EFFECT OF THE ETHANOL SEED EXTRACT OF *PICRALIMA NITIDA* (STAPF) TH. &H. DURAND) ON COUGH AND ITS COMPLICATIONS

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST. This work has not been submitted for any other degree.

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LIST OF ABBREVIATIONS

15-HPETE	15-hydroperoxyeicosatetraenoic acid
ACEI‘s	Angiotensin converting enzyme inhibitors
Ach	Acetylcholine
ATP	Adenosine triphosphate ATR
Atropine cAMP	Cyclic adenosine
monophosphate	
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disorder
DHC	Dihydrocodeine
DPPH	2, 2-diphenyl-1-picrylhydrazyl hydrate
EPM	Elevated plus-maze
GABA	Gamma amino butyric acid
GERD	Gastro esophageal reflux disease
GIRK	G-protein-coupled inwardly rectifying K^+ IASP
International Association for the Study of Pain	
i.p	Intraperitoneal
KNUST	Kwame Nkrumah university of science and technology
MCD	Mast cell degranulation
MPE	Maximum possible effect
NH_4Cl	Ammonium chloride
NMDA	N-methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drugs NTS
Nucleus tractus solitarius	
p.o	*Per os*

PNDS	Post nasal drip syndrome
PNE	*Picralima nitida* extract
PNS	Peripheral nervous system
RAR	Rapidly adapting receptors
SAR	Slowly adapting receptors
SCG	Sodium cromoglycate
TRP	Transient receptor potential

ABSTRACT

Seed extracts of *Picralima nitida* are used by some local folks in the management of cough. Previous reports concerning the pharmacological activities of the plants support such claim. Also chronic idiopathic cough leads to worrisome complications like pain and anxiety. The experiment therefore aimed at investigating primarily the effect of the ethanolic seed extract of *Picralima nitida* (PNE) on cough, its mode of action as well as the anxiolytic and analgesic effects after the secondary metabolites and safety profile had been investigated. The secondary metabolites present, and safety profile of PNE (10-2000 mg/kg) was ascertained by preliminary phytochemical screening and Irwin's test respectively. Percentage reduction in cough count and percentage increase in latency of cough offered by the extract was established by the Citric acid-induced cough model in which guinea pigs were treated with 100-500 mg/kg PNE or reference drug, dihydrocodeine. The expectorant properties of PNE (100-1000 mg/kg) was determined using the tracheal phenol red secretion, with ammonium chloride as a reference medication. The bronchodilator; muco-suppressant and mast cell stabilization effects of PNE (100-500 mg/kg) were ascertained using acetylcholine and histamine-induced bronchoconstriction models; ammonium chloride-induced (5 mg/kg; p.o) phenol red (500 mg/kg, i.p) secretion in mice; compound 48/80-induced (1 µg)mesenteric mast cell degranulation assay respectively. PNE's (1-50 mg/ml) antibacterial potential was ascertained on *S. aureus, S. pneumonia, S.typhi, E. coli* and *K. pneumonia* by the agar plate diffusion method and its antioxidant potential (0.01-0.3 mg/ml) by the DPPH free radical scavenging method. Percentage maximal possible analgesic effect in a tail flick test, and the total nociceptive score in acetic acid-induced abdominal writhes, after treatment of mice with PNE (100-500 mg/kg), diclofenac (10-100 mg/kg), and morphine (1-10 mg/kg) were also estimated. Finally, the anxiolytic effect of PNE (100-500 mg/kg) was determined using the open field and the elevated plus maze in mice. Phytochemical

screening revealed the presence of tannins, alkaloids, glycosides, saponins, steroids, terpenoids and anthraquinones. PNE (10-500 mg/kg) did not show any extract-related physical, pharmacological, and CNS toxicities; sedation was observed at doses 10002000 mg/kg. No mortality was recorded. PNE showed significant ($P \leq 0.05$) dosedependent reduction in cough count, and increased ($P \leq 0.01$) cough latency. PNE (1000 mg/kg) enhanced ($P \leq 0.05$) tracheal phenol red secretion as an indication of its expectorant activity. The extract inhibited both acetylcholine and Histamine-induced bronchoconstriction ($P \leq 0.05$). PNE (100-500 mg/kg) and Sodium cromoglycate (100 mg/kg) reduced ($P \leq 0.05$ - 0.001) tracheal phenol red secretion. The extract (100-500 µg/ml) dose-dependently ($P \leq 0.05$ - 0.0001) stabilized mast cell. PNE (10-50 mg/ml) had significant ($P \leq 0.05$), activity against *Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Streptococcus pneumonia,* and *Staphylococcus aureus* (minimum zone of inhibition 13.0±0.00 mm; maximum: 22.3±0.88 mm). PNE showed antioxidant effect as it enhanced 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging effect (EC_{50}= 6.53 $\times 10^{-2}$ mg/ml).PNE (100-500 mg/kg) just like Diazepam (0.1-1.0 mg/kg) increased open arm activities in the elevated plus maze ($P \leq 0.05$) as well as central zone exploration ($P \leq 0.05$) in the open field test. PNE (100–500 mg/kg) significantly ($P \leq 0.05$) and dose dependently increased tail withdrawal latencies, and nociceptive score. PNE has LD_{50}> 2000 mg/kg. It has an antitussive effect as well as an expectorant effect which occurs at a higher dose. It exhibits the antitussive effect through bronchodilator, mucus suppressant, mast cell stabilizing, antioxidant, anxiolytic and antibacterial effects. PNE will be effective against complications of idiopathic chronic cough like anxiety and pain.

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1.0 GENERAL INTRODUCTION, AIM AND OBJECTIVES

1.1 OVERVIEW

Cough is defined ―a forced expulsive maneuver, usually against a closed glottis and which is associated with a characteristic sound‖ (Morice *et al.,* 2007). Cough was at a point the most frequent reason for seeking an ambulatory health care visit (Schappert, 1991; Chung and Pavord, 2008). No wonder it was once reported that over $2,000 million was spent on over-thecounter (OTC) cough medicines by patients in the USA (Morice, 2002). Environmental pollutants contribute significantly to cough (Pierse *et al.,* 2006). At other times, cough usually presents with other conditions like Asthma, GORD etc. (Ford, 2006).

The management of symptomatic cough has usually been with the opioid antitussives like codeine, morphine etc. Not only has the efficacy of the opioid antitussive been questioned of late against cough in certain conditions but also a lot of concerns have been raised with regards to their adverse effects. This has made it necessary to get appropriate medicinal products that will have a better safety and efficacy profile.

Picralima nitida is a plant from the family Apocynaceae that seems very promising in the management of cough as it is being used traditionally for the condition. But without scientific evidence, very little can be said about efficacy of plants having sole evidence in the traditional setting since it may just infer community knowledge of existence and application of such substances (Zhang, 2000).

1.2 COUGH

Cough is defined ―a forced expulsive maneuver, usually against a closed glottis and which is associated with a characteristic sound‖ (Morice *et al.,* 2007). It was at a point the commonest

symptom for which medical advice was sought (Schappert, 1991). Many other respiratory reflexes seems to have a pattern similar to cough but are very different: sneezing and gagging can all be induced by stimulation of airway nerves just as cough, but there exist a difference in that whilst they are induced by stimulating other cranial nerves, cough is induced by stimulation of the tenth cranial nerve (Vagus nerve).

Cough can be partitioned into four-to five phases:

- Encoding phase when there is encoding of the action potential by the particular afferent nerve responding to the tussive stimulus followed by reconfiguration of the respiratory motor drive within the brainstem
- Inspiration phase when a deep inspiration then follows
- compressive phase characterized by closure of the larynx
- expulsive phase when the larynx opens allowing forceful expiration
- The restorative phase, during which a deep breath is taken.

The cough reflex has three components- the afferent system, which senses the cough-inducing stimulus, the central nervous system (CNS) and lastly the efferent system (Widdicombe, 1999).

1.2.1 COUGH SENSORS

The evidential cough afferents consist basically of the C-fibers, rapidly adapting receptor, and the touch-sensitive Aδ fibers (the cough receptor)

1.2.1.1 C-FIBERS

C-fibers constitute majority of airway afferents. Unlike other fibers, they are unmyelinated and are usually distinguished from rapidly adapting receptors (RAR) and slowly adapting receptors (SAR) by the fact that they do not respond to mechanical stimulation but rather respond well to chemical agents like bradykinin and capsaicin. Animal studies have revealed subclasses of the

C-fibers: In dogs, airway afferent C-fibers may be further subdivided into bronchial and pulmonary C-fibers. C-fibers are polymodal in that they respond to both chemical and mechanical stimulation, but they have a higher threshold for mechanical stimulation; consequently, they are inactive during respiration but are activated by chemical stimulants like capsaicin and bradykinin. Upon activation of the C-fibers by some of these agents, there can be peripheral release of neuropeptide without CNS involvement by a process called axonal reflex. Evidence for the involvement of C-fibers in cough is as follows:

- C-fiber selective stimuli like capsaicin, citric acid, bradykinin all evoke cough in conscious guinea pigs
- high dose Capsaicin desensitizes C-fibers, hence abolishes citric acid-induced coughing in guinea pigs, but has no effect on cough evoked by mechanical stimulation of the airway mucosa in these such animals
- lastly,neurokinin receptor antagonist are very good antitussive and neurokinins are highly expressed in C-fibers

1.2.1.2 RAPIDLY ADAPTING RECEPTORS (RARS)

RAR‘s terminate both in the extrapulmonary and intrapulmonary airway but are predominantly intrapulmonary. As their name implies, they adapt rapidly to sustained lung inflation. They are myelinated and respond to bronchospasm or obstruction that results from edema or mucus suppression but insensitive to direct chemical stimulus. Activation of RAR also induce bronchospasm and mucus secretion through parasympathetic pathways. The purported active role of RAR in cough has been questioned over the years due to the fact that:

- Direct stimulants of RAR are ineffective or only partially effective in causing cough.
- RARs are active throughout the respiratory cycle yet cough does not occur during breathing in normal humans.

From these facts, it has been concluded that they act synergistically with other afferent nerves in inducing cough

1.2.1.3 COUGH RECEPTOR

Identified by Canning in 2004, this receptor seems to be the main afferent that mediate cough. Though they are not the same as either the C-fiber or the RAR, there exist certain similarities between the cough receptor and these afferents. For instance cough receptor and RAR are all myelinated and adapt rapidly to stimulation. The cough receptor however does not respond to airway obstruction. The cough receptor is polymodal and is activated by punctate mechanical stimuli, acid, water, and the potassium channel blocker 4-aminopyridine.

1.2.2 CENTRALLY MEDIATED EVENTS

After sensors respond to stimulus, impulses are sent through the vagus nerve to the CNS. The first synaptic target in the CNS is the second-order neuron in the nucleus tractus solitarius (NTS). Varying afferent fibers interact with this second order neuron in the NTS, and the processing done by the second order neuron depend on the afferent fiber that it receives the impulse. This has effect on the intensity, frequency and the threshold for cough. In fact the particular afferent bringing the impulse, determines whether there will be suppression or in inhibition of cough. For instance stimulation of bronchopulmonary C-fiber, cardiac, and abdominal afferent nerve endings may down regulate the cough reflex. In instances where different sensory inputs come to the NTS, they will be processed with any other relevant inputs from even higher brain regions and outputs are appropriately coordinated by the NTS to distal synapses in order to elicit the desired response, be it suppression or modification of the cough response.

Centrally, the cerebral cortex is also known to influence the cough reflex (Lee *et al.*, 2002). The cough pathway is shown in Figure 1.1

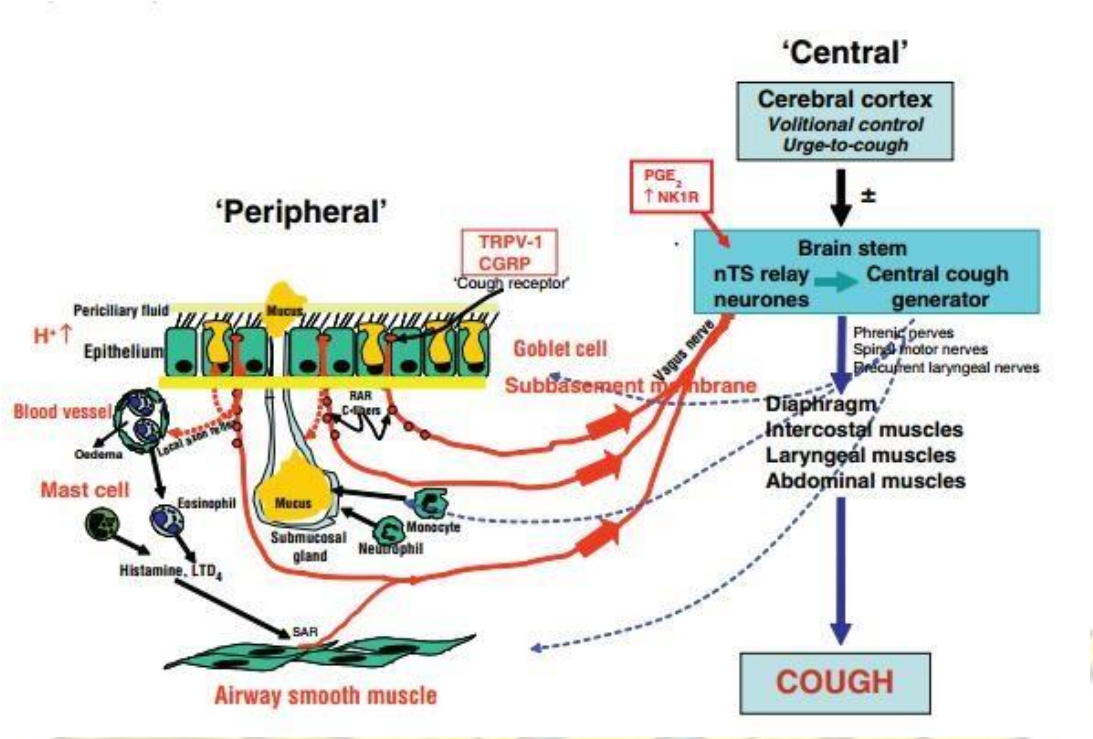

Figure 1.1: Schematic representation of the cough reflex's pathway; there is generation of impulses from the peripheral site which passes through the vagus nerve and terminate at the brain stem(central site) and finally back through efferent nerves to thoracic muscle for the reflex to be complete. The diagram also shows how the cerebral cortex modulate the activity of the cough reflex. (Adapted from Chung and Widdicombe, 2009)

1.2.3 EVALUATION OF ANTITUSSIVES

Antitussive drugs can be tested in different ways. There are basically three ways of testing antitussives in human and two ways in animals. In humans the evaluation can occur by one of these three ways:

- In healthy non coughing subjects using provocation of cough by irritants aerosol such as citric acid, capsaicin or distilled water (fog)
- in clients with cough due to airways' disease

- in clients with cough, induced by an irritant aerosol

In animals, the test can be ascertained by one of two ways:

- spontaneous cough due to disease or
- induced by an irritant

1.2.4 ANIMAL MODELS OF COUGH

The ultimate aim of an animal research is to develop a model that will help to study cough mechanisms and identify potential antitussives. Usually in development of drugs, if promise is shown in animal studies, clinical studies can be undertaken.

1.2.4.1 Animal species used in cough experiments

Various kind of animals can be used for antitussive experiments. Dogs, cats, guinea pigs, pigs, rats and rabbits have all at some point been used. There are a lot of issues that come with the use of some of these animals for cough research: it has been much debated whether the response that occur in rats and mice upon exposure of tussigenic agents could be classified as cough. There is a respiratory reflex produced from the larynx which is similar to cough, but whose pathway and regulation seem to be very different from cough (Korpas, 1972). It may then be that investigators using rats may be counting a respiratory reflex instead of cough. Use of mice is also faced by at least two challenges:

- Mice cannot generate the needed energy to cough and so they don‘t cough
- Mice lack RAR and intraepithelial nerve endings (Karlsson *et al.*, 1988).

The use of large animals like cat and pigs require capital for their purchasing, feeding and housing. Secondly, there need to be efficient training and taming of personnel and animals respectively since some of these animals are wild in nature, hence posing danger for the experimenter. With all these factors considered, the guinea pig seems to be the species preferred

and mostly used in antitussive experiments (Tatar *et al.*, 1997). It seems to mimic the human cough response and pathway though there are slight differences. For instance, human airway unlike the guinea pig airway has few Substance P containing neurons. Also the guinea pig is an obligate nose breather, which may also introduce differences in the cough reflex.

1.2.4.2 Tussigenic agents

These are the agents that induce the cough response. There are many ways of inducing cough in animals including chemical, electrical, mechanical changes in ion concentration or osmolarity in the mucosal surface fluid, or of sensory afferents or by stimulation of the CNS (Belvisi & Hele, 2004). In most instances, electrical stimulation of especially the superior laryngeal nerve has been used in studying central pathway and in identifying centrally acting antitussives. Mechanical stimulation is mostly employed in anesthetized animals. Chemical stimulation seems to be the commonest of the various methods listed above. Chemicals like capsaicin and citric acid have been used in numerous experiments to induce cough. Generally, the kind of stimulation will inform the experimenter as to the exact receptor these agents stimulate: whiles mechanical stimulation of tracheobronchial tree stimulates predominantly touch sensitive Aδ fibers, capsaicin and inhaled citric acid stimulates C-fibers (Widdicombe, 1996a). Differences may exist between agents acting on a similar sensor; though capsaicin and citric acid all stimulate C-fiber, citric acid allows repeated cough measurement without developing tachyphylaxis, whereas repeated exposure to capsaicin results in tachyphylaxis (Morice *et al*., 2001).

1.2.4.3 Protocol

The protocol used depends on the model that is being applied in the investigation.

In citric acid-induced cough model as a sample, animals should be housed under controlled conditions with frequent changes of bedding since the build-up of ammonia in cages has been

shown to influence the cough response to citric acid (Moreaux *et al*., 2000a). When guinea pigs are in turn placed in the chamber (~1L), they should be allowed to freely move. They are then exposed to aerosol of the stimulus at a specific rate. The exposure time is usually ≤10 min, depending on the tussive agent used. Animals are then closely monitored. Cough is recognized basically by three parameters:

- By observing the guinea pig as it coughs. An observer should be able to differentiate between coughing, sneezing and expiratory reflex. Cough is characterized by splaying of the front feet and forward stretching of the neck and opening of the mouth
- By sound. The cough sound can be amplified by microphones
- By pressure changes owing to the deep inspiration and explosive expiration occurring during cough. This can be detected by a chart recorder.

