Isolation and Chemical and Pharmacological Characterization of Potential Trace Amine-Associated Receptor Antagonist from Plant Sources

A thesis submitted in accordance with the conditions governing candidates for the degree of

DOCTOR OF PHILOSOPHY

Presented by

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ABSTRACT

The present study describes the preliminary evaluation of Philippine medicinal plants *Artemisia vulgaris, Chrysanthemum coronarium, Moringa oleifera, Sesbania grandiflora and Vitex negundo* for their antagonistic activity at selected biogenic amine receptors on smooth muscle of the airways, gastrointestinal tract and vascular system.

The antagonistic activity of these plants were studied against dose-response curves for contractions of the guinea pig ileum, trachea and aorta to 5-hydroxytryptamine (5-HT_2 receptors), methacholine (M_3 muscarinic receptors), histamine (H_1 receptors), phenylephrine (α_1-adrenoceptors) and β-phenylethylamine (trace amine-associated receptors, $TAAR_1$).

The methanolic extracts of *S. grandiflora* (flowers and leaves) revealed the presence of histamine H_1 receptor and muscarinic M_3 receptor antagonist in the ileum. The *A. vulgaris* chloroform (AV-$CHCl_3$) and methanol (AV-MeOH) extracts, and the acid-base extract of *V. negundo* (VN-E) showed histamine H_1 antagonism in the ileum and trachea. Further analysis of AV-$CHCl_3$ isolated two major components yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide. Yomogin a sesquiterpene lactone exhibited a novel histamine H_1 receptor antagonism in the ileum. Repeated exposure of aortic rings to phenylephrine and β-PEA CRCs produced significant increases in maximum vascular tension due to enhanced intracellular Ca^{2+} mobilization. Both the AV-$CHCl_3$ and VN-E inhibited this enhanced response. Further analysis of AV-$CHCl_3$ revealed that it is probably inhibiting the increase of vascular tone mediated via intracellular Ca^{2+} release regulated by ryanodine.

This study further validates the traditional use of *S. grandiflora, A. vulgaris* and *V. negundo* in the treatment of hyperactive gut, asthma and hypertension.

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Isolation and Chemical and Pharmacological Characterization of Potential Trace Amine-Associated Receptor Antagonist from Plant Sources

ABBREVIATIONS

AC:	Adenylyl cyclase
ACh:	Acetylcholine
AMP:	Adenosine monophosphate
ANOVA:	One-way analysis of variance
ATP:	Adenosine triphosphate
cAMP:	Cyclic adenosine monophospahte
^{13}C-NMR:	Carbon-13 Nuclear magnetic resonance
CNS:	Central nervous system
COSY:	Correlation spectroscopy
CRC:	concentration-response curves
DAG:	Diacylglycerol
DCM:	Dichloromethane
DMSO:	Dimethyl sufoxide
EC_{50}:	Effective concentration 50%
GPCR:	G protein-coupled receptors
GDP:	Guanosine diphosphate
GI:	gastrointestinal
GTP:	Guanosine-5'-triphosphate
HMBC:	Heteronuclear Multiple Bond Coherence
^{1}H-NMR:	Proton NMR
HSQC:	Heteronuclear Single Quantum Coherence

5-HT:	5-hydroxytryptamine or Serotonin
IP_3:	inositol 1,4,5-triphosphate
ISA:	Indirect symphatomimetic amines
MAO:	Monoamine oxidase
MLCK:	Myosin light chain kinase
NA:	noradrenaline
NMR:	Nuclear magnetic resonance
NO:	nitric oxide
eNOS:	Endothelial constitutive nitric oxide synthase
PE:	Phenylephrine
PEA:	Phenylethylamine
PENDANT	Polarization enhancement nurtured during attached nucleus testing
PI:	phosphatidylinositol
PKA:	Protein kinase A
PKC:	Protein kinase C
PLC:	Phospholipase C
SR	Sarcoplasmic reticulum
TA:	Trace amine
TAAR:	Trace amine-associated receptor

Chapter 1

General Introduction

1.1 Introduction

Discovery of new biologically active compounds is one of the motivating forces behind much of phytochemical research (Cseke *et al.*, 2006). The total number of plants species is estimated at around 250,000, however, only 6% have been evaluated for biological activity screening and 15% have been submitted for phytochemical screening (Fabricant *et al.*, 2001). Cseke et al. (2006) estimated that about 80% of the all medicines are originally derived from plant or "natural" sources. He further stated the high degree of certainty that plant-derived medicines and other useful compounds can still be discovered, characterized or evaluated for their novel bioactivity given the low percentage of plant species being examined.

In the present study five selected Philippine medicinal plants (*Artemisia vulgaris, Chrysanthemum coronarium, Moringa oleifera, Sesbania grandiflora and Vitex negundo*) known traditionally to cure an array of diseases were harvested in the Philippines and pharmacologically investigated for their possible role in diseases related to the airways, vascular system and gastrointestinal (GI) tract.

1.2 Biogenic Amines

Biogenic amines are substances containing an amine group that are derived from life processes (Figure 1.1) (Paxon *et al.*, 2005). Some of the major groups of compounds of biogenic amines include histamine, 5-hydroxytryptamine (5-HT) and the three catecholamines (dopamine, noradrenaline, and adrenaline) (Blaschko, 1957). In the central and peripheral nervous systems these compounds are also well-known hormones and neurotransmitters (Hashiguchi *et al.*, 2007; Rudnick *et al.*, 1993). Another group of endogenous biogenic amines which are present in very small amounts in the mammalian tissue are the trace amines which include β-phenylethylamine, tyramine, octopamine and tryptamine (Burchett *et al.*, 2006; Grandy, 2007; Zucchi *et al.*, 2006).

1.2.1. 5-HT (serotonin)

5-HT is found in the wall of the intestine, in blood platelets, and in the enteric and central nervous systems where it acts as a neurotransmitter (Gershon, 1999; Gershon *et al.*, 1981; Turetta *et al.*, 2002). Endogenous 5-HT is biosynthetically derived from the amino acid tryptophan were approximately 95% of the body's total 5-HT is confined and synthesized in the enterochromaffin cells in the gut where it is released and eventually taken and stored by blood platelets (Berger *et al.*, 2009; Racké *et al.*, 1995; Reimann *et al.*, 1994). In the CNS it is synthesized in the serotonergic neurons (Young *et al.*, 1989). Eventually 5-HT is metabolized by the enzymes monoamine oxidase A

and aldehyde dehydrogenase in the liver to form the product 5-hydroxyindoleacetic acid (5-HIAA) which is excreted in the urine (Figure 1.2) (Ruddell *et al.*, 2008).

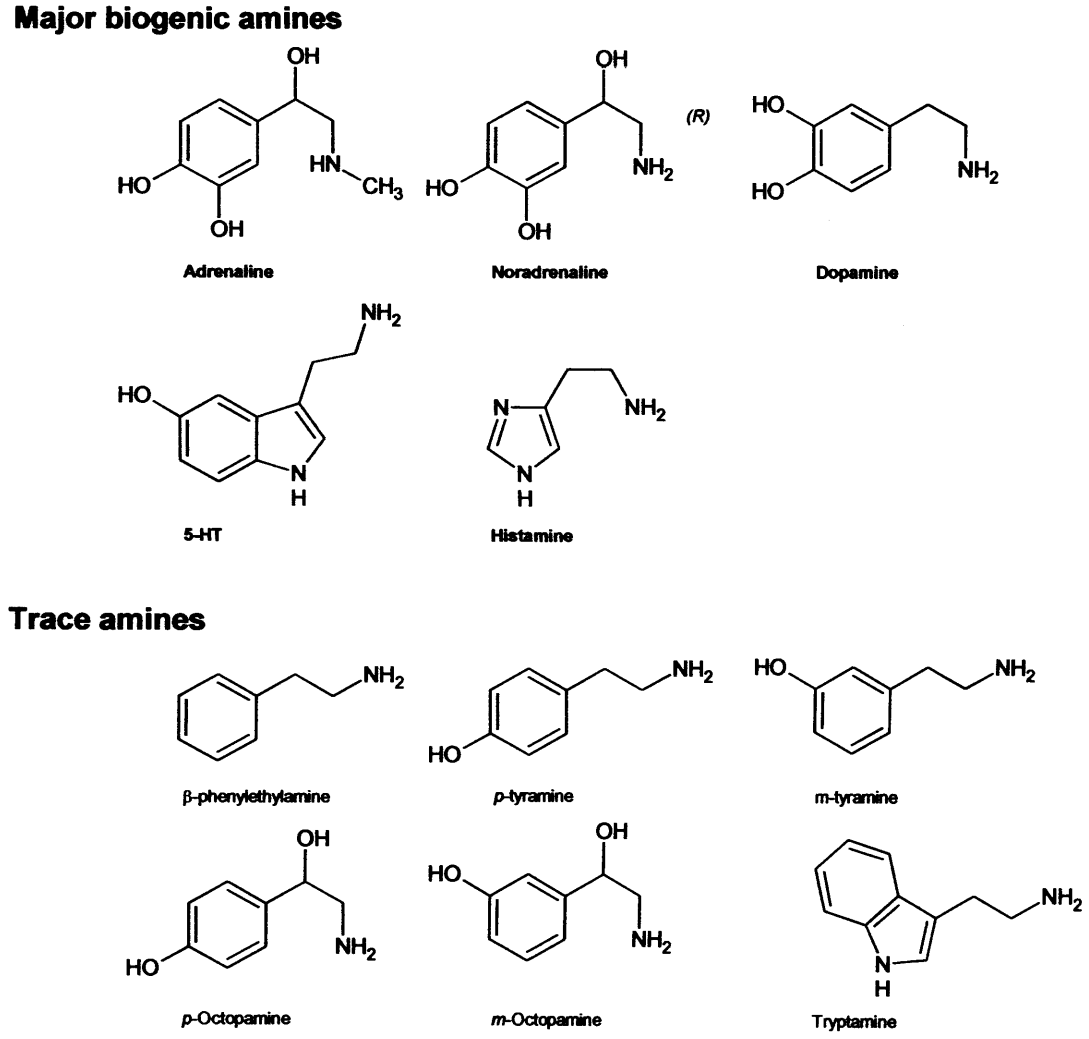


Figure 1.1. Structures of the biogenic amines (Zucchi *et al.*, 2006).

5-HT is known to play important roles in number of physiological processes such as initiation of secretory and peristaltic reflexes in the gut, smooth muscle contraction/ relaxation (Foxx-Orenstein *et al.*, 1996), platelet aggregation (Glusa *et al.*, 1989), stimulates nociceptive sensory nerve endings and can excite/inhibit neurons (Oliveira *et al.*, 2007; Steenwinckel *et al.*, 2009). It is also known to be associated with diseases like hypertension, pulmonary hypertension, migraine, nausea and vomiting, eating disorders and irritable bowel syndrome (Gilman *et al.*, 1990; Reimann *et al.*, 1994; Ruddell *et al.*, 2008; Wijngaarden *et al.*, 1990).

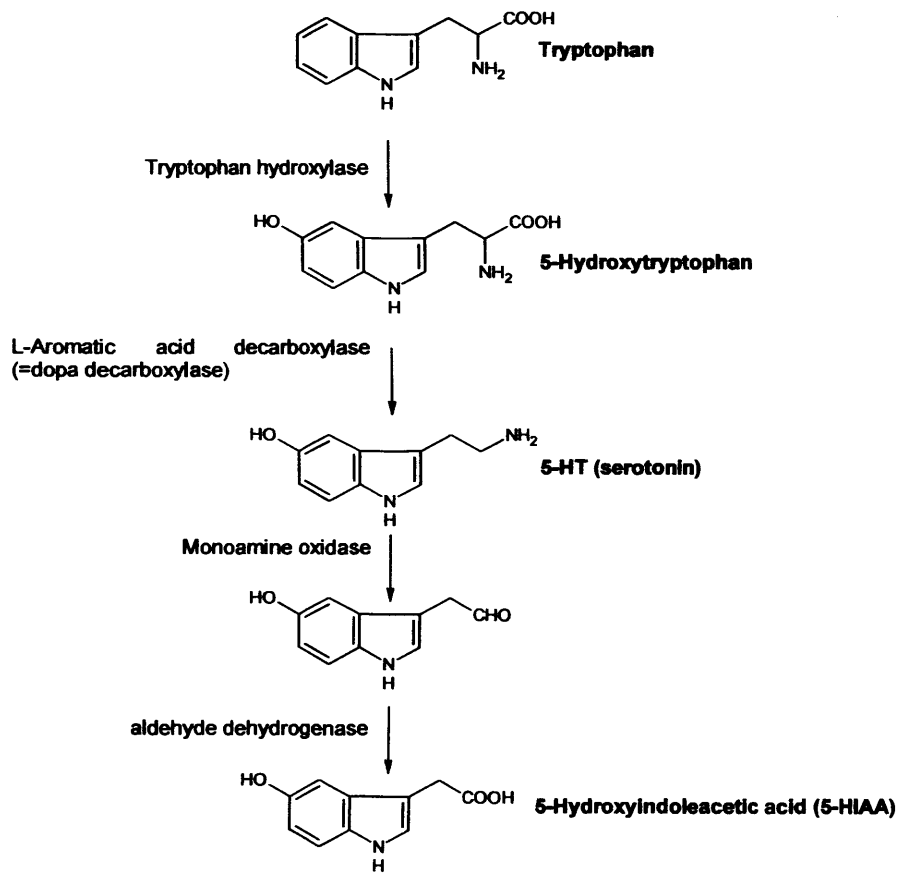


Figure 1.2. The pathway for the synthesis of 5-HT from tryptophan (Fitzpatrick, 1999)

1.2.2. Histamine

Histamine, a basic amine, is a product of the decarboxylation of the amino acid histidine by histidine decarboxylase (Cowan *et al.*, 1971). Once formed it is rapidly inactivated by histamine-N-transferase then mono amine oxidase (Figure 1.3) (Barnes *et al.*, 1998; Barnes *et al.*, 2004). It is distributed in most tissues but is located in high concentrations in the GI tract, lungs and skin (Bischoff *et al.*, 2005; Small, 2005). It is produced chiefly in mast cells and basophils (Middleton *et al.*, 1983; Newman *et al.*, 1980). Enterochromaffin-like cells found in the stomach are another important site for histamine storage and release (Rangachari, 1992). The compound plays major function in regulating immune responses (Akdis *et al.*, 2006), physiological functions in the gut (Rangachari, 1992) and is known as a neurotransmitter (Jacobs *et al.*, 2000; Yanai *et al.*, 2007). In the synapses the shortage of acetaldehyde dehydrogenase, used to catalyze the degradation of histamine, can result to the increase of histamine that triggers allergic reaction (Jayarajah et al., 2007). Food poisoning in spoiled food, such as fish, is mainly linked to the free histidine content which is converted to histamine in the presence of certain bacteria that releases histidine decarboxylase (Lehane *et al.*, 2000; Tapingkaea *et al.*, 2010).

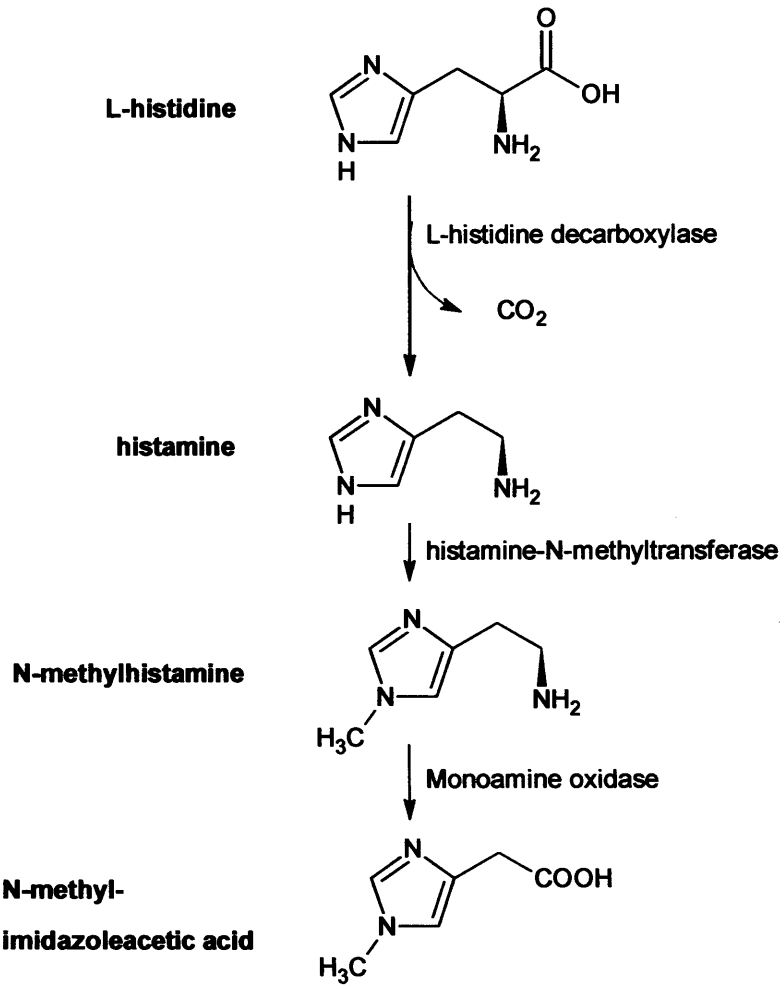


Figure 1.3. Biosynthesis and metabolism of histamine from the amino acid histidine (Small, 2005)

1.2.3. Catecholamines

Noradrenaline, adrenaline and dopamine are compounds containing a catechol structure (benzene ring with two adjacent hydroxyl groups) and an ethylamine substituent (Blaschko, 1957). Their biosynthetic pathways and metabolism are shown on Figure 1.4. Dopamine is formed by the hydroxylation and decarboxylation of tyrosine, noradrenaline from further hydroxylation of dopamine, and adrenaline from methylation of noradrenaline (Ganong, 1991). Phenylethalonamine-N-methyltransferase the enzyme that catalyses the conversion of adrenaline from noradrenaline is present in the cells of adrenal medulla (Blaschko, 1957; Ganong, 1991).

The catecholamine hormones (noradrenaline and adrenaline) are released into the circulatory system in periods of severe stress (McCarty *et al.*, 1991; Perry *et al.*, 2010). Adrenaline is mainly produced by chromaffin cells of the adrenal medulla whereas noradrenaline is found in post-ganglionic sympathetic neurons (Perry *et al.*, 2010; Ponti *et al.*, 1998). In the central and peripheral nervous system noradrenaline acts as a neurotransmitter (Wassall et al., 2009). Stimulation of the sympathetic nervous system generally results in the release of noradrenaline and adrenaline which activates α and β adrenoceptors to induce physiological responses (Ahlquist *et al.*, 1959; Gilman *et al.*, 1990; Ponti *et al.*, 1998). The most significant mechanism of termination of action of noradrenaline is by neuronal reuptake (uptake 1) of released noradrenaline back into the neurone and subsequently into the storage vesicles (Gilman et al., 1990). Noradrenaline

can also be removed by means of the extra neuronal uptake (uptake 2) (Figure 1.5) (Gilman et al., 1990).

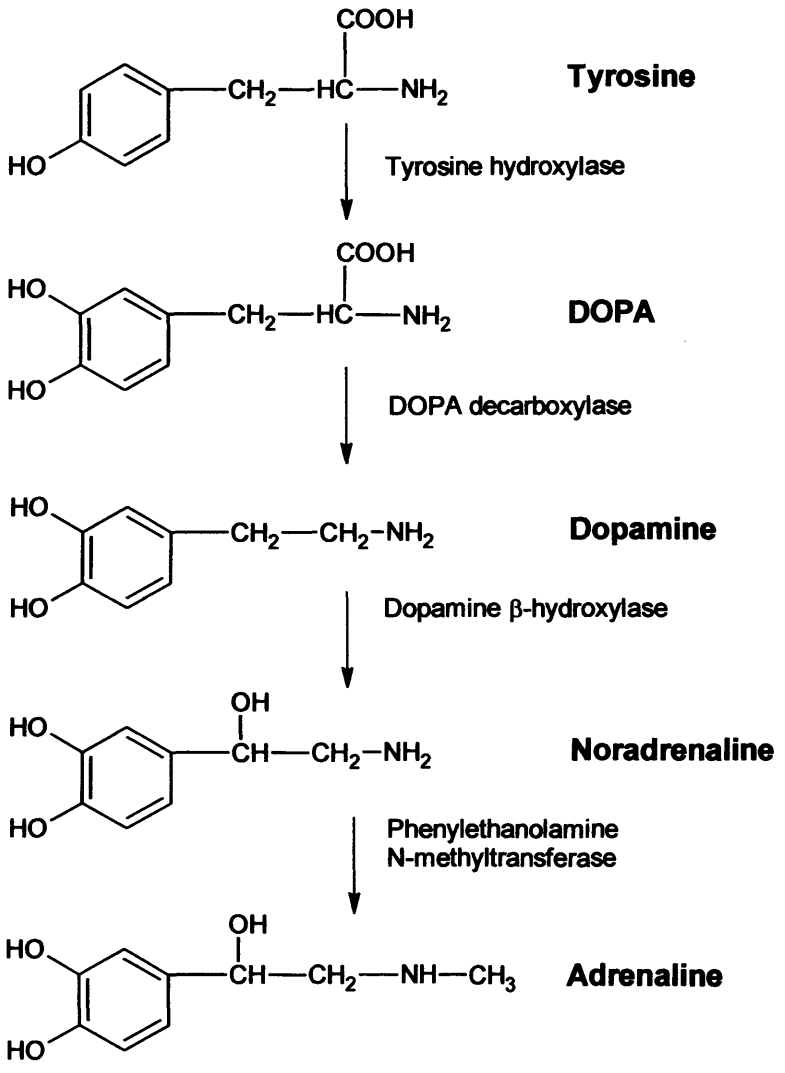


Figure 1.4. Biosynthesis and metabolism catecholamine (Blaschko, 1957; Gilman *et al.*, 1990; Rang *et al.*, 2007).

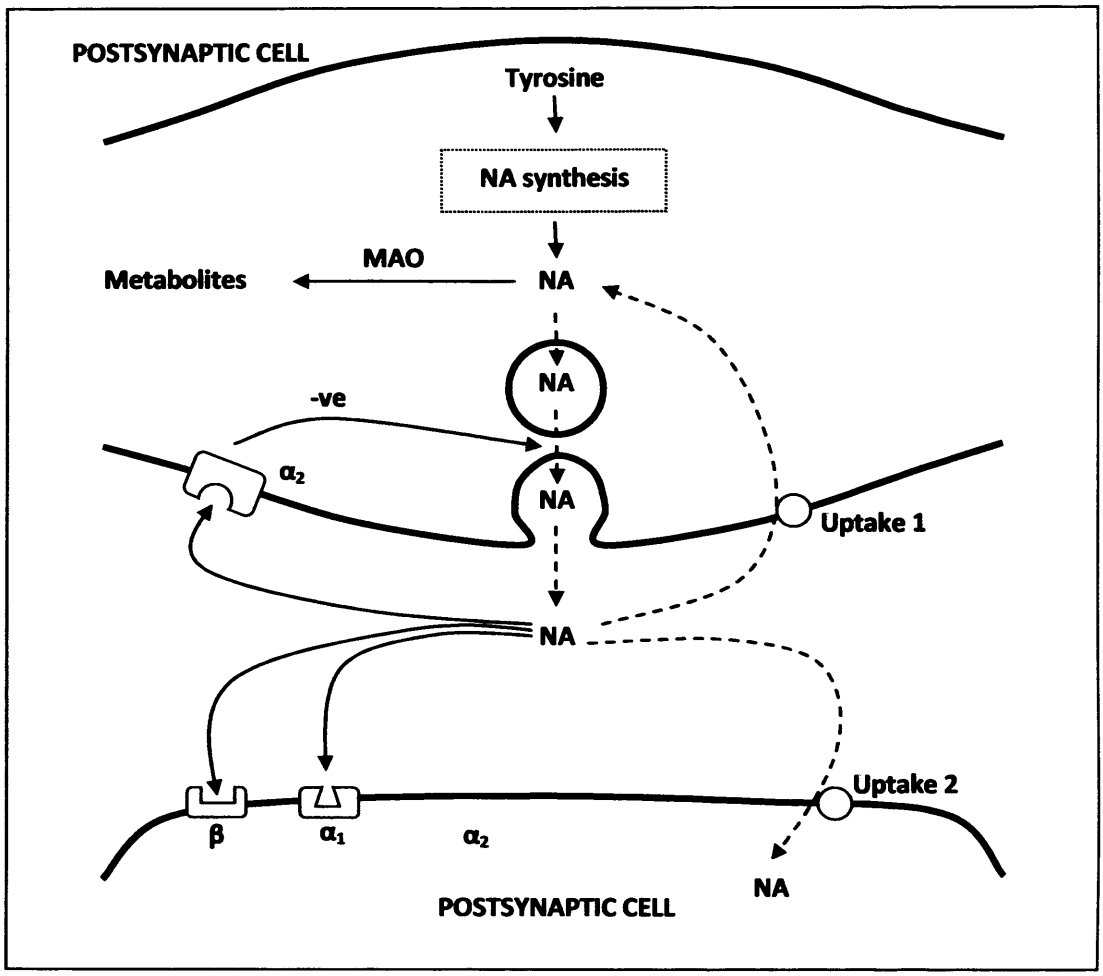


Figure 1.5. The synthesis, action and fate of noradrenaline at sympathetic neuroeffector junctions. NA – noradrenaline, MAO – mono amine oxidase. Adapted from Rang et al. (2007).

1.2.4. Trace amines

Trace amines are endogenous biogenic amines (Figure 1.6) with close similarity to the structure of major biogenic amines and overlapping functions with the aminergic pathways (Grandy, 2007; Premont et al., 2001). They are also found in other organisms like plants, bacteria and insects (Branchek *et al.*, 2003). In the diet they can be found in foods such as chocolates, cheese and wines (Branchek *et al.*, 2003).

Trace amines are derived from their parent amino acids through enzymatic decarboxylation and have very fast turnover rates resulting to their low nanomolar concentration (trace levels) in the body (Boulton, 1982; Grandy, 2007). In addition, these trace amines are known to cause amphetamine-like effects but such responses occur at micromolar concentrations (Berry, 2004; Broadley, 2010). Disorder like hypertension, migraine and coronary heart diseases has been implied to be caused by trace amine dysfunction (Branchek *et al.*, 2003; Broadley, 2010).

