril																														
	Pre										A										B								
	a	B	c	D	e	f	g	h	i	j	a	b	c	d	E	f	g	h	i	j	a	b	c	d	E	f	g	h	i	j
R1	1	1	0	0	2	1	0	1	2	2	2	0	1	0	0	1	0	1	0	0	1	0	0	1	1	1	1	0	1	1
R2	1	0	1	0	1	0	0	1	0	0	2	1	1	1	0	1	1	1	0	0	1	0	0	1	1	1	0	0	1	0

R3	1	0	0	0	0	0	0	0	0	0	2	1	1	1	0	2	0	1	0	0	1	0	1	1	1	1	0	0	1	1

	Whisker																													
	Pre										A										B									
	a	B	c	D	e	f	g	H	i	j	a	b	c	d	E	f	g	h	i	j	a	b	c	d	E	f	g	h	i	j
R1	0	0	2	0	1	0	0	2	0	0	2	1	1	1	1	1	1	1	0	0	0	1	0	1	2	1	1	2	0	0
R2	1	0	2	0	1	0	1	0	0	0	0	2	1	1	2	1	0	1	0	0	0	1	1	0	0	1	1	1	0	0
R3	0	0	0	0	1	0	1	2	0	0	2	1	1	1	0	2	1	0	0	0	1	0	1	1	0	1	1	1	0	0

	Ear																													
	Pre										A										B									
	a	B	c	D	e	f	g	H	i	j	a	b	c	d	E	f	g	h	i	j	a	b	c	d	E	f	g	h	I	j
R1	0	0	1	0	1	1	0	0	0	0	2	2	2	2	2	2	1	0	1	1	0	1	1	2	2	1	0	0	0	1
R2	0	0	0	0	1	0	0	2	0	0	1	2	2	2	2	1	1	2	1	2	2	1	0	0	1	1	0	0	1	1
R3	1	0	1	2	2	0	0	1	0	0	0	2	1	2	1	2	0	0	0	0	2	1	0	2	1	1	0	2	0	0

KEY: R: Rabbit; a: Observer 1; b: Observer 2; c: Observer 3; d: Observer 4; e: Observer 5; f: Observer 6; g: Observer 7; h: Observer 8; i: Observer 9; j: Observer 10; pre: pre-surgical values; A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical);

Appendix 3. 1b The pain scored by ten observers for different facial expression using the Rabbit Grimace Scale.

Orbital																													
			C										D									E							
A	b	c	d	e	f	g	h	I	j	a	b	c	d	e	f	g	h	i	j	a	b	c	d	e	f	G	h	i	J
0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0
1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	0	0	0	0	0	1	1
0	0	0	0	0	1	1	0	0	0	1	1	1	1	0	1	0	0	1	1	1	0	1	0	0	0	0	1	1	1
Cheek																													
			C										D									E							
A	b	c	d	e	f	g	h	I	j	a	b	c	d	e	f	g	h	i	j	a	b	c	d	e	f	G	h	i	j
1	0	2	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	1	1	1	1	1	0	0	0	0	0	0	0
2	1	2	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	0	1	0	0	0	0	0	1	1
0	0	1	0	1	1	1	0	1	1	1	1	1	1	0	1	1	0	0	0	2	0	1	0	0	0	0	0	1	1
Nostril																													
			C										D									E							
A	b	c	d	e	f	g	h	I	j	a	b	c	d	e	f	g	h	i	j	a	b	c	d	e	f	G	h	i	j
0	0	1	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	1	0	1	1	1	0	0	0	0	1	2	0
1	0	1	1	1	1	1	1	1	2	0	1	1	0	0	1	1	0	0	0	1	0	1	0	0	1	0	0	1	0

1	0	0	0	0	1	1	0	2	0	1	1	1	1	0	1	1	1	0	0	0	0	1	0	0	0	1	1	2	2

Whisker

										C										D									E								
A	b	c	d	e	f	g	h	I	j	a	b	c	d	e	f	g	h	i	j	a	b	c	d	e	f	G	h	i	j							
1	1	1	0	0	0	1	0	0	0	1	0	0	0	1	1	1	0	0	0	2	1	1	0	1	0	1	1	0	0							
1	1	2	1	1	1	1	1	0	0	1	1	1	1	2	1	1	0	1	1	1	0	1	1	0	1	1	1	0	1							
1	0	1	0	0	1	1	0	1	1	0	1	1	1	1	1	1	1	0	1	0	0	1	1	0	0	1	1	0	0							