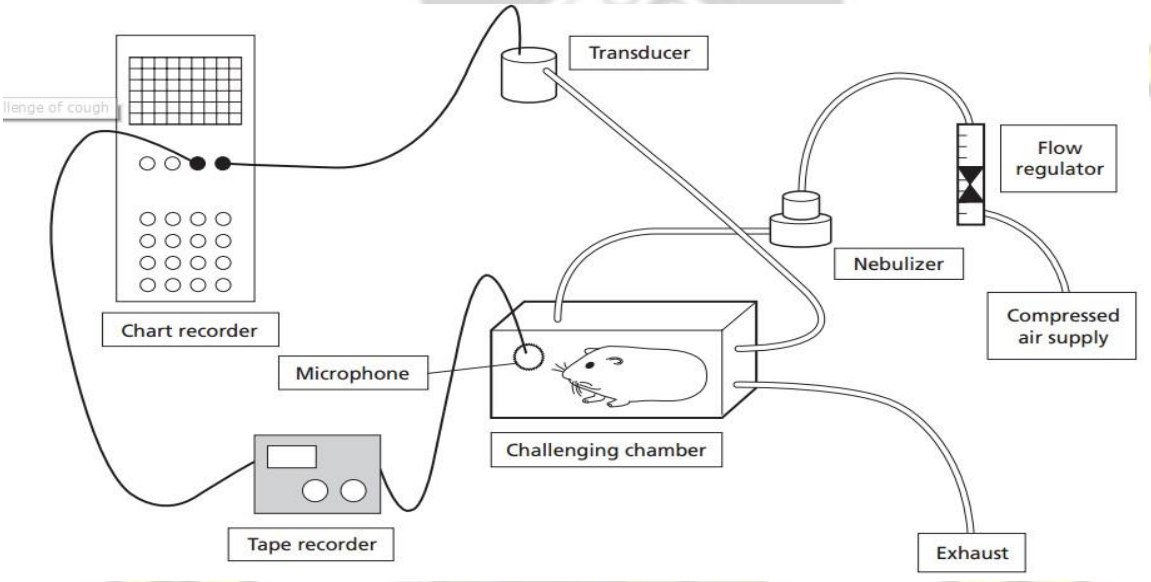

Figure 1.2: Schematic representation of a typical set-up used in investigating the tussive response of guinea pig to aerosolized chemicals: a nebulizer delivers the aerosolized chemical (eg citric acid) with the flow regulated. The animal responds by coughing with the sound amplified by a microphone and recorded on a chart recorder by the help of a transducer (adapted from Chung *et al*., 2003)

1.2.5 PHARMACOLOGICAL MODULATION

Various agents are being developed pharmacologically for cough taking into consideration the cough sensors and pathway involved in the reflex. Figure 1.3 is a diagrammatic representation of the sites of various pharmacological agents.

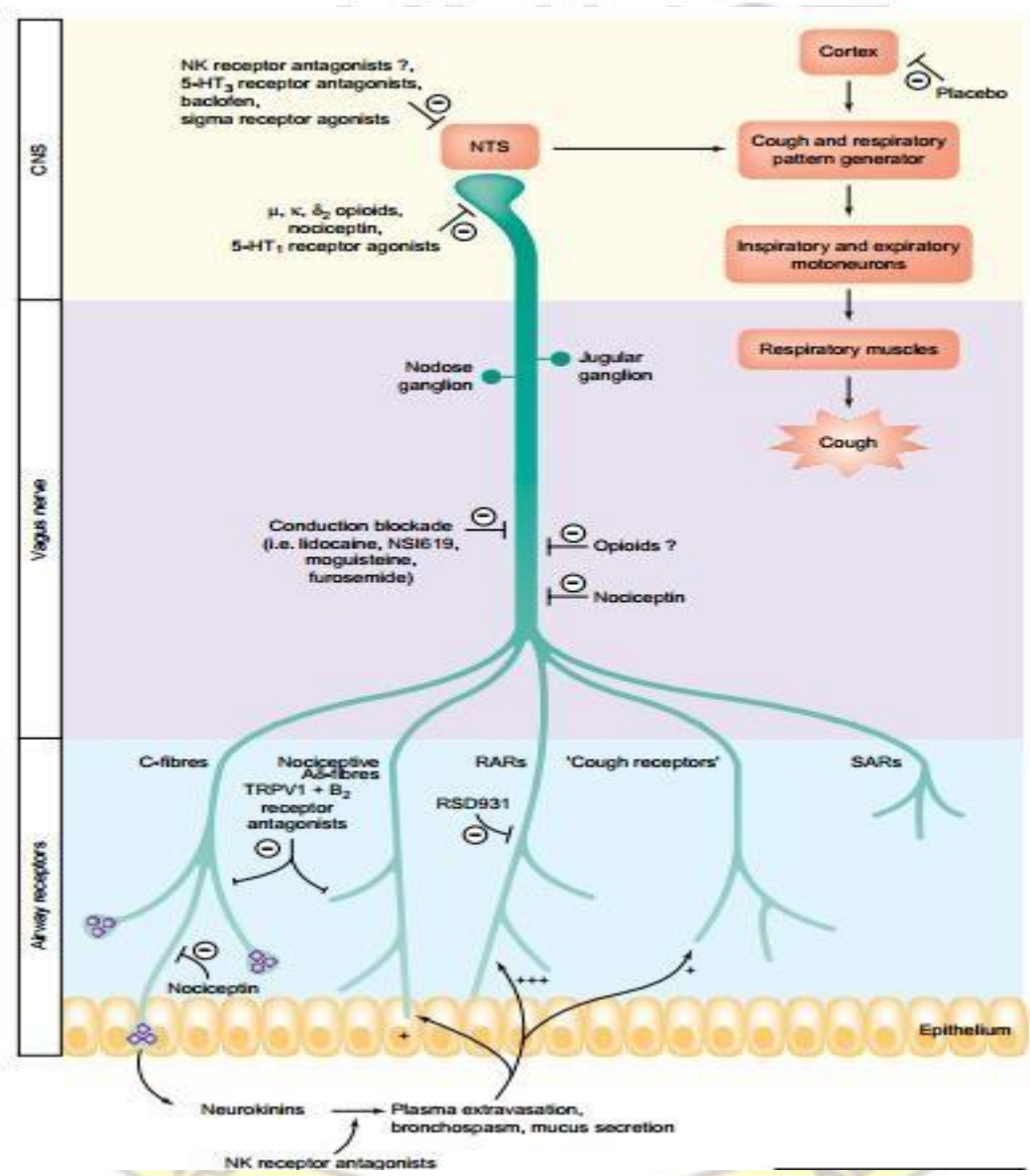

Figure 1.3: An illustration of how pharmacological agents affect the cough reflex: at the airway receptor level, C-fiber stimulation results in release of neurokinin which induces plasma extravasion etc. this in turn stimulate the RAR and rarely the cough receptor. Impulses then pass through the vagus to the NTS.
Θ indicates inhibition by these pharmacological agents at their respective sites (adapted from Reynolds *et al.*, 2004)

1.2.5.1 AGENTS THAT AFFECT TRP

Transient receptor potential (TRP) channels have been found to play significant role in cough. There are subfamilies in this group of channels including TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPML(mucolipin) and TRPP (polycystin) (Nilius *et al*., 2007). These channels especially TRPA and TRPV which are located on the Cfibers respond to tussive stimuli. TRPV1 is activated by agents like capsaicin, proton, heat (Caterina *et al.*,1997) and lipid mediators like 15-hydroperoxyeicosatetraenoic acid (15-HPETE). The role of TRPV1 in cough is supported by the fact that TRPV1 antagonist like capsazepine (Lalloo *et al*., 1995) have demonstrated significant cough suppressive effect in various models. Pharmacologists attach so much interest in finding agents that will have antagonistic effect against TRPV1 activity since retrospective experiments concerning TRPV1's shows that they inhibit pathologic cough without affecting the normal defensive cough. Apart from the TRPV1, TRPA1 are also another class of channels that get stimulated by agents like cigarette smoke. Menthol's antitussive effect has been attributed partly to antagonism of TRPA1 channels. Menthol additionally activates TRPM8 channels (McKemy *et al.*, 2002).

1.2.5.2 ION CHANNEL MODULATORS

Many ion channels do exist on the neurons. And most of these channels play significant role in cough. NS1619 is a potassium channel activator that has shown promising antitussive effect through hyperpolarization of neurons by causing activation of calcium-activated potassium channel. In *in vitro* experiment with guinea pigs, NS1619 inhibit both citric acid and bradykinin induced cough (Adcock *et al*., 1988). Another type of potassium channel that has also received much attention is the ATP-sensitive potassium channel. Agents like pinacidil and moguistene which are known openers of these channels have demonstrated antitussive properties. The loop diuretic, furosemide which mechanistically blocks the $Na^+/K^+/2Cl^-$ cotransporter expressed in

the peripheral terminals of the _cough' receptor in guinea pigs (Mazzone and McGovern, 2006), inhibits cough induced by low chloride solution. Local anesthetics are also another group of channel blockers that suppress cough. These drugs have long been known to act by blocking sodium channels. Local anaesthetics and their analogues have also shown to act independently on their local anesthetic effect by selectively acting on certain fibers: RSD 931 (carcainium chloride), a quaternary ammonium molecule inhibit capsaicin and citric acid induced cough in guinea pigs (Adcock *et al*., 2003). It has been demonstrated that RSD 931 act selectively on Aδfibres independent on their local anesthetic mechanism.

1.2.5.3 TACHYKINNIN ANTAGONISTS

Tachykinins are important mediators in cough. They are expressed in neurons, and with regard to cough is highly expressed in the C-fiber. The tachykinins of interest include substance P, neurokinin A and neurokinin B. These agents when released from a stimulated C-fiber, induces local effects like bronchoconstriction, vasodilation, edema and mucus secretion through an interaction with NK_1, NK_2 and NK_3. These effects can also in turn stimulate RAR's. Antagonism of all three neurokinin receptors have resulted in cough suppression (Daoui *et al*., 1998; Moreaux *et al*., 2000b). Though these tachykinin antagonist usually act in the periphery, possibility of a central mechanism can't also be ruled out. For instance, NK1antagonist CP99,994 significantly crosses the blood brain barrier to interfere with transmission in the NTS making it a potential cough suppressant.

1.2.5.4 BRADYKININ RECEPTOR ANTAGONIST

Bradykinin stimulates action potential discharge in airway C-fibers (Hargreaves *et al*., 1993) and such effects are blocked by their antagonists (Kajekar *et al*., 1999). Also a search for effective antitussive acting through bradykinin antagonism has become necessary since the

angiotensin converting enzyme inhibitors (ACEI's), one of the mainstay in heart failure treatment cause cough through elevation of bradykinin levels, and so cough sensitivity to capsaicin is increased following administration of ACEI's (Morice *et al*., 1987). Some pharmacological agents like HOE-140, a B2receptor antagonist has exhibited an antitussive effect (Featherstone *et al*., 1996).

1.2.5.5 LEUKOTRIENE RECEPTOR ANTAGONIST

Leukotrienes are eicosanoids produced through the lipoxygenase pathway. The exact mechanism by which leukotrienes contribute to the act of coughing has not clearly been elucidated but it is likely due to their action on the airway: bronchoconstriction, mucus secretion and edema. All these effects are known to effectively stimulate RAR. Another possible mechanism by which leukotrienes may possibly contribute to cough causation is by direct stimulation of cough afferent nerves (McAlexander *et al*., 1998). The typical leukotriene receptor antagonist Zafirlukast has demonstrated antitussive effects in humans (Dicpinigaitis *et al*., 2002).

1.2.5.6 OPIOID RECEPTOR AGONISTS

Opioid receptor agonists have traditionally been the symptomatic antitussives in use for centuries. They are usually limited by adverse effects like constipation, respiratory depression, sedation, nausea and physical dependence. This has called for a search into new antitussives that will be free from these effects. A lot of controversy has surrounded the classification of opioid receptors, but the current classical opioid receptors are μ, δ and κ (Gundlach *et al.,* 1986). These receptors are not only confined to the CNS as previously thought, but they are also located peripherally (Hughes *et al*., 1975). Evidence for peripheral effects of these opioids have been that, inhalations of aerosolized codeine and morphine exhibited cough suppression in animal

studies (Karlsson *et al.*, 1990). They work by acting on the μ receptors with resultant inhibition of adenylyl cyclase activity, which in turn closes calcium channels and opens potassium channels leading to hyperpolarisation, hence reducing sensory nerve activity. The NTS was the site that was considered to have the cough center, but recently, the raphe nuclei has also been included (Jakus *et al.*, 1998) and it is likely that it may be the site of action of most centrally acting antitussives.

Stimulation of all the three opioid receptors result in cough suppression. For instance codeine, U-50,488H, SB227122 being μ, δ and κ agonists respectively exhibit antitussive effect in animal models. There are subtypes for these receptors, and knowing them may be significant in predicting the effects of their respective agonist; delta-2 agonist are antitussives whiles delta-1 agonists have inhibitory role on cough suppression (Kamei, 2002).

1.2.5.7 SIGMA RECEPTOR AGONISTS

The sigma receptor, previously regarded as an opioid receptor, also like the classical opioid receptors have a significant role to play in cough. Dextromethorphan though inactive on opioid receptors, activates the sigma-1 receptor (Monnet, 2005) and that accounts for its antitussive effect. Dextromethorphan also has antagonist action on NMDA receptors (Franklin and Murray, 1992) also contribute to their antitussive effect. Another pharmacologic agent whose antitussive effect has been attributed to it v s dual mechanism just like dextromethorphan is Noscapine; it acts both as a sigma receptor agonist and a bradykinin receptor antagonist (Brown *et al.*, 2004).

1.2.5.8 GABA RECEPTOR LIGANDS

GABA is the main inhibitory neurotransmitter in the CNS and it is also present in the PNS as well. $GABA_A$ and $GABA_B$ are subtypes of this receptor. The $GABA_B$ receptor agonist,

especially baclofen exhibits antitussive effect both in animals and humans (Dicpinigaitis and Dobkin, 1997). A peripherally acting analogue of Baclofen, 3-aminopropylphosphinic has also been developed as an antitussive.

1.2.5.9 MAST CELL STABILIZERS

Disodium cromoglycate and nedocromil are mast cell stabilizers that have shown some promise as potential antitussives (Dixon *et al*., 1980) especially in conditions like Asthma. Though mast cells and their products seem to play a key role in cough, these drugs exhibit their antitussive effect mostly by mechanisms independent of their mast cell stabilizing property: they suppress neuron activation by inducing desensitization of the nerve (Jackson *et al*., 1992)

1.2.5.10 MUCOACTIVE AGENTS

These are agents that are known to have effect on mucus. Mucus play significant role in cough; mechanical perturbation caused by mucus can be induced in the airway. This can in turn activates RAR, one of the sensors involved in cough. Most mucoactive agents act through a physiological mechanism to relieve cough. mucoactives include expectorants, mucolytics, mucoregulatory and mucokinetic agents.

Expectorants are agents that increase the volume of airway secretion or add water to them in order to make them easy to be coughed out whiles mucolytic medications act to non-selectively decrease both the viscosity and elasticity of airway secretions. Example of an expectorant is Guaifenisin whiles that of mucolytics are acetylcysteine and carbocisteine. Mucoregulatory medications are known to reduce mucus hyper secretion through diverse mechanisms. Examples include anti-inflammatory, antibiotics and anticholinergic medications. Mucokinetic agents are medicines that enhance easy flow of mucus. Examples include bronchodilators and surfactants.

1.2.6 HERBAL ANTITUSSIVES

Herbal medicines have been used for centuries and have been found to be very effective for many conditions, even in those ones which orthodox medicines have failed. This has led to the upsurge in use of herbal medicines. In fact, it was at a point estimated that 80% of Africans rely on medicinal plants for their daily healthcare needs (Johnson *et al*., 2007). Apart from its efficacy, accessibilities and perceived relative safety of herbal medicines make them suitable for the indigenous population. Various plants have previously been found to be effective antitussives through different mechanisms. Table 1.1 lists different plant species with cough suppressant effect and their various actions which contribute to such cough suppressant effect.

Table 1.1 antitussive plants and their actions (Adapted from Chung and Widdicombe, 2009)

SPECIES	ACTIONS
Adhatoda vasica	Bronchodilator, expectorant and bitter
Allium sativum	Antibacterial
Glycyrrhizae glabra	Anti-cAMP
Panex quinquefolium	Antioxidant, antibacterial
Piper longum	Spicy
Theobroma cocoa	Bronchodilator
Sesamus indicum	Bronchodilator, mucolytic, surfactants
Zingiber officinale	Anti-inflammatory
Crocus sativus	Anti-inflammatory
Carum copticum	Bronchodilator, antihistamine
Fritillaria spp.	Anti-inflammatory, expectorant, antibacterial
Echinacea spp.	Anti-inflammatory, antioxidant, local anesthetic
Ephedra sinica	Bronchodilator

One plant that has shown promise in the community setting as an effective antitussive is *Picralima nitida* which is discussed shortly.

*Picralima nitida (P. nitida) (*Stapf.*)* T.A. Durand & H. Durand, belongs to the Apocynaceae family and is locally called Akuamma.

1.2.6.1 DESCRIPTION OF *PICRALIMA NITIDA*

P. nitida is an understorey tree which reaches up to 4-35m in height. The wood is pale yellow, hard, elastic, fine-grained and taking a high polish. *P. nitida* have ovoid shape fruit and are green in color. When these fruits fall on the ground, they turn to yellow and the seeds germinate on the ground with many seedlings. The leaves are broad (3-10cm) and oblong (6-20cm long) with tough tiny lateral nerves of about 14 to 24 pairs (Burkill, 1985).

Figure 1.4: A picture of dried seeds of *Picralima nitida*

1.2.6.2 ECOLOGICAL AND GEOGRAPHICAL DISTRIBUTION

The plant is widely distributed in high deciduous forest of West-Central Africa from Ivory Coast to West Cameroons, and extending across the Congo basin and Uganda (Burkill, 1985).

1.2.6.3 TRADITIONAL USES

The plant has been used in managing respiratory disorders: In most West African countries especially Nigeria, the seeds are used for pneumonia and cough. In Ghana the crushed seeds is used for chest complaints (Irvine, 1961).It is also used traditionally for infectious disorders: *P. nitida* ranks first among four plants used to treat typhoid fever by indigenous communities in the eastern region of Cameroon (Yakeu *et al*., 2012). The seeds are also used for malaria management. The leaves are used as a vermifuge (Iwu, 1993) and in Ivory Coast, a decoction of the plant is used for the management of yellow fever (Burkill, 1985). *Picralima nitida* is used for pain and inflammatory disorders: The fruits are traditionally used for muscular pain management (Yakeu *et al*., 2012). It has also been found to be effective in dysmenorrhea (JE Ajanohoun, 1996). Apart from the fruits, the leaves are also used into the ear for otitis (Iwu, 1993).In Ghana, the seed-decoction is given as an enema (Irvine, 1961). The bark also used as a purgative. Topically, the seeds are applied externally for the treatment of abscesses.