Trace amines (β-PEA) bearing sufficient resemblance to noradrenaline can go through the presynaptic nerve terminals by uptake 1(Gilman *et al.*, 1990; Rang *et al.*, 2007). Once inside the sympathetic neurons trace amine can liberate noradrenaline to produce indirect symphatomimetic (ISA) effects (Figure 1.7) (Gilman *et al.*, 1990; Rang *et al.*, 2007). Pharmacological responses of ISAs such as vasoconstriction can be triggered from the stimulation of noradrenaline release from sympathetic neurons to activate α-adrenoceptors in postsynaptic cells (Broadley, 2010; Gilman *et al.*, 1990; Rang *et al.*, 2007). In addition the release of noradrenaline can also stimulate β-adrenoceptors resulting in vasodilatation (Shafiei *et al.*, 1999) or bronchodilatation (Barnes, 1993).

Other action of ISAs in the periphery includes raised arterial pressure, inhibition of gut motility and increased heart rate and myocardial force of contraction (Broadley *et al.*, 2009; Gilman *et al.*, 1990; Rang *et al.*, 2007). In the gut the expected action of sympathomimetic amines such as adrenaline and noradrenaline acting on the α_1-adrenoceptor or β_1-adrenoceptor is relaxation (Ahlquist *et al.*, 1959; Broadley *et al.*, 2009; Innes *et al.*, 1969; Ponti *et al.*, 1998). However, in the guinea pig and rat isolated gut preparations, tyramine and β-PEA were shown to cause contractions which is opposite of the sympathomimetic affects (Broadley *et al.*, 2009; Innes *et al.*, 1969).

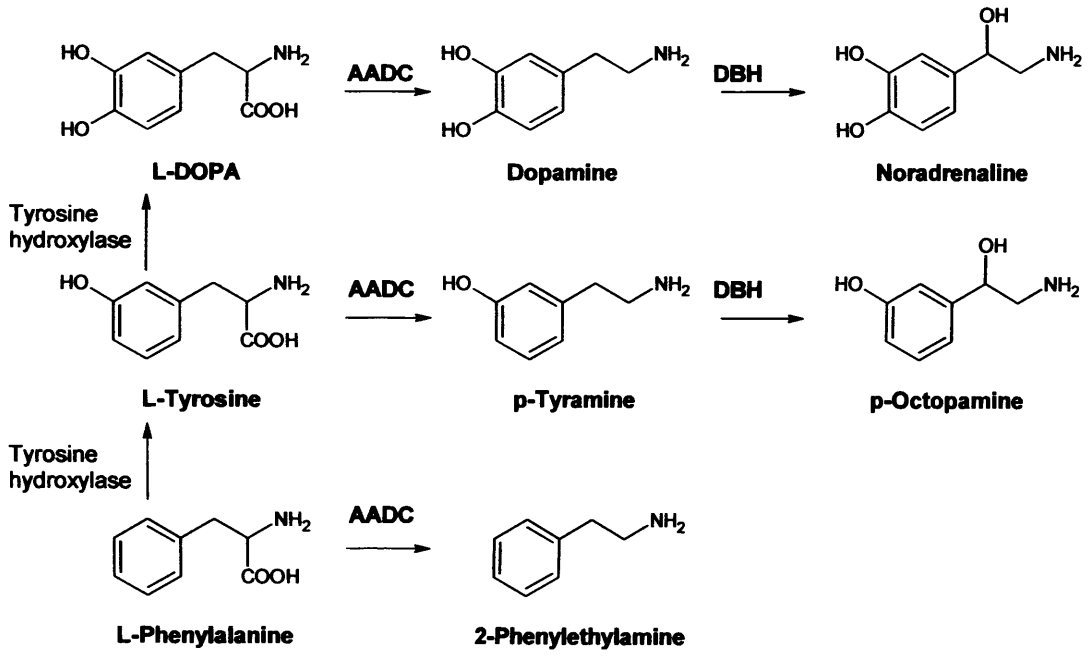


Figure 1.6. Relationship between trace amine and neurotransmitter synthetic pathways (Berry, 2009).

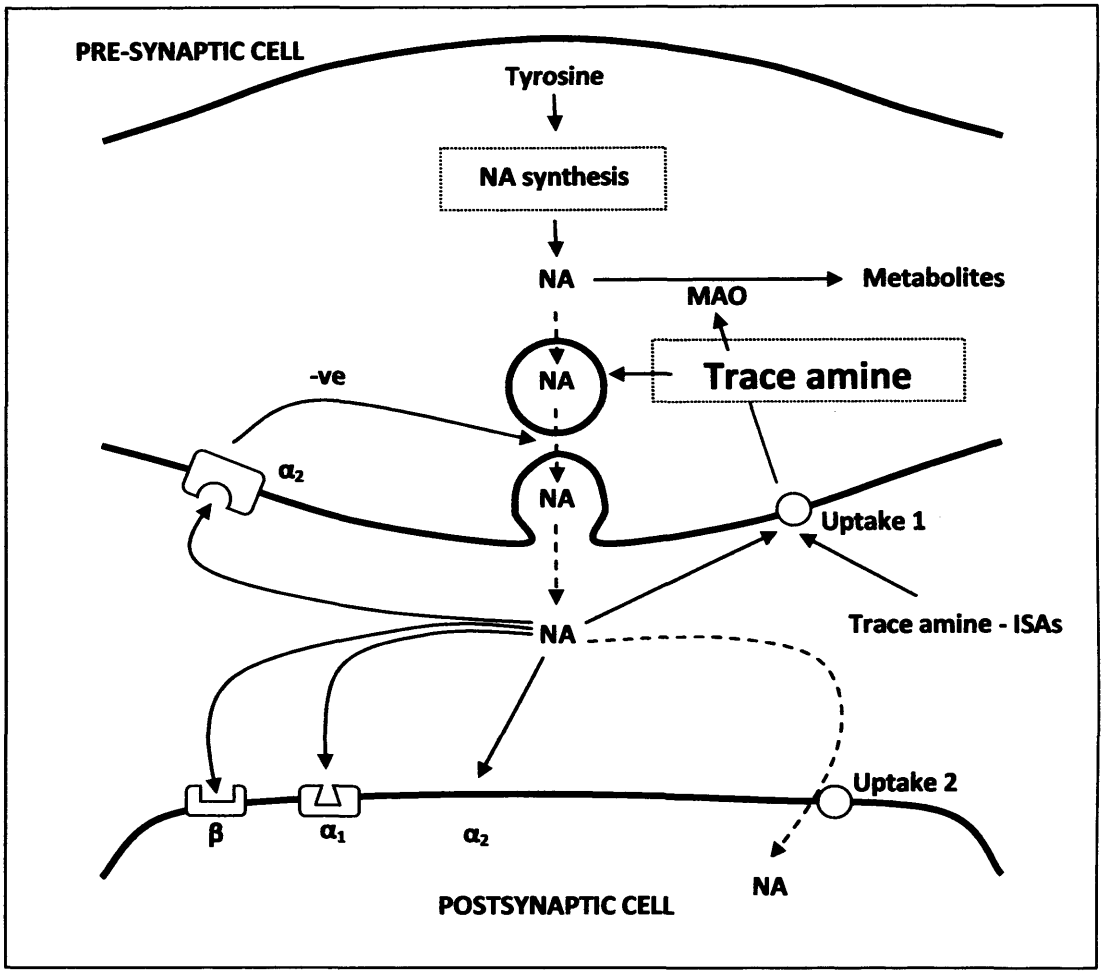


Figure 1.7. The indirect sympathomimetic effects of trace amines at sympathetic neuroeffector junctions. NA- noradrenaline, MAO – monoamine oxidase, ISA – indirect symphatomimetic effects (Gilman *et al.*, 1990; Rang *et al.*, 2007).

1.3 Acetylcholine

An ester of choline and acetic acid (Figure 1.8) it acts as a chemical transmitter in the central nervous system (CNS), peripheral somatic and parasympathetic autonomic nervous systems (Koppen *et al.*, 2003; Racké *et al.*, 2006). It is found in synaptic vesicles in high concentrations largely in cholinergic neurons (Racké et al., 2006). Its synthesis involves the reaction of choline and acetyl-coenzyme A (acetyl-CoA) catalyzed by choline acetyltransferace (Ganong, 1991; Racké *et al.*, 2006). Once formed it is rapidly removed from the synapse by acetylcholine hydrolysis catalyzed by acetylcholinesterase (Racké et al., 2006). Parasympathetic nerve activity releases acetylcholine which causes increased gut motility (Olsson *et al.*, 2010), bronchoconstriction (Racké et al., 2006), decreased heart rate and increase production of saliva and mucus (Ganong, 1991). In high doses, acetylcholine can cause convulsions and tremors (Itoh, 1995). Deficient levels in the somatic nerves innervating skeletal muscles can contribute to motor dysfunction (Ganong, 1991) such as myasthenia gravis (Kawaguchi *et al.*, 2004).

Its action in parasympathetic nerves is mediated via muscarinic receptors (Koppen *et al.*, 2003; Tobin, 2002). Acetylcholine and its derivatives activity can be attributed to the presence of quaternary ammonium group with positive charge and the ester group with partial negative charge (Baker et al., 1971). Muscarinic receptor antagonists have a similar structure to acetylcholine but with a bulky aromatic component in place of the acetyl group (Sauerberg et al., 1991).

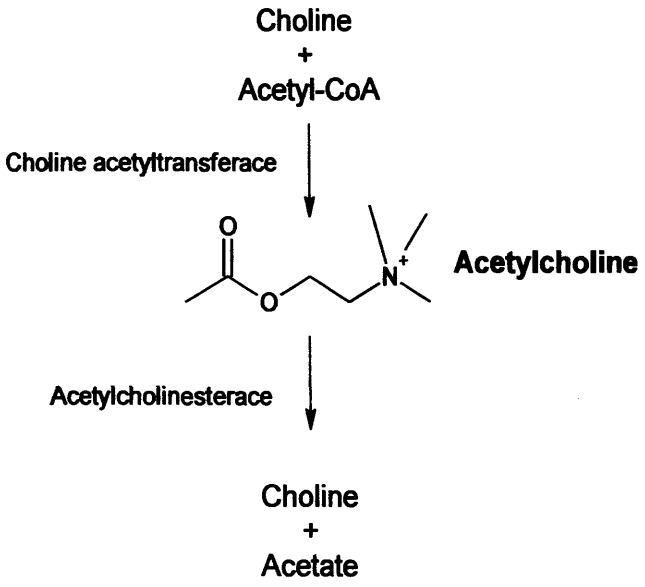


Figure 1.8. Acetyl choline metabolism (Ganong, 1991).

1.4 G protein-coupled receptors

The biogenic amines described above all exert their effects on the GI tract, cardiovascular and respiratory systems through G-protein coupled receptors and signal transduction to the tissue response (Brunton *et al.*, 2005; Rang *et al.*, 2007). Chemical signalling is the ability of a living cell to receive and acts on signals detected by highly specific and sensitive receptors (Krauss, 2003). One type of this signalling mechanism is through the activation of G protein-coupled receptors (GPCRs) or seven-transmembrane-spanning (heptahelical) receptors (Nelson et al., 2005). Among membrane-bound receptors, the GPCRs are undoubtedly the most diverse (Bockaert *et al.*, 1999). They are the binding sites for naturally occurring ligands (biogenic amines) and/or exogenously introduced analogues (Kristiansen, 2004).

Heterotrimeric G-proteins hold three subunits designated α, β, and γ (Hur *et al.*, 2002). In the resting state of the receptor the G_{α}, with a bound GDP (guanosine diphosphate), is complexed with $G_{\beta\gamma}$ (Hur *et al.*, 2002). Once a particular specific ligand attach to the GPCR a conformational modification arises in its structure that initiates the exchange of intracellular bound GDP to GTP (guanosine triphosphate) (Gilman *et al.*, 1990; Hamm *et al.*, 1996; Lodish *et al.*, 2008). The GTP-protein then dissociates from the occupied receptor which can either act directly on an ion channel, mitogen-activated protein kinases (MAPKs), or bind to a nearby enzyme that triggers the production of secondary messengers (Gilman et al., 1990; Lodish et al., 2008). The molecular differences within the alpha subunits achieved specificity of each type of receptors that produces a distinct cellular response (Hur *et al.*, 2002). There are four main classes of

G-protein ($G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha 12/13}$ and $G_{\alpha q/11}$) of pharmacological importance which they show selectivity with respect to receptors and effectors with which they couple (Table 1.1) (Hamm *et al.*, 1996; Hur *et al.*, 2002; Kristiansen, 2004).

There are two different sequences of second-messenger reactions that can be stimulated when a GTP-protein acts on an enzyme. One is set in action when the GTP-protein acts on the AC (adenylyl cyclase), (Figure 1.9), which activates the production of second messenger cAMP (cyclic adenosine monophospahte) from ATP (adenosine triphosphate). This results in the activation of PKA (protein kinase A) which initiates the phosphorylation of many cellular proteins including enzymes involved in energy and metabolism and enzymes which promote muscle contraction in heart. The other second-messenger reaction series begins when the GTP-protein acts on PLC (phospholipase C), (Figure 1.10). This triggers the dissociation of PI (phosphatidylinositol) to the second messengers IP_3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). Intracellular IP_3 disperses in the cytosol to the endoplasmic reticulum, where it binds to specific IP_3 receptors and causes the release of intracellular stores of Ca^{2+}. Ca^{2+} with the help of DAG activates PKC (protein kinase C) which also causes phosphorylation of other enzymes resulting to smooth muscle contraction (Gilman *et al.*, 1990; Hamm, 1998; Hamm *et al.*, 1996; Hur *et al.*, 2002; Krauss, 2003; Kristiansen, 2004; Lodish *et al.*, 2008; Nelson *et al.*, 2005; Rang *et al.*, 2003; Selbiea *et al.*, 1998; Warber *et al.*, 2006).

Table 1.1. The main types of G-protein α subtypes and their functions (Rang *et al.*, 2007).

G-protein	Biogenic amines	Receptors	Main Effectors
α_s	catecholamines, histamine, 5-HT	β_1, β_2, β_3-adrenergic receptors, histamine H_2 receptors, serotonin 5-HT4	Stimulates adenylyl cyclase, ↑cAMP
α_i	catecholamines, histamine, 5-HT, acetylcholine, includes also opiods and cannabinoids	α_2-adrenoceptors, serotonin 5-HT_1 and 5-HT_5, histamine H_3 and H_4, muscarinic M_2 and M_4	Inhibits adenylyl cyclase, ↓cAMP
$\alpha_{12/13}$	catecholamines, histamine, 5-HT, acetylcholine, includes also opiods and cannabinoids		Limited effects mainly due to βγ subunits
$\alpha_{q/11}$	catecholamines, histamine, 5-HT, acetylcholine	α_1-adrenoceptors, histamine H_1, serotonin 5-HT_2, muscarinic M_1, M_3 and M_5	Activates phospholipase C, ↑IP_3 and ↑DAG

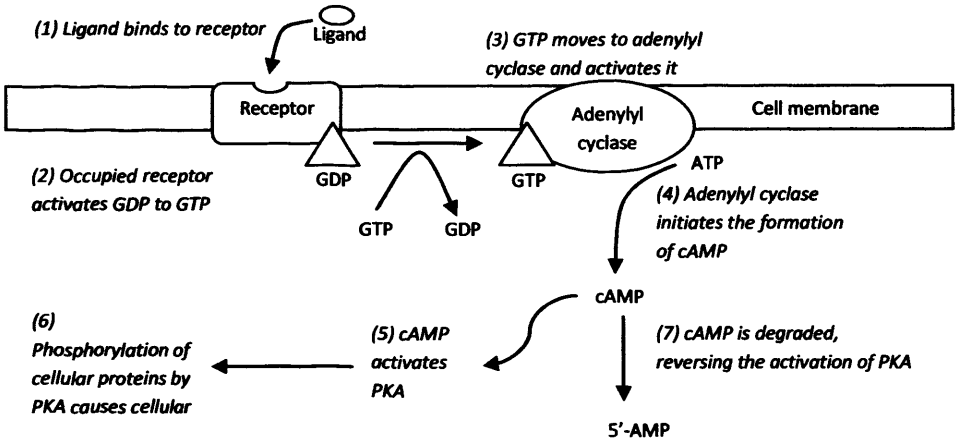


Figure 1.9. The Adenylyl cyclase / cAMP system (AC-cAMP system). △ - G-protein. Adapted from (Gilman *et al.*, 1990; Lodish *et al.*, 2008; Nelson *et al.*, 2005)

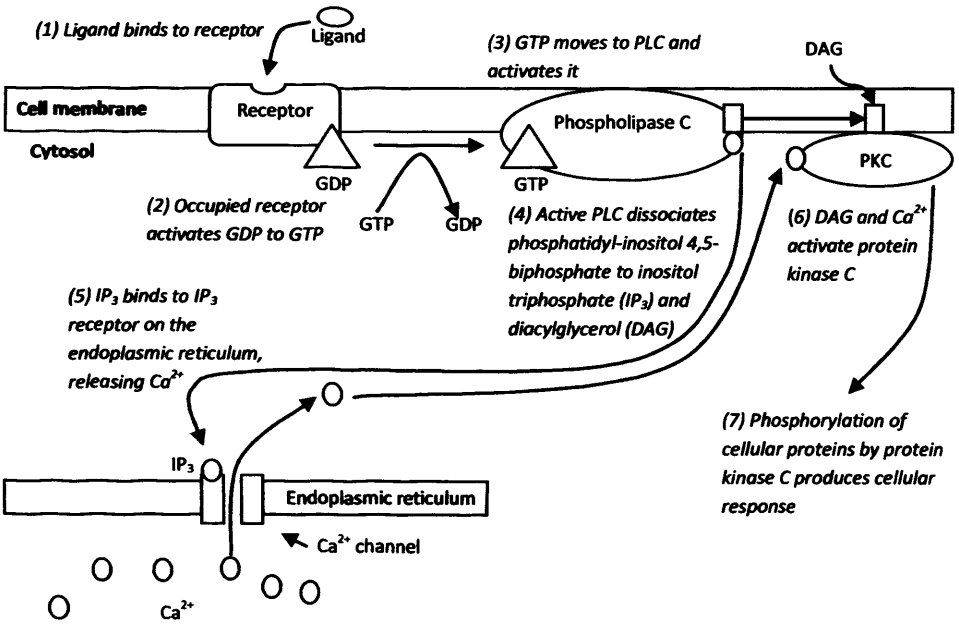


Figure 1.10. The Phospholipase C / Inositol phosphate system (PLC-IP_3 system). △ - G-protein. Adapted from (Gilman *et al.*, 1990; Lodish *et al.*, 2008; Nelson *et al.*, 2005).

1.3.1. 5-HT receptors

All 5-HT receptors are GPCR that activate secondary messenger cascades to produce stimulation or inhibition of responses, except 5-HT_3 which is a ligand-gated ion channel (Hoyer et al., 2002; Marsden et al., 1988). At present seven classes of pharmacologically and structurally distinct 5-HT (5-HT_{1-7}) receptor have been identified. Further subtypes of $5HT_1$ (A-F) and 5-HT_2 (A-C) are also recognized (Barnes *et al.*, 1999; Brunton *et al.*, 2005; Ganong, 1991; Hoyer *et al.*, 2002; Rang *et al.*, 2007; Raymond *et al.*, 2001).

In the gut 5-HT provides an important role in the regulation of GI processes (Gershon, 2004). Released from enterochromaffin cells in the gut, 5-HT produces diverse sensory and motor functions in the GI tract through a variety of receptors found in the submucosal and myenteric neurons (Kim *et al.*, 2000; Nemeth *et al.*, 1989). 5-HT_1 receptors occur mainly in the brain where they act as neurotransmitter release modulator (Peroutka, 1984). They are coupled to $G\alpha_{i/o}$ which reduces cAMP through the inhibition of adenylate cyclase (Raymond et al., 2001). Activation of 5-HT_{1B} in the vascular smooth muscle can lead to pulmonary vasoconstriction (Rang *et al.*, 2007; Raymond *et al.*, 2001). 5-HT_{1D} subtype expressed in the cerebral blood vessels can regulate vasoconstriction in the brain and is assumed to be important in migraine (Raymond et al., 2001). When stimulated, 5-HT_4 receptors located on enteric cholinergic neurons results also in smooth muscle contraction through acetylcholine release (Sikander et al., 2009). 5-HT can also stimulate of 5-HT_4, $5HT_{1A}$ or $5HT_{1D}$

receptors expressed on nitrergic neurons which release nitric oxide (NO) which results in smooth muscle relaxation (Sikander et al., 2009).

5-HT_2 receptors are composed of three GPCRs (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors) with similar pharmacology and transduction pathway (Boess *et al.*, 1994; Marsden *et al.*, 1989; Raymond *et al.*, 2001). 5-HT_2 receptors operate through the IP_3/DAG pathway (Boess *et al.*, 1994) and are expressed in the smooth muscle of blood vessels (Ullmer et al., 1995) and of the GI tract (Gershon, 2004), the CNS (Marsden et al., 1989), and platelets (Pletscher, 1987). The 5-HT_{2A} subtype is expressed throughout the smooth muscle, CNS and blood platelets (Raymond et al., 2001). It mediates several physiological processes like smooth muscle contraction in the gut (McLean et al., 2007) and bronchi (Szarek et al., 1993; Szarek et al., 1995), vasoconstriction (McLennan *et al.*, 1984)/vasodilatation (Verbeuren et al., 1991), platelet aggregation (Glusa et al., 1989) and neuronal excitation (Beubler et al., 1993; Fozard, 1984). In the gut, the complementary smooth muscle contraction largely mediated by 5-HT_2 and relaxation resulting from stimulation of other 5-HT receptors plays an important part in the regulation of GI motility or peristalsis (Boess *et al.*, 1994; Gershon, 1999; McLean *et al.*, 2007; Raymond *et al.*, 2001). Other physiological processes mediated by 5-HT includes intestinal secretion (Beubler et al., 1993), nausea and vomiting (Sanger *et al.*, 2006).

1.3.2. Histamine receptors

Histamine receptors are the class of GPCRs that are targets of the endogenous ligand histamine (Leurs *et al.*, 1995). This class of GPCRs are composed of four known receptors (H_{1-4}) (Leurs *et al.*, 1990; Leurs *et al.*, 1995). The histamine H_1-receptor is directly coupled with the G-protein G_q and causes direct contractile actions on smooth muscles present in the ileum and trachea through the PLC/IP_3 system (Leurs et al., 1995). On the other hand, activation of H_1-receptors results in relaxation of the vascular smooth muscle due to the production and release of an endothelium derived relaxant factor nitric oxide (Beyak *et al.*, 1995; Hide *et al.*, 1988; Yang *et al.*, 2002). The Histamine H_2-receptor triggers the AC/cAMP system that causes relaxation of the vascular smooth muscle and is directly coupled with the G-protein G_s (Monczor et al., 2006). In the regulation of GI motility H_1 which modulates the smooth muscle contraction coexist in a complementary manner with H_2 receptors that induces relaxation (Mullera *et al.*, 1993; Poli *et al.*, 2001; Valle *et al.*, 1997). H_3 receptors are coupled with G_i G-protein and act on neurons where they presynaptically inhibit the release of neurotransmitters like 5-HT, acetylcholine and noradrenaline (Poli et al., 2001; Yokotani et al., 2000).

Table 1.2. The four classes of histamine receptors (Akdis *et al.*, 2006; Small, 2005)

Histamine receptors	Effects	Secondary messenger	G proteins
H_1	smooth muscle contraction of GI tract and airways Vasodilatation via NO release	$\uparrow Ca^{2+}$, activation of Phospholipase C via PLC/IP_3 pathways	$G_{q/11}$
H_2	Smooth muscle relaxation	$\uparrow$cAMP, stimulation of AC	G_s
H_3	Presynaptic inhibition of transmitter release	$\downarrow$cAMP, inhibition of AC and inhibition of Ca^{2+} influx	$G_{i/o}$
H_4	Highly expressed in bone marrow, promotes chemotaxis in eosinophils and mast cells	$\downarrow$ncAMP, inhibition of AC	$G_{i/o}$

1.3.3. Adrenergic receptors

The adrenergic receptors are a class of GPCRs that are targets of the endogenous catecholamines, adrenaline and noradrenaline (Minneman, 2007). These receptors are further classified into α-adrenergic receptors and β-adrenergic receptors based on studies with selective agonist and antagonist interactions (Ahlquist *et al.*, 1959; Minneman, 2007). With further subdivision into α_1, α_2, β_1, β_2, β_3 receptors (Brody *et al.*, 1998; Minneman, 2007; Minneman *et al.*, 1981).

The activation of α_1-adrenergic receptors produced its effects by activating PLC which causes an increase in IP_3 and Ca^{2+} (Cotecchia et al., 1990). This triggers cellular responses like vasoconstriction in blood vessels (Shaul et al., 1990) and relaxation of gastrointestinal smooth muscle (Ahlquist *et al.*, 1959; Innes *et al.*, 1969; Ponti *et al.*, 1998). Phenylephrine is a nasal decongestant that reduces mucus formation and inflammation in the nose and sinuses though its action as a selective α_1-adrenoceptor that results to vasoconstriction of blood vessels in these areas (Brunton *et al.*, 2005; Görnemann *et al.*, 2009; Johnson *et al.*, 2008; Lui *et al.*, 2000). α_2-adrenergic receptors which are negatively coupled to AC reduces *c*AMP formation as well as inhibiting Ca^{2+} channels resulting to contraction of vascular smooth muscle (Aburto *et al.*, 1995; Brunton *et al.*, 2005; Rang *et al.*, 2007). α_2-adrenoceptors are also located on autonomic nerve endings (Figure 1.11) where their stimulation by released noradrenaline causes inhibition of further transmitter release (i.e. negative feedback) (Table 1.3) (Aburto *et al.*, 1993; Cotecchia *et al.*, 1990; Gilman *et al.*, 1990).

β_2-receptors (Table 1.3) cause smooth muscle relaxation through the AC-*c*AMP system resulting to bronchodilatation (Green et al., 1995) and vasodilatation (Kazanietz *et al.*, 1991; Lui *et al.*, 2000). Propranolol is a non-selective β-adrenoceptor inhibitor that is used in the treatment of hypertension (Wu *et al.*, 1995).

Table 1.3. Major physiological effect mediated by adrenergic receptors associated with the smooth muscle present in blood vessels, bronchi and gastrointestinal tract. Adapted from (Rang *et al.*, 2007).