Ear

										C										D									E								
A	b	c	d	e	f	g	h	I	j	a	b	c	d	e	F	g	h	i	j	a	b	c	d	e	f	G	h	i	j							
2	1	2	2	1	1	0	2	1	1	0	2	2	2	2	1	1	2	2	1	2	1	1	2	2	1	1	0	1	1							
2	1	2	2	2	1	0	2	1	1	2	2	2	2	2	1	0	2	2	2	0	0	1	1	2	1	0	0	0	1							
0	2	2	1	1	1	1	1	1	0	2	2	2	2	2	1	0	1	2	2	0	0	1	1	2	0	0	0	1	1							

KEY: R: Rabbit; a: Observer 1; b: Observer 2; c: Observer 3; d: Observer 4; e: Observer 5; f: Observer 6; g: Observer 7; h: Observer 8; i: Observer 9; j: Observer 10; pre: pre-surgical values; A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical);

Appendix 3.2a The pain scored by ten observers for different treatment agents using the Rabbit Grimace Scale.

		pre										A										B									
		a	b	c	d	e	f	g	h	i	j	a	b	c	d	e	f	g	h	i	j	a	b	c	d	e	f	g	h	i	j
I	R1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
	R2	0	0	0	0	0	0	1	1	0	0	1	1	1	2	2	1	0	0	0	0	1	0	0	0	0	1	1	0	0	0
	R3	0	0	0	0	0	0	0	1	0	0	2	2	2	1	2	2	0	0	1	1	1	0	1	0	1	0	0	1	0	0
III	R1	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	1	0	0	0	0	0	1	0	0
	R2	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	2	1	0	0	0	0	0	0	1	0	0	0	0
	R3	1	0	0	0	0	0	0	1	0	1	0	2	1	1	1	2	0	1	1	1	1	0	1	1	0	0	0	0	0	1
III	R1	1	1	0	0	2	1	0	1	2	2	2	0	1	0	0	1	0	1	0	0	1	0	0	1	1	1	1	0	1	1
	R2	1	0	1	0	1	0	0	1	0	0	2	1	1	1	0	1	1	1	0	0	1	0	0	1	1	1	0	0	1	0
	R3	1	0	0	0	0	0	0	0	0	0	2	1	1	1	0	2	0	1	0	0	1	0	1	1	1	1	0	0	1	1
IV	R1	0	0	2	0	1	0	0	2	0	0	2	1	1	1	1	1	1	1	0	0	0	1	0	1	2	1	1	2	0	0
	R2	1	0	2	0	1	0	1	0	0	0	0	2	1	1	2	1	0	1	0	0	0	1	1	0	0	1	1	1	0	0
	R3	0	0	0	0	1	0	1	2	0	0	2	1	1	1	0	2	1	0	0	0	1	0	1	1	0	1	1	1	0	0
V	R1	0	0	1	0	1	1	0	0	0	0	2	2	2	2	2	2	1	0	1	1	0	1	1	2	2	1	0	0	0	1
	R2	0	0	0	0	1	0	0	2	0	0	1	2	2	2	2	1	1	2	1	2	2	1	0	0	1	1	0	0	1	1
	R3	1	0	1	2	2	0	0	1	0	0	0	2	1	2	1	2	0	0	0	0	2	1	0	2	1	1	0	2	0	0