*1.2.6.*4 CHEMICAL CONSTITUENTS OF PNE

Phytochemical studies have been conducted on *Picralima nitida*; phytochemicals that have been found to be present include alkaloids, glycosides, saponins, tannins, terpenoids, flavonoids and steroids. The specific alkaloids are akuammine, pseudoakuammine, akuammidine, akuammicine, akuammigine, pseudoakuammigine, akuammiline, akuammenine, picraphylline, picracine, picraline, picralicine, picratidine, picranitine, burnamine, pericalline and pericine (Henry, 1972; Ansa-Asamoah *et al*., 1990).

*1.2.6.*5 PREVIOUS STUDIES ON THE PLANT

By the carrageenan induced paw oedema, pseudoakuammigine exhibited anti-inflammatory. A similar results has also been demonstrated by the total alkaloidal extract. (Duwiejua *et al.*,

1995;Duwiejua *et al.*,2002). The alkaloid, Pseudo-akuammigine as well as the crude ethanol seed extract have all exhibited analgesic effect. (Menzies *et al.,* 1998; Ansa-Asamoah *et al*., 1986) The methanol fruit extract produced antipyrexia. (Ezeamuzie *et al*., 1994).

Both the methanol and chloroform seed extracts were all effective in reducing ulcer index. The fractions of the methanol extract also has anti-ulcer effect. (Mabeku *et al.,* 2008).

Various extracts of the plant: ethanol, chloroform, cold water, hot water and butanol extracts of various part of the plant have been tested against certain bacteria and some of these extracts have exhibited activity against organisms like *E. coli, P. aeruginosa, Bacillus subtilis, S. aureus and Salmonella kintambo.* (Nkere and Iroegbu, 2005). The antifungal activities of ethanol and aqueous leaf extracts of P. nitida were evaluated in three fungal species*: Aspergillus flavus, C. albicans and Microsporum canis.* These extracts exhibited activity against all these organisms with the exception of *Microsporum canis.* (Ubulom *et al*., 2012).

The seed, stem bark and fruit rind extracts showed significant activity against resistant clones of *Plasmodium falciparum* (Iwu *et al.,* 2002). An activity against the erythrocytic stage has also been established using the extracts mentioned above. Similarly, the ethanol seed extract has been shown *in vivo* antiplasmodial activity in both early and established infections (Okokon *et al.,* 2007).The chloroform seed extract was shown to be active against *Leishmania donovani* (Iwu *et al*., 1992). The boiling water extract of *P. nitida* bark had effect against *Trypanosoma brucei* in rats (Wosu *et al*., 1989).The aqueous and ethanol extract of the seed exhibited concentration and time dependent effect against larvae of *Anopheles gambiae* (Ubulom *et al*., 2012).

Ethanol and butanol seed extract of the plant exhibited hypoglycemic effect in diabetic pregnant rats. Alkaloids and glycosides extracts obtained from the seed have also demonstrated significant hypoglycemic effect. Blood glucose lowering effect of the coconut water extract of P. nitida seeds in alloxan-induced diabetic rats and rabbits has been demonstrated (Salihu *et al.*, 2009; Adegoke *et al.*, 2013). The leaves have also shown hypoglycemic effect in various studies.The methanol root bark extract was shown to exhibit antioxidant effects (Erharuyi *et al*., 2012). The *in vitro* antioxidant assay of methanol and hydro ethanol extracts of the stem bark and leaves were also conducted and the results showed that they significantly reduced the levels of malondialdehyde and hydrogen peroxide. Different extractions made from the seed were also found to exhibit antioxidant activity (Fakeye *et al.*, 2000).

1.3 JUSTIFICATION OF THE STUDY

Cough is usually a defensive respiratory reflex that helps to protect the airway. In such a case, the cough reflex will relieve the unwanted sensation caused by the foreign agent without the need for therapeutic intervention. But in pathological states, cough occur repeatedly over a long period of time unless pharmacological interventions are sought. Persistent cough as an isolated symptom was at a point accounting for 10 to 38% of new referrals to respiratory specialists (Irwin *et al.*, 1990, McGarvey *et al.*, 1998a).Because chronic cough is disturbing to the patient, it monumentally affects the patient‘s quality of life (Birring *et al*., 2003b; French *et al*., 1998). This has made it necessary for the search into effective pharmacological agents. A lot of medicines have been used over the past centuries. For instance the 1899 edition of Merck‘s Manual of the Materia Medica, gave 61 possible remedies for cough (Eccles, 2009). Due to various reasons especially with the issue of unwanted adverse effects, very few are still in use; codeine happens to be the main stay in cough management. The use of codeine (Bolser and Davenport, 2007; Taylor *et al.*, 1993) and Dextromethorphan (Paul *et al*., 2007; Taylor *et al.*,

1993) as ―gold standards‖ for cough have been found to be questionable. This makes it necessary to get better antitussives.

Cough just like other medical conditions lead to complications (Irwin, 2006). In most instances, the general rule is to treat the underlying condition (in this case cough) and the complication will likely resolve automatically. However there are certain instances where there seem to be no known cause of certain incidences of cough making treatment very difficult. Any of such cases are described as ―idiopathic cough‖ (Irwin *et al*., 1990; Poe *et al*., 1989; McGarvey *et al*., 1998b; Fujimura *et al*., 2005) and 20-40% of chronic cough cases in the UK are idiopathic(Morice *et al*., 2004). So in idiopathic cough, coughing becomes part of the client hence coming with all the fatal complications of cough (Irwin, 2006); notable, worrisome and widely reported among them being pain (Pascual *et al*., 2008; Sands *et al.*, 1991; Moncada and Graff-Radford, 1993; Perini and Toso, 1998;Irwin & Curley, 1991) and anxiety (McGarvey *et al.*, 2006; Everett *et al.*, 2007;Vernon *et al.*, 2009). Under such circumstance, management of the complication becomes imperative; consequently, unlike many pathological conditions where discovery of drugs focus primarily on highly efficacious agents with few adverse effects, the search for cough medicines will be looking not only at agents that can be used in place of the existing medicines but also those that can manage cough complications as well.

Picralima nitida is one of such plants that shows promise as an effective antitussive. Apart from the evidence obtained from the indigenous population with regard to its effectiveness in cough, other pharmacological properties confirmed on the plants by retrospective researchers give good reasons why the antitussive effect should be confirmed experimentally; the alkaloids in the plant have demonstrated significant activity on opioid receptors (Menzies *et al*., 1998). Moreover the anti-inflammatory (Duwiejua *et al*., 1995; Duwiejua *et al*.,2002), antibacterial (Nkere and

Iroegbu, 2005; Kouam *et al*., 2011; Fakeye *et al.*, 2000) as well as its stimulatory effect on the β2 adrenoreceptors in the trachea may all contribute somewhat to such potential antitussive effect.

1.4 AIM/SPECIFIC OBJECTIVES

The purpose of the study is to evaluate the effect of ethanol seed extract of *Picralima nitida* (PNE) on cough and its complications.

To achieve this;

- The phytochemical constituents of the plant would be investigated
- Irwin test which is a primary screening test would be conducted

The effect of PNE on cough would be ascertained by evaluating its

- Antitussive and expectorant effects

Pharmacological mechanisms responsible for such antitussive and expectorant effect would also be ascertained by investigating the effect of PNE on;

- acetylcholine-induced bronchoconstriction,
- histamine-induced bronchoconstriction,
- ammonium chloride-induced mucus secretion
- certain bacteria strains,
- mast cell degranulation,
- Reactive oxygen species.

Lastly, PNE‘s activity against complications from chronic cough will also be evaluated by,

- Evaluating the analgesic effect using tail flick test and the acetic acid-induced writhing assay and
- Finally, the anxiolytic effect using elevated plus maze and open field test

CHAPTER TWO

2.0 MATERIALS AND METHOD

2.1 PLANT COLLECTION

In January 2013, the berries of the plant were collected from the KNUST botanical garden in Kumasi. The authenticity of the plant were confirmed by Dr. Kofi Annan, a Senior Lecturer at the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences in KNUST.

2.2 EXTRACTION

The pods of *Picralima nitida* were opened, and the seeds removed, air-dried, and milled into powder. A 3 kg quantity of the powder was extracted with 70% ethanol by cold maceration over a 72-hour period. The extract obtained was concentrated at 40^{o}C and under low pressure using a rotary evaporator (Rotavapor R-210, Buchi, Switzerland) to a syrupy mass. This was then dried in a hot air oven (Gallenkamp, UK) maintained at 40^{o}C to obtain 0.389 kg (% yield: 12.97%) of a solid mass of *Picralima nitida* extract labeled as PNE which was reconstituted in normal saline and was used in this study.

2.3 MATERIALS

2.3.1 DRUGS AND CHEMICALS

Phenol red and sodium chloride were obtained from BDH Chemicals Ltd, Poole, England; sodium hydroxide from Avondale, England; ammonium chloride from Philip Harris, HydeCheshire; citric acid from Fisons Scientific Equipment, Loughborough; dihydrocodeine (DHC) from Bristol Laboratories Ltd., UK. Histamine dihydrochloride, acetylcholine chloride, mepyramine and atropine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO,

USA). sodium cromoglycate (SCG) from Ashford Laboratory Ltd., Macau; Ketotifen fumarate from Novartis Pharma AG (Basle, Switzerland); compound 48/80, ovalbumin (OVA) and toluidine blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA); acetic acid, morphine (PhytoRiker, Accra, Ghana); Diclofenac sodium was purchased from Troge, Hamburg, Germany; diazepam and caffeine from Sigma-Aldrich Inc., St. Louis, MO, USA.

2.3.2 ANIMALS

Guinea-pigs (190-390 g), mice (15-25 g) and Sprague-Dawley rats (130g) were obtained from the Animal Unit of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST; and fed on standard rodent pellet diet obtained from Agricare Ltd, Tanoso in Kumasi The animals were given water *ad libitum*. The animals were kept in the experimental area of the Departmental animal house at room temperature for 10 days prior to experimentation. All procedures and techniques used in these studies were in accordance with accepted principles for laboratory animal use and care (EU directive of 1986: 86/609/EEC). All protocols used were approved by the Departmental Ethics Committee.

2.4 METHODS

2.4.1 PHYTOCHEMICAL SCREENING

Phytochemical tests were performed on PNE to determine the presence of tannins, saponins, glycosides, alkaloids, flavonoids, steroids and terpenoids.

2.4.1.1 Glycosides

About 200 mg of PNE was warmed with 5 ml dilute H_2SO4 on a water bath for 2 minutes. It was then cooled and filtered. The filtrate was made alkaline with 2 to 5 drops of 20% NaOH and

1 ml each of Fehlings solution A and B was then added to the filtrate and heated on the water bath for 2 minutes and observed for the appearance of a brick-red precipitate. (Sofowora, 1993).

2.4.1.2 Saponins

An amount of 0.2 g of PNE was shaken with few milliliters of water and the mixture observed for the presence of a froth which does not readily break upon standing. (Sofowora, 1993).

2.4.1.3 Tannins

About 0.5g of PNE was boiled with 25 ml of water for 5 minutes. It was then cooled, filtered and the volume of the filtrate adjusted to 25 ml with water. To 1ml of the filtrate, 10 ml of water and 5 drops of 1% lead acetate was added. The colour and amount of precipitate, if any, was noted and recorded. The procedure was repeated using 5 drops of 1% ferric chloride (Trease and Evans, 1989).

2.4.1.4 Terpenoids

An amount of 0.5g of PNE was extracted with 2 ml of chloroform in a test tube followed by addition of 1ml of concentrated sulphuric acid. The presence of terpenoids was identified by appearance of a reddish-brown coloration at the interface (Sofowora, 1993).

2.4.1.5 Steroids

About 0.5g PNE was extracted with 2 ml of chloroform in a test tube followed by addition of acetic anhydride. Concentrated sulphuric acid was added to the walls of the test tube.

Appearance of a blue colour at the interface indicates the presence of steroids (Sofowora, 1993).

2.4.1.6 Flavonoids

About 0.5 g of PNE was extracted separately extracted with 2 ml of chloroform in a test tube and 2 ml of methanol added dissolve it. Concentrated hydrochloric acid was then added together

with four pieces of magnesium ribbons. A reddish or pink colour indicates the presence of flavonoids (Trease and Evans, 1989).

2.4.1.7 Anthraquinones

About 0.5 g of PNE was each extracted separately with 10 ml of benzene and filtered. About 5 ml of 10% ammonia was added to the filtrate and shaken. A reddish or pink colouration is a positive test (Trease and Evans, 1989).

2.4.1.8 Alkaloids

About 0.5 g of PNE was boiled with 10 ml of dilute HCl in a test tube for 5 mins. The supernatant was filtered and 3 drops of Dragendorff‘s reagent (potassium bismuth iodide solution) added to 1 ml of the filtrate in the test tube. The mixture was then shaken and observed for the appearance of an orange-red precipitate (Sofowora, 1993).

2.4.2 PRIMARY SCREENING

In the Irwin_s test, mice were treated with either distilled water or PNE (10, 30, 100, 300, 1000 and 2000 mg/kg p.o) and then observed at times 0, 15, 30, 60, 120, 180 minutes and 24 h. The animals were observed for obvious toxic symptoms; basically central nervous system (CNS) related behaviours and autonomic functions.

2.4.3 ANTITUSSIVE EFFECT

The antitussive effect of PNE was investigated using the citric acid-induced cough model (Yuebin *et al.,* 2009) with modifications. Guinea pigs were individually placed in a perspex chamber (24 × 14 × 24 cm) and exposed to 15% citric acid, delivered by an ultrasonic nebulizer, for 5 min. The animals were then monitored visually within this exposure time for cough; the latency and counts, of which, were taken as the basal values. The cough count was also taken

for five minutes outside the chamber, post citric acid-exposure, as a way of monitoring its recovery. The guinea pigs were put into five groups, I-V, (n=5) and treated as follows: Group I, vehicle, Group II, Dihydrocodeine (20mg/kg), Groups III-V PNE (100, 300 and 500 mg/kg respectively). An hour later, the animals were again exposed to the citric acid and the latency of cough and cough count taken. The procedure was repeated at hour 2 and 3 after treatments. Antitussive activity was then evaluated in each guinea-pig as the percentage in reduction in the number of coughs, and percentage increase in latency of cough in comparison with the previously established control basal value, calculated as shown:

Percentage reduction in cough count = [1 – (C2/C1)] × 100;

(Where, C1 = basal values, and C2 = total number of coughs after treatment).

Percentage increase in latency of cough = [(L2/L1)-1] × 100

(Where, L1 = basal values, and L2 = latency of coughs after treatment)

2.4.4 EXPECTORANT EFFECT

The expectorant effect was determined using the tracheal phenol red secretion (Engler and Szelenyi, 1984; Zhang *et al*., 2009). Mice, made to acclimatize for a week in the experimental laboratory, were grouped in six (n=5). Group 1, the control, was administered normal saline. Group 2 received 1000 mg/kg ammonium chloride *per os*, while Groups 3-6 received respectively 100, 300, 500, and 1000 mg/kg PNE respectively. Treatments were for four (4) consecutive days. One hour after drug administration on day 4, all animals were injected intraperitoneally with 5% phenol red in normal saline (0.1 ml/10 g). Thirty minutes after phenol red injection, animals were sacrificed by cervical dislocation and their tracheae removed and each put into 2 ml normal saline immediately. After 15 min ultra-sonication, 2 ml of 5%

$NaHCO_3$ was added to the saline and the optical density of each mixture was measured at 558 nm using a UV/Viscible spectrophotometer (UV-7501).

2.4.5 BRONCHODILATOR EFFECT

The bronchodilator effect was investigated with the acetylcholine and histamine-induced bronchoconstriction model (Kumar & Ramu, 2002; Kumar *et al*., 2010) with modifications. In Acetylcholine-induced bronchoconstriction, twenty five guinea pigs were each placed in a perspex chamber (24 × 14 × 24 cm) and exposed to an atomised mist of 0.5% Acetylcholine (ACh) aerosol using a nebulizer. As exposure to Ach causes respiratory distress and cough due to bronchoconstriction which then leads to convulsion, the time to onset of respiratory distress and cough i.e. pre-cough time (PCT) was recorded as basal values for each animal. After 24 h full recovery, the guinea pigs were grouped into five (n=5) and given the following treatments: Group I, 2 ml/kg normal saline (control); Group II, 5 mg/kg Atropine; Groups III-V, 100, 300, or 500mg/kg PNE. After an hour, the animals were exposed to acetylcholine aerosol and PCT were established.

The protection offered by the treatment against bronchoconstriction and cough was calculated as follows:

Percentage protection = [1 – (PCT1/PCT2)] × 100;

(Where, PCT1 = basal PCT, and PCT2 = PCT after treatment).

The same protocol was used in histamine-induced bronchoconstriction using 0.8% histamine and 8 mg/kg Mepyramine.

2.4.6 MUCUS-SUPPRESSANT EFFECT

The mucus suppression effect was determined using the ammonium chloride-induced phenol red secretion as described previously (Engler and Szelenyi, 1984). Mice were made to

acclimatize in the experimental laboratory for a week and then allotted to five groups (n=5). They then received the following pre-treatments: Group I, 2 ml/kg normal saline (p.o); Group II, 100 mg/kg Sodium cromoglycate (i.p) for 15 minutes; Groups III-V were pre-treated with 100, 300, and 500 mg/kg of PNE orally for 30 minutes respectively. Tracheal mucus secretion was then induced with 5 mg/kg ammonium chloride *per os*. Animals were then injected with 500 mg/kg phenol red, intraperitoneally, 30 minutes later. The trachea was excised from each mouse and cleared of adhering tissues, after sacrificing it by cervical dislocation, 30 minutes after the phenol red injection. Each excised trachea was washed in 3 ml physiological saline; sodium hydroxide (0.3 ml NaOH, 1M) was then added to stabilize the pH of the lavage fluid. The absorbance of the mixture was then taken at a wavelength of 460 nm using a spectrophotometer (T90+ UV/VIS Spectrometer – PG Instruments Ltd). A calibration curve for phenol red was determined; from which concentrations of phenol red secreted by mice tracheae were
extrapolated.