Tissues and Effects	α_1	α_2	β_1	β_2
Blood vessels	Constrict	Constrict/Dilate	-	Dilate
Bronchi	Constrict	-	-	Dilate
Gastrointestinal tract	Relax	Relax, inhibit neurotransmitter release	-	Relax
Heart			↑Rate ↑Constrict	
Second messenger and effectors	PLC activation ↑IP3 ↑DAG ↑Ca^{2+}	↓cAMP ↓Calcium channel ↑K+ channel	↑cAMP	↑cAMP

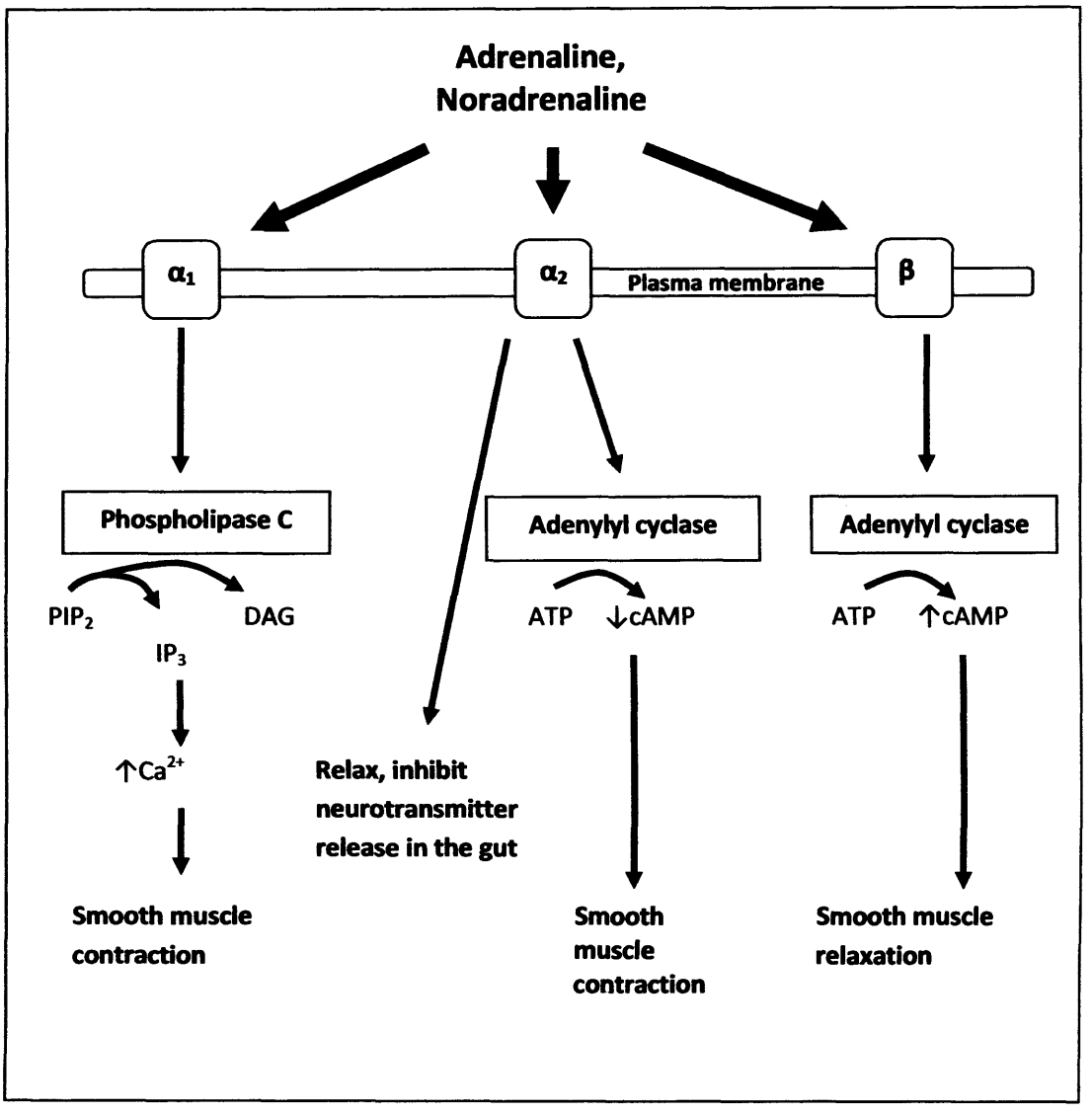


Figure 1.11. The mechanism of action of adrenergic receptors (Brunton *et al.*, 2005; Rang *et al.*, 2007).

1.3.4. Trace amine-associated receptors

Trace amine-associated receptors (TAAR) are a class of G protein-coupled receptors (Berry, 2004; Zucchi et al., 2006). Although the existence and distribution of TAs such as octopamine were well known in invertebrates and in mammals, TAAR were only identified in 2001 (Berry, 2004; Xie *et al.*, 2009; Zucchi *et al.*, 2006). TAAR receptors have been of interest in many years as putative endogenous receptors for trace amines, and other similar compounds like amphetamine, methamphetamine and metabolic derivatives of the major biogenic amines (Broadley, 2010). Currently three subclasses of TAAR have been identified based on phylogenic and ligand-binding pocket analysis (Berry, 2009). Both $TAAR_1$ and $TAAR_4$ are sensitive to TAs and in transfected cell lines appear to couple with Gs that activates AC resulting in cellular increase in cAMP levels (Table 1.4, Figure 1.12) (Berry, 2009; Frascarelli *et al.*, 2008; Xie *et al.*, 2009). Whether there is a similar coupling of the natural TAARs is not known.

Table 1.4. Classification of trace amine-associated receptors (TAARs) (Berry, 2009; Lindemann *et al.*, 2005).

TAAR Subclasses	Receptor	Sensitive to TAs
Group 1	$TAAR_1 - TAAR_4$	$TAAR_1$ and $TAAR_4$
Group 2	$TAAR_5$	No
Group 3	$TAAR_6 - TAAR_9$	No

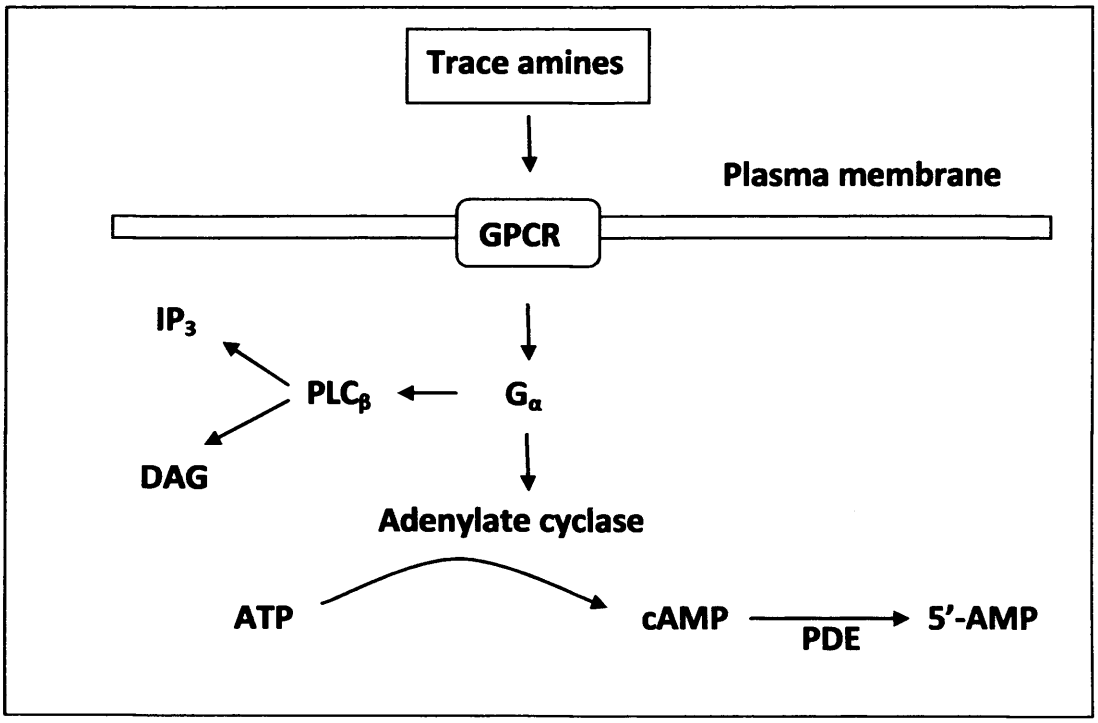


Figure 1.12. Trace amines signal transduction pathway in transfected cell lines (Berry, 2009; Xie *et al.*, 2009).

1.3.5. Muscarinic receptors

Muscarinic receptors are a class of GPCRs that have five molecular subtypes which mediate the effects of acetylcholine. The M_1, M_3, and M_5 receptors are primarily coupled to the rise of intracellular Ca^{2+} which results from the stimulation of G protein Gq which leads to the activation of PLC and release of IP_3. The M_2 and M_4 receptor activation generally results in the inhibitions and reduction of cAMP through the AC-cAMP systems which switch off smooth muscle relaxation related to cAMP increase and have anti-adrenergic effects as well as negative chronotrophy in the heart (Brunton *et al.*, 2005; Caulfield, 1993; Eglen *et al.*, 2001; Hulme *et al.*, 1990; Kitazawa *et al.*, 2007; Koppen *et al.*, 2003; Racké *et al.*, 2006).

The M_1, M_2 and M_3 are well characterized (Caulfield, 1993; Koppen *et al.*, 2003). M_1 receptors are found mainly on peripheral neurons and on gastric parietal cells and CNS (Caulfield, 1993; Rang *et al.*, 2007). M_2 receptors occur mainly on presynaptic terminals of peripheral and central neurons, and also in the heart (Caulfield, 1993; Rang *et al.*, 2007). In the smooth muscles in the gut M_2 is abundantly coexpressed with M_3 receptors (M_2:M_3 = 3:1 to 5:1) (Caulfield, 1993; Kitazawa *et al.*, 2007). Despite the dominant expression of M_2 receptors on smooth muscle, contraction and stimulation in the GI tract is largely mediated by M_3 receptors which results from the increase of intracellular Ca^{2+} (Eglen, 2001; Ehlert, 2003). M_3 receptors also mediate vasodilatation which results from the release of nitric oxide from the endothelial cells (Ehlert, 2003; Kitazawa et al., 2007).

1.5 Natural products

For thousands of years, man has used plant natural products as "remedies for diseases, spices, narcotics, dyes and poison" (Bratt, 2000). Although these compounds were used at first in their crude forms, their bioactive components were only isolated and pharmacologically evaluated beginning in the nineteenth century (Heinrich, 2010). Examples of plant-derived drugs related to treatment of diseases of the airways, gastrointestinal tract and vascular systems are given below:

Ma huang or ephedra (*Ephedra sinica* or *E. equisetina*) has been used for thousands of years in China for asthma, hay fever treatment, and for the common cold (Abourashed *et al.*, 2003; Chen *et al.*, 2010). The main components of this plant are ephedrine and pseudoephedrine (Figure 1.13) which stimulate the brain, increase heart rate, constrict blood vessels, and expand bronchial tubes (Abourashed et al., 2003; Warber et al., 2006). Their action via the α- and β-adrenoceptors of the sympathetic nervous system either directly or indirectly through the release of neuronal noradrenaline (Chen *et al.*, 2010) is common with the structurally related trace amines (see Figure 1.7) (Broadley, 2010).

Figure 1.13. The main components of Ephedra (*Epidra sinica*)

Cathinone (Figure 1.14) is a bioactive alkaloid from the Khat plant (Goudie, 1985). The leaves are chewed for their central stimulant effects (Al-Motarreb *et al.*, 2010; Broadley, 2010). The pharmacological effects of cathinone include amphetamine-like and phenyl-ethylamine-like stimulant actions like euphoria, exhilaration, hyperactivity and restlessness (Broadley, 2010; Goudie, 1985). The compound can also increase blood pressure, increase heart rate and can elicit vasoconstriction (Al-Motarreb *et al.*, 2010; Baker *et al.*, 2007; Broadley, 2010).

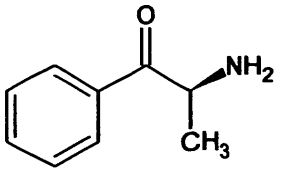


Figure 1.14. Cathinone

Methylxanthines are a group of alkaloids extracted from coffee, tea and cocoa. Theophylline, caffeine and theobromine (Figure 1.15) are three pharmacologically bioactive compounds belonging to this group (Moritoki *et al.*, 1976). These compounds and some of their synthetic derivatives relax the bronchial smooth muscle through the increase of cAMP levels via phosphodiesterase inhibition (Clarke *et al.*, 1989; Cushley *et al.*, 1985; Gong *et al.*, 1986; Krzanowski *et al.*, 1988), increase heart muscle contractility, and increase blood pressure through vasoconstriction (Rang *et al.*, 2007).

Theophylline is used for asthma therapy (Lam *et al.*, 1990). Caffeine also acts as a direct vasoconstrictor through the release of intracellular Ca^{2+} (Leijten *et al.*, 1984).

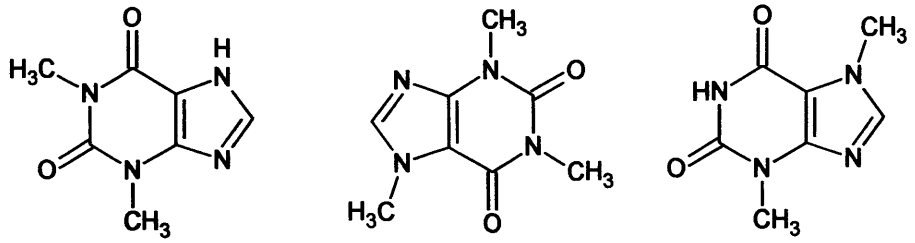


Figure 1.15. The three pharmacologically active Methylxanthines

The two naturally occurring alkaloids atropine and hyoscine (Figure 1.16) are extracted from deadly nightshade (*Atropa belladonna*) and thorn apple (*Datura stramonium*) respectively (Miraldia et al., 2001; Yun et al., 1992). Both compounds are competitive antagonist for the muscarinic acetylcholine receptor and known for their action as antispasmodics to reduce GI hypermotility and bronchodilation (Rang *et al.*, 2007).

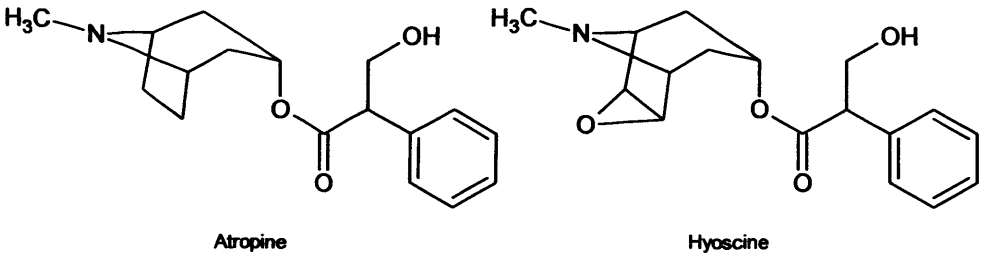


Figure 1.16. Atropine and hyoscine the deadly nightshade (*Atropa belladonna*) and thorn apple (*Datura stramonium*)

Another example is morphine (Figure 1.17) which is the principal alkaloid of the opium poppy (*Papaver somniferum*) known for its effective narcotic analgesic effect and used extensively for the treatment the moderate to severe pain (Andersen *et al.*, 2003; Brielmann *et al.*, 2006). It can also decrease the rhythmic contractions of the intestine giving an overall effect of constipating (Rang et al., 2003). This effect is through μ opiod receptor located prejunctionally on parasympathetic neurons, stimulation of which causes inhibition of acetylcholine release (Tjon *et al.*, 1995).

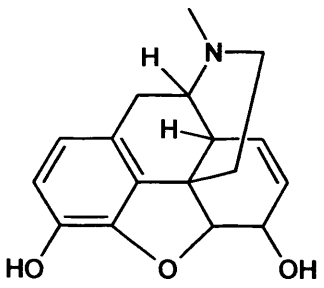


Figure 1.17. Morphine

1.6 Philippine medicinal plants

The Philippine forests originally occupied approximately 70% of its land area (Revilla et al., 2000). According to Myers et al. (2000) this primary vegetation which is equivalent to 300,800 km^2 was reduced to 9,023 km^2 in the late nineteenth century mainly due to illegal logging. They further noted that out of this remaining primary vegetation only 3,940 Km^2 were officially protected. In addition, they also recorded that out of 7,620 Philippine plants species 5,832 were endemic.

1.6.1. *Sesbania grandiflora*

Sesbania grandiflora, a nitrogen-fixing ornamental leguminous tree, belongs to the family Fabaceae (Bodhipadma et al., 2006). The plant is considered native to Southeast Asian countries like the Philippines but it has been well distributed also in southern Florida, West Indies, and southern Mexico through most countries of Central America down to South America (Duke, 1983).

According to Duke (1983), the plant is reported to be febrifuge (reduce fever), emetic (induce vomiting), diuretic, emmenagogue (induce menstruations), laxative and a tonic. He further reported that in Southeast Asian countries different parts of the plants have different uses: In the Philippines, the pounded bark was used for haemoptysis (coughing up blood) and the flowers were considered to be a vegetable and believed to lower blood pressure; Cambodians uses the bark for treatment of dysentery (inflammation of the intestine resulting to severe diarrhoea), consider the flowers to be

emollient (soften skin) and laxative; and Malayans use crushed leaves for contusions and sprains.

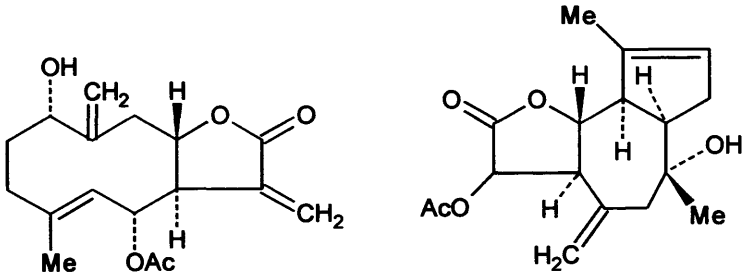
In the preliminary pharmacological screening of *S. grandiflora* extracts done by Fojas et.al. (1982), they reported that it causes histamine-like contractions of the isolated guinea pig ileum which was blocked by mepyramine, hypotension in cats and CNS depression in mice. Furthermore, Subramanian et al. (2003) reported also that the different plant fractions exhibited significant analgesic, anti-diarrhoeal, antifungal and antibacterial activity.

Among the associated compounds reported to be present in the plant includes several sterols or phytosterols (Bhattacharjee *et al.*, 1958), terpenoids (Das *et al.*, 1999; Das *et al.*, 2002) and saponins (Tiwari *et al.*, 1964a; Varshney *et al.*, 1971). Several flavonoids and related compounds with rearranged flavonoid skeleton were also identified, among the classes of flavonoids present including flavones, flavonols, flavonone, isoflavonone, and anthocyanins (Andal *et al.*, 1986; Das *et al.*, 1998; Saxena *et al.*, 1999a; Saxena *et al.*, 1999b). The presence also of grandiflorol known to reduce the blood cholesterol level was noted (Ramesh *et al.*, 2006; Tiwari *et al.*, 1964b; Tiwari *et al.*, 1964c). In addition simple alkaloids like tryptophan, indole acetic acid (Bhowmick *et al.*, 1988) and imidazole-4-ethylamine (Fojas et al., 1982) were reported to be present in the plant.

1.6.2. *Chrysanthemum coronarium*

Chrysanthemum coronarium is an ornamental plant and vegetable belonging to the family *Asteraceae* (refer to appendix). The plant is not considered poisonous but excessive consumption may result to intoxication (Ragasa et al., 1998).

In the phytochemical study of *C. coronarium* several groups of compounds were found to be present in the plant. Examples of such groups includes terpenes (Song et al., 2003b), sesquiterpene (El-Masry et al., 1984; Lee et al., 2003b; Lee et al., 2003c; Lee et al., 2003d; Lee et al., 2002), diterpenes (Ragasa et al., 1998), triterpenes and sterols (Choi et al., 2007; Song et al., 2003a), terpenoids (Lee et al., 2003a), flavonoids (Gins et al., 2000), quinines (Gins et al., 2000), antioxidant quinic acids derivatives (Chuda et al., 1996), insect antifeedant and plant growth inhibitors (Tada *et al.*, 1984), and thiophene (Ragasa *et al.*, 1997; Romo de Vivar *et al.*, 1974). In addition, the terpenes dihydrochrysanolide and cumambrin A (Figure 1.18) were proven to have anticarcinogenic property (Lee et al., 2003d; Lee et al., 2002) and have been shown to lower blood-pressure in rats (Hong et al., 1999).



Dihydrochrysanolide **Cumambrin A**

Figure 1.18. Structures of dihydrochrysanolide and cumambrin A from *C. coronarium.*

1.6.3. *Vitex negundo*

Vitex negundo locally known as "lagundi" is a shrub that grows and is widely distributed in the Philippines (Dayrit *et al.*, 1987). In 1996, the Philippine Department of Health approved the manufacture and distribution of the plant in the form of tablets as a remedy for asthma, cough, colds and fever (Mendoza, 2010). Traditional uses of the plant extracts include antibacterial, antifungal, anti-inflammatory, anti-allergy, anti-asthma, analgesic, anti-tumor, anticonvulsant, anti-oxidant and antinociceptive (Bansod *et al.*, 2009; Dharmasiri *et al.*, 2003; Ismail, 2010; Zaware *et al.*, 2010).

In the phytochemical investigations of *V. negundo,* several groups of secondary metabolites were identified. The seeds contain known terpenoids and several labdane-type diterpenes (Figure 1.19) in which negundoin C and E were reported to be effective nitric oxide (NO) production inhibitors (Zheng *et al.*, 2010a). In addition several phenylnapthalene-type lignan derivatives also show similar inhibitory effects on NO

production (Ono et al., 2004; Zheng et al., 2009a). Analgesic, anti-inflammatory and anti-nociceptive properties of the seed extracts were also noted (Chawla et al., 1992; Zheng et al., 2009b). Flavonoids (Subramanian *et al.*, 1979), flavanones (Achari et al., 1984), cytotoxic and antifungal flavones (Banerji *et al.*, 1969; Diaz *et al.*, 2003; Sathiamoorthy *et al.*, 2007) and flavonoid-glycoside derivatives were also isolated (Li *et al.*, 2009; Misra *et al.*, 1980). An alkaloid vitedoamine A (Figure 1.20) (Li et al., 2009), leucoanthocyanidins (Subramanian *et al.*, 1978) and phenols (Rao et al., 1977) was also isolated. Antimicrobial activity of the plants was also reported (Ragasa et al., 1999).

Phytochemical investigations carried out on the leaves also resulted in the isolation of several iridoids, flavonoids, flavone glygocides, phenols (Banerji *et al.*, 1969; Dayrit *et al.*, 1994; Sharma *et al.*, 2009; Shen *et al.*, 2009; Subramanian *et al.*, 1979), and stilbenes (Banerji *et al.*, 1988). Dayrit *et al.* (1987) also reported the presence of active fractions that causes the relaxation of cat's trachea.

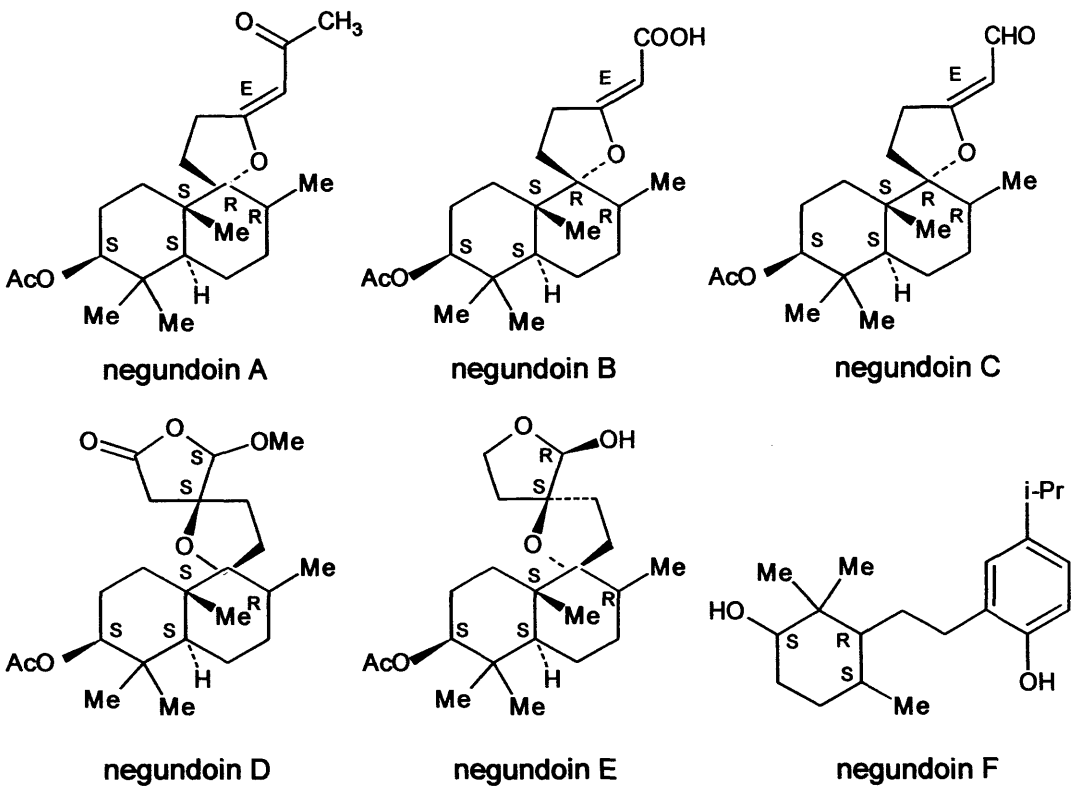


Figure 1.19. Diterpenes from the seeds of *V. negundo* (Zheng *et al.*, 2010a).

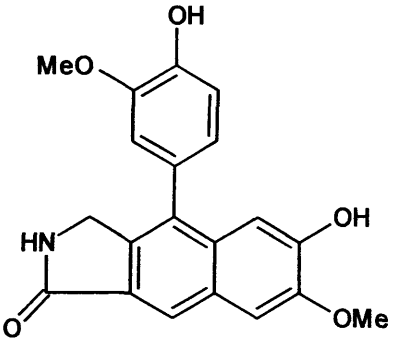


Figure 1.20. Vitedoamine A isolated from *V. negundo* (Li et al., 2009).

1.6.4. *Moringa oleifera*

Moringa oleifera known as "*Malungay*" or referred to as "horseradish tree" is cultivated as nutritious vegetables in the Philippines (Guevara *et al.*, 1999). The plant is distributed in tropical and subtropical countries like Southeast Asia, India, some parts of Africa and Arabia, Central America, North and South America, and the Caribbean islands (Anwar et al., 2007).