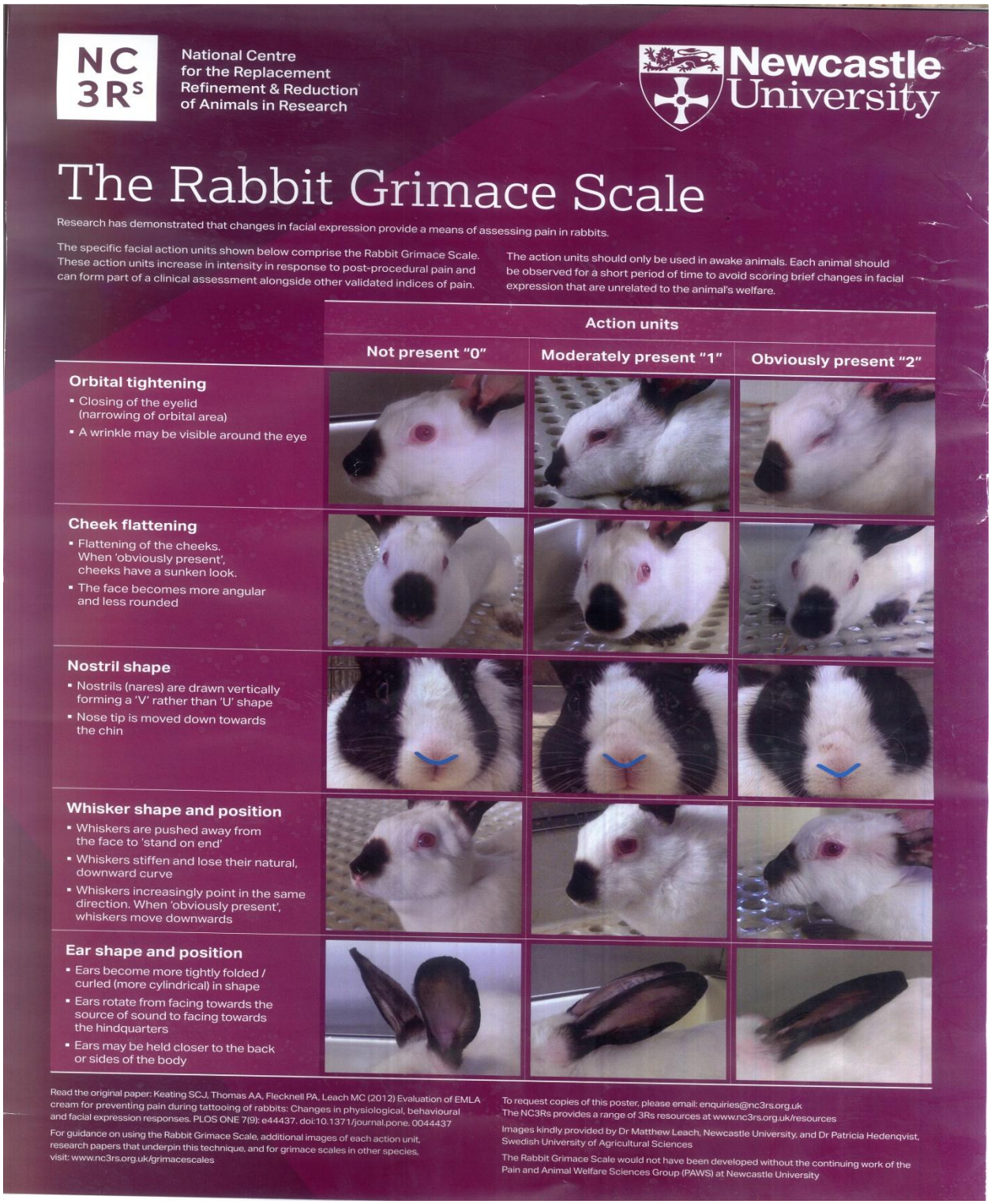
KEY: R: Rabbit; a: Observer 1; b: Observer 2; c: Observer 3; d: Observer 4; e: Observer 5; f: Observer 6; g: Observer 7; h: Observer 8; i: Observer 9; j: Observer 10; pre: pre-surgical values; A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical);

Appendix 3. 2b The pain scored by ten observers for different treatment agents using the Rabbit Grimace Scale.