2.4.7 MAST CELL STABILIZATION EFFECT

The mast cell stabilizing effect was ascertained with the Compound 48/80-induced mesenteric mast cell degranulation as described by Balaji *et al*., 2014 with modifications. A Sprague Dawley rat was sacrificed and its intestinal mesenteries were excised into several pieces and kept in petri dishes containing Tyrode solution in groups (n=5). The mesenteries were then subjected to the following treatment: Petri dish 1=normal saline; Petri dish 2=20 µg/ml ketotifen furamate; Petri dish 3= 100 µg/ml PNE; Petri dish 4=250 µg/ml PNE; Petri dish 5=500 µg/ml PNE. The petri dishes were then incubated at 37 °C for 15 min after which 1 ml of compound 48/80 solution (10 µg/ml) was added and incubated at 37 ºC for 10 min. The mesenteric pieces were then fixed in 10% buffered formalin and processed histologically in xylene and acetone and later stained

with 0.1% toluidine blue and observed under a Leica DM 750 microscope (Leica Microsystems CM5 GmbH, Wetzlar - Germany) for both intact and degranulated cells. The percentage mast cell degranulation for each treatment was estimated.

2.4.8 ANTIOXIDANT EFFECT

The anti-oxidant property of PNE was evaluated using the DPPH radical scavenging effect as described by Blois, 1958. PNE (0.01, 0.03, 0.1, 0.3 mg/ml) and ascorbic acid (0.01, 0.03, 0.1, 0.3 mg/ml) were prepared in methanol. One ml of the test substance was added to 3 ml methanolic solution of DPPH in a test tube. The reaction mixtures were kept at 25ºC for 1 h. The absorbance of the residual DPPH was then determined at 517 nm using a Cecil UV/VIS spectrophotometer (Model: CE 2041, Milton, England). The measurements were done in replicates. One (1) millilitre methanol added to 3.0 ml DPPH solution, incubated at 25ºC for 1 h served as control and methanol (99.8%) was used as blank. The percentage scavenging effect was calculated as follows:

$$\% \text{ scavenging} = [(A_C - A_T)/A_C] \times 100$$

Where A_C = Absorbance of control, and A_T = Absorbance of test)

The procedure was done three times. The concentration of the test drug required to cause a 50% scavenging effect (IC_{50}) was estimated.

2.4.9 ANTIBACTERIAL EFFECT

Investigation into the antibacterial property of PNE was carried out using the agar well diffusion method as described by Okeke *et al*., 2001 with modifications. Ten (10) test tubes (labeled I-X) each containing 20 ml nutrient agar was stabilized. The molten agars were inoculated with 0.2 ml each of the following organisms: Test tubes 1 and 2-*Staphylococuss aureus*; Test tubes 3 and 4-*Streptococcus pneumonia*; Test tubes 5 and 6-*Salmonella typhi*; Test tubes 7 and 8-*Escherica*

coli; Test tubes 9 and 10-*Klebsiella pneumonia*. The seeded agar were poured into sterile petri dishes and allowed to set. Using a sterile cork borer number 7 (British), five wells were created in each of the ten petri dishes. Various concentrations of PNE were prepared and poured in the wells to three-quarter (3/4) full as follows: Petri dishes 1, 3, 5, 7 and 9 each received 0.05, 0.5, 5,

25 mg/ml PNE in four of the five wells created. Petri dishes 2, 4, 6, 8 and 10 received 0.1, 1, 10, 50 mg/ml PNE in four of the five wells created. The petri dishes were covered and left on the bench for 45 min in order to allow effective diffusion of the extract. They were then incubated at 37 °C for 24 hours, after which they were examined for zones of growth inhibition around the wells. Amoxicillin (1%) was used as a positive control in the test against *Staphylococcus aureus*, *Streptococcus pneumonia*, *Escherica coli* and *Klebsiella pneumonia.* Ciprofloxacin (0.1 %) was the control in the test against *Salmonella typhi*. The results are the mean of 3 replicates.

2.4.10 ANXIOLYTIC EFFECT OF PNE

2.4.10.1 Elevated plus maze

This test has widely been used to measure anxiety in rodents especially mice (Pellow *et al*., 1985). The apparatus was made of plexiglas and consisted of two open arms (30 cm × 5 cm × 0.5 cm) (30 cm×5 cm) and two closed arms (30 cm × 5 cm × 15 cm). These arms extend from a central square platform (5×5 cm). The maze was elevated to a height of 60 cm above the floor and placed in a lit room. Mice were divided into ten groups (n=6) and received the following treatment: vehicle-control, PNE (100, 300, 500 mg/kg), diazepam (0.1, 0.3, 1.0 mg/kg) and Caffeine (3, 10, 30 mg/kg). So diazepam and caffeine served as reference anxiolytic and anxiogenic drugs respectively. The vehicle, PNE and Caffeine were orally administered to their respective animals an hour before the experiment, whiles the Diazepam was given

intraperitoneally 30 minutes before the experiment. At the start of the experiment, animals were individually placed at the center of the maze, facing one of the enclosed arm and their behavior videotaped for 5 min with a digital camera placed 100 cm above the maze. After each test, the maze was carefully cleaned up with 10% ethanol solution. Behavioral parameters were scored from the videotapes as follows: 1) number of closed and open arm entries—(absolute value and percentage of the total number); 2) time spent in exploring the open and closed arms of the maze — absolute time and percentage of the total time of testing 3) number of head-dips (absolute value and percentage of the total number)—protruding the head over the ledge of either an open (unprotected) or closed (protected) arm and down toward the floor; 4) number of stretch-attend postures (absolute value and percentage of the total number)—the mouse stretches forward and retracts to original position from a closed (protected) or an open (unprotected) arm. The behaviour was tracked by JWatcher TM Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sidney, Australia available at http://www.jwatcher.ucla.edu/.).

2.4.10.2 Open field

The open field method has previously been described by Kasture *et al*., (2002). The test was conducted in clear Plexiglas boxes (40 ×40 ×30 cm^3). The floor of this box was divided by red lines into 16 equal squares. For behavioral analysis, the arena of the open field was designated as (i) corner (one of the four corner squares); (ii) periphery (the squares along the walls); or (iii) center (the four inner squares). The animals were divided into ten groups (n=6), and received either the extracts (100, 300 or 500 mg/kg, p.o.), the vehicle or the reference drugs diazepam (0.1, 0.3 or 1 mg/kg, i.p.). Thirty minutes after i.p and 1hour after oral administration, the animals were placed at the center of the field and were allowed to explore for 5minutes. This was recorded by a video camera which was suspended 100cm above the arena. Behavioral parameters for all the tests were scored from videotapes with the aid of the public domain

software JWatcher™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia. Available at http://www.jwatcher.ucla.edu/). Number of entries as well as the duration of stay in individual zones are the parameters assessed.

2.4.11 ANALGESIC EFFECT OF PNE

2.4.11.1 Tail flick

The extreme end of the tail (3 cm) of mice was immersed in a water of temperature 50 ± 0.5 ºC. The time (in seconds) taken for the mouse to flick its tail was regarded as the tail withdrawal latency. A cut off latency of 10 s was set in order to prevent tissue damage.

Selected mice were randomly assigned into ten groups, I-X, (n=5) and treated as follows: Group I, vehicle (control), Groups II-IV, diclofenac treatment (10, 30 and 100 mg/kg, i.p, respectively); Groups V-VII, morphine treatment (1, 3 and 10 mg/kg, i.p, respectively); Group VIII-X, PNE treatment (100, 300 and 500 mg/kg, p.o respectively). Thirty minutes after intraperitoneal administration, and one hour after oral after treatment of the drugs, their tail was immersed in the water bath at time 30, 60, 90, 120, 150, 180 min and the post-drug latency was determined. The percentage maximal possible effect (% MPE) was calculated from the reaction times using the following formula:

$$\% \text{ MPE} = [(T_2-T_1) / (T_0-T_1)] \times 100$$

Where T_1 and T_2 are the pre- and post- drug treatment latency times, and T_0 is the cut-off time.

2.4.11.2 Acetic acid-induced writhing

The test was done with the method as described by Amresh *et al.*, (2007) with slight modifications. Mice were divided into seven groups, I-VII, (n=5) and received the following treatment: Group I, vehicle (control); Groups II-IV, diclofenac treatment (10, 30, or 100 mg/kg, i.p respectively); Groups V-VII, PNE treatment (100, 300, or 500 mg/kg, p.o, respectively). An hour (for orally administered drugs) or 30 minutes (for drugs administered intraperitoneally)

later, the mice were injected with acetic acid (0.6 %, 10 ml/kg, i.p.) and each placed individually in a perspex chamber (testing chamber: 15 cm × 15 cm × 15 cm). A mirror was inclined at 45° below the floor of the chamber allowed a complete view of the mice. Ten (10) minutes after acetic acid administration, responses were captured (for 20 min) by a camcorder (EverioTM, model GZ-MG1300, JVC, Tokyo) placed directly opposite the mirror and attached to a computer for analysis. Acetic acid induced writhing is characterized by extension of the abdomen combined with the outstretching of the hind limbs. The behaviour was tracked by JWatcher TM Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sidney, Australia available at http://www.jwatcher.ucla.edu/.) and the number of writhes were recorded

CHAPTER THREE

3.0 RESULTS

3.1 PHYTOCHEMICAL SCREENING

Table 3.1 gives the results obtained from the phytochemical screening of PNE. PNE was found to contain saponins, alkaloids, tannins, steroids, glycosides, anthraquinones and terpenoids. Flavonoids were however not seen in PNE

Table 3.1: Results of preliminary phytochemical screening of PNE

Phytoconstituents	Present (+)/ Absent (-)
Saponins	++
Alkaloids	+++
Tannins	++
Steroids	+
Flavonoids	–
Glycosides	+
Anthraquinones	+
Terpenoids	++

3.2 PRIMARY SCREENING

Cage side examination of mice revealed no physical signs of toxicity (such as unkemptness, hair loss or ulcers on the skin), stimulation of autonomic function, or CNS excitation or depression with PNE treatment at doses of 10, 30, 100 and 300 mg/kg relative to control mice. However, mild sedation was observed at doses of 1000 and 2000 mg/kg. No mortality was recorded at all doses.

Table 3.2 Primary Observation Test of *Picralima nitida* in mice

DOSE (mg/kg)	MORTALITY	EFFECT
	D/T	
Control	0/6	No effect
10	0/6	No effect
30	0/6	No effect
100	0/6	No effect
300	0/6	No effect
1000	0/6	Sedation (+) and hypo activity(+)
2000	0/6	Sedation (++) and hypo activity (++)

D/M: number of deaths /number of mice treated. Grade of signs observed: (+) present or slightly increased, (++) moderately increased.

3.3 ANTITUSSIVE

Administration of citric acid induced cough in the guinea-pigs. Both dihydrocodeine (20 mg/kg) and PNE (100, 300, and 500 mg/kg) dose-dependently reduced ($P \leq 0.05$) cough count, and increased significantly ($P \leq 0.01$) the latency of cough (Figure 3.1).

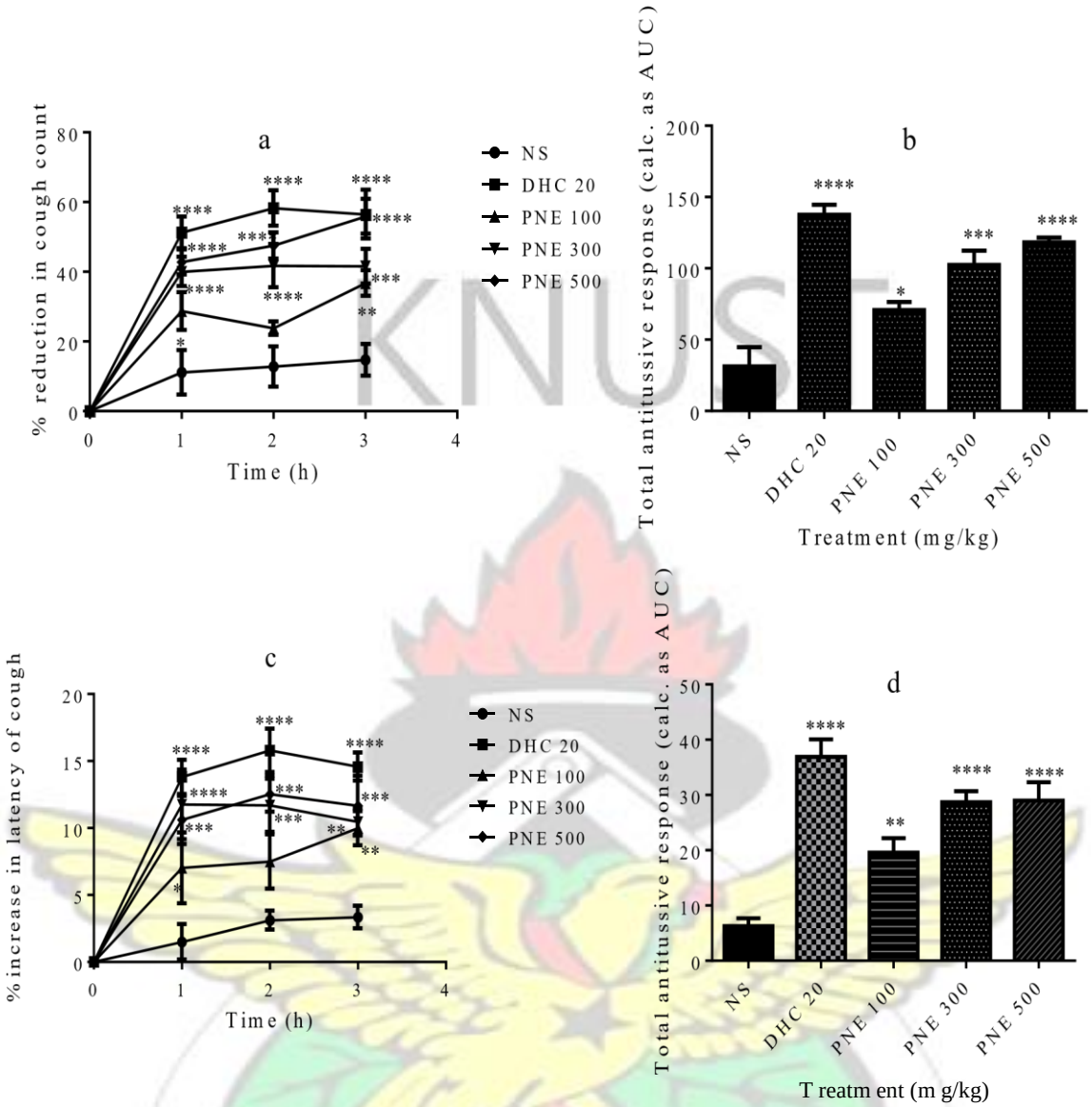

Figure 3.1 : Effects of 100, 300, 500 mg/kg PNE, 20mg/kg Dihydrocodeine (DHC), and normal saline on the time course curve of (a) % reduction in cough count and (c) % increase in latency to cough and (b and d) their AUC's respectively) in the citric acidinduced cough test. Data plotted are mean ± SEM, (n=5). ****P≤ 0.0001; ***P≤ 0.001; **P ≤ 0.01; *P ≤ 0.05; compared to vehicle-treated group (ANOVA followed by Dunnett's multiple comparisons test)

3.4 EXPECTORANT

Both PNE ($P \leq 0.05$) and ammonium chloride ($P \leq 0.01$) showed significant increase in phenol red secretion relative to the control (Figure 3.2)

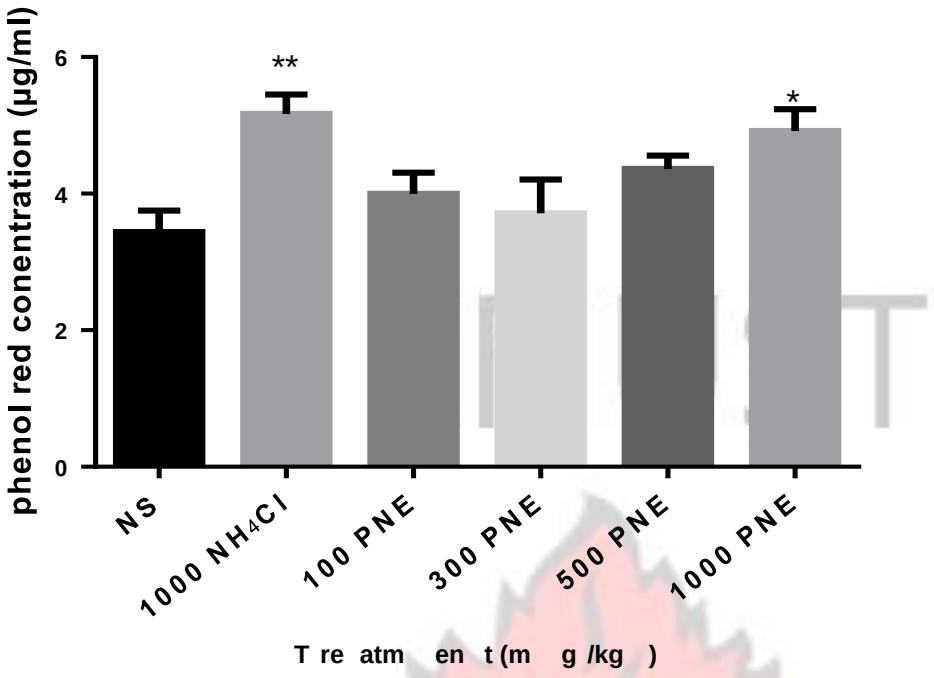

Figure 3.2: Effect of PNE (100, 300, 500, 1000 mg/kg), ammonium chloride (1000 mg/kg) and normal saline on tracheal phenol red secretion in mice as a measure of the expectorant effect. Values plotted are means ± SEM (n = 5). ns implies P > 0.05; **P ≤ 0.01; *P ≤ 0.05 compared to vehicle-treated group, (ANOVA followed by Dunnett's *post-hoc* test).

3.5 BRONCHODILATOR EFFECT

With acetylcholine and histamine-induced bronchoconstriction, Atropine and PNE (100, 300, 500 mg/kg) offered significant protection to acetylcholine-induced bronchoconstriction by increasing significantly (P ≤0.05 -0.0001) PCT, compared to the control. The percentage protection offered by PNE was dose-dependent (Figure 3.3). Similarly, mepyramine and PNE (300, 500mg/kg) reduced significantly (P ≤ 0.01-0.0001) bronchoconstriction induced by histamine (Figure 3.4).