Traditional uses of the plant as an alternative medicine includes anti-allergy (Mahajan et al., 2009), for treatment of gastrointestinal motility disorders (Gilani et al., 1994), antispasmodic (Caceres et al., 1992), hypotensive (Faizi et al., 1998; Faizi et al., 1994; Gilani et al., 1994; Hameed-Un-Nisa et al., 1998), anti-ulcer (Dahiru *et al.*, 2006; Debnath *et al.*, 2007), antihypertensive (Anwar et al., 2007; Dangi et al., 2002), possesses anti-diarrhoeal, anti-inflammatory and diuretic properties (Hameed-Un-Nisa et al., 1998), anti-arthritis (Mahajan, Banerjee et al. 2009; Mahajan and Mehta 2009), antifungal (Chuang et al., 2006; Jha et al., 2009) and anti tumor activities (Guevara et al., 1999; Murakami et al., 1998).

In the phytochemical study of *Moringa oleifera*, several groups of compounds were found to be present. Flavonoids such as rutin, quercetin glucoside, kaempferol, kaempferol rhamnoglucoside (Anwar et al., 2007; Atawodi et al., 2010; Coppin et al., 2008) and flavanone glycosides (Jangwan *et al.*, 2008; Manguro *et al.*, 2007) were noted to be present on stems and bark. β-sitosterol known to reduce blood levels of cholesterol was also reported (Anwar et al., 2007; Faizi et al., 1998). The plant hormone zeatin was isolated along with caffeoylquinic acid (Anwar et al., 2007). The

presence of several compounds of thiocarbamates and isothiocyanate glycosides were also identified (Faizi *et al.*, 1998; Faizi *et al.*, 1997; Faizi *et al.*, 1994; Guevara *et al.*, 2000; Murakami *et al.*, 1998; Tewari *et al.*, 2006). In the study of the plant for its hypotensive property mustard oil glycosides niazinin A (I) and niazinin B (II) and niazimicin C (III) (Figure 1.21) and niaziminin A and B were isolated (Faizi et al., 1998; Faizi et al., 1994; Faizi et al., 1992; Gilani et al., 1994).

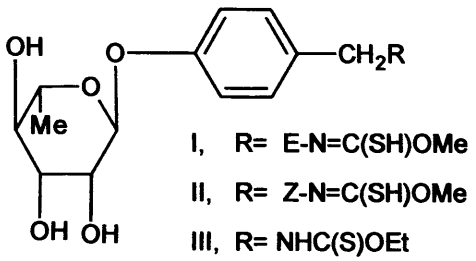


Figure 1.21. Mustard glycosides from *M. oleifera.*

1.6.5. *Artemisia vulgaris*

Artemisia vulgaris, commonly known as mugwort or St. John's plant, is a persistent weed growing wild native to Asia, Europe and North America (Lee *et al.*, 1998; Linley, 2002; Tigno *et al.*, 2000b). The plant is widely used in the Philippines among practitioners of alternative medicine, in particular for its anti-hypertensive actions (Tigno *et al.*, 2000b). It has also been suggested to have other medicinal activities such as "antispasmodic, carminative, anti-inflammatory and anti-helminthic properties" (Quisimbing, 1978; Tigno *et al.*, 2000b), and has been used in the treatment of painful menstruation (dysmenorrhoea) and in the induction of labour or miscarriage (Lee et al., 1998).

The *A. vulgaris* and closely related species ('Vulgares' group) comparative analysis of polyacetylenes resulted in a comprehensive survey of typical derivatives (Figure 1.22) (Drake *et al.*, 1974; Wallnofer *et al.*, 1989). The isolation and characterization of a coumarin, 6-methoxy-7,8-methylenedioxycoumarin (Figure 1.23) was also reported (Murray *et al.*, 1986). In the comprehensive analysis of the flavonoids in *A. vulgaris* by Lee, Chung et al. (1998), they isolated and evaluated twenty known flavonoids (Figure 1.24) for their estrogenic activity. They reported that presence of the weak estrogens eriodictyol and apigenin may explain for the use of the plant as a natural emmenogogue, an agent for inducing menstrual flow (Lee *et al.*, 1998).

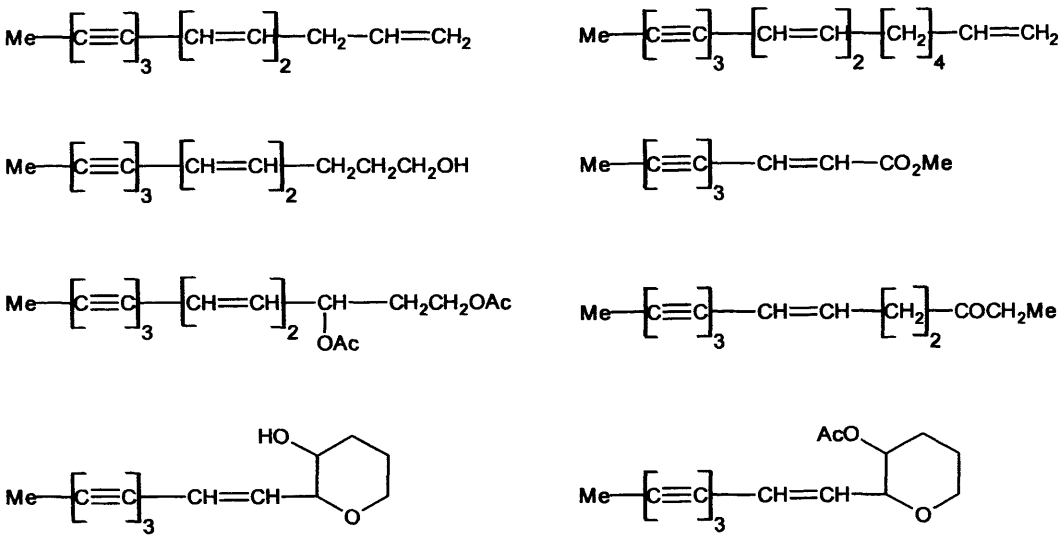


Figure 1.22. Acetylenic products found in *A. vulgaris* L. (Drake *et al.*, 1974)

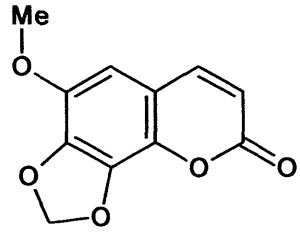
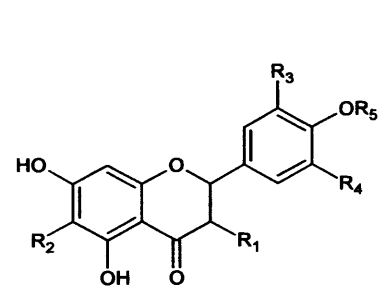
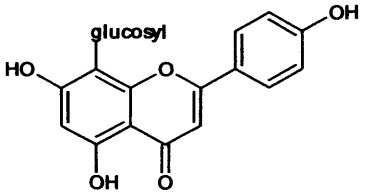


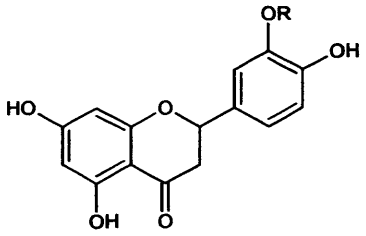
Figure 1.23. 6-Methoxy-7,8-methylenedioxycoumarin from *A. vulgaris* (Murray *et al.*, 1986).



(a) $R_1=R_2=R_5=H$, $R_3=R_4=OCH_3$
(b) $R_1=R_4=R_5=H$, $R_2=R_3=OCH_3$
(c) $R_1=R_4=R_5=H$, $R_2=OCH_3$, $R_3=OH$
(d) $R_1=R_2=R_4=H$, $R_3=OH$, $R_5=CH_3$
(e) $R_1=R_2=R_4=R_5=H$, $R_3=OCH_3$
(f) $R_1=OH$, $R_2=R_4=R_5=H$, $R_3=OCH_3$
(g) $R_1=R_2=R_3=R_4=R_5=H$
(h) $R_1=OH$, $R_2=R_3=R_4=R_5=H$
(i) $R_1=R_2=R_4=R_5=H$, $R_3=OH$
(j) R_1=O-rhamno-glucosyl, $R_2=R_4=R_5=H$, $R_3=OH$



(k)



(l) $R=H$ (m) $R=CH_3$

Figure 1.24. Estrogenic Flavonoids from *A. vulgaris* L. (a) Tricin, (b) Jaceosidine, (c) Eupalofin, (d) Diosmetin, (e) Chrysoeriol, (f) Isorhamnetin, (g) Apeginin, (h) Kaempferol, (i) Luteolin, (j) Rutin, (k) Vitexin, (l) Eriodictyol, and (m) Homoeriodictyol (Lee *et al.*, 1998).

Several sesquiterpenes were also reported to be present in the plant. Vulgarin (Figure 1.25), a sesquiterpene lactone, was reported to be present (Geissman *et al.*, 1961). In addition to the presence of eudasmane dialcohol, two sesquiterpene acids with a eudasmane framework was also reported (Figure 1.26) (Marco et al., 1990).

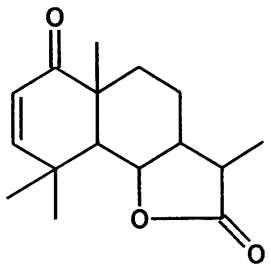


Figure 1.25. Vulgarin, a sesquiterpene lactone from *A. vulgaris* (Geissman *et al.*, 1961).

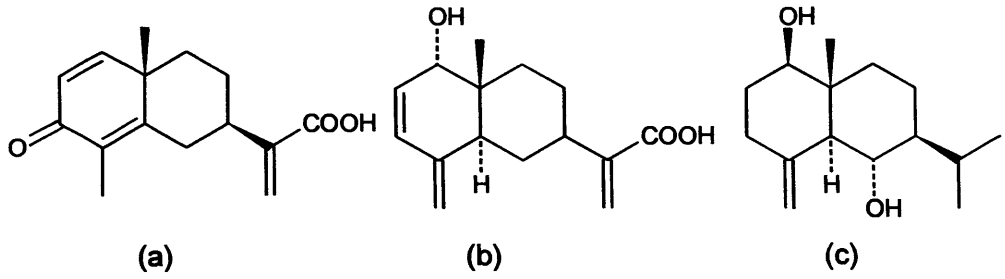


Figure 1.26. Sesquiterpenes with eudesmane framework (a) 3-Oxoeudesma-1,4,11(13)-trien-7αH-12-oic acid, (b)1α-Hydroxyeudesma-2,4(15),11(13)-trien-5α,7αH-oic acid and (c) Eudesmane dialcohol (Marco et al., 1990).

Furthermore the presence of 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide, yomogin and [3-(1-benzodioxol-5-yl)oxiran-2-yl]methanol (Figure 1.27) was also reported on the Philippine species of *A. vulgaris* during the evaluation of plants extracts action to the antihypertensive property on cardiovascular haemodynamics (Tigno *et al.*, 2000b).

The presence of caffeoylquinic acids, 3,5-di-o-caffeoylquinic acid and 1,5-di-O-caffeoylquinic acid (Figure 1.28), were also isolated from the flowering tops of *A. vulgaris* (Carnat et al., 2000).

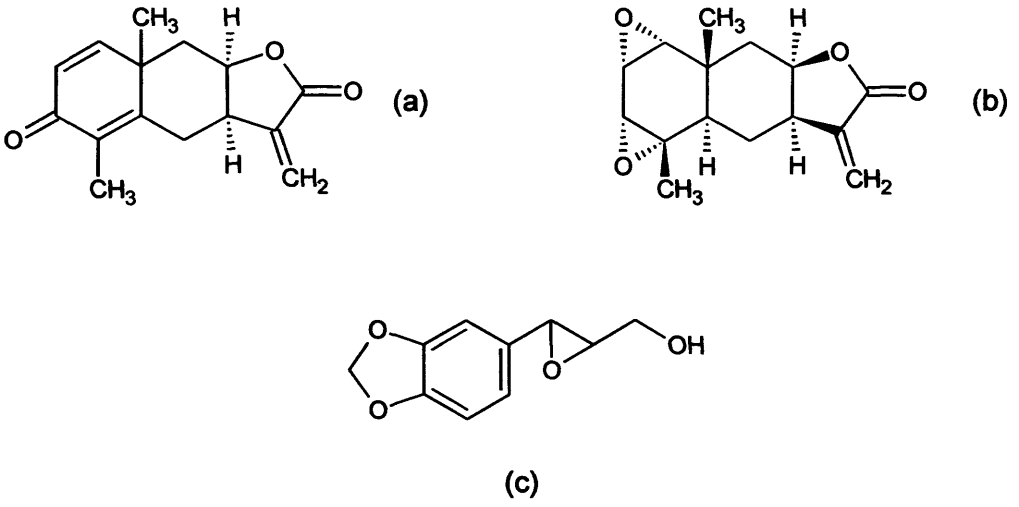


Figure 1.27. Compounds isolated from Philippine *A. vulgaris* (a) Yomogin, (b) 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide and (c) [3-(1,3-benzodioxol-5-yl)oxiran-2-yl]methanol (Tigno *et al.*, 2000b).

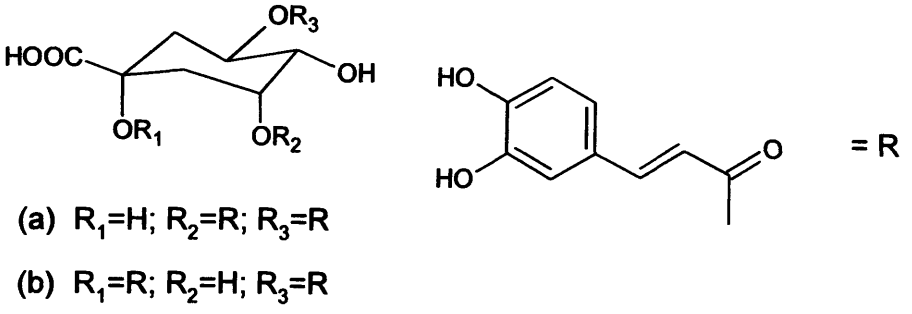


Figure 1.28. The two major caffeoyquinic acids, (a) 3,5-di-O-caffeoyquinic acid and (b) 1,5-di-O-caffeoyquinic acid from *A. vulgaris* (Carnat et al., 2000).

1.7 Aims of the thesis

Trace amine associated receptors (TAAR) are the targets for the pharmacological action of trace amines in the brain and on peripheral tissues (Berry, 2004; Lindemann *et al.*, 2005). Responses to typical trace amines such as tyramine and β-phenylethylamine (β-PEA) have been identified in the heart (Frascarelli *et al.*, 2008), blood vessels, gastrointestinal tract (Broadley *et al.*, 2009) and trachea (Baker *et al.*, 2007; Hawthorn *et al.*, 1984; Hawthorn *et al.*, 1985; Herbert *et al.*, 2008). In blood vessels, tyramine, β-PEA, and synthetic and naturally occurring amphetamine derivatives such as ecstasy and cathinone (the active constituent of Khat) cause vasoconstriction (Baker *et al.*, 2007). In the coronary vasculature (Baker *et al.*, 2007) (Herbert *et al.*, 2008) and rat aorta (Fehler *et al.*, 2010), these amines cause vasoconstriction which is not blocked by the α_1-adrenoceptor antagonist, prazosin or the neuronal uptake, inhibitor cocaine. It is therefore concluded that the response is not an indirect symphatomimetic action (Broadley, 2010) but may be due to stimulation of TAARs. In the ileum, tyramine and β-PEA cause contraction, a response opposite to that expected of an indirect sympathomimetic amine (Broadley *et al.*, 2009; Innes *et al.*, 1969). This contraction was also not blocked by adrenoceptor antagonists or 5-HT_2 receptor antagonists suggesting that TAAR might be involved (Broadley *et al.*, 2009). In the guinea pig trachea, β-PEA also caused contraction, which is opposite to the bronchodilatation expected of a symphathomimetic amine (Hawthorn *et al.*, 1985). This response was not inhibited by H_1 and muscarinic receptor antagonists, chlorpheniramine and atropine, and attributed to a phenylethylaminergic receptor or TAAR (Broadley, 2010).

To confirm the role of TAARs in these contractile responses of the ileum, trachea and blood vessels it is necessary to use as antagonist of these receptors. However, no such antagonist had been identified at the start of this work. Thus one aim of this thesis was to attempt to identify a compound with TAAR antagonistic activity from plants of Philippine origin. As a clue to this type of activity to ascertain plant selection, it was predicted that such activity would lower blood pressure by blockade of trace amine-mediated vasoconstriction. It might also cause bronchodilatation and relax the gut by blockade of the airways and gut contractions due to trace amine stimulation. Thus, plants were selected that had local medicinal uses for treating hypertension, asthma and increased gut motility associated with diarrhoea. The plants selected were therefore *Artemisia vulgaris, Chrysanthemum coronarium, Moringa oleifera, Sesbania grandiflora* and *Vitex negundo.*

A secondary aim was to identify activity in these plants against the major biogenic amine receptors. The receptors therefore examined, were those for histamine H_1, 5-HT (5-HT_2), noradrenaline (α_1-adrenoceptor), acetylcholine (muscarinic M_3) and β-PEA ($TAAR_1$) using histamine, 5HT, phenylephrine, methacholine and β-PEA, respectively, as the standard agonists.

Below are the summaries of objectives of the present study:

- To prepare crude extracts of *Artemisia vulgaris, Chrysanthemum coronarium, Moringa oleifera, Sesbania grandiflora* and *Vitex negundo.*

- To established reproducibility of responses of the guinea pig ileum, trachea and aorta to histamine, 5-HT, noradrenaline, methacholine and β-PEA and the effect of vehicle for the crude extracts.
- To identify activity of the crude extracts against the standard agonist.
- To separate components of the crude extracts showing activity and further test for receptor activity.
- To isolate and chemically identify any active components of active fractions.

Chapter 2

Pharmacological methods: control responses to standard agonist on guinea pig ileum, trachea and aorta

2.1 Introduction

In this chapter the details of materials and methods used for preliminary pharmacological work for evaluating the antagonistic activity of crude plant extracts against selected biogenic amines will be described. Subsequent Chapters will present methods and protocols specific to those chapters. Chloroform and methanol crude extraction of plant components of *S. grandiflora* and *C. coronarium* will be presented in Chapter 3. Acid-base crude extraction of plant components of *V. negundo* and *M. oleifera* will be presented in Chapter 4. Isolation of fractions of *A. vulgaris* chloroform crude extract, chemical analysis, structure elucidation and a bioassay guided fractionation, for its histamine antagonist activity will be presented in Chapter 5.

Biogenic amines are endogenous substances derived from biological processes (Blaschko, 1957). They include the major biogenic amines such as catecholamine, histamine and 5-HT, and the trace amines (β-PEA, octopamine and tyramine) which are present in the mammalian tissues at very low (nanomolar) concentrations (Borowsky et al., 2001). These biogenic amines along with acetylcholine (ACh) interact with specific G-protein coupled receptors (GPCR) to produces various specific responses of tissues (Kristiansen, 2004). In the regulation of gastrointestinal, airways and vascular system function, the major biogenic amines have been recognized to play essential roles (Brunton *et al.*, 2005). For the trace amines however their pharmacological effects are usually attributed to their overlapping function with the aminergic pathways (Borowsky *et al.*, 2001; Zucchi *et al.*, 2006). Currently the lack of specific antagonists for TAAR

hinders further pharmacological study on its signalling mechanism (Zucchi et al., 2006).

The present study was undertaken to examine the contractile effects of 5-HT as an agonist of 5-HT_2 receptors, methacholine as an agonist for the muscarinic M_3 receptors, histamine as an agonist of histamine H_1 receptors, and phenylephrine as an agonist of α_1-adrenoceptors on smooth muscle. These receptors directly couple to the G-protein G_q and largely elicits stimulatory responses though the activation of phospholipase C (PLC) which initiates intracellular Ca^{2+} release though the increase of inositol-triphosphate (IP_3) (Hamm, 1998; Hamm *et al.*, 1996; Taylor *et al.*, 1991). In addition, β-PEA which usually causes smooth muscle relaxation or contraction through indirect sympathomimetic effect (Broadley et al., 2009) was also examined as an agonist of trace amine-associated receptors (TAAR). Furthermore, the reproducibility of contractile responses when repeating cumulative exposures of 5-HT, methacholine, histamine, phenylephrine and β-PEA on smooth muscle was tested. Since DMSO is used to dissolve plant extracts for evaluation of their activity on the tissues, concentration-response curves were also obtained in the absence and presence of DMSO to determine whether it affected the responses.

2.2 Aims

- To study the effect of 5-HT, methacholine, histamine and β-PEA employing cumulative concentration-response curves (CRCs) for the contractile responses of guinea pig isolated ileum.
- To study the effect of histamine and β-PEA employing cumulative concentration response curves (CRCs) for the contractile responses of guinea pig isolated tracheal spiral.
- To study the effect of phenylephrine and β-PEA employing cumulative concentration response curves (CRCs) for the contractile responses of guinea pig isolated aortic rings.
- To determine the reproducibility of repeated CRCs in guinea pig ileum, trachea and aorta
- To determine the effect of the vehicle for plants extracts (DMSO) on CRCs in guinea pig ileum, trachea and aorta

2.3 Methods and Material

Protocols for pharmacological evaluation

2.3.1 Tissue preparation

Male Dunkin-Hartley guinea-pigs, 200-300 g, were killed by a blow to the back of the head and then exsanguinated under running water. The ileum, trachea and thoracic aorta were excised and placed in Krebs-bicarbonate solution of composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2.2H_2O$ 1.9, $MgSO_4.7H_2O$ 1.2, $KH_2PO_4.2H_2O$, 1.2, $NaHCO_3$ 25, glucose 11.7 (Ford *et al.*, 1999).

2.3.2 Ileum segments

The ileum was selected 10 cm from the stomach. Following the removal of adhering fat and connective tissue, the ileum was cut in 2 cm segments which were suspended in a 50-mL heated tissue bath (37°C) with Krebs-bicarbonate solution continuously gassed with 5% CO_2 in oxygen. One end was attached to a tissue holder and the other by means of a cotton thread to a transducer. A resting tension of 0.5 g was applied and the tissues left to equilibrate for 30 min before drug addition (Figure 2.1) (Grassby *et al.*, 1987; Rubinstein *et al.*, 1985).

2.3.3 Tracheal spirals

After removing the adhering fat and connective tissue, the trachea was cut spirally (2 mm wide) into lengths of 3-4 cm. The strips were then set up with one cartilage end attached to a tissue holder and the other to a transducer by means of a cotton thread. They were immersed in warmed Krebs-bicarbonate solution continuously gassed with 5% CO_2 in oxygen in 50-mL organ baths and maintained at 37°C. A resting tension of 1.5 g was applied and the tissues left to equilibrate for 60 min before drug addition (Figure 2.2) (Hawthorn et al., 1985).

2.3.4 Aortic rings

The thoracic aorta was cleared of connective tissue in-situ and then excised. Rings (5 mm) were then cut and mounted under 1-g tension in 50-mL tissue baths containing Krebs-bicarbonate solution continuously gassed with 5% CO_2 in oxygen and maintained at 37°C. The tissues were left to equilibrate for 60 min before drug addition (Figure 2.3) (Ford *et al.*, 1999).

2.3.5 Cumulative concentration-response curves (CRCs)

To construct cumulative CRCs, successive concentrations of agonist were added to the 50 mL tissue bath in half logarithmic increments, after the peak effect was reached for the preceding concentration, at least until the maximum response was recorded. All experiments were repeated at least four times on tissues from four different guinea pigs.

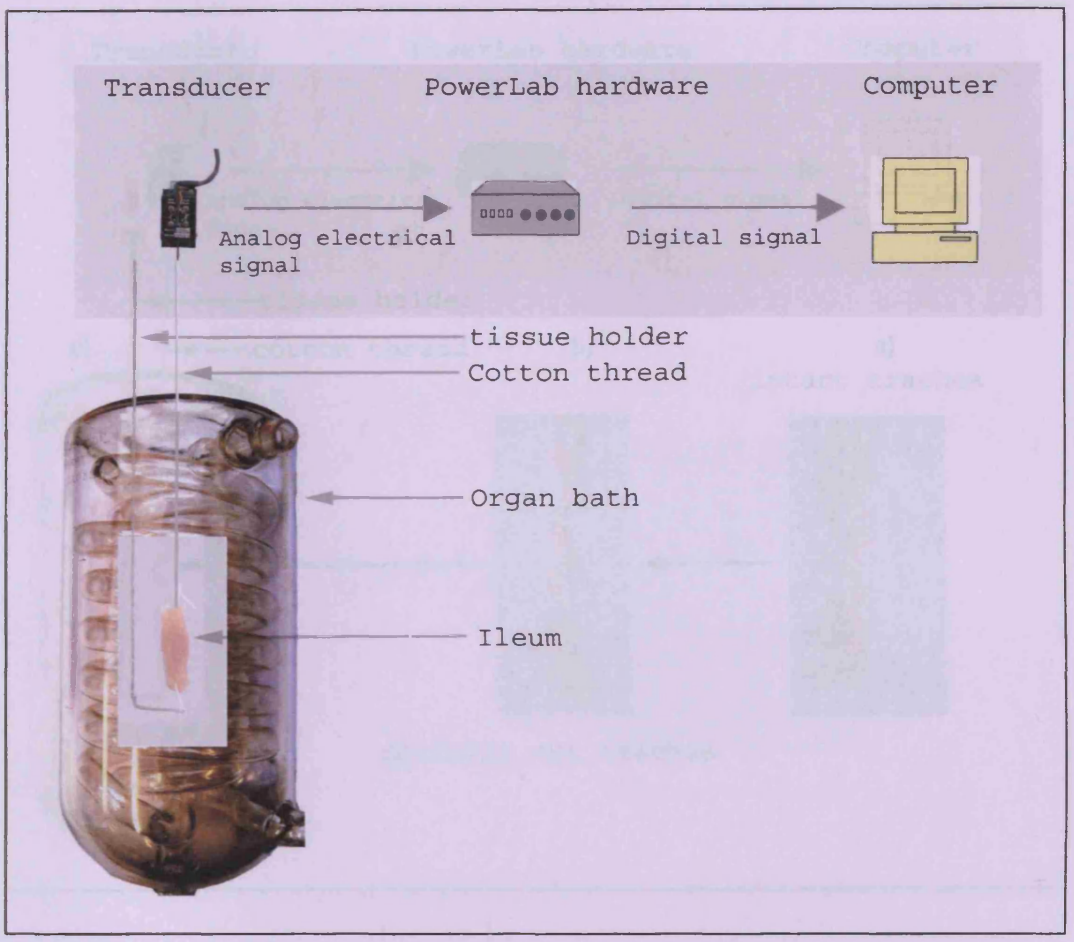


Figure 2.1. Organ bath with guinea pig ileum in place held on a fixed tissue holder. Measurement and analysis of the isometric tension via an isometric transducer attached by a cotton string to one end of the ileum and was connected to an isometric transducer. The results were recorded on a computer.