		C										D										E									
		a	b	c	d	e	f	g	h	i	j	a	b	c	d	e	f	g	h	i	j	a	b	c	d	e	f	g	h	i	j
I	R1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0
	R2	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	0	0	0	0	0	1	1
	R3	0	0	0	0	0	1	1	0	0	0	1	1	1	1	0	1	0	0	1	1	1	0	1	0	0	0	0	1	1	1
II	R1	1	0	2	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	1	1	1	1	1	0	0	0	0	0	0	0
	R2	2	1	2	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	0	1	0	0	0	0	0	1	1
	R3	0	0	1	0	1	1	1	0	1	1	1	1	1	1	0	1	1	0	0	0	2	0	1	0	0	0	0	0	1	1
IIII	R1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	1	0	1	1	1	0	0	0	0	1	2	0
	R2	1	0	1	1	1	1	1	1	1	2	0	1	1	0	0	1	1	0	0	0	1	0	1	0	0	1	0	0	1	0
	R3	1	0	0	0	0	1	1	0	2	0	1	1	1	1	0	1	1	1	0	0	0	0	1	0	0	0	1	1	2	2
IV	R1	1	1	1	0	0	0	1	0	0	0	1	0	0	0	1	1	1	0	0	0	2	1	1	0	1	0	1	1	0	0
	R2	1	1	2	1	1	1	1	1	0	0	1	1	1	1	2	1	1	0	1	1	1	0	1	1	0	1	1	1	0	1
	R3	1	0	1	0	0	1	1	0	1	1	0	1	1	1	1	1	1	1	0	1	0	0	1	1	0	0	1	1	0	0
V	R1	2	1	2	2	1	1	0	2	1	1	0	2	2	2	2	1	1	2	2	1	2	1	1	2	2	1	1	0	1	1
	R2	2	1	2	2	2	1	0	2	1	1	2	2	2	2	2	1	0	2	2	2	0	0	1	1	2	1	0	0	0	1
	R3	0	2	2	1	1	1	1	1	1	0	2	2	2	2	2	1	0	1	2	2	0	0	1	1	2	0	0	0	1	1

KEY: R: Rabbit; a: Observer 1; b: Observer 2; c: Observer 3; d: Observer 4; e: Observer 5; f: Observer 6; g: Observer 7; h: Observer 8; i: Observer 9; j: Observer 10; pre: pre-surgical values; A: Normal saline (oral); B: Meloxicam (0.1mg/kg; oral); C: *C. ambrosioides* extracts (500mg/Kg; oral); D: Ketoprofen (Liberal; topical); E: *C. ambrosioides* extracts (Liberal; topical);

Appendix 4: The Rabbit Grimace Scale



NC 3Rs
National Centre for the Replacement Refinement & Reduction of Animals in Research

Newcastle University

The Rabbit Grimace Scale

Research has demonstrated that changes in facial expression provide a means of assessing pain in rabbits.

The specific facial action units shown below comprise the Rabbit Grimace Scale. These action units increase in intensity in response to post-procedural pain and can form part of a clinical assessment alongside other validated indices of pain.

The action units should only be used in awake animals. Each animal should be observed for a short period of time to avoid scoring brief changes in facial expression that are unrelated to the animal's welfare.

	Action units		
	Not present "0"	Moderately present "1"	Obviously present "2"
Orbital tightening ▪ Closing of the eyelid (narrowing of orbital area) ▪ A wrinkle may be visible around the eye			
Cheek flattening ▪ Flattening of the cheeks. When 'obviously present', cheeks have a sunken look. ▪ The face becomes more angular and less rounded			
Nostril shape ▪ Nostrils (nares) are drawn vertically forming a 'V' rather than 'U' shape ▪ Nose tip is moved down towards the chin			
Whisker shape and position ▪ Whiskers are pushed away from the face to 'stand on end' ▪ Whiskers stiffen and lose their natural, downward curve ▪ Whiskers increasingly point in the same direction. When 'obviously present', whiskers move downwards			
Ear shape and position ▪ Ears become more tightly folded / curled (more cylindrical) in shape ▪ Ears rotate from facing towards the source of sound to facing towards the hindquarters ▪ Ears may be held closer to the back or sides of the body			

Read the original paper: Keating SCJ, Thomas AA, Flecknell PA, Leach MC (2012) Evaluation of EMLA cream for preventing pain during tattooing of rabbits: Changes in physiological, behavioural and facial expression responses. PLOS ONE 7(9): e44437. doi:10.1371/journal.pone. 0044437

For guidance on using the Rabbit Grimace Scale, additional images of each action unit, research papers that underpin this technique, and for grimace scales in other species, visit: www.nc3rs.org.uk/grimacescales

To request copies of this poster, please email: enquiries@nc3rs.org.uk
The NC3Rs provides a range of 3Rs resources at www.nc3rs.org.uk/resources

Images kindly provided by Dr Matthew Leach, Newcastle University, and Dr Patricia Hedenqvist, Swedish University of Agricultural Sciences

The Rabbit Grimace Scale would not have been developed without the continuing work of the Pain and Animal Welfare Sciences Group (PAWS) at Newcastle University