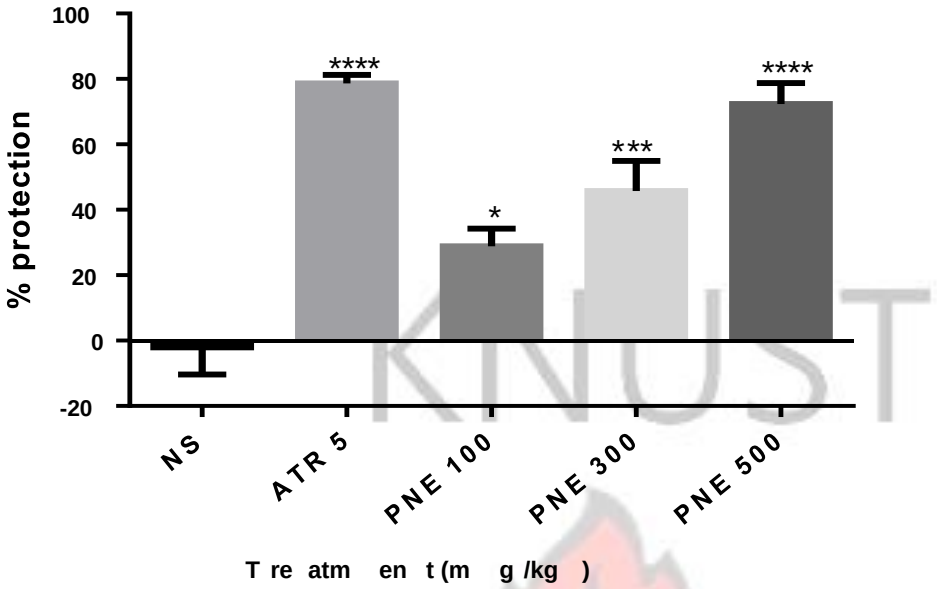

Figure 3.3: Effect of PNE (100, 300, 500 mg/kg *p.o*), Atropine (5 mg/kg) and normal saline on acetylcholine-induced bronchoconstriction. Values plotted are means ± SEM, (n = 5). ****P ≤ 0.0001;*P ≤ 0.001; *P ≤ 0.05; compared to vehicle-treated group (ANOVA followed by Dunnett's multiple comparisons test)**

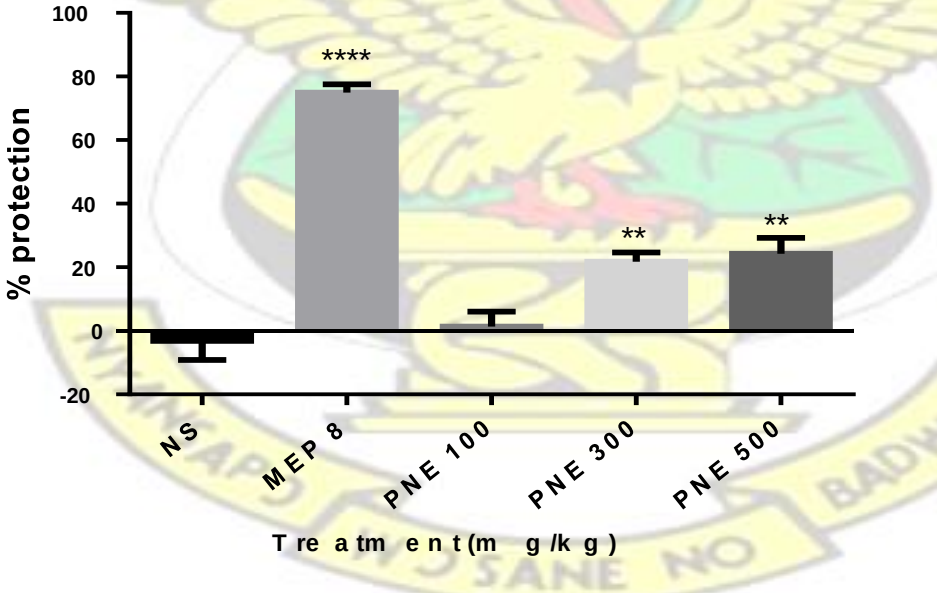

Figure 3.4: Effect of PNE (100, 300, 500 mg/kg *p.o*), Mepyramine (8 mg/kg) and normal saline on bronchospasm induced by histamine. Values plotted are means ± SEM, (n = 5). ns implies P > 0.05; ****P ≤ 0.0001; **P ≤ 0.01 compared to vehicle-treated group (ANOVA followed by Dunnett's multiple comparisons test)

3.6 MUCUS SUPPRESSANT EFFECT

The 100, and 300 mg/kg doses of PNE significantly reduced tracheal phenol red secretion compared to the control, with the 100 mg/kg dose showing a better effect ($P \leq 0.001$) than the 300 mg/kg ($P \leq 0.05$) dose. Sodium cromoglycate also caused significant reduction ($P \leq 0.01$) in tracheal phenol red secretion (Figure 3.5).

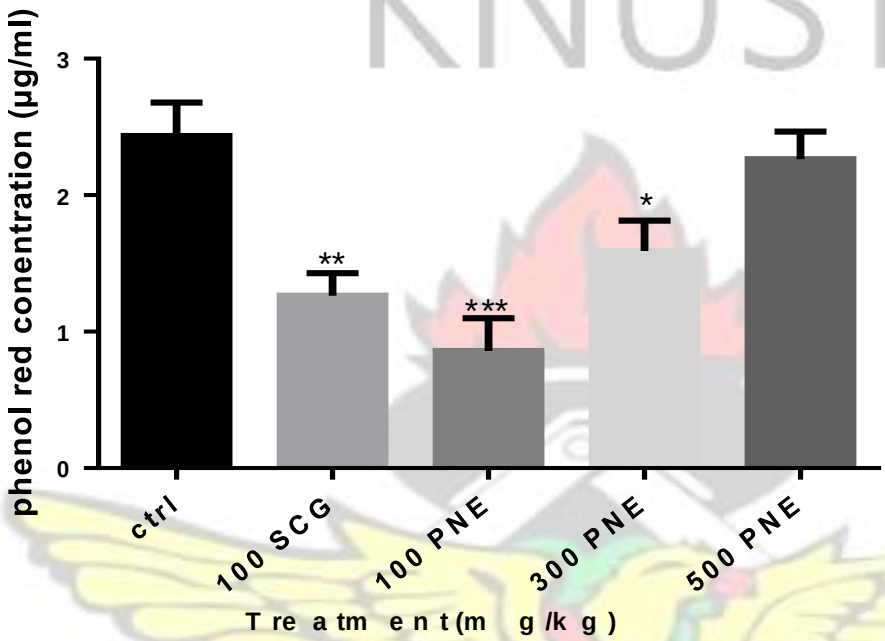

Figure 3.5: Effect of PNE (100, 300, 500 mg/kg), sodium cromoglycate (100mg/kg), and normal saline on ammonium chloride-induced tracheal phenol red secretion as a measure of muco-suppressant effect. Values plotted are means ± SEM of n = 5. Ns implies P > 0.05;*P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05 compared to vehicle-treated group; (ANOVA followed by Dunnett's *post-hoc* test)**

3.7 ANTIOXIDANT ACTIVITY

The EC_{50} obtained for PNE was 0.06530 mg/ml and that for the reference antioxidant, Ascorbic acid was 0.001070 mg/ml (figure 3.6)

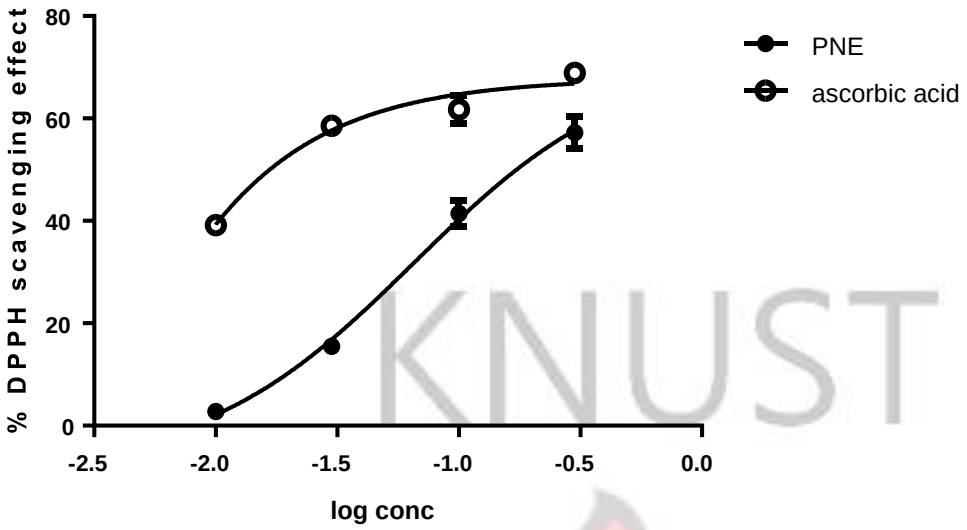

Figure 3.6: Free radical scavenging ability of PNE (0.01-0.3 mg/ml) compared to ascorbic acid (0.01-0.3mg/ml) in the DPPH radical assay. Values plotted are means + SEM, n=3.

3.8 MAST CELL STABILIZING EFFECT

Treatments with PNE and sodium cromoglycate were able to reduce significantly ($P \leq 0.01$) mast cell degranulation induced by compound 48/80 relative to the control (Figure 3.7).

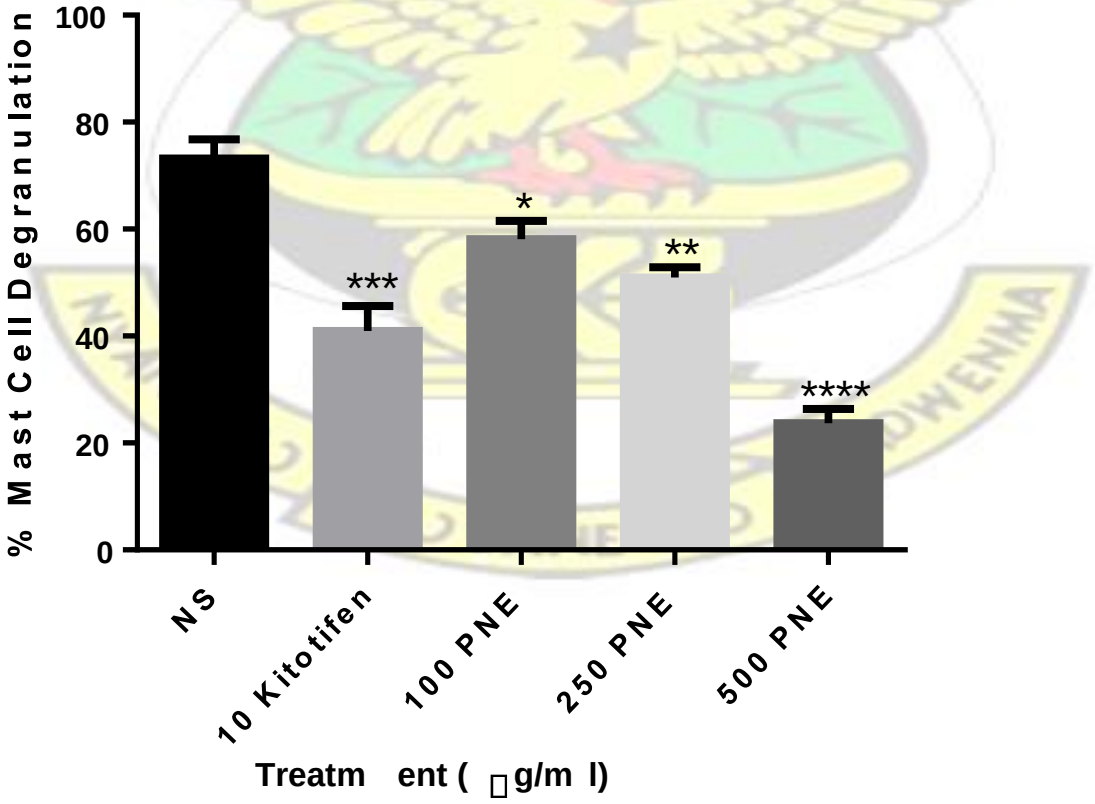

Figure 3.7: Effect of PNE (100, 250, 500 µg/ml), Ketotifen (10 µg/ml), and normal saline on mast cell degranulation induced by Compound 48/80. Values plotted are means ± SEM, (n = 3).ns implies P >0.05; ****P ≤ 0.0001;*P ≤ 0.001; **P ≤ 0.01 compared to vehicletreated group; (ANOVA followed by Dunnett's *post-hoc* test)**

3.9 ANTIBACTERIAL

PNE at concentrations of 0.05-1 mg/ml showed no antibacterial activity against *Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Streptococcus pneumonia,* and *Staphylococcus aureus*. Significant (P ≤ 0.05) antibacterial activity was however observed from 5-50 mg/ml on all the microorganisms except for *Escherichia coli* and *Klebsiella pneumoniae,* on which 5 mg/ml PNE had no activity. The lowest effect was against *Salmonella typhi* at 5 mg/ml with a zone of 13.0±0.00 mm whiles the highest response was observed against *Streptococcus pneumonia* at 50 mg/ml with a zone of 22.3±0.88 mm (Table 3.3).

Table 3.3: Effect of 0.05-50 mg/ml PNE on *Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Streptococcus pneumonia and Staphylococcus aureus*.

CONC. (mg/ml)	*S. aureus*	*S. pneumonia*	*S. typhi*	*E. coli*	*K. pneumoniae*
0.05	-	-	-	-	
0.1	-	-	-	-	-
0.5	-	-	-	-	-
1	-	-	-	-	-
5	14.67±0.33	14.00±0.00	13.00±0.000	-	-
10	17.00±0.58*	15.33±0.33ns	15.00±0.56ns	13.33±0.33	14.33±0.33

25	18.67±0.88**	17.67±0.67**	17.67±0.88***	14.00±1.16ns	16.00±0.00**
50	20.00±0.00***	22.33±0.88****	19.33±0.33***	16.67±0.33*	17.67±0.33***

Values quoted as zones of inhibitions are means ± SEM; n = 3. (-) indicates "no zones of inhibition were observed. Diameter of borer: 11 mm. ns P > 0.05, *P ≤0.05, **P ≤0.01, ***P ≤0.001, ****P≤0.0001 (ANOVA followed by Bonferroni's *post hoc* test)

3.10 ANXIOLYTIC EFFECT OF PNE

3.10.1 Elevated plus maze

PNE (100, 300, 500 mg/kg) enhanced, dose-independently, activities in the open arm by increasing percentage of entry into open arms ($F_{3, 19}$= 12.97, P ≤ 0.0001; Figure 3.8f) and percentage time spent in open arms ($F_{3, 19}$ = 2.148, P = 0.1278; Figure 3.9 f). There was also reduction in risk assessment by decreasing both the percentage protected head dips ($F_{3, 19}$ = 6.635, P = 0.0030; 3.10 c) and percentage protected stretch attend postures ($F_{3, 19}$ = 2.552, P=0.0860; Figure 3.10 f). Diazepam (0.1-1 mg/kg) also dose dependently and significantly increased percentage of open arm entries ($F_{3, 22}$ = 8.677, P=0.0005; Figure 3.8 e) and percentage time spent in open arm ($F_{3, 22}$ = 9.085, P=0.0004; Figure 3.9 e). Diazepam also reduced risk assessment by decreasing both the percentage protected head dips ($F_{3, 22}$ = 10.43, P = 0.0002; Figure 3.10 b) and percentage protected stretch attend postures ($F_{3, 22}$= 4.071, P = 0.0192; Figure 3.10 e). These effects confirmed the anxiolytic effects of both PNE and diazepam.

With regard to Caffeine, it increased open arm avoidance by reducing percentage of open arm entries ($F_{3, 21}$ = 0.1934, P = 0.8997; Figure 3.8 d) and percentage time spent in open arms ($F_{3, 21}$ = 3.673, P = 0.0285; Figure 3.9 d). Not only that, Caffeine increased both the percentage protected head dips ($F_{3, 21}$= 0.4492, P = 0.7205; Figure 3.10 a) and percentage protected stretch

attend postures ($F_{3, 23}$= 3.764, P = 0.0247; Figure 3.10 d), all indicative of anxiogenic effect of caffeine

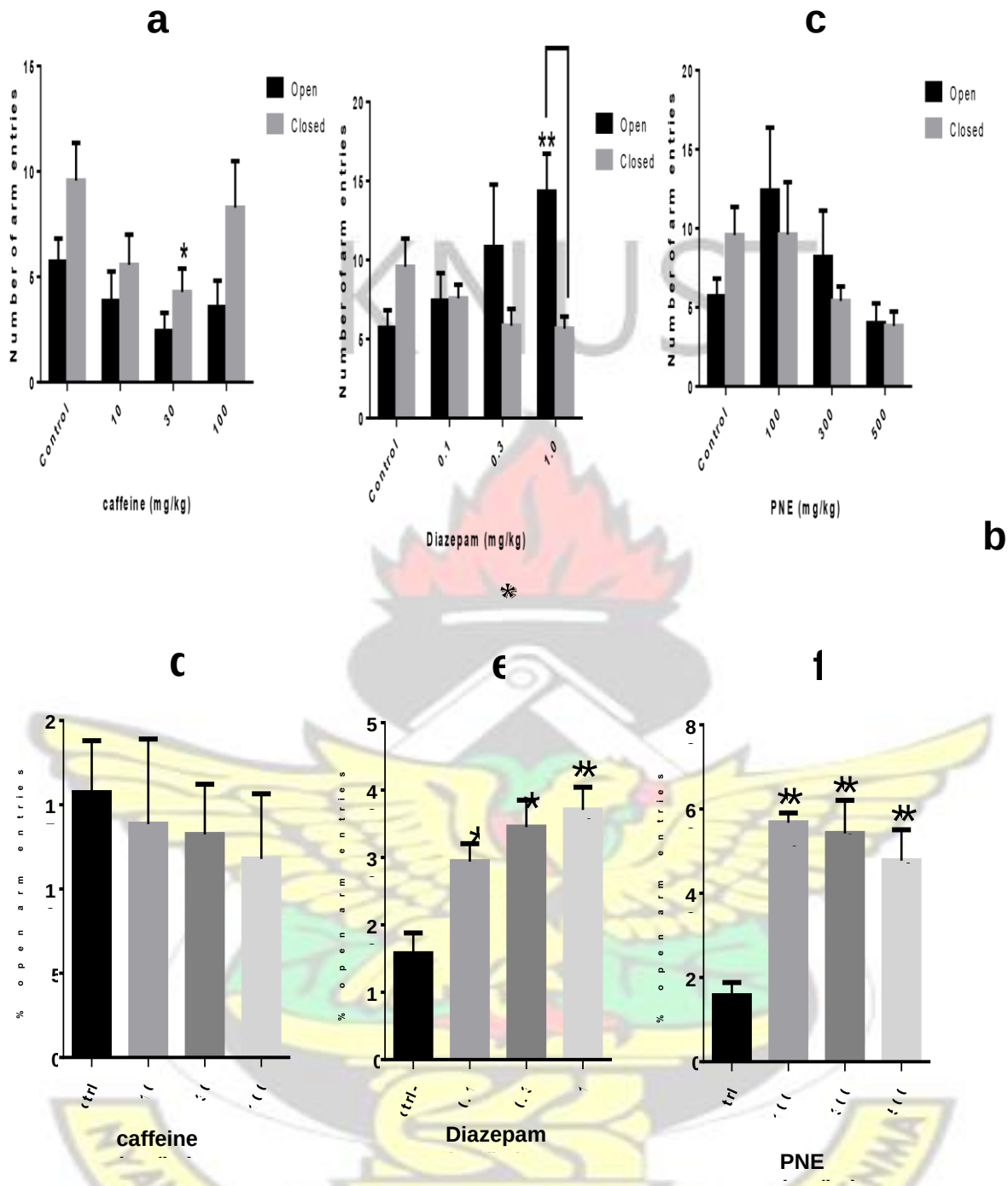

Figure 3.8: Effect of Caffeine (10, 30, 100 mg/kg), Diazepam (0.1, 0.3, 1 mg/kg), PNE (100, 300, 500 mg/kg) on the number of arm entries for caffeine (a), diazepam (b), PNE (c) and the % number of arm entries for caffeine (d), diazepam (e), PNE (f) in the elevated pluz maze. Data are presented as group mean± SEM (n=6). Significantly different from control: *P ≤0.05, **P ≤0.01, ***P ≤0.001, ****P≤0.0001. Also significantly different significant difference when the zonal entries were compared to each other in a, b and c. *P ≤0.05. (Two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