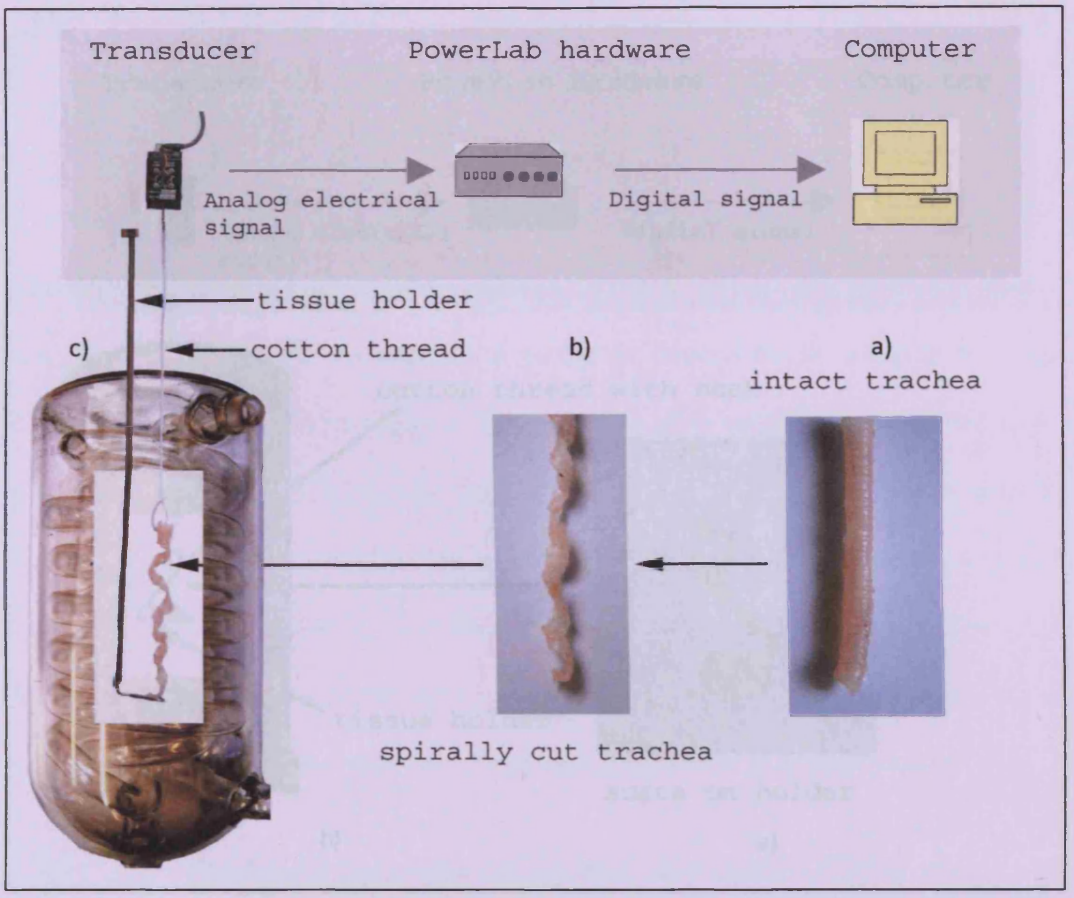


Figure 2.2. a) Intact guinea pig trachea. b) Spirally cut trachea. c) Organ bath with guinea pig trachea in place, held on a fixed tissue holder. Measurement and analysis of the isometric tension via an isometric transducer attached by a cotton string to the one end of the trachea. The results were recorded on a computer.

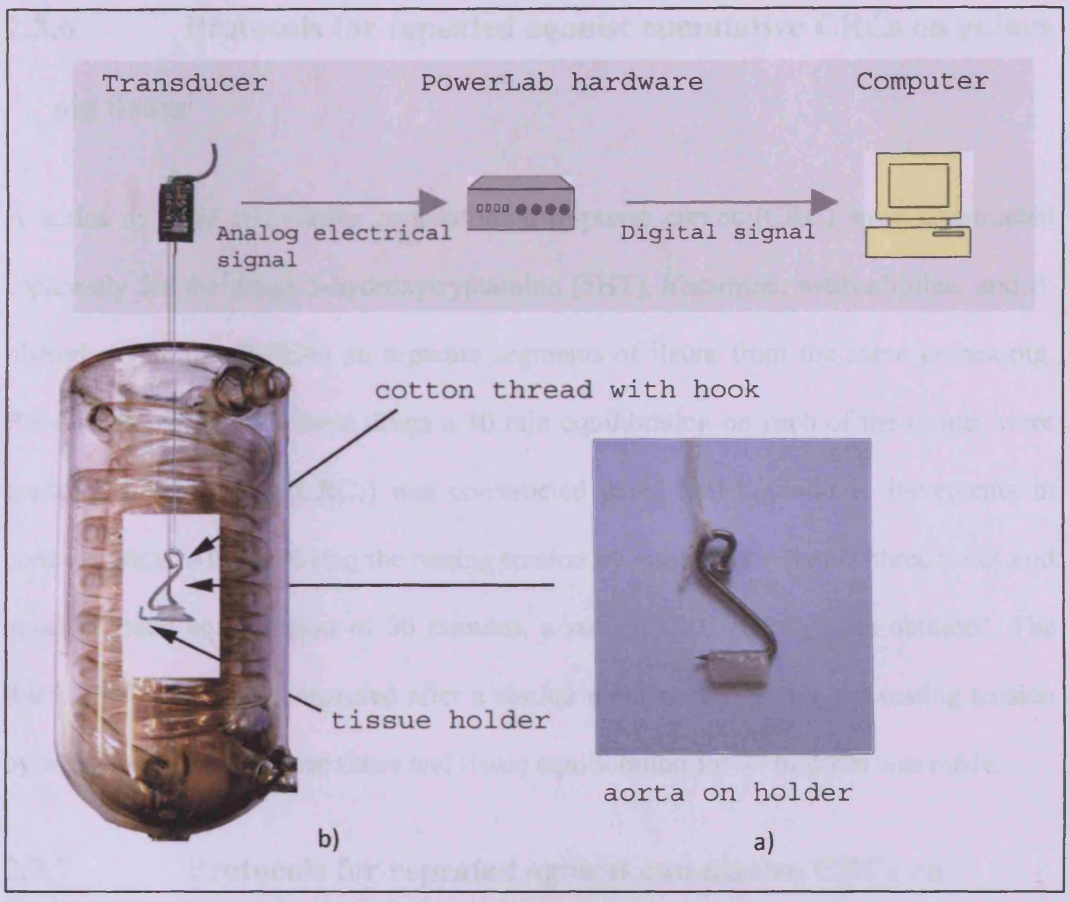


Figure 2.3. a) Guinea pig aorta on holder. b) Organ bath with guinea pig aorta in place, held on a fixed tissue holder. Measurement and analysis of the isometric tension via an isometric transducer attached by a cotton string to the upper mobile hook was. The results were recorded on a computer.

2.3.6 Protocols for repeated agonist cumulative CRCs on guinea pig ileum

A series of three cumulative concentration-response curves (CRC) were constructed separately for the drugs 5-hydroxytryptamine (5HT), histamine, methacholine, and β-phenylethylamine (β-PEA) on separate segments of ileum from the same guinea-pig. Prior to the addition of these drugs a 30 min equilibration on each of the tissues were made. The first CRC (CRC_1) was constructed using half-logarithmic increments in concentration. After restoring the resting tension by washing the tissues three times and another tissue equilibration of 30 minutes, a second CRC (CRC_2) was obtained. The third CRC (CRC_3) was repeated after a similar method of restoring the resting tension by washing the tissue three times and tissue equilibration for 30 minutes was made.

2.3.7 Protocols for repeated agonist cumulative CRCs on guinea-pig tracheal spirals

A series of two cumulative concentration-response curves for tracheal spirals were constructed separately for contractile responses to histamine and β-PEA. The tissues were left to equilibrate for 60 min before drug addition. The first CRC was constructed using half-logarithmic increments in dose. After restoring the resting tension by washing the tissues and a total tissue equilibration of 60 minutes, a second CRC was obtained. A similar series of two cumulative CRCs for β-PEA were constructed wherein $1x10^{-6}$ M of propranolol was added 10 min prior to the construction of the first and second curves.

2.3.8 Protocols for repeated agonist cumulative CRCs on guinea-pig aorta

A series of two cumulative concentration-response curves for aortic rings were constructed separately for contractile responses to phenylephrine and β-PEA. The tissues were left to equilibrate for 60 min before drug addition. The first CRC was constructed using half-logarithmic increments in dose. After restoring the resting tension by washing the tissues and a total of tissue equilibration of 60 minutes, a second CRC was obtained.

2.3.9 The effect of DMSO

DMSO will be used throughout this study to dissolve plant extracts and 0.1 mL used in the tissues. To establish that DMSO has no effect on the reproducibility of the cumulative CRC for each agonist on a particular guinea pig tissue a separate set of controls were made by applying 0.1 mL DMSO 20 minutes prior to the construction of the second cumulative CRC.

2.3.10 Data measurement and analysis

Tension measurements were made by the use of Dynamometer UF1 isometric force transducer, 57 g sensitivity range, and displayed on Powerlab Chart 5 (ADI Instruments, Oxfordshire, UK). The peak responses in grams for each dose measured from the baseline before each CRC were recorded. The mean contractile maximum (±S.E.M) responses for the first (CRC_1), second (CRC_2) and third (CRC_3) CRCs were

compared using Student's t-test. The n value represents the number of guinea pigs providing ileum, trachea or aorta.

The contractile response values were expressed also as a percentage of the maximum contractile response obtained in CRC_1 set to 100%. These transformed values were then plotted as mean (± SEM) responses. Mean responses at individual doses of CRC_1 and CRC_2, or CRC_2 and CRC_3 were compared using repeated measures ANOVA followed by Bonferroni post-hoc test. The contractile responses were expressed as a percentage of their own maximum contractile response set to 100%. Values from these transformations were used to obtain the true EC_{50} (-log molar concentration to produce 50% maximum response). True -log EC_{50} (±S.E.M) were compared between CRC_1 and CRC_2, or between CRC_2 and CRC_3 using Student's t-test. The dose-ratio was expressed as EC_{50} of CRC_1 / EC_{50} of CRC_2 in individual experiments and mean values (±S.E.M) calculated. Pseudo EC_{50} values were also calculated as -log molar concentration producing 50% of the maximum response of CRC_1 in individual experiments and mean (±SEM) responses values were calculated. Where the response of CRC_2 or CRC_3 did not reach 50%, then pseudo EC_{20} or EC_{30} values were compared.

To measure the change in resting baseline after the addition of plant extracts, the resting baseline (±SEM) before CRC_2 was expressed as a percentage of the baseline before the plant extract addition.

All statistical analysis and plotting of the data were performed on GraphPad Prism 5.01.

2.3.11 Drugs and solutions

Acetyl-β-methylcholine chloride, 5-hydroxytryptamine salt (5-HT), histamine diphosphate salt, dimethylsulfoxide (DMSO), (R)-(-)-phenylephrine hydrochloride, β-phenylethylamine hydroxide (β-PEA) and (±)-propranolol hydrochloride were from Sigma-Aldrich (Poole, Dorset, UK). All chemicals for the Krebs-bicarbonate buffer (analytical grade) were purchased from Fisher Scientific (Leicestershire, UK). All drugs were dissolved in distilled water prior to their use.

2.4 Results

2.4.1 Contractile responses of guinea pig ileum to repeated CRCs for 5-HT – Absolute controls

The addition of low concentrations of 5-HT in cumulative CRCs caused concentration-related contractions on guinea pig ileum (Figure 2.4).

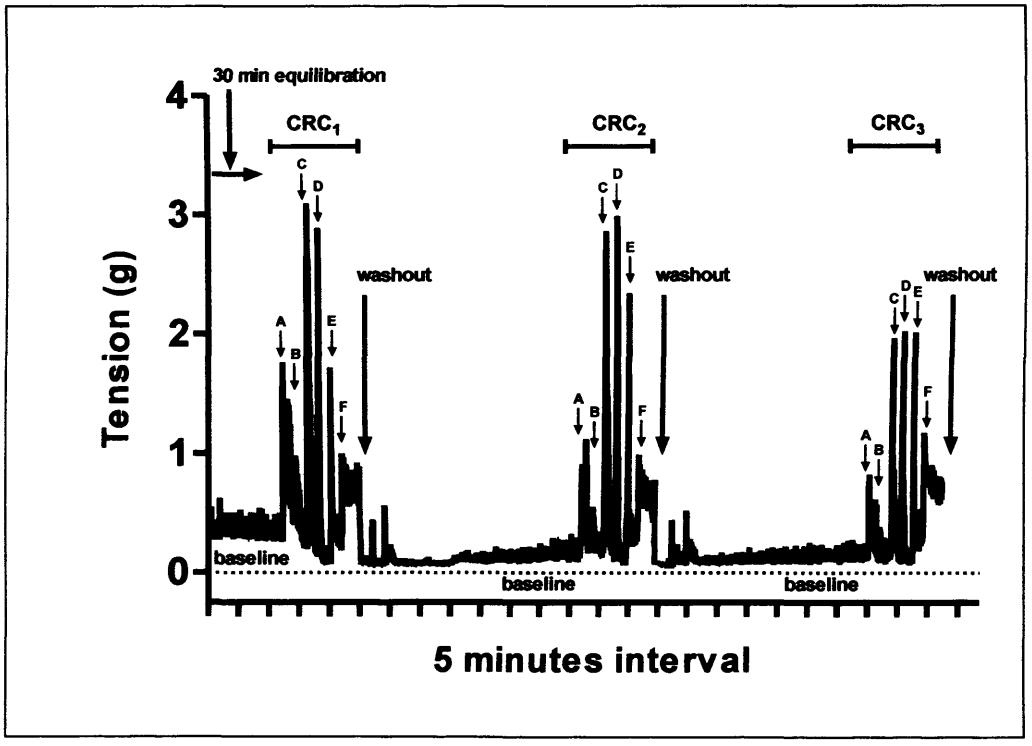


Figure 2.4. Representative chart recording showing a series of three cumulative concentration-response curves (CRC) for the contractile response of guinea pig ileum to 5-HT (A = $1x10^{-7}$ M, B = $3x10^{-7}$ M, C = $1x10^{-6}$ M, D = $3x10^{-6}$ M, E = $1x10^{-5}$ M, F = $3x10^{-5}$ M).

The mean maximum contractile responses to 5-HT (n=4) in CRC_2 (0.79±0.46 g) were not significantly different (P>0.05) from CRC_1 (0.82±0.44 g) and CRC_3 (0.74±0.35 g) (Table 2.1). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed no significant differences on the maximum contractile responses obtained in CRC_1, CRC_2 and CRC_3 (Figure 2.5.a). Furthermore the true -log EC_{50} values on CRC_2 (-6.29 ± 0.17) was not significantly different from CRC_1 (-6.56 ± 0.17) and CRC_3 (-6.00±0.17) (Table 2.1). The dose ratio between CRC_1 and CRC_2 was 1.96±0.34 (Table 2.1).

2.4.2 Contractile responses of guinea pig ileum to 5-HT – DMSO control

To test the effect of DMSO on the reproducibility of mean contractile response of guinea pig ileum (n=5) against 5-HT, 0.1 mL DMSO was applied prior to the construction of CRC_2 (Figure 2.4). The mean contractile response maximum of 5-HT on CRC_2 (2.62±0.22 g) was not significantly different (P>0.05) from CRC_1 (2.54±0.25 g) but a significant (P<0.01) reduction of the maximum response was observed with CRC_3 (1.77±0.30 g) compared with CRC_2 after washout of DMSO (Table 2.2). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed that 5-HT contractile response values of CRC_2 (100.92±7.89%) showed no significant difference (P>0.05) from CRC_1 (91.38±7.47%) but a significant (P<0.05) reduction of the maximum of CRC_3 (69.06±11.89%) was observed (Figure 2.5.b). In addition the true -log EC_{50} values for CRC_2 (-6.49±0.18) was not significantly

different at (P>0.05) from CRC_1 (-6.42±0.28) and CRC_3 (-6.47±0.09) (Table 2.2). The dose ratio between CRC_1 and CRC_2 was 1.43±0.58 (Table 2.2).

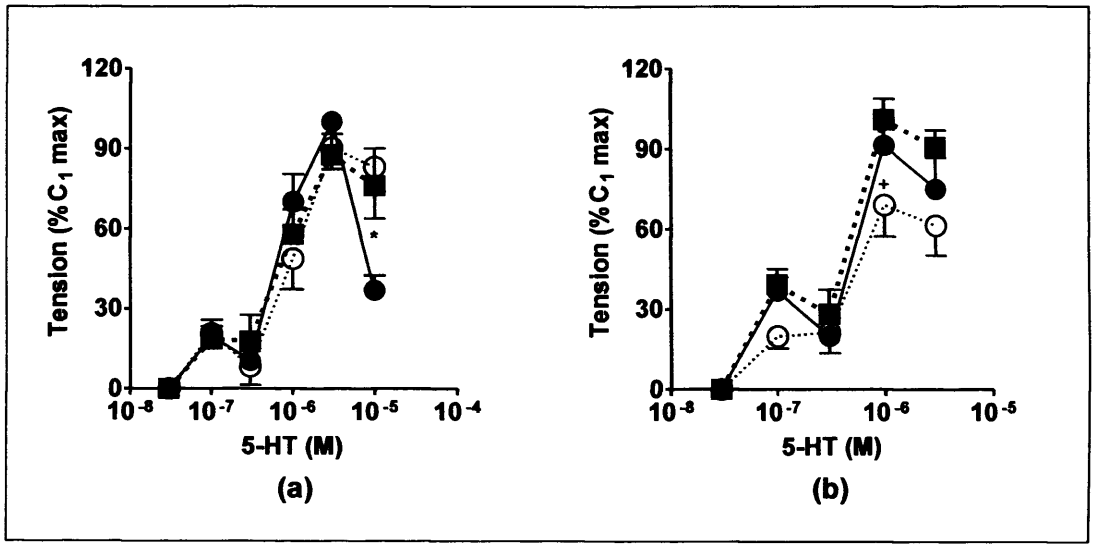


Figure 2.5. Mean cumulative CRCs for 5-HT contractile responses of the guinea pig ileum. (a) Repeated CRCs for 5-HT, n=4, (b) effect of DMSO added during CRC_2 on 5-HT (n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 vs. CRC_2, and CRC_2 vs. CRC_3 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. * Significant (P<0.05) differences between CRC_1 and CRC_2, and $^+$ between CRC_2 and CRC_3. ●—● CRC_1, ■–■ CRC_2 after 30 min tissue equilibration after the wash out of first curve with or without DMSO, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve.

2.4.3 Contractile responses in guinea pig ileum to repeated CRCs for methacholine – Absolute control

The addition of methacholine in cumulative CRCs caused concentration-dependent contractions on guinea pig ileum (Figure 2.6).

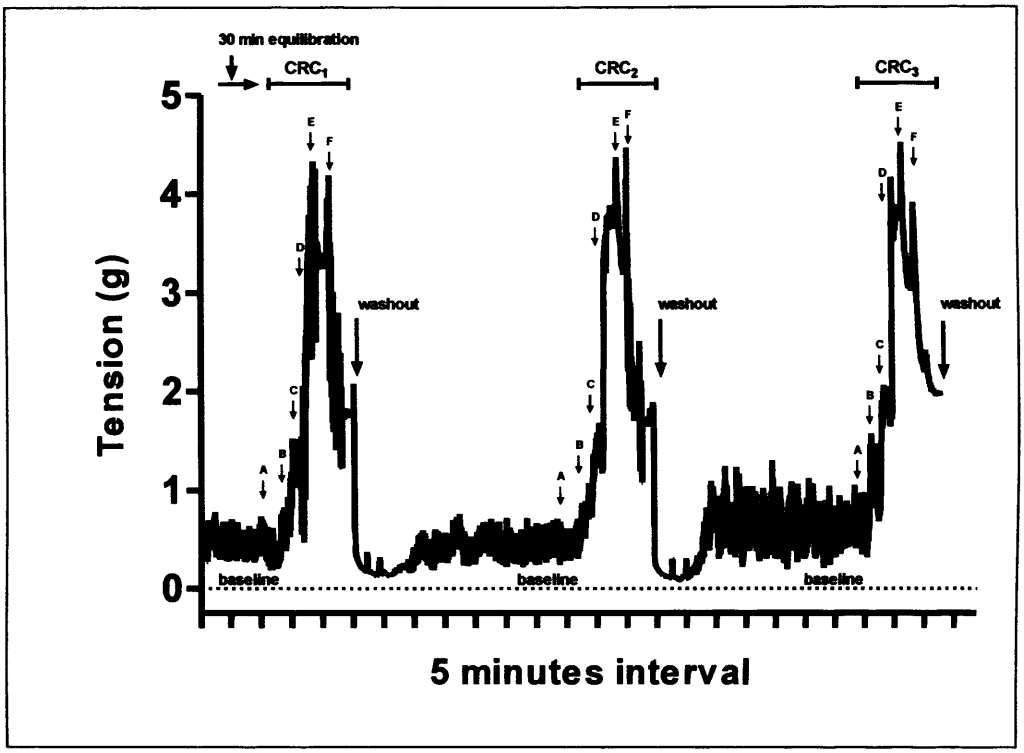


Figure 2.6. Representative chart recording showing repeated of cumulative concentration-response curves (CRC) for the contractile response of guinea pig ileum to methacholine (A = $1x10^{-8}$ M, B = $3x10^{-8}$ M, C = $1x10^{-7}$ M, D = $3x10^{-7}$ M, E = $1x10^{-6}$ M, F = $3x10^{-6}$ M).

The mean contractile response maximum of methacholine (n=4) for CRC_2 (3.41±0.34 g) was not significantly different (P>0.05) from CRC_1 (3.46±0.46 g) and CRC_3 (3.44±0.53 g) (Table 2.1). Contractile responses expressed as a percentage of the maximum obtained in CRC_1 set to 100% further showed no significant differences (P>0.05) at each dose between CRC_1, CRC_2 and CRC_3 (Figure 2.7.a). The true -log EC_{50} value for CRC_2 (-7.27±0.11) was also not significantly different (P>0.05) from CRC_1 (-7.16±0.09) and CRC_3 (-7.18±0.11) (Table 2.1). The dose ratio between CRC_1 and CRC_2 was 1.31±0.16 (Table 2.1).

2.4.4 Contractile responses in guinea pig ileum to methacholine – DMSO Control

To test the effect of the presence of DMSO on the reproducibility of mean contractile response of guinea pig ileum (n=4) against methacholine, 0.1 mL DMSO was applied before the construction of CRC_2 (Figure 2.6). The mean maximum contractile response of methacholine for CRC_2 (3.22±0.57g) was not significantly different (P>0.05) from CRC_1 (3.25±0.50 g) and CRC_3 (3.24±0.41 g) (Table 2.2). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% also showed no significant difference (P>0.05) between CRC_1, CRC_2 and CRC_3 at each dose (Figure 2.7.b). The true -log EC_{50} value for CRC_2 (-7.43±0.21) was also not significantly different at (P>0.05) from CRC_1 (-7.37±0.05) and CRC_3 (-7.31±0.19) (Table 2.2). The dose ratio between CRC_1 and CRC_2 was 1.03±0.30 (Table 2.2).

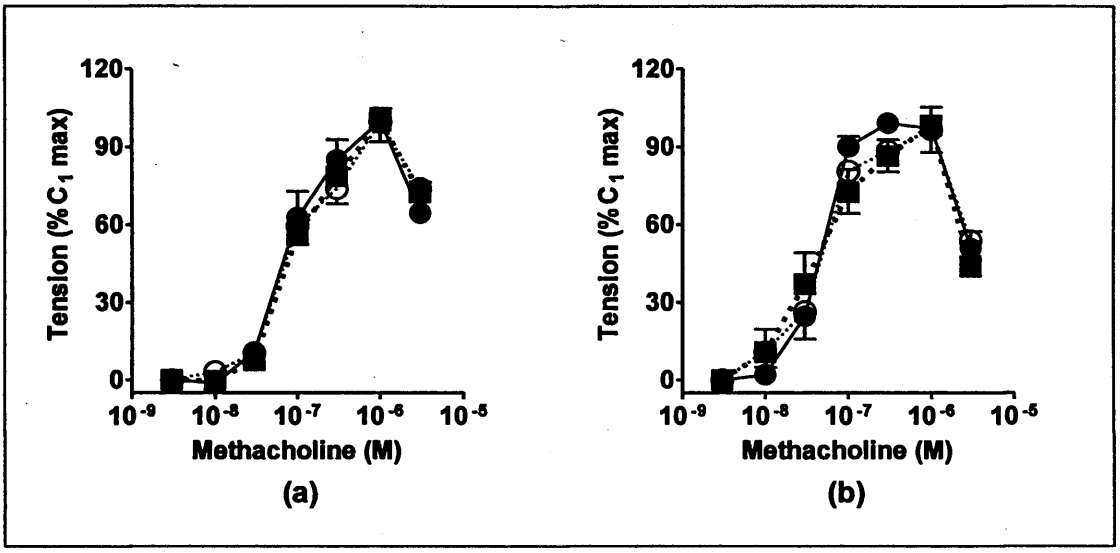


Figure 2.7. Mean cumulative CRCs for the contractile responses of the guinea pig ileum to methacholine. (a) Repeated CRCs for methacholine, n=4, (b) effect of DMSO added during CRC_2 on methacholine (n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 vs. CRC_2, and CRC_2 vs. CRC_3 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. No significant ($P>0.05$) differences were seen at each dose between CRC_1, CRC_2 and CRC_3 of 5-HT. ●—● CRC_1, ■--■ CRC_2 after 30 min tissue equilibration after the wash out of first curve with or without DMSO, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve.

2.4.5 Contractile responses in guinea pig ileum to repeated CRCs for histamine – Absolute control

The addition of low concentrations of histamine in cumulative CRCs caused concentration-related contractions on guinea pig ileum (Figure 2.8).

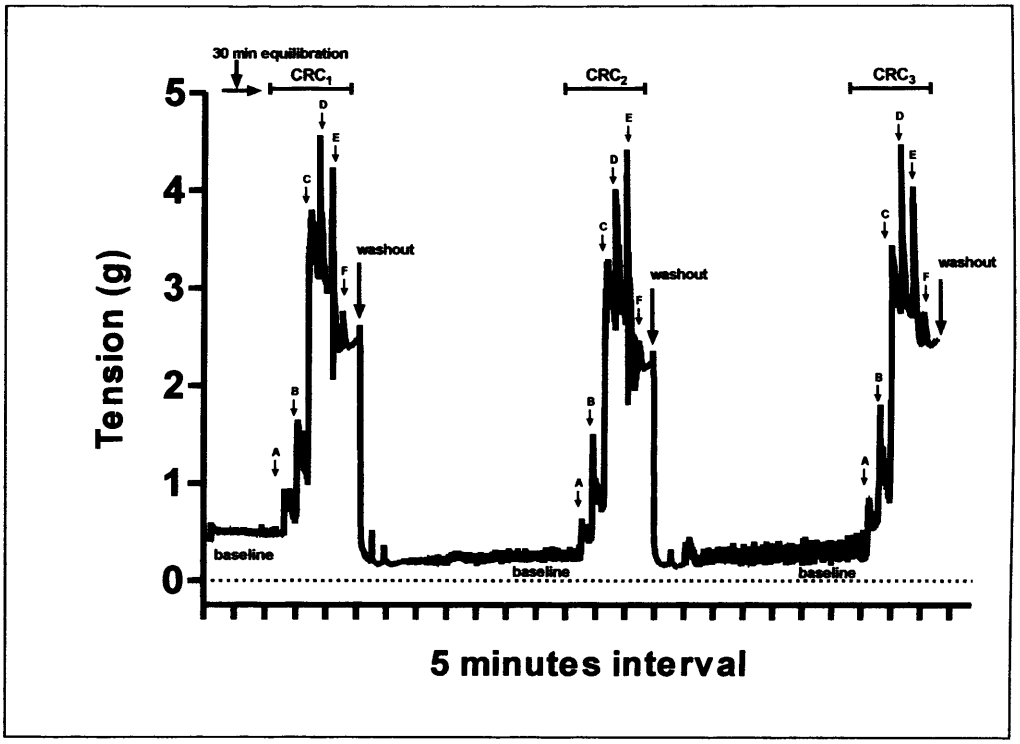


Figure 2.8. Representative chart recording showing repeated of cumulative concentration-response curves (CRC) for the contractile response of guinea pig ileum to histamine (A = $1x10^{-7}$ M, B = $3x10^{-7}$ M, C = $1x10^{-6}$ M, D = $3x10^{-6}$ M, E = $1x10^{-5}$ M, F = $3x10^{-5}$ M).

The mean maximum contractile response of histamine (n=5) for CRC_2 (3.46±0.88 g) was not significantly different (P>0.05) from CRC_1 (3.14±0.76 g) and CRC_3 (3.06±0.89 g) (Table 2.1). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed no significant difference (P>0.05) for CRC_2 compared with CRC_1 and CRC_3 at each dose (Figure 2.9.a). The true -log EC_{50} values for CRC_2 (-5.77±0.17) was also not significantly different (P>0.05) from CRC_1 (-5.44±0.13) and CRC_3 (-5.59±0.12) (Table 2.1). The dose ratio between CRC_1 and CRC_2 was 0.93±0.57 (Table 2.1).

2.4.6 Contractile responses of guinea pig ileum to histamine – DMSO control

To test the effect of DMSO on the mean contractile response of guinea pig ileum (n=4) to histamine, 0.1 mL DMSO was applied prior to the construction of CRC_2 (Figure 2.8). The mean maximum contractile response of histamine for CRC_2 (3.35±0.83 g) was not significantly different (P>0.05) from CRC_1 (3.17±0.55 g) and CRC_3 (3.33±0.72 g) (Table 2.2). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed no significant difference (P>0.05) between CRC_2 at each dose compared with CRC_1 and CRC_3 (Figure 2.9.b). The true -log EC_{50} value for CRC_2 (-6.07±0.24) was also not significantly different (P>0.05) from CRC_1 (-6.38±0.24) and CRC_3 (-6.03±0.26) (Table 2.2). The dose ratio between CRC_1 and CRC_2 was 2.41±0.83 (Table 2.2).

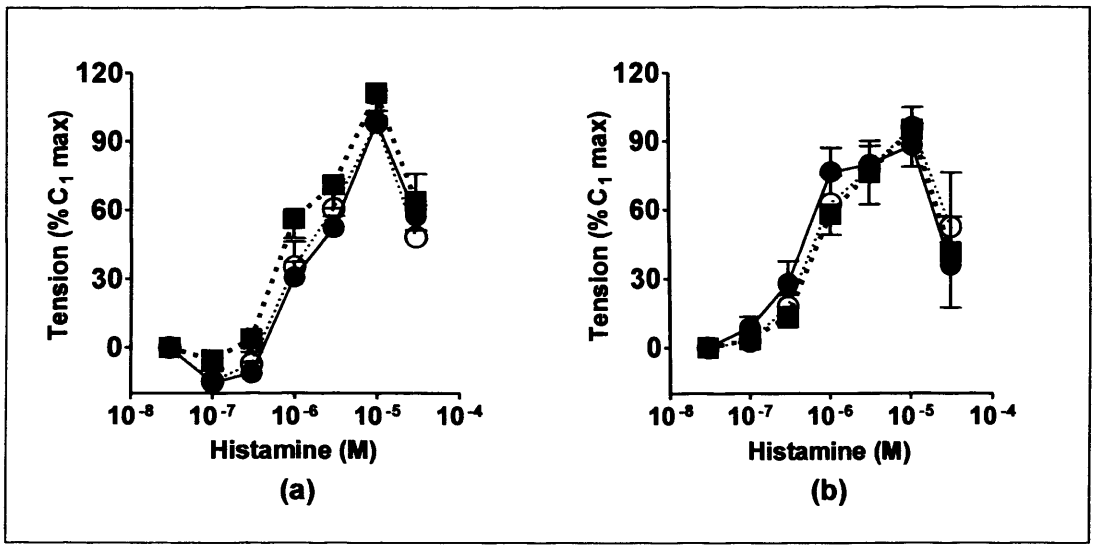


Figure 2.9. Mean cumulative CRCs for the contractile responses of the guinea pig ileum to histamine. (a) Repeated CRCs for histamine (n=4), (b) effect of DMSO added during CRC_2 on histamine (n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 vs. CRC_2, and CRC_2 vs. CRC_3 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. No significant ($P>0.05$) differences were seen at each dose between CRC_1, CRC_2 and CRC_3 of 5-HT. ●—● CRC_1, ■--■ CRC_2 after 30 min tissue equilibration after the wash out of first curve with or without DMSO, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve

2.4.7 Contractile responses of guinea pig ileum to repeated CRCs for β-PEA – Absolute control

The addition of low concentrations of β-PEA in cumulative CRCs caused concentration-related contractions on guinea pig ileum (Figure 2.10).

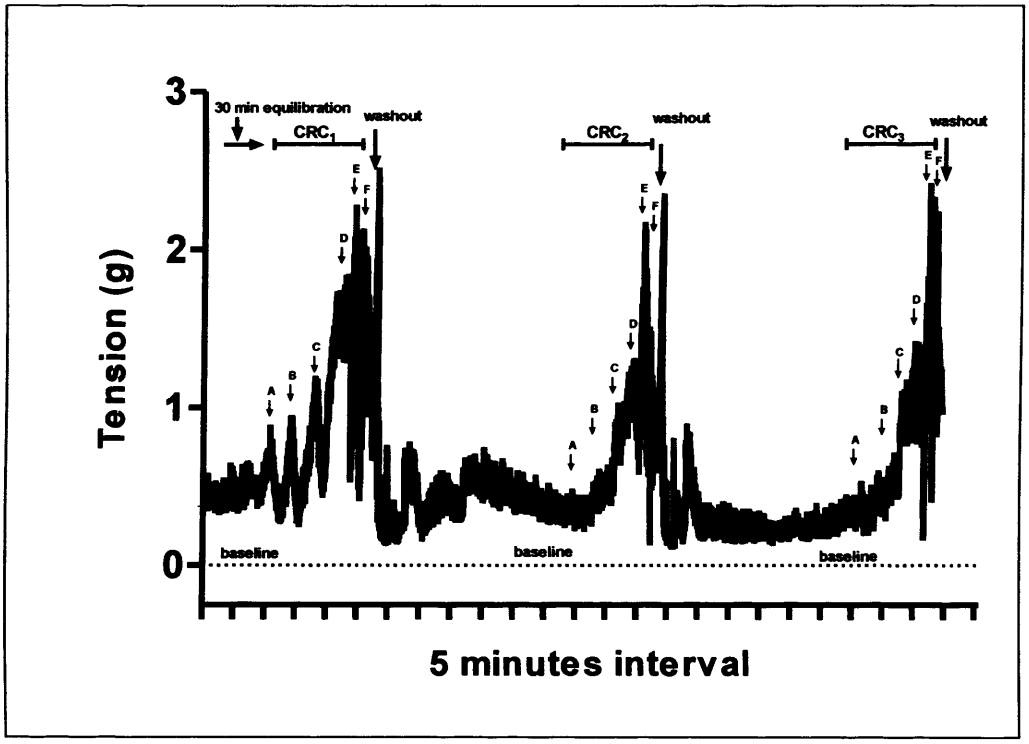


Figure 2.10. Representative chart recording showing repeated of cumulative concentration-response curves (CRC) for the contractile response of guinea pig ileum to β-PEA (A = $1x10^{-5}$ M, B = $3x10^{-5}$ M, C = $1x10^{-4}$ M, D = $3x10^{-4}$ M, E = $1x10^{-3}$ M, F = $3x10^{-3}$ M).

The mean maximum contractile response of β-PEA for CRC_2 (1.09±0.37 g) was not significantly different (P>0.05) from CRC_1 (1.06±0.32 g) and CRC_3 (0.76±0.25 g) (Table 2.1). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed no significant difference (P>0.05) for CRC_2 compared with CRC_1 and CRC_3 at each dose (Figure 2.11.a). The true -log EC_{50} value for CRC_2 (-3.30±0.37) was also not significantly different at (P>0.05) from CRC_1 (-3.69±0.57) and CRC_3 (-3.50±0.34) (Table 2.1). The dose ratio between CRC_1 and CRC_2 was 5.04±3.11 (Table 2.1).

2.4.8 Contractile responses of guinea pig ileum to β-PEA – DMSO Control

To test the effect of DMSO on the mean contractile response of guinea pig ileum (n=4) against β-PEA, 0.1 mL DMSO was applied prior to the construction of CRC_2 (Figure 2.10). The mean maximum contractile response for β-PEA on CRC_2 (1.57±0.30g) was not significantly different (P>0.05) from CRC_1 (1.43±0.34 g) and CRC_3 (1.47±0.42 g) (Table 2.2). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed no significant difference (P>0.05) between CRC_1 and CRC_2 at each dose (Figure 2.11.b). The true -log EC_{50} values for CRC_2 (-3.87±0.28) was also not significantly different (P>0.05) from CRC_1 (-4.16±0.24) and CRC_3 (-3.91±0.17) (Table 2.2). The dose ratio between CRC_1 and CRC_2 was 4.35±0.25 (Table 2.2).

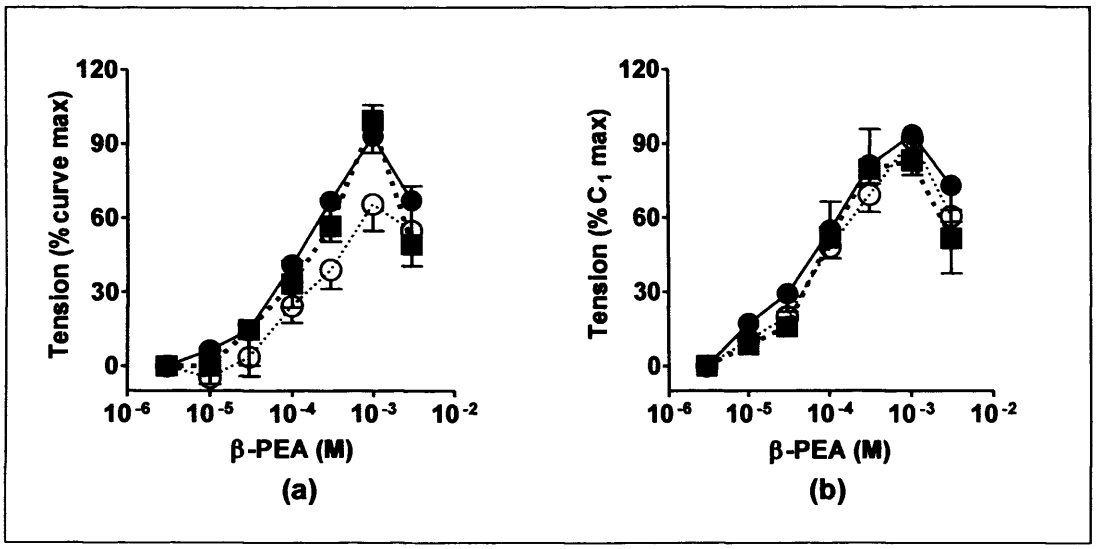


Figure 2.11. Mean cumulative CRCs for the contractile responses of the guinea pig ileum to β-PEA. (a) Repeated CRCs for β-PEA (n=4), (b) effect of DMSO added during CRC_2 on β-PEA (n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 vs. CRC_2, and CRC_2 vs. CRC_3 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. No significant ($P>0.05$) differences were seen at each dose between CRC_1 and CRC_2, and CRC_2 and CRC_3 of 5-HT. ●—● CRC_1, ■–■ CRC_2 after 30 min tissue equilibration after the wash out of first curve with or without DMSO, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve.

Table 2.1. Summary of maximum and the true -log EC_{50} of mean cumulative CRC for constrictor response of the guinea pig ileum to the agonist's 5-HT, methacholine, histamine and β-PEA – Absolute control. Maximum responses are mean (±S.E.M.) contractions (g). True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (± S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 vs. CRC_2, and CRC_2 vs. CRC_3 were compared using their corresponding values by paired Student t-test. No significant ($P>0.05$) differences on mean maximum responses and -log EC_{50} of CRC_1 vs. CRC_2, and CRC_2 vs. CRC_3 were seen on 5-HT, methacholine, histamine and β-PEA.

Agonist		CRC_1	CRC_2	CRC_3	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
5-HT	**Max (g)**	0.82±0.44	0.79±0.46	0.74±0.35		4
	-log EC_{50}	-6.56±0.17	-6.29±0.17	-6.00±0.17	1.96±0.34	
Methacholine	**Max (g)**	3.46±0.46	3.41±0.34	3.44±0.53		4
	-log EC_{50}	-7.27±0.11	-7.16±0.09	-7.18±0.11	1.31±0.16	
Histamine	**Max (g)**	3.14±0.76	3.46±0.88	3.06±0.89		5
	-log EC_{50}	-5.44±0.13	-5.77±0.17	-5.59±0.12	0.93±0.57	
β-PEA	**Max (g)**	1.06±0.32	1.09±0.37	0.76±0.25		4
	-log EC_{50}	-3.69±0.57	-3.30±0.37	-3.50±0.34	5.04±3.11	

Table 2.2. Summary of maximum and the true -log EC_{50} of mean cumulative CRC for constrictor response of the guinea pig ileum to the agonist's 5-HT, methacholine, histamine and β-PEA – DMSO control. Maximum responses are mean (±S.E.M.) contractions (g). True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (± S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 vs. CRC_2, and CRC_2 vs. CRC_3 were compared using their corresponding values by paired Student t-test. Significant ($P<0.01$, **) differences on mean maximum contractile responses for 5-HT.

Agonist		CRC_1	CRC_2	CRC_3	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
5-HT	Max (g)	2.54±0.25	2.62±0.22	1.77±0.30**		5
	-log EC_{50}	-6.42±0.28	-6.49±0.18	-6.47±0.09	1.43±0.58	
Methacholine	Max (g)	3.25±0.50	3.22±0.57	3.24±0.41		4
	-log EC_{50}	-7.37±0.05	-7.43±0.21	-7.31±0.19	1.03±0.30	
Histamine	Max (g)	3.17±0.55	3.35±0.83	3.33±0.72		4
	-log EC_{50}	-6.38±0.24	-6.07±0.24	-6.03±0.26	2.41±0.83	
β-PEA	Max (g)	1.43±0.34	1.57±0.30	1.47±0.42		4
	-log EC_{50}	-4.16±0.24	-3.87±0.28	-3.91±0.17	4.35±3.25	

2.4.9 Contractile responses of guinea pig tracheal spirals to repeated histamine cumulative CRCs – Absolute control

The addition of histamine in cumulative CRCs caused concentration-related contractions on the guinea pig trachea (Figure 2.12).

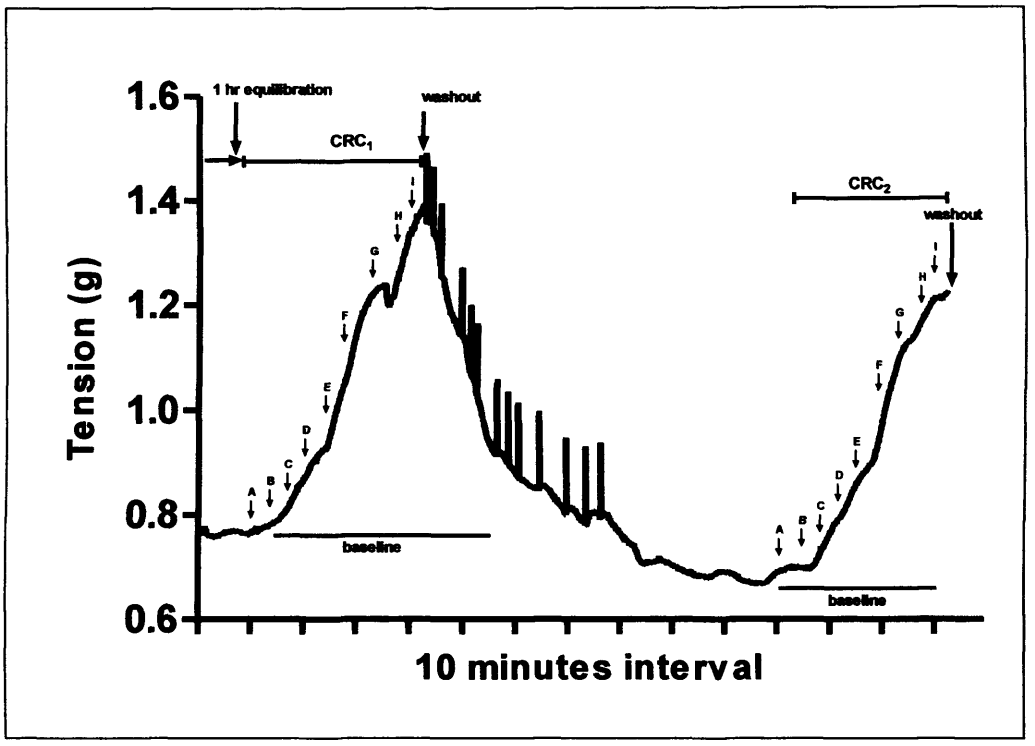


Figure 2.12. Representative chart recording showing repeated of cumulative concentration-response curves (CRC) for the contractile response of guinea pig trachea to histamine (A = $1x10^{-7}$ M, B = $3x10^{-7}$ M, C = $1x10^{-6}$ M, D = $3x10^{-6}$ M, E = $1x10^{-5}$ M, F = $3x10^{-5}$ M, G = $1x10^{-4}$ M, H = $3x10^{-4}$ M, I = $1x10^{-3}$ M).

The mean maximum contractile response of the trachea to histamine for CRC_1 (1.42±0.53 g) was not significantly different (P>0.05) from CRC_2 (1.27±0.43 g) (Table 2.3). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% was significantly less (P<0.05) on CRC_2 ($3x10^{-6}$ M , 29.34±5.72%) than CRC_1 ($3x10^{-6}$ M , 51.53±9.04%) vs. (Figure 2.13.a). The true -log EC_{50} value of CRC_1 (-5.57±0.18) was not significantly different (P>0.05) from CRC_2 (-5.19±0.14) (Table 2.3). The dose ratio between CRC_1 and CRC_2 was 3.22±1.29 (Table 2.3).

2.4.10 Contractile responses in guinea pig tracheal spirals to histamine – DMSO control

To test the effect of DMSO on the reproducibility of mean contractile response of guinea pig trachea (n=5) to histamine, 0.1 mL DMSO was applied prior to the construction of CRC_2 (Figure 2.12). The mean maximum contractile response to histamine for CRC_2 (0.85±0.10 g) was not significantly different (P>0.05) from CRC_1 (0.89±0.13 g) (Table 2.3). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed no significant (P>0.05) difference between CRC_1 and CRC_2 at each dose (Figure 2.13.b). The true -log EC_{50} value of CRC_2 (-5.27±0.32) was also not significantly different (P>0.05) from CRC_1 (-6.02±0.28) (Table 2.3). The dose ratio (12.92±8.47) observed between CRC_1 and CRC_2 indicates a variable but not significant shift of the CRC to the right (Table 2.3).

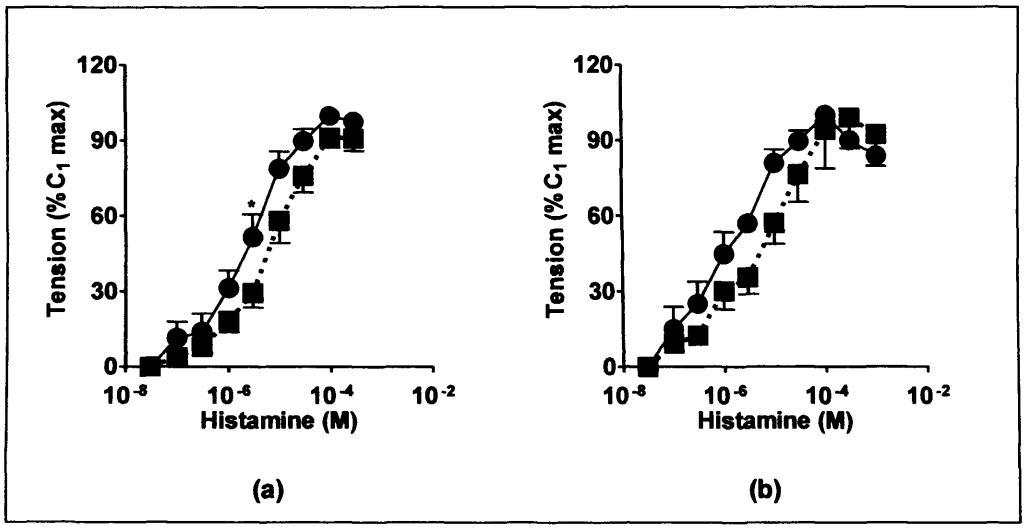


Figure 2.13. Mean cumulative CRCs for the contractile responses of the guinea pig trachea to histamine. (a) Repeated CRCs for histamine (Absolute control, n=5). (b) Effects of DMSO added during CRC_2 (n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. * Significant difference ($P<0.05$) were observed on $3x10^{-6}$ M histamine. ●—● CRC_1 after 60 min tissue equilibration and ■--■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1. 0.1 mL DMSO was added 20 minutes prior to the construction of CRC_2 in the DMSO control.

2.4.11 Contractile responses of guinea pig tracheal spirals to repeated β-PEA cumulative CRCs – Absolute control

The addition of β-PEA in cumulative CRCs caused concentration-related relaxation responses at lower concentrations in the guinea pig trachea (Figure 2.14, Figure 2.15). This was followed by contractile responses at higher concentrations.

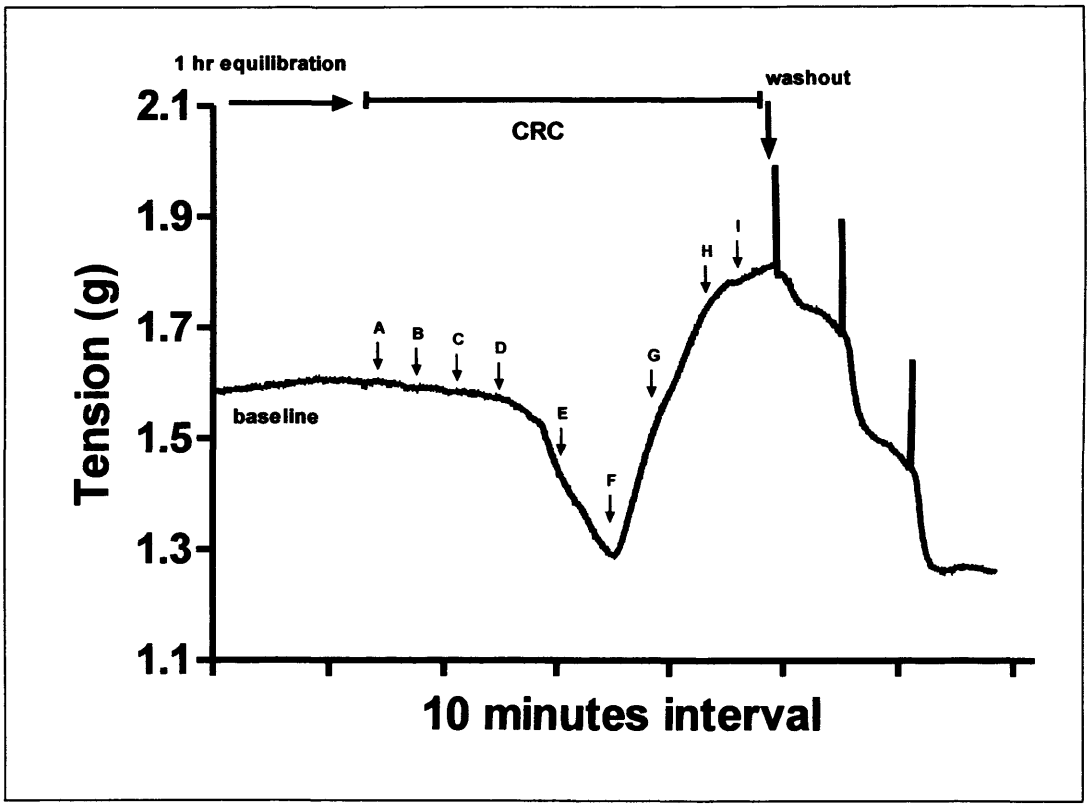


Figure 2.14. Representative chart recording showing a cumulative concentration-response curve (CRC) for the relaxation and contractile responses of guinea pig trachea to β-PEA (A = $1x10^{-6}$ M, B = $3x10^{-6}$ M, C = $1x10^{-5}$ M, D = $3x10^{-5}$ M, E = $1x10^{-4}$ M, F = $3x10^{-4}$ M, G = $1x10^{-3}$ M, H = $3x10^{-3}$ M, I = $1x10^{-2}$ M).