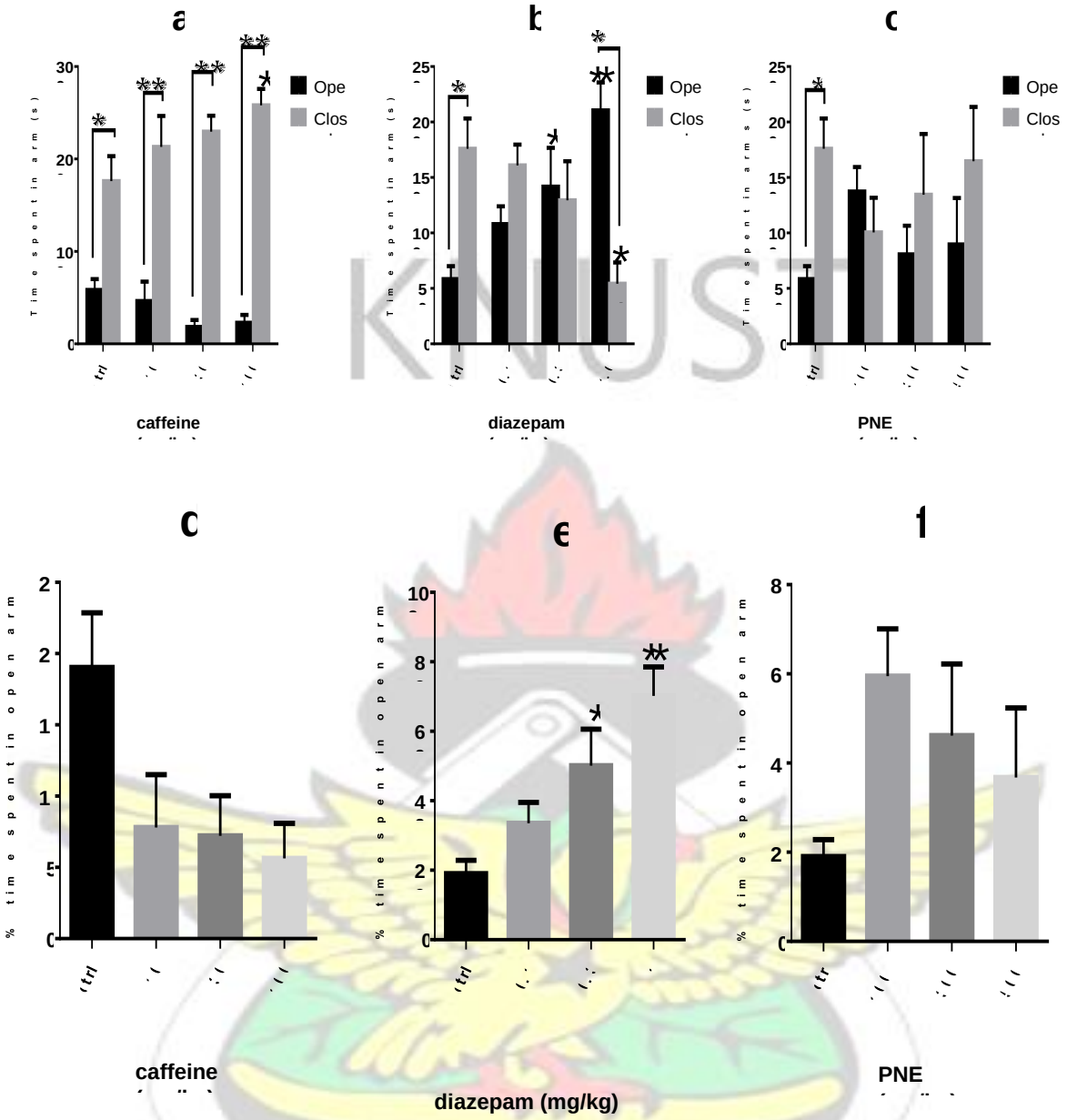

Figure 3.9: Effect of Caffeine (10, 30, 100 mg/kg), diazepam (0.1, 0.3, 1 mg/kg), PNE (100, 300, 500 mg/kg) on the time spent in various arms for caffeine (a), diazepam (b), PNE (c) and the % time spent in open arm for caffeine (d), diazepam (e), PNE (f) in the elevated pluz maze. Data are presented as group mean±SEM (n=6). Significantly different from control: *P≤0.05, **P≤0.01, ***P≤0.001. Also significantly different when the zonal entries were compared to each other as done in a, b and c. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001. (Two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

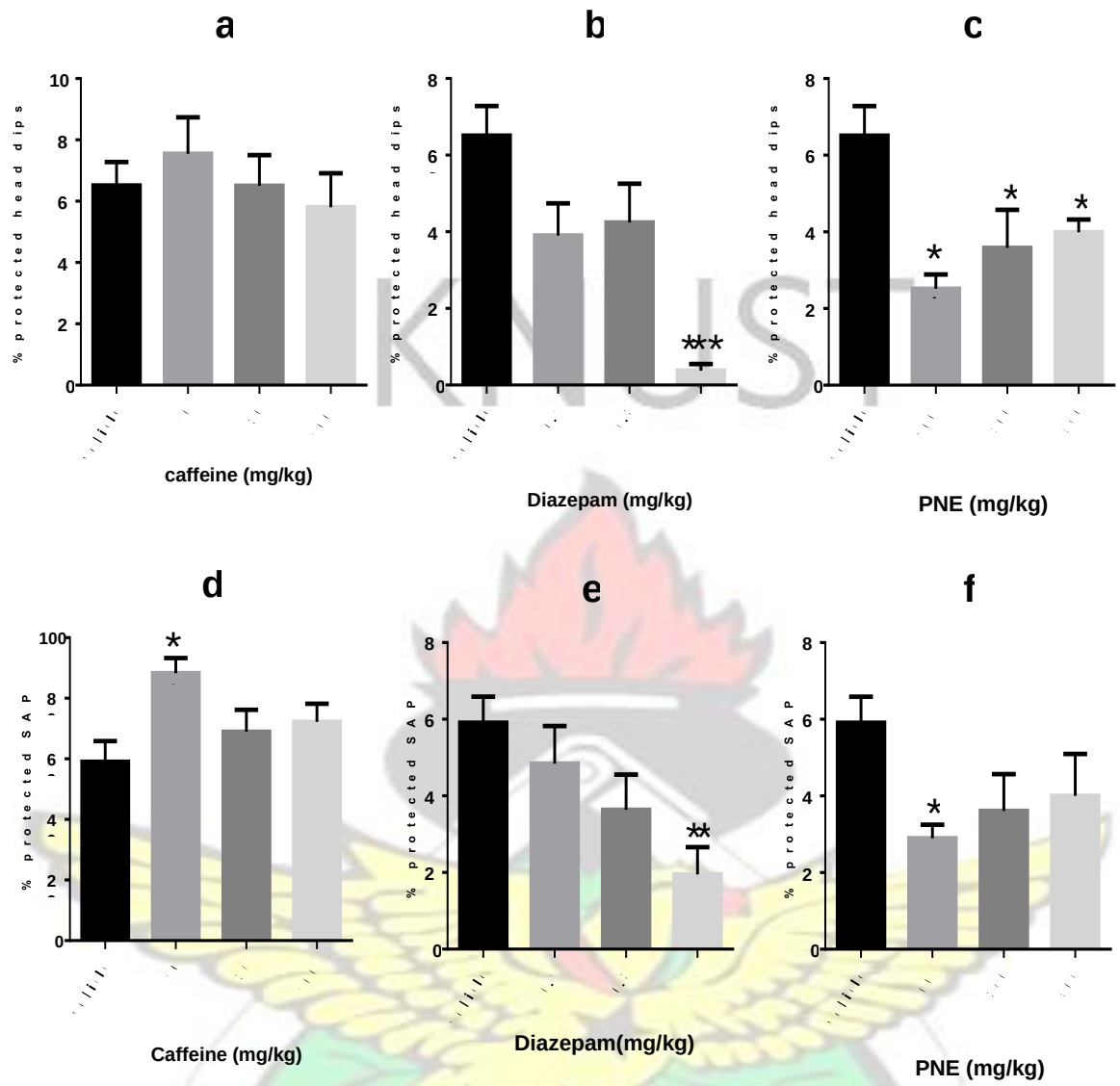

Figure 3.10: Effect of caffeine (10-100 mg/kg), diazepam (0.1-1 mg/kg.) and PNE (100-500 mg/kg) on the percentage protected head-dips (a) percentage protected stretch-attend postures (b) in the elevated plus-maze over a 5 min test period in the mice. Each bar represents mean±SEM, (n=6). Significantly different from control: *P≤0.05, **P≤0.01, ****P≤0.0001. P values for group comparisons were obtained by one-way ANOVA followed by Student-Newman–Keuls test compared to vehicle-treated group

3.10.2 Open field

PNE (100, 300, 500 mg/kg) dose-independently increased the frequency of central zone entries, time spent in central zone, percentage entry into central zone ($F_{3, 18} = 4.216$, P= 0.0201; Figure

3.11 d), percentage time spent in central zones ($F_{3, 18}$ = 3.337, P=0.0427; Figure 3.12 d).These observations support the claim that PNE acts as an anxiolytic.

The reference anxiolytic, Diazepam (0.1-1mg/kg) dose dependently increased the frequency of central zone entries, time spent in central zone, percentage entry into central zone ($F_{3, 26}$ =6.318, P=0.0023; Figure 3.11 c) and percentage time spent in central zones ($F_{3, 26}$ = 2.793, P=0.0603; Figure 3.12 c).

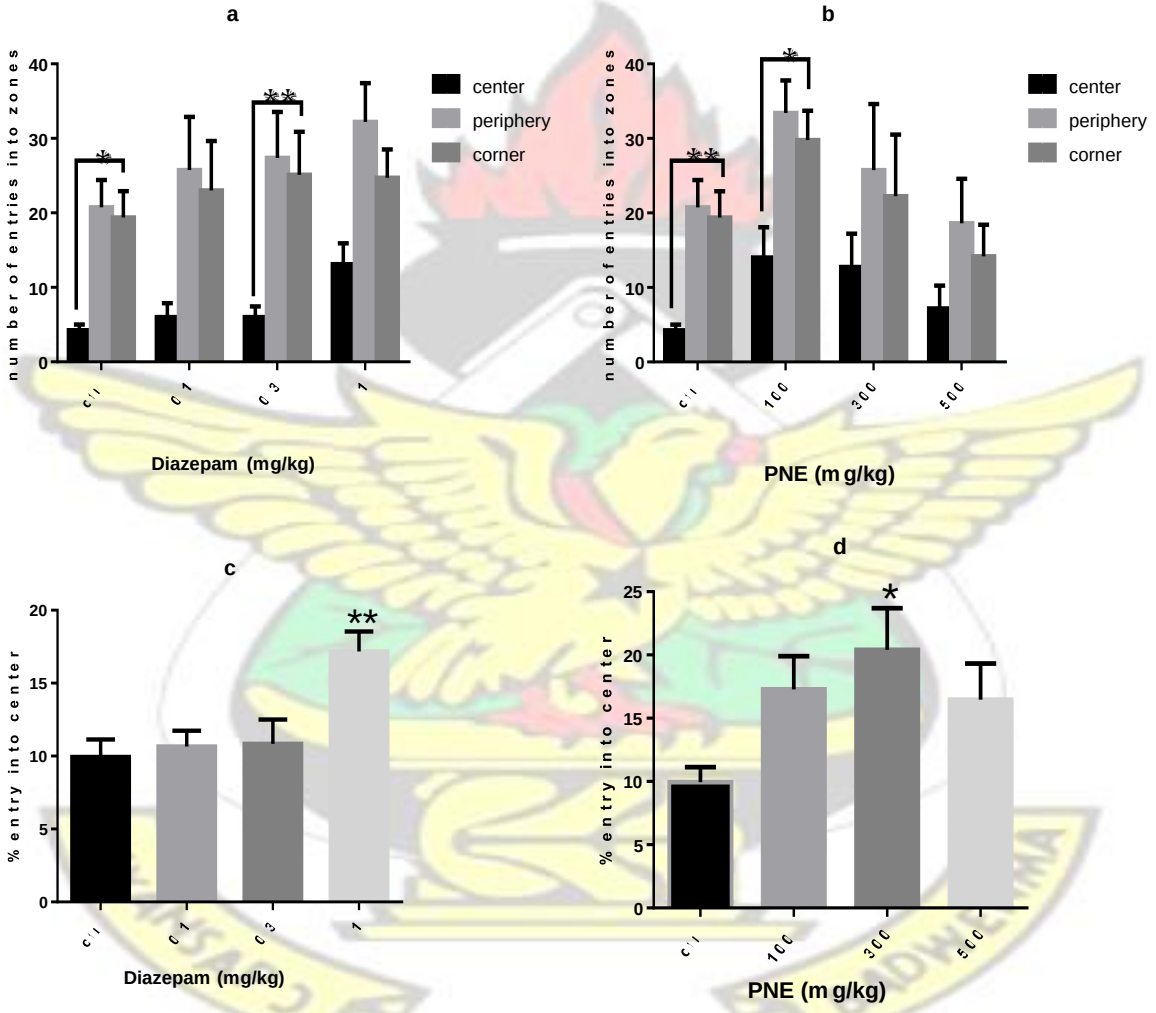

Figure 3.11: Effect of diazepam (0.1, 0.3, 1mg/kg), PNE (100, 300, 500mg/kg) on the number of entries into zones for diazepam (a), PNE (b) and the % entry into central zone for diazepam (c), PNE (d) in the open field test. Data are presented as group mean± SEM (n=6). Significantly different from control: *P ≤0.05, **P ≤ 0.01. Also significantly different when the zonal entries were compared to each other as done in a and b *P ≤ 0.05, **P ≤ 0.01. (Two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

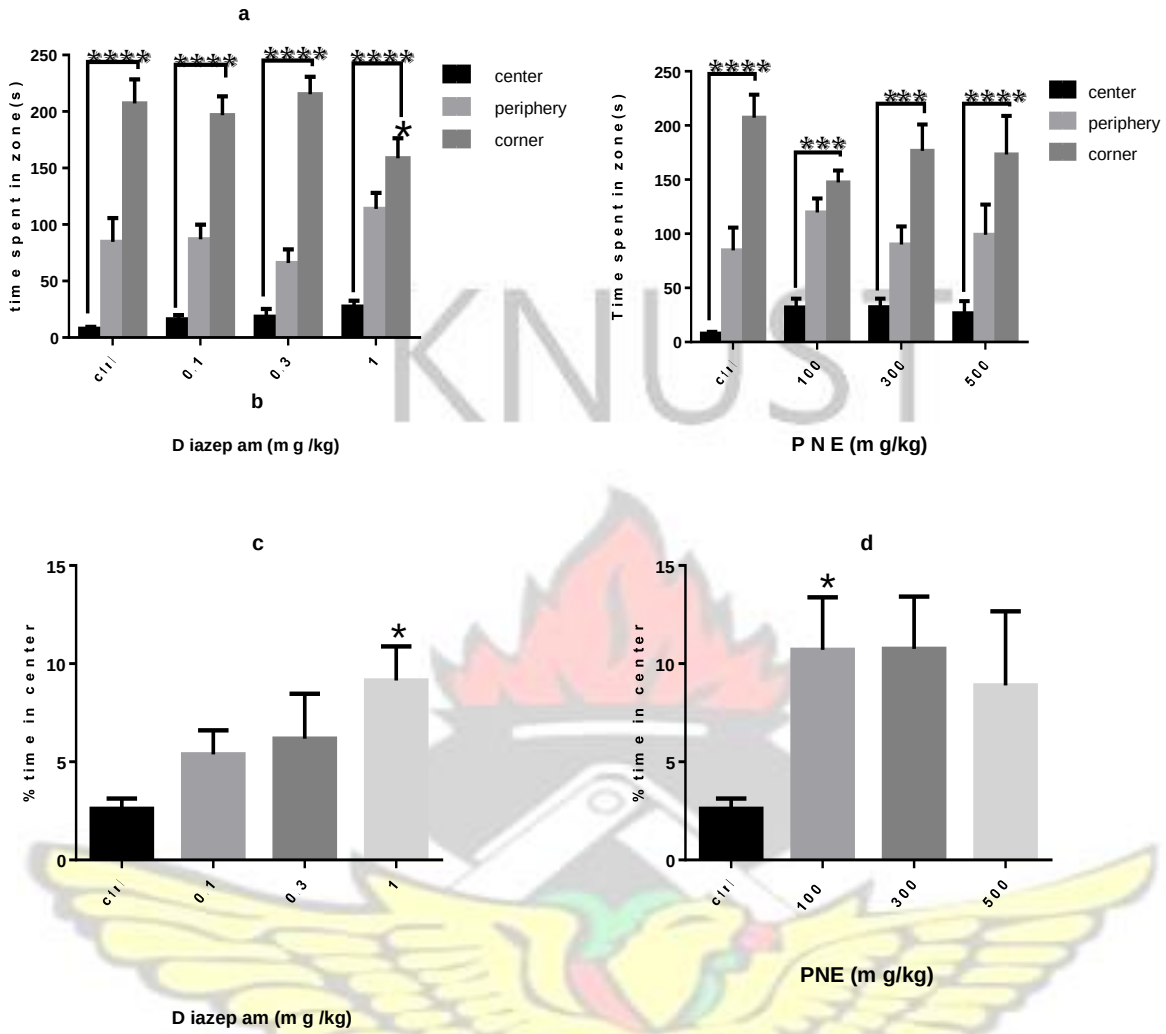

Figure 3.12: Effect of diazepam (0.1, 0.3, and 1 mg/kg), PNE (100, 300, and 500 mg/kg) on the time spent in various arms for diazepam (a), PNE (b) and the % time spent in open arm for diazepam (c), PNE (d) in the open field test. Data are presented as group mean± SEM (n=6). Significantly different from control: *P ≤0.05. Also significantly different when the zonal entries were compared to each other as done in a and b. ***P ≤0.001, ****P≤0.0001. (Two-way repeated measures ANOVA followed by Bonferroni's *post hoc* test).