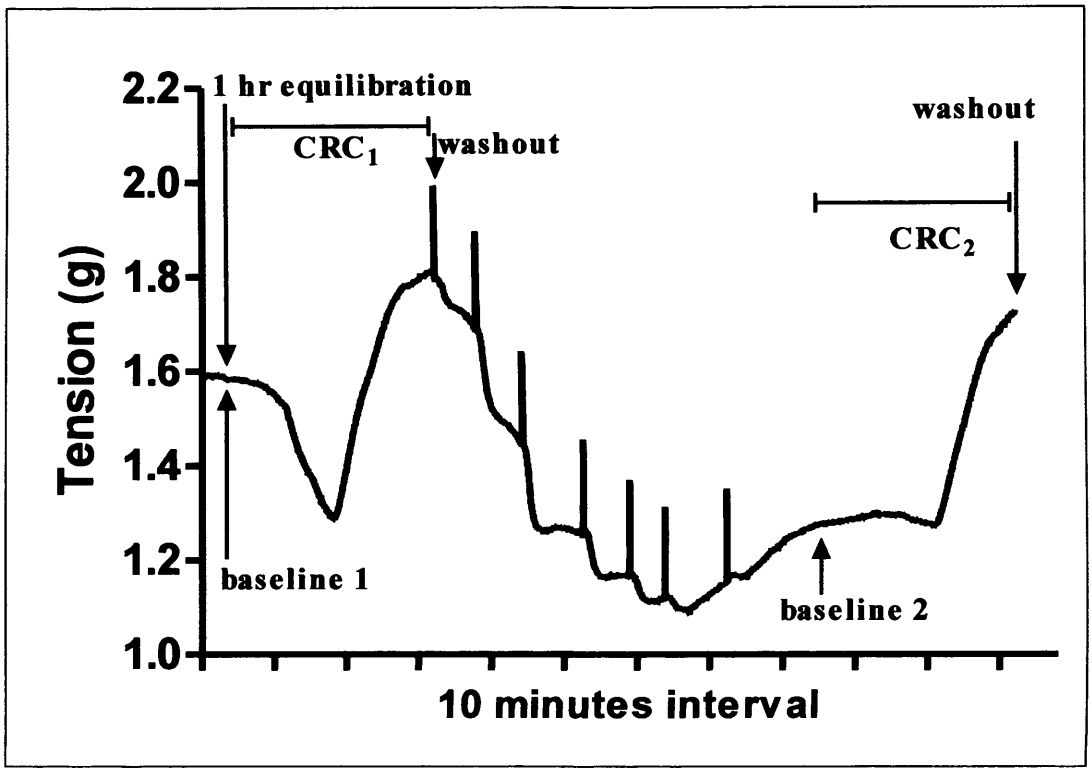


Figure 2.15. Representative chart recording showing repeated cumulative concentration-response curve (CRC) for the contractile response of guinea pig trachea to β-PEA. Refer to Figure 2.15 for β-PEA concentrations used for each CRC.

The mean contractile response maximum of β-PEA (n=4) on CRC_1 (0.51±0.17 g) was not significantly different (P>0.05) from CRC_2 (0.61±0.20 g) (Table 2.3). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed no significant difference (P>0.05) between CRC_1 and CRC_2 at each dose (Figure 2.16). The true -log EC_{50} values of CRC_2 (-2.86±0.29) was also not significantly different at (P>0.05) from CRC_1 (-2.80±0.28) (Table 2.3). Finally a

relatively low dose ratio (1.21±0.26) between CRC_1 and CRC_2 was observed (Table 2.3).

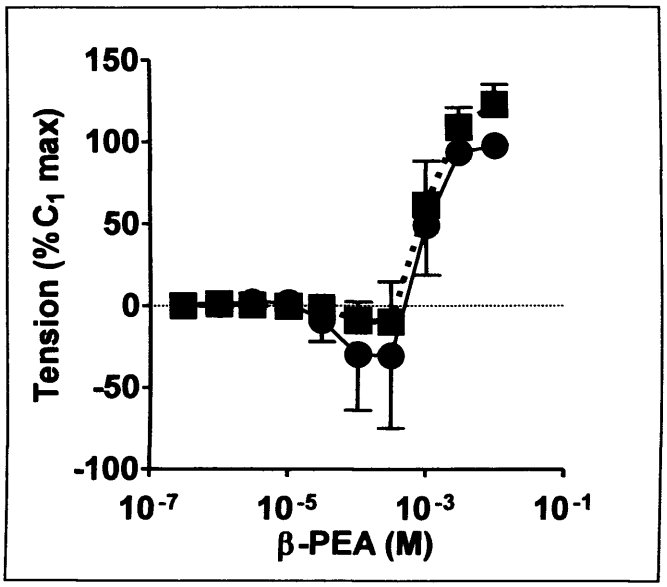


Figure 2.16. Mean cumulative CRCs for responses for repeated β-PEA (n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. ●—● CRC_1 after 60 min tissue equilibration and ■--■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1.

2.4.12 Effect of propranolol on contractile responses of guinea pig tracheal spirals to β-PEA

In an attempt to remove the relaxation response, experiments were repeated in the presence of the β-adrenoceptor antagonist propranolol. In the presence of $1x10^{-6}$ M propranolol, the relaxation response observed at lower concentrations of β-PEA (Figure 2.14) was abolished (Figure 2.17).

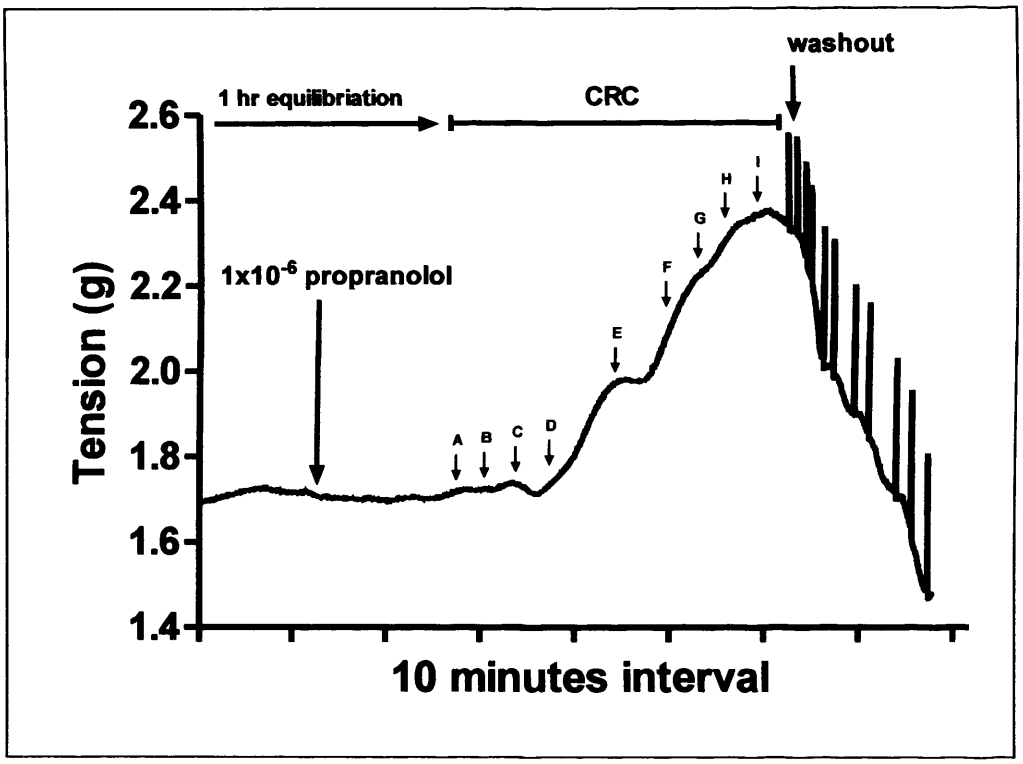


Figure 2.17. Representative chart recording showing a cumulative concentration-response curve (CRC) for β-PEA on the guinea pig trachea to in the presence of propranolol (A = $1x10^{-6}$ M, B = $3x10^{-6}$ M, C = $1x10^{-5}$ M, D = $3x10^{-5}$ M, E = $1x10^{-4}$ M, F = $3x10^{-4}$ M, G = $1x10^{-3}$ M, H = $3x10^{-3}$ M, I = $1x10^{-2}$ M).

The mean contractile response maximum of β-PEA was not significantly different ($P>0.05$) in the absence (0.51±0.17 g, n=4) and in the presence of propranolol (0.61±0.26 g, n=4). Contractile responses expressed as percentage of the maximum, however, showed significant differences ($P<0.01$) at concentrations below the maximum (Figure 2.18). The $-\log EC_{50}$ value of 2.68±0.26 was significantly ($P<0.05$) different from -3.75±0.17 in the presence of propranolol (Figure 2.18).

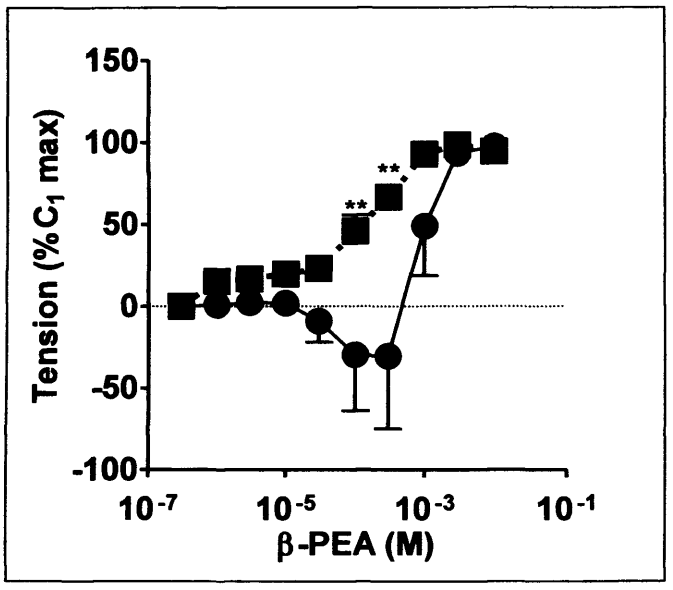


Figure 2.18. Mean cumulative CRCs for contractile responses of the guinea pig trachea to β-PEA. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of the β-PEA CRC with (n=4) and without propranolol (n=4) were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant ($P<0.01$, **) differences. ●—● CRC after 60 min tissue equilibration and ■--■ CRC after 60 min tissue equilibration with $1x10^{-6}$ M propranolol.

2.4.13 Contractile responses in guinea pig tracheal spirals to repeated β-PEA cumulative CRCs in the presence of propranolol – Absolute control

A separate set of control experiments were performed for the β-PEA CRC treated with $1x10^{-6}$ M propranolol 10 minutes before each CRC (Figure 2.19).

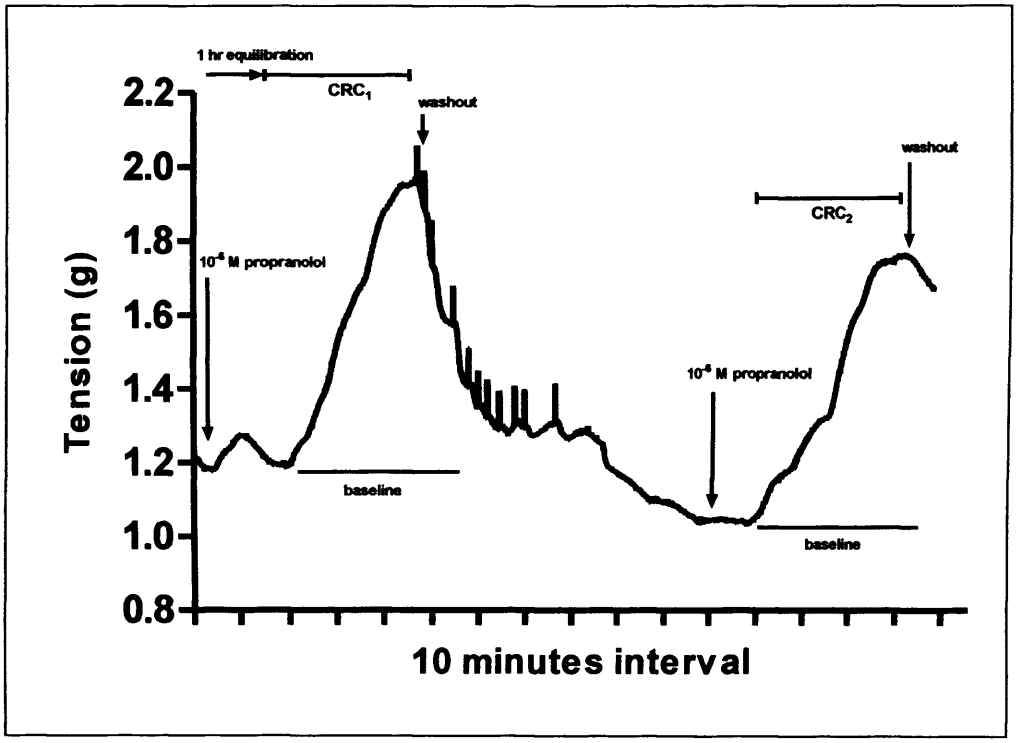


Figure 2.19. Representative chart recording showing repeated cumulative concentration-response curve (CRC) for the contractile response of guinea pig trachea to β-PEA in the presence of propranolol ($1x10^{-6}$ M). Refer to Figure 2.17 for β-PEA concentrations used for each CRC.

The mean contractile response maximum of β-PEA (n=5) showed no significant difference (P>0.05) between CRC_1 (0.66±0.26) and CRC_2 (0.90±0.40 g) (Table 2.3). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed a non-significant potentiation (P>0.05) of the maximum from CRC_1 ($3x10^{-3}$ M, 98.85±0.66%) to CRC_2 ($3x10^{-3}$ M, 128.10±13.59) (Figure 2.20.a). The true –log EC_{50} values of CRC_1 (-3.81±0.30) was not significantly different (P>0.05) from CRC_2 (-3.76±0.26) (Table 2.3). Finally the dose ratio between CRC_1 and CRC_2 was 1.15±0.15 (Table 2.3).

2.4.14 Contractile responses of guinea pig tracheal spirals to repeated β-PEA cumulative CRCs in the presence of propranolol – DMSO control

To remove the relaxation observed at low doses of β-PEA, $1x0^{-6}$ M propranolol (β-adrenoceptor blocker) was added 10 minutes prior to the construction of each β-PEA CRC (Figure 2.19) which remained present throughout the CRC. 0.1 mL DMSO was added 20 minutes prior to the construction of CRC_2. The mean maximum contractile response on CRC_1 (0.76±0.08 g) was not significantly different (P>0.05) from CRC_2 (0.73±0.06 g) (Table 2.3). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% however showed a significant relaxation (P<0.01) from CRC_1 to CRC_2 at concentrations $1x10^{-4}$ M and $3x10^{-4}$ M (Figure 2.20.b) before the maximum response. In addition, the true –log EC_{50} value of CRC_1 (-4.12±0.25) was significantly different (P<0.05) from CRC_2 (-3.21±0.18) (Table 2.3).

The dose ratio (10.81±5.00) between CRC_1 and CRC_2 indicates a slight shift of the slope of the curve to higher concentrations (Table 2.3).

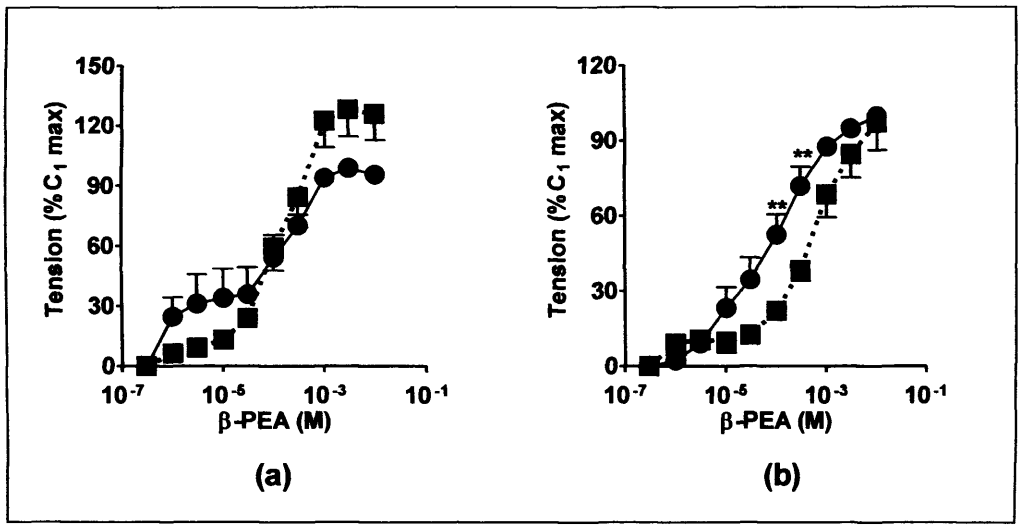


Figure 2.20. Mean cumulative CRCs for the contractile responses of guinea pig trachea in the presence of propranolol ($1x10^{-6}$ M) to β-PEA (a) absolute control, n=5, (b) DMSO control, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant differences (**, $P<0.01$). ●—● CRC_1 after 60 min tissue equilibration and ■--■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1. 0.1 mL DMSO was added 20 minutes prior to the construction of CRC_2. Propranolol ($1x10^{-6}$ M) was added 10 minutes before the construction of each CRC in (a) and (b).

Table 2.3. Absolute control and DMSO control: Trachea. Summary of maximum an EC_{50} of mean cumulative CRC on constrictor responses (g) of guinea pig aorta using histamine and β-PEA. Responses are the mean (± S.E.M.) contractions. Mean responses (± S.E.M.) of CRC_1 and CRC_2 were compared by paired Student t-test. Significant (*, $P<0.05$) differences between CRC_1 and CRC_2. 0.1 mL DMSO was applied 20 minutes prior to the construction of each CRC_2 on the DMSO control. [£] $1x10^{-6}$ M propranolol applied prior to each CRC.

Tissue	Agonist		CRC_1	CRC_2	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
Absolute control	Histamine	Max (g)	1.42±0.53	1.27±0.43		5
		-log EC_{50}	-5.57±0.18	-5.19±0.14	3.22±1.29	
	β-PEA	Max (g)	0.51±0.17	0.61±0.20*		4
		-log EC_{50}	-2.86±0.29	-2.80±0.28	1.21±0.28	
	β-PEA[£] + propranolol	Max (g)	0.66±0.26	0.90±0.40		5
		-log EC_{50}	-3.82±0.17	-3.82±0.14	1.15±0.15	
DMSO control	Histamine	Max (g)	0.89±0.13	0.85±0.10		4
		-log EC_{50}	-6.02±0.28	-5.27±0.32	12.92±8.47	
	β-PEA[£] + propranolol	Max (g)	0.76±0.08	0.73±0.06		4
		-log EC_{50}	-4.12±0.25	-3.21±0.18*	10.81±5.00	

2.4.15 Contractile responses of guinea pig aortic rings to repeated phenylephrine cumulative CRCs – Absolute control

The addition of phenylephrine in cumulative CRCs caused concentration-related contractions (Figure 2.21).

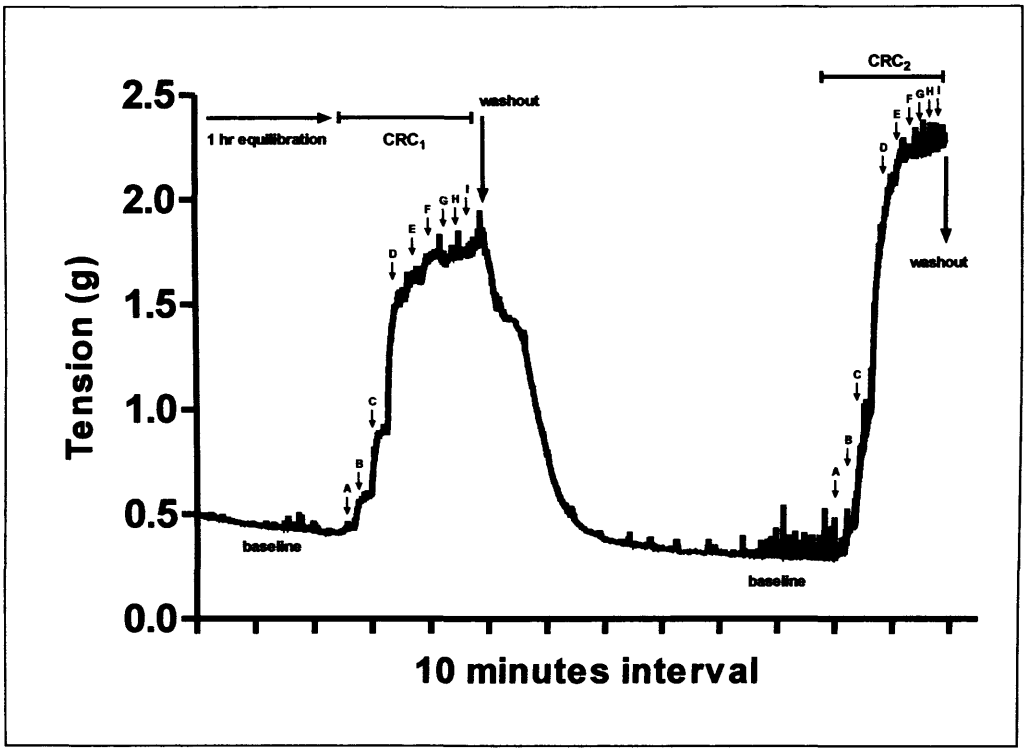


Figure 2.21. Representative chart recording showing repeated cumulative concentration-response curve (CRC) for the contractile response of guinea pig aorta to phenylephrine (A = $1x10^{-7}$ M, B = $3x10^{-7}$ M, C = $1x10^{-6}$ M, D = $3x10^{-6}$ M, E = $1x10^{-5}$ M, F = $3x10^{-5}$ M, G = $1x10^{-4}$ M, H = $3x10^{-4}$ M, I = $1x10^{-3}$ M).

The mean maximum contractile response of phenylephrine (n=5) for CRC_1 (1.07±0.16 g) was significantly ($P<0.05$) potentiated in CRC_2 (1.35±0.15 g) (Table 2.4). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed a significant ($P<0.05$) potentiation of the maximum from CRC_1 ($1x10^{-3}$ M, 100.00±0.00%) to CRC_2 ($1x10^{-3}$ M, 128.66±9.29%) (Figure 2.22.a). However true –log EC_{50} value of CRC_1 (-5.48±0.12) was not significantly different ($P>0.05$) from CRC_2 (-5.25±0.09) resulting in a low dose ratio (1.92±0.54) (Table 2.4).

2.4.16 Contractile responses in guinea pig aortic rings to repeated phenylephrine cumulative CRCs – DMSO Control

To test the effect of DMSO on the reproducibility of mean contractile response of guinea aorta treated with phenylephrine (n=5), 0.1 mL DMSO was applied prior to CRC_2. The mean contractile response maximum of phenylephrine on CRC_1 (1.19±0.24 g) was significantly increased ($P<0.05$) in CRC_2 (1.39±0.29 g) (Table 2.4). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed a significant potentiation ($P<0.01$) of the maximum from CRC_1 ($1x10^{-3}$ M, 98.25±1.75%) to CRC_2 ($1x10^{-3}$ M, 120.32±5.41%) (Figure 2.22.b). The true –log EC_{50} value of CRC_1 (-5.67±0.18) however was not significantly different ($P>0.05$) from CRC_2 (-5.76±0.29) (Table 2.4). The dose ratio between CRC_1 and CRC_2 was 0.95±0.27 (Table 2.4).

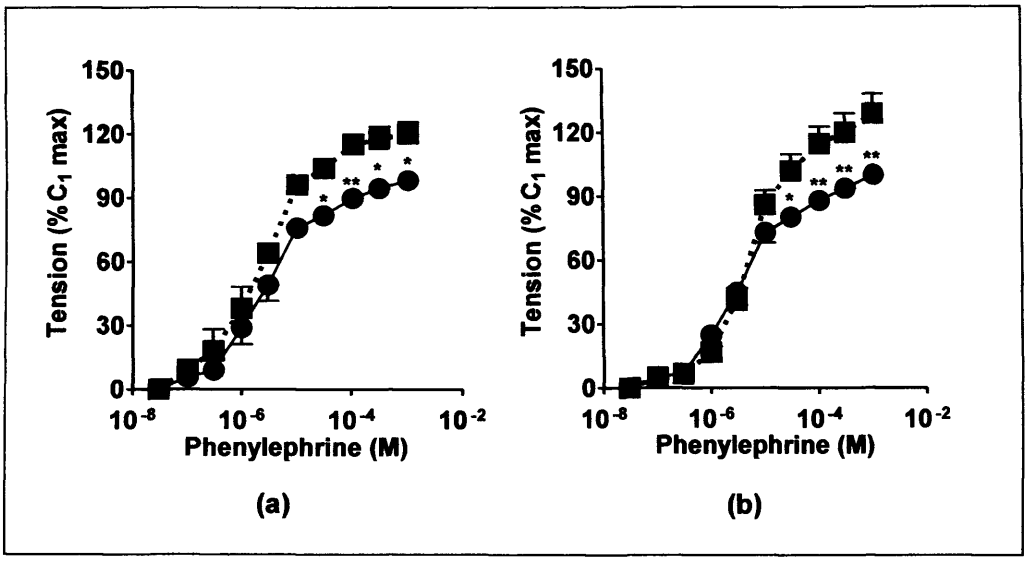


Figure 2.22. Mean cumulative CRCs for the contractile responses of guinea pig aorta to phenylephrine (a) absolute control, n=4, and (b) DMSO control, n=5. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant differences (*, $P<0.05$ or **, $P<0.01$). ●—● CRC_1 after 60 min tissue equilibration and ■--■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1.

2.4.17 Contractile responses in guinea pig aortic rings to repeated β-PEA cumulative CRCs – Absolute Control

The addition of low concentrations of β-PEA in cumulative CRCs caused concentration-related contractions (Figure 2.23).