3.11 ANALGESIC EFFECT OF PNE

3.11.1 Tail flick

Both PNE and the reference drugs caused an increase in the tail withdrawal latency, calculated as a percentage of the maximum possible effect (% MPE). Two-way ANOVA revealed a significant effect of drug treatments on the tail withdrawal latencies (PNE: $F_{3, 56} = 41.28$, $P \leq$

0.0001; Diclofenac: $F_{3, 56}$ = 57.18, P ≤ 0.0001; Morphine: $F_{3, 56}$ = 74.13, P≤ 0.0001) (Figure 3.13 a, c, e). PNE (100–500 mg/kg) dose dependently increased ($F_{3, 8}$ = 44.25, P < 0.0001) tail withdrawal latencies. Diclofenac (10-100mg/kg) likewise exhibited increased tail withdrawal latencies ($F_{3, 8}$ = 25.93, P = 0.0002). Morphine (1-10mg/kg) also showed a significant ($F_{3, 8}$ = 46.38, P ≤ 0.0001) and dose dependent increase in tail withdrawal latencies (figure 3.13 b, d, and f)

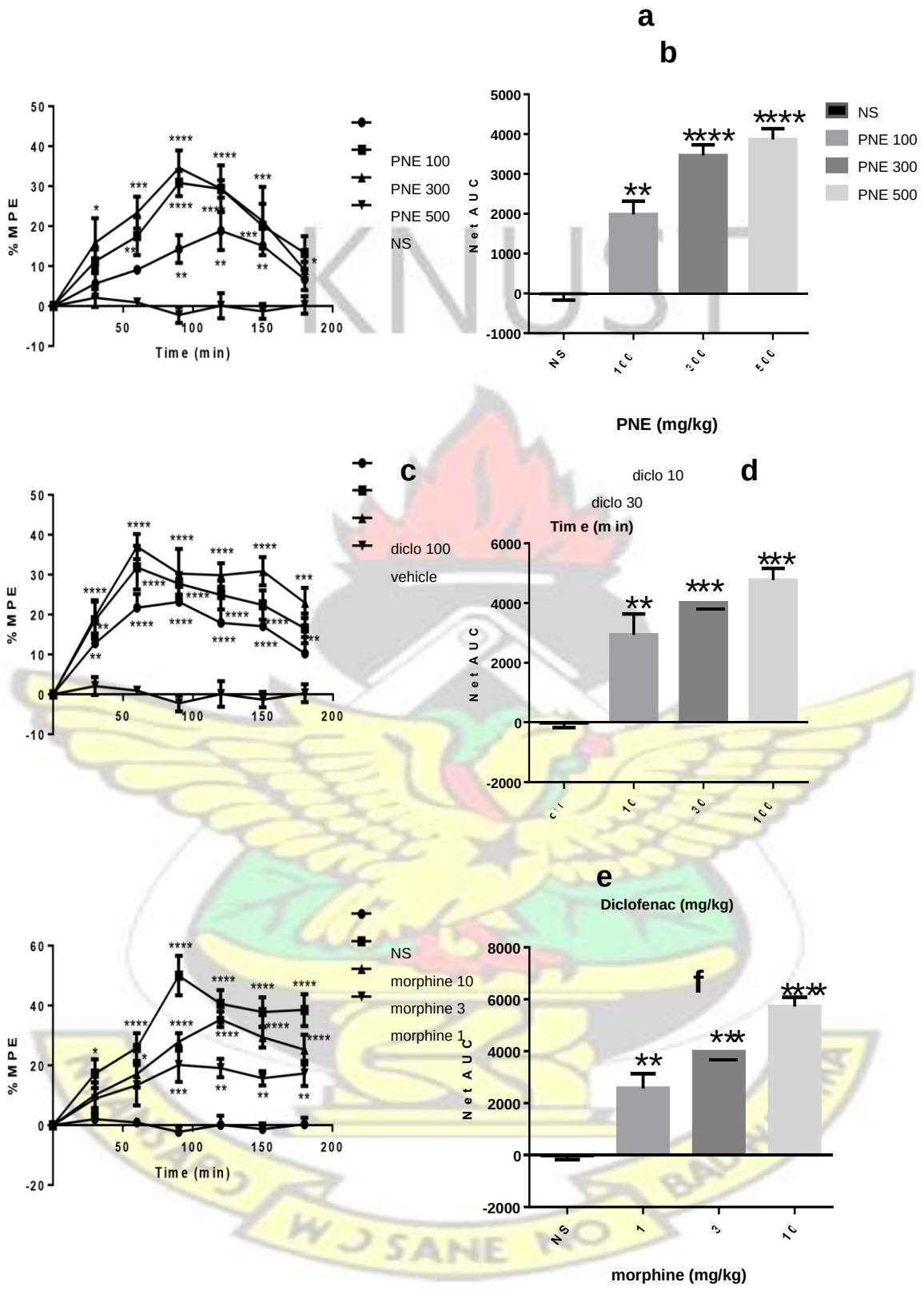

Figure 3.13: Effect of PNE (100, 300, 500mg/kg), Diclofenac (10, 30, 100mg/kg), Morphine (1, 3, 10mg/kg) on the time course curve (a, c, e) of the tail flick test and the AUC (b, d, f) in rats. Values plotted are mean±SEM, (n=5). ****P ≤ 0.0001;*P≤ 0.001; **P ≤ 0.01;**

***P≤0.05 compared to vehicle-treated group.(ANOVA followed by Dunnett's multiple comparisons test).**

3.11.2 Acetic acid-induced writhing

Acetic acid injected intraperitoneally produced writhing, characterized by exaggerated extension of the abdomen combined with the outstretching of the hind limbs. PNE (100, 300, 500mg/kg) and Diclofenac (10, 30, 100mg/kg) suppressed this writhing (Figure 3.14). Two-way ANOVA revealed a significant effect of drug treatments on the number of writhes (PNE: $F_{3, 60} = 68.67$, $P \leq 0.0001$; Diclofenac: $F_{3, 60} = 135.7$, $P \leq 0.0001$) (Figure 3.14 a, c). PNE significantly and dose dependently reduced abdominal writhes over the 20 minutes observed ($F_{3, 12} = 23.52$, $P < 0.0001$) (Figure 3.14 b). Diclofenac also significantly reduced abdominal writhes ($F_{3, 12} = 47.09$, $P < 0.0001$) (Figure 3.14 d).

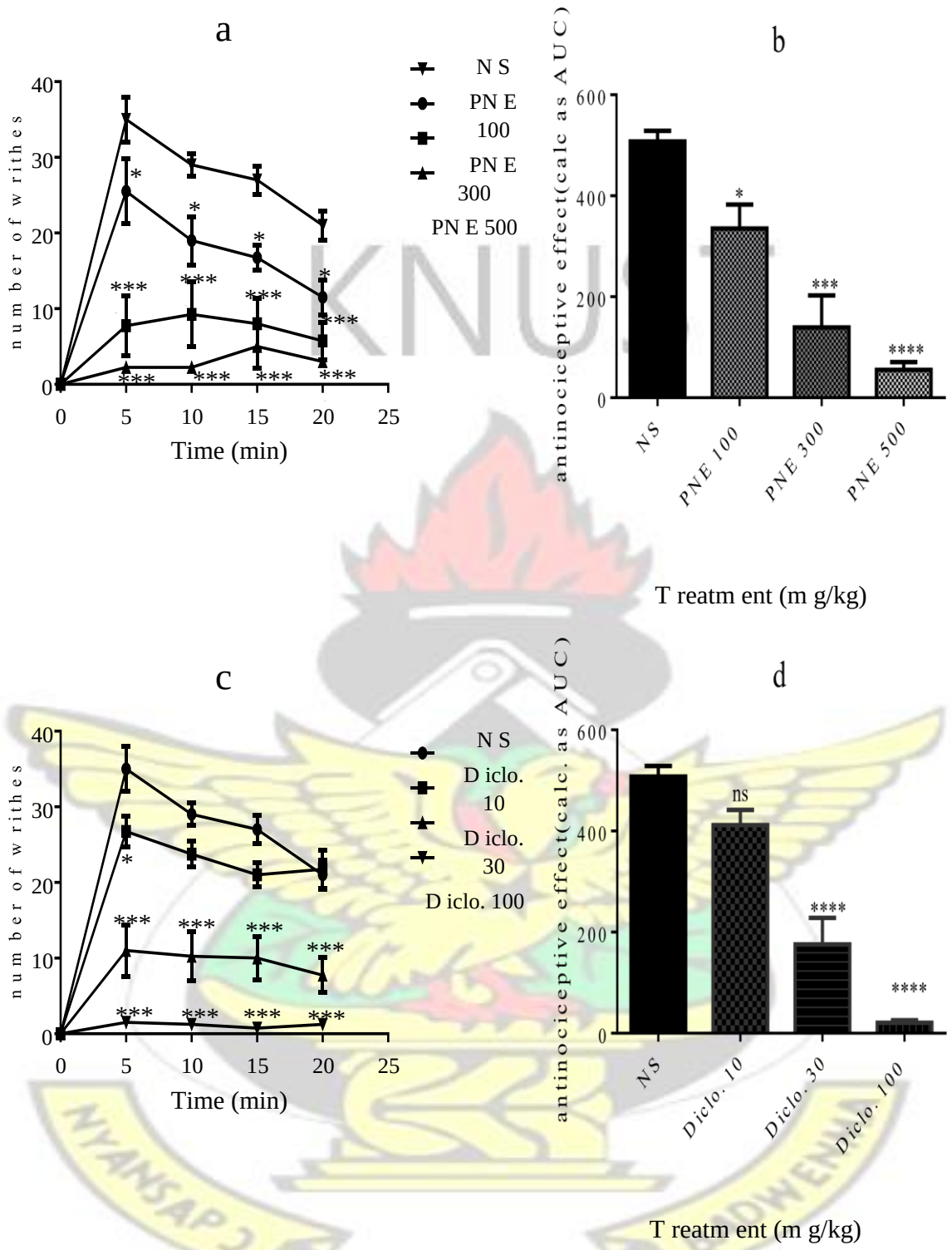

Figure 3.14: Effect of PNE (100, 300, 500mg/kg), Diclofenac (10, 30, 100mg/kg) on the time course curve of acetic acid-induced abdominal writhes (a, c) and the total nociceptive score (calculated as AUC) (b, d) in the mice. Data are expressed as mean ± SEM, (n=5). ****P ≤ 0.0001;*P ≤ 0.001; **P ≤ 0.01; *P ≤0.05 compared to vehicle-treated group. (ANOVA followed by Dunnett's multiple comparisons test).**

CHAPTER FOUR

4.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 DISCUSSION

The research focused on determining the effects of PNE on cough and also the various mechanisms that contribute to the antitussive and the expectorant effects. Lastly the effect of the PNE on anxiety and pain as complications of cough were studied. Before endeavouring on such pharmacological studies, the phytochemical and safety studies were done.

Plants have been used for medicinal purposes for years. The phytochemicals in the plants which are known to be responsible for these purposes are termed secondary metabolites. So conducting phytochemical screening on PNE is a way of ascertaining the presence or otherwise of these active principles responsible for the pharmacological activities. From the test, the extract has in it alkaloids, glycosides, tannins, saponins, terpenoids, steroids and anthraquinones. Retrospective experiments have given similar secondary metabolites (Mabeku *et al*., 2008; Obasi *et al*., 2012). Alkaloids as secondary metabolites play monumental role in plants. *Picralima nitida* alkaloids have shown activity on opioid receptors (Menzies *et al*., 1998). This may explain why *Picralima nitida* has antitussive effect. Apart from the alkaloids, other secondary metabolites will all have a role in one way or the other in making the plant exhibit the effects it has, for instance various tannins have exhibited antimicrobial activity against broad spectrum organisms (Chung *et al*., 1993; Burapadaja, 1995).

The Irwin test is a systematic observational procedure for assessing and scoring the effects of drugs on the behavioral and physiological state of rodents. This method (Irwin, 1968) help scientists to detect potential adverse effects of candidates on the central nervous system (CNS) before human testing. From the test, the extract had no deleterious effects for the doses that were

given to the animals. Not only that, no autonomic and CNS signs were seen except at the doses of 1000 and 2000mg/kg where sedation became evident. The effects of the *Picralima nitida* alkaloids on opioid receptors (Menzies *et al*., 1998) could be a factor, considering the fact that the typical opioids cause sedation as one of their side effects. It is to be noted however that the LD_{50} of the extract will be greater than 2000mg/kg since no death were recorded at that dose.

Various cough models including chemical, mechanical and electrical stimulation of animal airway receptors have been developed over the years. Animals that have previously been used include mice, guinea pigs, rats, rabbits, cats and dogs. Limitations usually comes with using certain irritants in some animals for cough studies. Use of mice in antitussive studies have been questioned because they lack RAR, intraepithelial nerve endings (Karlson *et al*., 1988) and the needed energy to cause cough. The use of guinea pigs on the other hand are the mainstay in antitussive investigation because they possess the needed afferents and can produce cough just like humans. In the experiment, cough was detected with a characteristic sound and by stretching of limbs accompanied by inspiration and then expiration (Morice *et al*., 2007). These criteria were taken so as to distinguish it from other respiratory reflexes like sneezing and expiratory reflex.

Citric acid as a tussigenic agent is known to stimulate Trasient Receptor Potential Vanilloid 1 (TRPV1) on the C-fibers and Acid Sensing Ion Channel (ASIC) on the touch-Sensitive AδFibers (Kollarik *et al.,* 2007), but inhaled citric acid stimulates predominantly C-fibers. This can then cause release of tachykinnins to mediate bronchoconstriction, mucus secretion which in turn stimulates RAR (Bonham *et al.,* 1996; Canning *et al*., 2001). The impulse will eventually be conveyed through the vagus nerve to the CNS and then back to respiratory muscle through the efferent pathway to cause cough. From the results obtained, dihydrocodeine was found to be

effective. It was used as the positive control because it is the second most specific antitussive of the commonly used opioids (Eddy *et al*., 1969). Mechanistically, it acts on the μ opioid receptors to suppress the cough reflex (Kamei, 1996). The extract also exhibited a dose dependent antitussive effect with both the cough count and latency of cough similar to dihydrocodeine. The significant antitussive effect observed can be attributed to the activity of the alkaloids of the extract on the opioid receptors (Menzies *et al.*, 1998).

To determine the expectorant activity of PNE, the trachea phenol red secretion assay was used. This model is developed on a principle that when phenol red is injected after an expectorant is given consecutively, there will be enhancement of phenol red secretion from the trachea. PNE at doses of 100, 300, and 500 mg/kg did not cause significant phenol red secretion from the trachea. Ammonium chloride and 1000 mg/kg PNE however caused significant tracheal phenol red secretion. Ammonium chloride is known to cause irritative action on the bronchial mucosa, leading to the production of excess fluid in the tracheobronchial airways for easier clearance of mucus (Lin *et al*., 2008).

The RAR is another cough receptor that has well been studied and is known to play a role in cough modulation. Unlike the C-fiber, it is very difficult to get a chemical which directly stimulates the RAR. Rather, stimulation of RAR relies on indirect effects like bronchoconstriction and presence of mucus (Canning *et al.*, 2001). That explains the essence of the histamine/acetylcholine-induced bronchospasm and mucus-secretion suppression experiments. In investigating the bronchodilator effect of the extract, Guinea pigs were used because their airway have been known to be sensitive to histamine and acetylcholine which are regarded as mediators of bronchoconstriction. Moreover, there exist a similarity between theirs and that seen in humans (Agrawal *et al*., 1991).

Histamine causes bronchoconstriction by stimulation of H_1 receptors on smooth muscles in the airways (Simons, 1999). Histamine also induces airway smooth muscle contraction, reflex hyperpnoea and bronchoconstriction indirectly via stimulation of lung irritant receptors through vagal (cholinergic) pathways (Mills *et al.*, 1969; Canning *et al*., 2001). There are wide range of bronchodilators, each with its own mechanism. The fact that PNE inhibited bronchoconstriction induced by acetylcholine and histamine (in a manner similar to atropine and mepyramine) could also bring to mind that PNE could have component acting via antimuscarinic or antihistaminic mechanism to inhibit bronchoconstriction. Antihistamine activity could also alleviate cough. Older-generation antihistamines have been proposed to possess antitussive effects via a peripheral indirect mechanism involving cholinergic mechanisms (Pratter, 2006). Consistent with this premise is the fact that some antihistamines possess anticholinergic actions (Orzechowski *et al.*, 2005). That notwithstanding, the possibility of the extract acting directly on the bronchial wall as an agonist to cause relaxation cannot be disregarded.

Mucus is also another stimulant of RAR (Jonzon *et al.*, 1986; Bonham *et al.*, 1996) apart from bronchoconstriction. In the mucus suppressant experiment, the ammonium chloride served to induce secretion of phenol red after which its absorbance was determined. The design of the experiment was to determine the extent to which the various treatment will suppress such secretion. Sodium cromoglycate is known to prevent the release of inflammatory mediators, such as histamine from mast cells and the inhibition of calcium influx and chloride channels, and thus helps prevent the release of preformed cytokines from inflammatory cells (Heinke *et al*., 1995). From the experiment, it can be said that the extract has a mucus suppressant effect at the doses given. The presence of excess mucus give the impression of sensitization (Widdicombe, 1996b). By the extract‘s ability to inhibit the secretion of mucus from the airway,

the stimulation of RAR will be tampered with and that will contribute to the antitussive effect of the plant.

Elsewhere the anti-inflammatory activity of *Picralima nitida* has been established (Duwiejua *et al.*,1995). Since chronic airway inflammation can lead to mucus secretion, the mucus suppressant effect can be attributed to its anti-inflammatory effect. This does not in any way rule out the possibilities of other mechanisms. For instance airway surface epithelial cells and submucosal gland cells express muscarinic receptors (Basbaum *et al*., 1984; Barnes *et al*., 1983), and so atropine, the prototypical muscarinic antagonist inhibits mucus secretion (Groth *et al.*, 1992). Considering the fact that the extract exhibited an inhibitory activity against acetylcholineinduced bronchoconstriction with effects similar to atropine, a potential anticholinergic activity of the extract which possibly contribute to its mucus suppressant activity cannot also be overlooked.

In testing the anti-oxidant effect, DPPH, 2, 2-diphenyl-1-picrylhydrazyl hydrate a stable radical having a characteristic violet colour (and maximum absorption at 517 nm) and which accepts an electron or hydrogen in the presence of a suitable free radical scavenger (reducing agent). So the anti-oxidant activity is measured by the test drug‘s ability to reduce this DPPH to its reduced form, 2, 2-diphenyl-1-picrylhydrazyl, which is yellow in colour. The absorbance of the residual DPPH will then be measured; agents with high antioxidant activity will therefore give lower absorbance values and higher %scavenging activity. From the preliminary investigations conducted in the laboratory, similar concentrations of PNE and ascorbic acid were used. From the IC_{50} values obtained, ascorbic acid exhibited a higher antioxidant activity than the extract. All the same, the antioxidant effect of PNE as demonstrated from this very experiment is a good outcome: Oxidants can cause damage by interacting with antiproteases or other processes leading to the development of chronic lung damage (Repine *et al.*, 1997). Cough and phlegm

are symptoms frequently accompanying chronic obstructive pulmonary disease. This may be caused by oxidative stress–mediated inflammation and tissue damage (Heffner and Repine, 1989). Intake of fruit and vegetable, which are major sources of antioxidants, have been associated with higher lung function and reduced symptoms of cough with phlegm (Smit *et al.*, 1999; Schunemann *et al.*, 2001).

The compound 48/80-induced mast cell degranulation is use to test mast cell stabilizing effects of potential therapeutic agents. Compound 48/80 causes mast cell degranulation (Ennis *et al*., 1980) by increasing intracellular Ca^{2+}, which is required for degranulation in the mast cell (Tasaka *et al*., 1986). In the experiment, the cells were then stained with toluidine blue and the count taken. Reduction in number of degranulated mast cell is used as a measure in predicting the mast cell stabilizing effect of the various treatments. From the experiment, kitotifen and PNE all stabilized the mast cell from degranulation. It has been proven that acid (tussinogen) acts on both afferent fibers and mast cell in cough induction. The experiment demonstrate that the antitussive effect of the extract can be due to its mast cell stabilization effect; the extract by stabilizing mast cells, prevent release of leukotriene and histamine which can in turn stimulate C-fibers. In fact antileukotrienes are effective in cough variant asthma (Dicpinigaitis *et al*., 2002).Secondly, direct stimulation of C-fiber by acid cause release of tachykinins which acts on mast cells to cause degranulation. These demonstrate how mast cell stabilization contributes to cough suppression. This give an indication of the fact that the extract work through peripheral mechanisms. Moreover, stabilizing mast cells will prevent release of neurotrophins which when present results in plasticity of afferent nerves (Leon *et al*., 1994; Braun *et al*., 1999).

Antibiotics have been tested on different bacterial strains using different methods like the agar well diffusion and micro dilution method. In using the agar well diffusion, PNE had activity against all the strains of bacteria used. Various factors were taken into consideration in the

selection of the microorganism for the experiment. In all, two gram positive and three gram negative organisms were used. Community-acquired pneumonia accounts for about 6% of all ambulatory visits with a chief complaint of cough (Metlay *et al*., 1998). *Streptococcus pneumonia* was found to be the main culprit followed by *Staphylococcus aureus*; this explains why those two gram positive organism were chosen for the experiment. Some enteric gram negative bacilli are known to also cause extra-intestinal infections like pneumonia which has cough as a manifestation; *Klebsiella pneumonia* and *Escherichia coli*are two of such organisms (Pek and Boushey, 2004). From the results, the extract had activity against gram positive organism than the gram negative ones. The MIC obtained support this deduction; the MIC of the extract against *staphylococcus aureus, streptococcus pneumoniae* and *salmonella typhi* were all found to be 5mg/ml, but the zones were higher for the gram positive organisms than for *Salmonella typhi* indicating a greater degree of inhibition for the former. For the other two gram negative organisms, the extract had a MIC of 10mg/ml. From the results, PNE will be a good remedy for cough arising from bacterial infection of the airway. This will relieve the hypersensitivity and any other problem caused by these bacterial infections.

The present study on the plant has demonstrated a very good outcome: PNE has anxiolytic and antinociceptive effects. It is remarkable that these effects were seen in all the models deployed. The extract‘s effect on Pain and anxiety were considered since the two effects happen to be worrisome complications in chronic idiopathic cough. Anxiety however is known to modulate urge-to-cough (Davenport, 2008).

Cough and pain have many things in common; they originate in afferent nervous systems that detect and signal real or impending threats to the organism. As with pain, cough can also be evoked in experimental animals by stimulation of nociceptive C_fibres as well as by faster conducting Aδ_fibres (Canning *et al*., 2004). Several stimulants known to selectively stimulate

nociceptive C_fibres (e.g. capsaicin, bradykinin) also evoke cough in laboratory animals and humans (Fuller, 1991). Central sensitization that has been known to cause allodynia also results in hypertussive states.

From the results obtained from the tail flick test, the extract showed significant antinociceptive effect at all the doses given. This was also observed for both diclofenac and morphine. The tail flick test as a thermal is considered to be a spinal reflex, but it could also involve higher neural structures and so the method basically identifies centrally-acting analgesics (Jensen and Yaksh, 1986; Le Bars *et al*., 2001). And so the extract can be said to be acting through a centrally mediated pathway by elevating pain threshold of animals towards heat. The writhing test helps in identifying central and peripheral analgesic compounds (Le Bars *et al.*, 2001). The extract may act either of the two pathways. The peripheral mechanisms can be reduction of sensitization by such agents like prostaglandins. The probable non-sensitizing role of the extract was demonstrated in the antitussive experiment. That may account for the peripheral mechanism for the antinociceptive effect of the extract.

The extracts showed anxiolytic effects in both the elevated plus maze and the open field method. The Open field test works on the principle that animals removed from their normal environment and placed in a novel environment (in this case the central portion of the field) express anxiety and fear. This is evidenced by the fact that ambulation and exploration time in the center of the open field will be decreased whiles peripheral movement or thigmotaxis will be increased (Bhattacharya, 1994; Bhattacharya and Mitra, 1991). In other words, the open-field model examines anxiety-related behavior characterized by the normal aversion of the animal to an open, brightly lit area (Asano, 1986; Choleris *et al*., 2001). Typical anxiolytic drugs are known to reverse these effects whiles anxiogenic drugs will worsen it. In fact, anxiety behavior

in the open field is triggered by two factors: individual testing and agoraphobia (Prut and Belzung, 2002); this explains why the test work best in rodents. And so in the Open field test, effects on the reaction of the rodents to a stressful event is what is measured but not on exploration. Therefore, anxiolytic treatments do not themselves increase exploration in the open field but they decrease the stress-induced inhibition of exploration behavior. From the experiment both PNE and diazepam exhibited an anxiolytic effect by increasing the number of entries and the time spent in the central zone relative to other zones. With regard to this effect, PNE unlike Diazepam did not clearly show a dose dependent effect both for the percentage time spent in center and percentage entry into central zone.

Previously many CNS-acting drugs have shown anxiolytic activity in the open field test. Notable among them are drugs that have effect on serotonin neurotransmission (Angrini *et al*., 1998) and also drugs acting on $GABA_A$ receptors (Fonseca *et al*., 1976; Britton and Britton, 1981).That notwithstanding, an effect on $GABA_A$ receptors seems to be the most probable mechanism: most of these $GABA_A$ agonist like the Benzodiazepines at higher doses exhibit sedative effect (Rang *et al*., 2007), and from the Irwin test, higher doses of the extract had a sedative effect; secondly, certain GABAergic drugs like Baclofen exhibit antitussive effect (Dicpinigaitis and Dobkin, 1997).

The elevated plus maze test is one of the most popular tests of all currently available animal models of anxiety (Rodgers *et al*., 1997) because it uses natural stimuli (fear of a novel, brightly lit open space and fear of balancing on a relatively narrow, raised platform) that can induce anxiety in humans (Dawson and Tricklebank, 1995; Jung *et al*., 2000). It also has a strong predictive validity for screening anxiolytic drugs (Rodgers *et al*., 1997). Anxiolytic drugs increase whiles anxiogenic drugs specifically decrease the number of entries into the open arms

and the time spent there. Apart from these, other validated ethological measures of risk assessment such as stretched–attend postures and head-dipping were also considered in the experiment (Rodgers *et al*., 1997; Rodgers and Johnson, 1995).

From the experiment, diazepam exerted an anxiolytic activity consistent with results from previous experiments (Dalvi and Rodgers, 1996). PNE similarly exhibited an anxiolytic activity by increasing the percentage entries into open arms and also reducing percentage protected head dips and stretch attend posture. There was however not much effect on the percentage time spent in the open arm

The anxiolytic activity of PNE as confirmed by the open field and the elevated plus maze is important as far as the antitussive work is concerned; the respiratory sensation called ―the urgeto-cough‖ can be said to be lowered by the extract's ability to reduce anxiety. This deduction is justifiable on the ground that anxiolytics reduce the urge-to-cough (Davenport *et al*., 2009). In other words the anxiolytic activity of PNE contributes to its antitussive effect apart from its potential of reducing cough complications.

The effect of *Picralima nitida* alkaloids has been studied and their stimulatory effect on opioid receptors has been shown (Menzies *et al*., 1998). This is important not only for confirming the antitussive effect but also the bronchodilator effect since opioids are known to inhibit nonadrenergic non-cholinergic nerve-mediated bronchoconstrictor responses both *in vivo* in guinea pig airways (Belvisi *et al*., 1988) and *in vitro* in guinea pig bronchi (Frossard and Barnes 1987).

Bronchodilators like prostaglandin E2, adrenaline, and adenosine, adversely sensitize C-fibers to capsaicin and bradykinin through direct effects on their peripheral nerve terminals (Lee *et al*., 2001; Ho *et al.,* 2000). This will make the use of such bronchodilators questionable in cough

amelioration. In the evaluation of the antitussive effect, citric acid, a C-fiber stimulant was used in the induction of cough. It was however realized that there was a significant reduction in cough count. Since the extract has been shown to act peripherally, it will reduce sensitization of the Cfiber in the periphery. The mast cell stabilizing effect established elsewhere also give an indication of a non-sensitizing effect of the extract since mast cell stabilizers with antitussive effects suppress neuron activation by reducing the activity of peripheral nerves (Dixon *et al.,* 1980; Jackson *et al*., 1992). These clearly shows that unlike other bronchodilators, PNE likely reduce sensitization of C-fibers.

It seems obvious from the work, that PNE may be effective against asthma. According to Rang *et al.* (2003), there are two categories of anti-asthma drugs: anti-inflammatory drugs and bronchodilators. It has previously been confirmed that the crude extract as well as an alkaloid, Pseudo-akuammigine of *Picralima nitida* have all shown anti-inflammatory activities (Duwiejua *et al.,* 1995;Duwiejua *et al.* 2002). From the work, the anti-oxidant and the mast cell stabilizing effect established could all contribute to such anti-inflammatory property of *Picralima nitida*. Apart from the anti-inflammatory effect, there is an evidence that *Picralima nitida* has a bronchodilator effect: from the current research, this was confirmed by the extract's ability to inhibit both histamine and acetylcholine induced bronchospasms; the ability of *Picralima nitida* alkaloids to activate opioid receptors (Menzies *et al*., 1998) may also give an indication of the bronchodilator effect since opioids inhibit bronchoconstrictor responses (Frossard and Barnes 1987; Belvisi *et al*., 1988); the fact that *Picralima nitida* exhibits an anti-inflammatory and bronchodilator effects clearly implies that it will likely be an effective anti-asthmatic plant.

Since the cough reflex is usually elicited from the periphery through stimulation of certain sensors like the rapidly adapting receptor (RAR) and the C-fiber, there have always been the search for pharmacological agents that can reduce the stimulation of these sensors:

C-fiber stimulation is tampered in the presence of a mast cell stabilizer since the mast cells release histamine and leukotrienes which can all stimulate the C-fibers (Mazzone and Undem, 2009); also since reactive oxygen species (ROS) have excitatory effect on C-fibers (Taylor-clark *et al.*, 2008) antioxidants will be effective in reducing C-fiber activity; there is also a potential reduction in C-fiber stimulation as a result of the anti-bacterial effect: bacterial infection is known to reduce the level of endopeptisase (Borsson *et al.*, 1989) which are enzymes that break down peptides, especially Substance P which gets released from C-fiber. Considering the fact that Substance P can augment C-fiber activity (Mazzone and Undem, 2009) and that of the RAR through the mucus secretion and the bronchospasm it induces (Canning *et al.*, 2001), suppressing bacterial proliferation like that of PNE, will enhance the level of endopeptidase, which will in turn reduce substance P mediated events. This reduces the hypersensitivity which is usually induced by infection.

With regard to the RAR, mucus is known to be a stimulant of this sensor (Canning *et al.*, 2004).The mucus suppressant effect demonstrated from the work will contribute to the reduction of RAR activity; the mast cell stabilizing effect will also eventually lead to reduced stimulation of RAR in that, the mast cell upon degranulation releases mediators like histamine and leukotrienes (Chung and Widdicombe, 2009) which can cause bronchoconstriction and enhanced mucus secretion. These two local effects have the potential of enhancing RAR activity

(Mazzone and Undem, 2009); the antibacterial effect will also tend to suppress stimulation of RAR: airway bacterial infection especially when it is chronic leads to airway inflammation which subsequently causes mucous gland hypertrophy and hyperplasia of goblet cells (Rubin, 2003). There is then an increase in bronchial mucus secretion which capably stimulates RAR; PNE will also reduce stimulation of RAR through its bronchodilator effect, since bronchoconstriction stimulates RAR; lastly, reducing the C-fiber activity indirectly reduce RAR activity since C-fiber stimulation causes releaseof tachykinins like substance P, neurokinin A, neurokinin B (Chung and Widdicombe, 2009) which induce local effects like bronchoconstriction and mucus secretion, consequently stimulating RAR activity. As a result, the anti-oxidant, antibacterial and mast cell stabilizing which are known to reduce the C-fiber activity as explained earlier will also reduce RAR activity.

The strategy for effective chronic cough management will be to get drugs that target specific diseases. The main causes of chronic cough include Asthma, GORD and PNDS. Drugs for managing cough under such conditions are termed disease specific antitussives. The results from this work give good reasons why PNE may be effective as a disease specific antitussive:

Clinically, bronchodilators like the β2 agonist are useful in certain cough-variant Asthma (Fujimura *et al.,* 1994); elsewhere, the bronchodilator effect of the extract has been confirmed. The mast cell stabilizing effect of PNE can be a reason why it will likely be effective in Gastrooesophageal reflux cough (GORC) and PNDS: activation of mast cells in the lower respiratory tract was indicated to be a possible mechanism underlying GORC (Wang *et al.*, 2010).On the PNDS, the mast cell stabilizing effect established can contribute to its anti-inflammatory activity (Duwiejua *et al.,* 1995). Anti-inflammatory drugs which are known to have effect on mast cells like the corticosteroids have shown activity against PNDS (Morice *et*

al., 2006). Moreover, antihistamines are part of the agents that have shown promise in the management of PNDS

(Pratter, 2006); the potential of PNE exhibiting an antihistaminic effect has elsewhere been explained in this document. This means that PNE may be effective against PNDS-induced cough. All these demonstrate that PNE is likely to be effective in all the three main causes of chronic cough i.e asthma, GORD, PNDS.

As explained earlier, the anxiolytic activity of PNE is likely due to its GABAergic effect. This may also account for why higher dose of the extract exhibited a sedative effect as confirmed from the Irwin test. Previously, the alkaloids of P. nitida have shown activity on opioid receptors (Menzies *et al.,* 1998) and one of the primary side effects of the opioid drugs is sedation. Everything then points to the fact that PNE has a sedative effect which can result from its effect either on the opioid receptors or its possible GABAergic action. The sedative effect seen of PNE will be of great importance since dry irritating cough is worrisome to patients, and it will always be to the patient‘s advantage if a drug that calms the system is given.

4.2 CONCLUSION

From the results obtained, PNE showed antitussive and expectorant activities. This gives an indication of PNE‘s ability to manage both dry and chesty cough depending on the dose.

Bronchodilator, mucus suppressant, mast cell stabilizing, antibacterial and the anxiolytic effects may partially or fully contribute to the antitussive effect of PNE.

The anxiolytic and analgesic effects as shown from the experiment means that PNE will reduce anxiety and pain which usually happen as complications of idiopathic chronic cough.

4.3 RECOMMENDATIONS

- ✓ *Picralima nitida* can be used in the community for both productive and unproductive cough.
- ✓ *Picralima nitida* does not rely only on the organoleptic property to suppress cough. It should be made known to other researchers in the ―cough field‖ that *Picralima nitida* acts through pharmacological means to suppress cough.

4.4 PROPOSED FURTHER WORK

- ✓ The phytochemicals responsible for the individual effects established should be identified
- ✓ Other antitussive models like the Ammonia induced cough, Mechanically induced cough, Micro injection of citric acid, electrical stimulation of the superior laryngeal nerve, Antitussive evaluation using aerosol of extract, ACEI induced cough (bradykinin mediated) should all be evaluated.
- ✓ Other possible modes of action should be exploited

CHAPTER FIVE

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