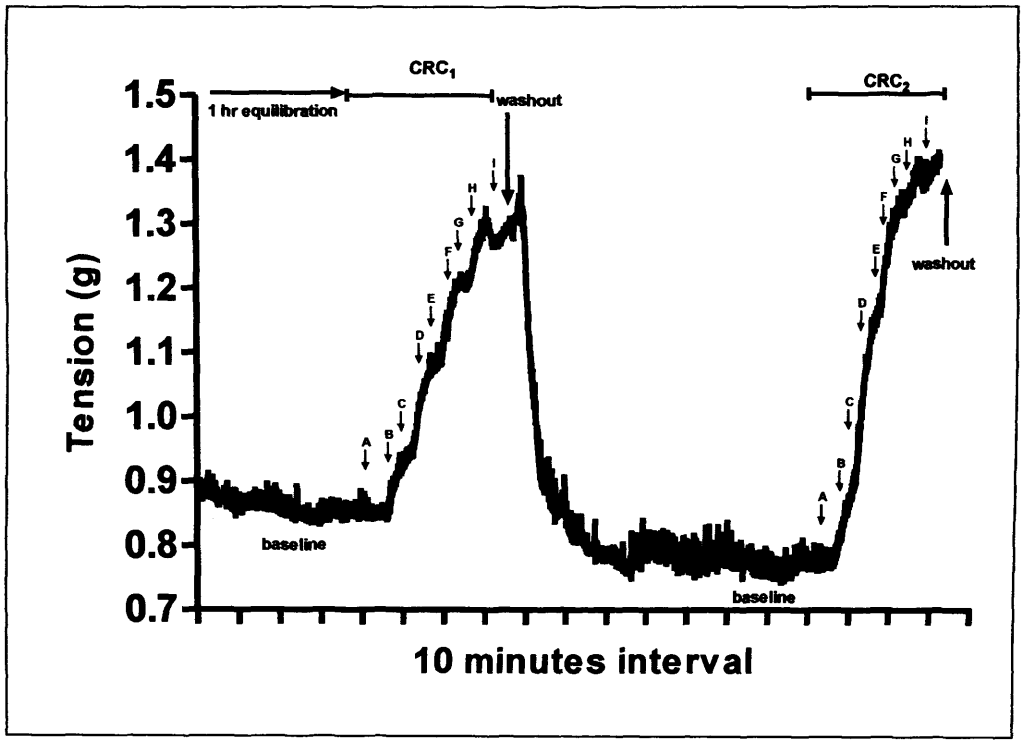


Figure 2.23. Representative chart recording showing a series of cumulative concentration-response curve (CRC) for the contractile response of guinea pig aorta to β-PEA (A = $1x10^{-6}$ M, B = $3x10^{-6}$ M, C = $1x10^{-5}$ M, D = $3x10^{-5}$ M, E = $1x10^{-4}$ M, F = $3x10^{-4}$ M, G = $1x10^{-3}$ M, H = $3x10^{-3}$ M, I = $1x10^{-2}$ M).

The mean maximum contractile response of β-PEA (n=5) on CRC_1 (0.70±0.14 g) was significantly less ($P<0.05$) than from CRC_2 (1.07±0.18 g) (Table 2.4). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% also showed a significant potentiation ($P<0.01$) from CRC_1 to CRC_2 at $3x10^{-4}$-$1x10^{-2}$ M β-PEA range (Figure 2.22.a). The true –log EC_{50} value of CRC_1 (-3.49±0.19) was not significantly different ($P>0.05$) from CRC_2 (-3.51±0.14) (Table 2.4) and the dose ratio between CRC_1 and CRC_2 was 1.00±0.16 (Table 2.4).

2.4.18 Contractile responses in guinea pig aortic rings to repeated β-PEA cumulative CRCs – DMSO Control

To test the effect of DMSO on the reproducibility of mean contractile response of guinea aorta treated with β-PEA, 0.1 mL DMSO prior to CRC_2 was applied. The mean contractile response maximum of β-PEA (n=5) on CRC_1 (0.46±0.07 g) was not significantly different ($P>0.05$) from CRC_2 (0.60±0.05 g) (Table 2.4). However, contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed significant potentiation ($P<0.05$) from CRC_1 to CRC_2 at $3x10^{-3}$-$1x10^{-2}$ M β-PEA range (Figure 2.24.b). The true –log EC_{50} values of CRC_1 (-3.45±0.20) was not significantly different ($P>0.05$) from CRC_2 (-3.48±0.17) (Table 2.4) and the dose ratio between CRC_1 and CRC_2 was 1.06±0.27 (Table 2.4).

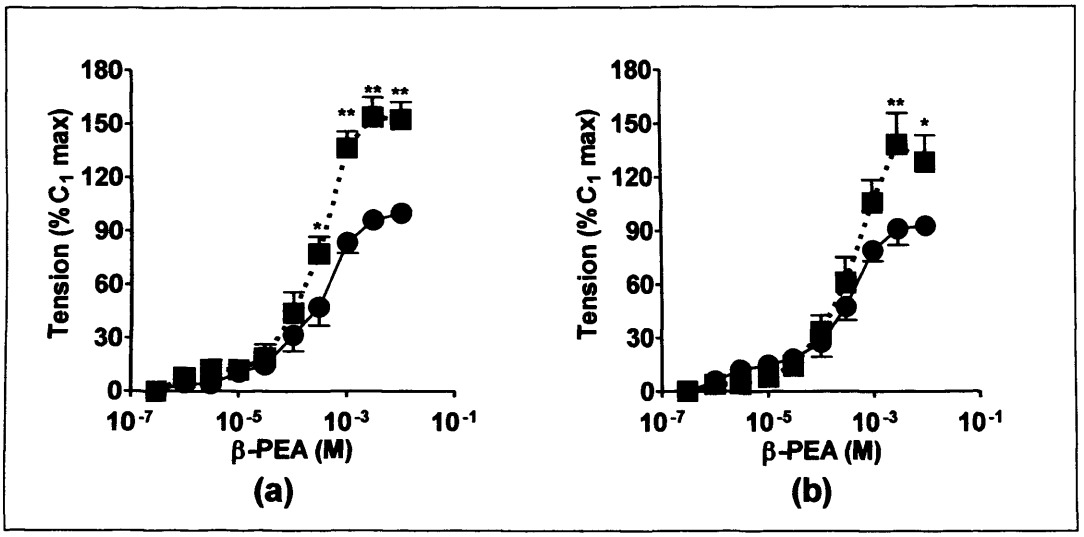
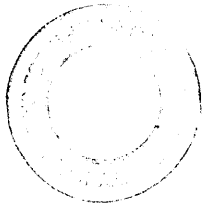


Figure 2.24. Mean cumulative CRCs for the contractile responses of guinea pig aorta to β-PEA (a) Absolute control, n=4, and (b) DMSO control, n=4, treated with DMSO. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant differences (**, $P<0.01$). ●—● CRC_1 after 60 min tissue equilibration and ■--■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1. 0.1 mL DMSO was added 20 minutes prior to the construction of CRC_2.

Table 2.4. Absolute control and DMSO control: Aorta. Summary of maximum and EC_{50} of mean cumulative CRC on constrictor responses (g) of guinea pig aorta using phenylephrine and β-PEA. Responses are the mean (± S.E.M.) contractions. Mean responses (± S.E.M.) of CRC_1 and CRC_2 were compared by paired Student t-test. Significant differences (*, $P<0.05$). Note: 0.1 mL DMSO was applied 20 minutes prior to the construction of each CRC_2 on the DMSO control.

	Agonist		CRC_1	CRC_2	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
Absolute control	**Phenylephrine**	**Max (g)**	1.07±0.16	1.35±0.15*		4
		-log EC_{50}	-5.48±0.12	-5.25±0.09	1.92±0.54	
	β-PEA	**Max (g)**	0.70±0.14	1.07±0.18*		5
		-log EC_{50}	-3.49±0.19	-3.51±0.14	1.00±0.16	
DMSO control	**Phenylephrine**	**Max (g)**	1.19±0.24	1.39±0.29*		5
		-log EC_{50}	-5.61±0.16	-5.73±0.26	0.87±0.22	
	β-PEA	**Max (g)**	0.46±0.07	0.60±0.05		5
		-log EC_{50}	-3.45±0.20	-3.48±0.17	1.06±0.27	



2.5 Discussion

2.5.1 Contractile responses of guinea pig ileum in repeated CRCs to 5-HT

5-HT caused concentration-dependent contractile responses of the guinea pig ileum (Figure 2.4). Physiological processes in the gut mediated by 5-HT includes increased GI motility, peristalsis (Olsson *et al.*, 2010), intestinal secretion (Beubler et al., 1993), nausea and vomiting (Sanger *et al.*, 2006). Previous studies show that 5-HT elicits smooth muscle contraction largely due to its direct action on 5-HT_2 receptors (Cohen *et al.*, 1985; Dickenson *et al.*, 1994). Directly coupled to the G-protein G_q, 5-HT_2 receptors can activate phospholiphase C (PLC) which initiates the formation of inositol triphosphate (PI_3) which results in the increase of the cytoplasmic Ca^{2+} via the release of intracellular Ca^{2+} (Boess *et al.*, 1994). In the gut 5-HT provides an important role in the regulation of GI processes (Gershon, 2004). Released from enterochromaffin cells 5-HT can respond to diverse sensory and motor functions in the GI tract through variety of receptors found in the submucosal and myenteric neurons (Kim *et al.*, 2000). When stimulated, 5-HT_3 or 5-HT_4 receptors located on enteric cholinergic neurons results also in smooth muscle contraction through acetylcholine release (Sikander et al., 2009).

In the present study cumulative additions of 5-HT gave almost identical repeated CRCs both in the absence and presence of DMSO (Figure 2.5). However further repetition of a third CRC showed significant decrease in the maximal response which might be due to 5-HT receptor desensitisation. Several recent studies revealed that GPCRs are prone

to agonist induce heterologous desensitization due to receptor phosphorylation by protein kinase A (PKA) or protein kinase C (PKC), or with homologous desensitization which promotes receptors binding to arrestin that prevents G-protein coupling (Allen et al., 2008). Activation of $5HT_2$ receptors by phosphorylation of PKC generally results to reduced agonist effect due feedback inhibition mediated by PKC (Rahman *et al.*, 1993) or through phosphorylation of GPCR kinases (GRKs) which promotes binding to arrestin (Schmid *et al.*, 2009). The inhibition of maximal responses on the third curve will not however affect the determination of the antagonistic action to 5-HT of plant extracts which are only present at CRC_2. CRC_3 are constructed to determine if reversibility on contractile responses to 5-HT occurs after plant extract washout.

2.5.2 Contractile responses of guinea pig ileum to methacholine

Methacholine caused concentration-related contractile responses on the guinea pig ileum (Figure 2.6). In the periphery muscarinic receptors play crucial roles in physiological functions like visceral smooth muscle contraction, activation of glandular secretion and heart rate control by parasympathetic stimulation (Kitazawa et al., 2007). A synthetic derivative of acetylcholine (ACh) it acts as a non-selective muscarinic receptor agonist (Scaf, 1971). Its possession of a charged amine group makes it insoluble to lipid cell membranes resulting to poor absorption in the GI tract and low penetration thought the blood-brain barrier (Brunton *et al.*, 2005). In the body methacholine is metabolised at a relatively slow rate due to its low reactivity to acetylcholinesterases compared to acetylcholine (Rang *et al.*, 2007; Scaf, 1971).

In the GI tract, parasympathetic stimulation is well recognized to result in gut contraction (Caulfield, 1993). The direct stimulation of muscarinic receptors, M_2 and M_3 subtypes, causes visceral smooth muscle contraction in the gut (Kitazawa et al., 2007) where M_2 and M_3 subtypes are abundantly expressed (Eglen et al., 1996). M_3 receptors largely causes smooth muscle contraction although their population in the mammalian gut accounts only for 30% compared to 70% of M_2 receptors of the total muscarinic receptors (Darroch *et al.*, 2000; Ponti *et al.*, 1998). Muscarinic M_3 receptor stimulation is linked through the activation of G_q which initiates IP_3-mediated intracellular Ca^{2+} release resulting to PKC activation (Eglen et al., 2001; Lecci et al., 2002). M_2 receptors coupled to G_i type receptor can also inhibit the AC activity leading to a decrease cAMP synthesis which switches off cAMP-mediated relaxation (Eglen et al., 2001; Lecci et al., 2002). Hyperactivity of the smooth muscle occurs in irritable bowel syndrome, asthma and chronic obstructive pulmonary disease is associated with increased sensitivity to muscarinic receptor stimulation and both M_2 or M_3 receptors blockade have been proven a useful approach in identifying therapeutic agents (Eglen et al., 2001).

In the present study, the presence or absence of DMSO in repeated cumulative CRCs showed superimposed and identical curves obtained for methacholine (Figure 2.7). So for future experiments DMSO will be used to dissolve plant extracts for antagonist activity screening against methacholine. CRC_3 will be constructed to determine if reversibility on contractile responses to methacholine can be observed after plant extract washout.

2.5.3 Contractile responses in guinea pig ileum and trachea to histamine

Histamine caused concentration-dependent contractions in the isolated guinea pig ileum and trachea (Figure 2.8 & Figure 2.12). Histamine smooth muscle contraction is largely due to stimulation of the histamine H_1-receptor subtype, coupled to G_q which stimulates PLC and activates secondary signal transduction cascades resulting to intracellular Ca^{2+} release via the IP_3 pathway, and increased levels of DAG which activates PKC (Timmerman *et al.*, 2009). In allergic conditions, histamine is one of the major compounds released from mast cells (Leurs *et al.*, 1991a). Mast cells coupled from the CNS through the myenteric neurons and submucous enteric neurons are involved in the induction of stress-induced adjustment of GI functions (Schultheiss et al., 2005). In the airways, histamine release results in various asthmatic symptoms through the stimulation of H_1-receptor which are responsible for broncoconstriction and increased pulmonary vascular resistance (Leurs et al., 1991a; Leurs et al., 1990). On the other hand, activation of H_1-receptors results also in relaxation of the vascular smooth muscle due to the production and release of an endothelium derived relaxant factor, nitric oxide (NO) (Leurs et al., 1991a).

In the present study, guinea pig ileum repeated cumulative CRCs to histamine revealed almost identical and superimposed curves (Figure 2.9). So the effects of inhibitors which are added in CRC_2 can be readily evaluated. CRC_3 are constructed to determine if any reversibility on the contractile response occur after the inhibitor washout. DMSO

which is used to dissolve plant extracts prior to its antagonistic activity evaluation against histamine showed no significant effects on the repeatability of the curves.

In the guinea pig trachea, the contractile maximum showed a reproducible action to repeated CRCs to histamine (Figure 2.13). Although a small non-significant shift of the slope of the curves to the right was observed this does not alter the reproducibility of the CRCs. This reduction of histamine efficacy in repeated dosage exposure was shown previously as a consequence to homologous H_1 receptor desensitization (Leurs et al., 1990; Leurs et al., 1991b). In the presence of DMSO a significant small shift of the curve to higher concentrations was observed but because of the relatively small dose ratio obtained, plant extract dissolved in DMSO can be straightforwardly evaluated.

2.5.4 Contractile responses of guinea pig ileum, trachea and aorta to β-PEA

β-PEA caused concentration-related contractile responses on the guinea pig ileum, trachea and aorta (Figure 2.10, Figure 2.14 and Figure 2.23). It manifests smooth muscle contraction at micromolar concentrations but is present only in trace amounts in the body due to its rapid metabolism by mono amine oxidase B (Zucchi et.al, 2006). In the periphery, β-PEA and other trace amines are regarded as indirectly acting symphathomimetic amine inducing pharmacological effects through the release of noradrenaline from sympathetic neurons (Broadley, 2010; Knoll et al., 1996). On the gut and trachea this activity would manifest as relaxation or bronchoconstriction (Rang *et al.*, 2007). However in the present study β-PEA caused contraction of both ileum and

trachea. In the aorta vasoconstriction is through the stimulation of α_1-adrenoceptors (Broadley, 2010; Hansen et al., 1980; Hawthorn et al., 1985). Opposing actions like vasodilatation via the stimulation of β-adrenoceptors is also a possibility (Broadley *et al.*, 2009). These contractile responses are not therefore due to symphatomimetic actions but have thought to be due to stimulation or phenylethylaminergic receptors or Trace amine-associated receptors (Broadley, 2010; Hawthorn et al., 1985). In the case of aorta, although α_1-adrenoceptors may explain vasoconstriction it has been shown that the α_1-adrenoceptor antagonist prazosin does not inhibit the vasoconstriction by β-PEA in rat aorta (Fehler *et al.*, 2010) and by other trace amines in coronary arteries (Herbert *et al.*, 2008). In guinea pig aorta, the related trace amine 101athinone (Al-Motarreb *et al.*, 2010) and ecstasy (Baker *et al.*, 2007) are also inhibited by prazosin. Therefore it can be assumed that the vasoconstriction by β-PEA in guinea pig aorta is not mediated via α_1-adrenoceptors but via trace amine associated receptors.

In the present study on the guinea pig ileum repeated exposure to β-PEA showed almost identical CRC (Figure 2.11). In the CRC_3 however a non-significant reduction of maximal responses was observed which might be probably due to repeated exposure of TAARs to β-PEA resulting to desensitization. This inhibition of responses will not however affect the evaluation of plant extracts which are present only in CRC_2. In the presence of DMSO non-significant alteration of the repeated CRCs was observed. So for future work the effects of plant extracts dissolved in DMSO will be readily evaluated.

In the guinea pig trachea, a biphasic effect was observed (Figure 2.14). At low concentrations of β-PEA a relaxation took place followed by contractions at higher concentrations. The relaxation can be attributed by the stimulation of β-adrenoceptors since it was blocked by propranolol (Figure 2.17) which confirms previous findings (Hawthorn et al., 1985). In the presence of propranolol, repeated β-PEA CRCs showed a significant potentiation of the maximum contractile effect which was inhibited in the presence of DMSO (Figure 2.20). In addition the presence of DMSO significantly shifted the slope of the curves to the right. The potentiation caused by repeated exposure in the presence of propranolol to β-PEA will not however affect the evaluation of the antagonistic activity of plant extracts since they were prepared in DMSO. Given the relatively small dose ratio obtained the effects of inhibitors or plants extracts can be readily evaluated.

Repeated β-PEA in the aorta caused a significant potentiation of the contractile responses on repeated exposure (Figure 2.24). In the presence of DMSO this potentiation by repeated exposures to β-PEA was maintained in CRC_2. Based on this assessment the antagonistic activity of plant extracts dissolved in DMSO can be readily evaluated for β-PEA.

2.5.5 Contractile responses of guinea pig aorta in repeated CRCs to phenylephrine

Phenylephrine (PE) caused concentration-dependent constrictor responses on the guinea pig aorta. The aortic smooth muscles contractions to phenylephrine are due to α_1-adrenoceptor which produces largely its contractile effects via intracellular calcium release in the sarcoplasmic reticulum brought about by increase in IP_3 and DAG concentrations mediated through the stimulation of PLC (Ford *et al.*, 1999; Fox *et al.*, 1985). Another mechanism which also results to smooth muscle contraction is partly due extracellular Ca^{2+} influx via receptor-operated channels (Ford *et al.*, 1999). PE is used mainly as a decongestant, its effectiveness results from vasoconstriction of nasal blood vessels which decrease the blood flow to the sinusoidal vessels resulting in decreased mucosal edema (Corboz et al., 2008; Morissette et al., 2007). It is also used to increase blood pressure in patients with hypotension without increasing the heart rate or contractility (Alahuhta et al., 1992; Nagashima et al., 1997). PE is used also to dilate the pupil in the form of eye drops (Eyeson-Annan et al., 1998).

In the present study repeated exposure of the aortic rings to PE caused potentiation of the contractile responses to PE and an increase on the maximum response. This increase in vascular tone may be due to priming-up of intracellular Ca^{2+} storage sites caused by repeated exposure to the agonist (McCarron et al., 2006; Mellentin et al., 2007). In the SR Ca^{2+}-binding proteins like calsequestrin and/or calreticulin can actively impound Ca^{2+} (Burns *et al.*, 1993). Based on Ca^{2+} affinity to Ca^{2+}-binding proteins it can be argued that during the first PE challenge, Ca^{2+} uptake is lesser

because its attraction to Ca^{2+}-binding proteins is stronger. During the second PE challenge more Ca^{2+} is being released due to Ca^{2+} weaker affinity to Ca^{2+}-binding proteins resulting to stronger contractile responses.

A thorough analysis on intracellular and extracellular Ca^{2+} uptake and reuptake on guinea pig aorta using PE as agonist will be presented in Chapter 6. Although there is a significant potentiation of the maximal response on repeated exposure to PE, with or without DMSO, contractile responses relative to the curve own maximum showed identical and superimposed curves. So for future experiments on assessment of antagonistic activity of plant extracts the repeated CRC in guinea pig aorta to PE will be used.

The potentiation of β-PEA acting via TAARs could also be through the same mechanisms.

Chapter 3

Pharmacological effects of *Sesbania grandiflora* and *Chrysanthemum coronarium* extracts on responses of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA

3.1 Introduction

The present study was undertaken to examine the possible antagonistic activity of crude extracts of *S. grandiflora* and *C. coronarium* on responses of guinea-pig ileum mediated via 5-HT_2 receptor, muscarinic M_3 receptor, histamine H_1 receptor and the Trace amine-associated receptor (TAAR). Phytochemical study of both of these plants revealed the occurrence of bioactive components mostly derivatives of flavonoids and terpenes that exhibited therapeutic uses in various diseases of the gastrointestinal tract and cardiovascular system (refer to section 1.6.1 and 1.6.2).

Previous ethno-pharmacological survey and phytochemical reports have shown that different parts of *S. grandiflora* have beneficial use as laxative, emetic (induce vomiting), antipyretic and tonic (Duke, 1983). The flowers are used as a vegetable and are also believed to cause hypotension (Fojas et al., 1982). Other parts such as the leaves, roots and bark have been reported to have analgesic property and anti-inflammatory activity and are used for the treatment of sprains and contusions and also in diarrhoea and dysentery (Duke, 1983; Subramanian *et al.*, 2003).

For *C. coronarium*, the leaves are consumed as a vegetable and reported to have laxatives properties (Ragasa *et al.*, 1997). Cumambrin A, a sesquiterpene-lactone, was also isolated from the *C. coronarium* and has been shown to cause hypotension in rats (Lee et al., 2003b; Lee et al., 2003c).

3.2 Aims

- Collection and preparation of crude extract of *S. grandiflora* (leaves and flowers) and *C. coronarium* leaves.
- To study the antagonistic property of *S. grandiflora* (leaves and flowers) crude extracts against 5-HT, methacholine, histamine and β-PEA employing repeated cumulative concentration response curves (CRCs) for the contractile responses of guinea pig isolated ileum.
- To study the antagonistic property of *C. coronarium* leaves crude extracts against 5-HT, methacholine, histamine and β-PEA employing repeated cumulative concentration response curves (CRCs) for the contractile responses of guinea pig isolated ileum.

3.3 Material and Methods

The main methods and experimental protocols described in Chapter 2 were retained throughout this study unless otherwise stated.

3.3.1 Collection of *S. grandiflora* and *C. coronarium*

Sesbania grandiflora and *C. coronarium* were collected from Bayombong, Nueva Vizcaya, Philippines in September 2007 for pharmacological evaluation (Table 3.1). Voucher specimens were submitted to the Philippine National Museum for plant identification (See appendix). Plants samples were purchased from local and commercial sources and sent intact from Nueva Vizcaya, Philippines to Cardiff University, Wales UK.

Table 3.1. Collection of *Sesbania grandiflora* and *Chrysanthemum coronarium*.

Family	*Fabaceae*	*Asteraceae*
Species	*Sesbania grandiflora*	*Chrysanthemum coronarium*
Place	Bayombong, Nueva Vizcaya, Philippines	Bayombong, Nueva Vizcaya, Philippines
Date	September 2007	September 2007
Parts used	Flowers and leaves	Leaves
Drying method	Air drying method (cabinet)	Air drying method (cabinet)
Collected by:	Gaudencio M. Natividad	Gaudencio M. Natividad

3.3.2 Protocols for chloroform and methanol plant extraction

Air dried plant parts pulverized to powder was soaked with an excess amount of hexane for at least 24 hours to remove most of the plants fatty acids, pigments and non-polar components. The mixture was filtered and the filtrate was collected and concentrated in a rotary evaporator yielding the "crude hexane fraction". The residue was further extracted with an excess amount of chloroform for another 24 hours. After filtration and solvent evaporation of collected filtrates the "chloroform crude extract" was obtained. Further extraction of the residues with excess amount of methanol for another 24 hours followed. The mixture obtained after filtration and solvent evaporation of the collected filtrates yields the "crude methanol extract" (Figure 3.1). The crudes extracts were then weight and stored in a freezer at -20°C.

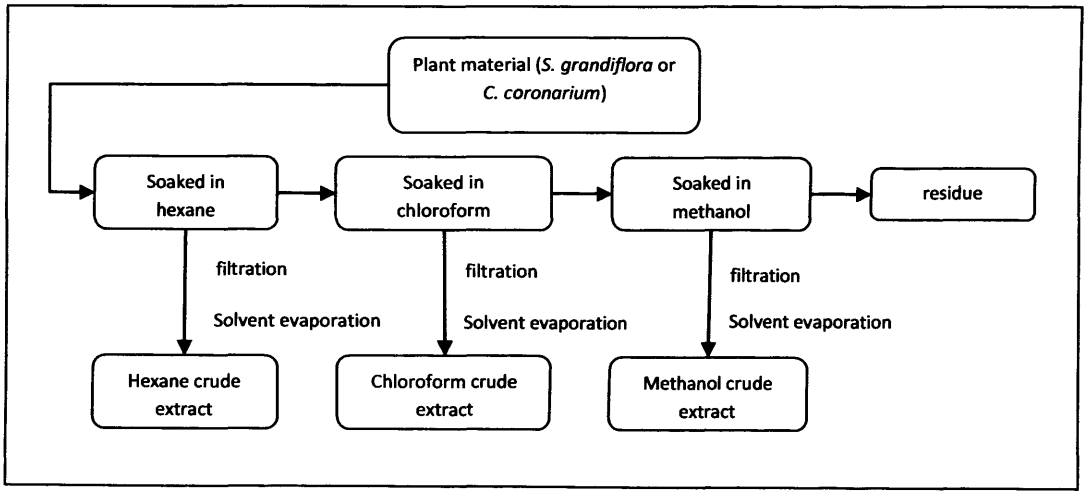


Figure 3.1. Simplified diagram for chloroform and methanol crude extraction of *S. grandiflora* and *C. coronarium*.

3.3.3 Experimental protocol

After equilibration, a series of three cumulative CRCs for 5-HT, histamine, methacholine or β-PEA in each section of guinea pig ileum were obtained in the absence and presence and after washout of different crude extracts of *S. grandiflora* and *C. Coronarium*. One milligram of each of the crude extracts was dissolved in 0.1 mL DMSO and incubated individually with the tissue for 20 minutes prior to the construction of each CRC_2. The approximate concentration of the plant extract in a 50 mL bath was therefore equivalent to 0.02 mg/mL.

3.4 Results

3.4.1 Extraction of *S. grandiflora* and *C. coronarium*

Air-dried *S. grandiflora* (flowers and leaves) and *C. coronarium* (leaves) were individually subjected to extraction protocols described in section 3.3.2. An initial weight of 98.00 g of *S. grandiflora* flowers yielded 3.23 g of chloroform layer and 3.11 g methanol layer. *Sesbania grandiflora* leaves (141.00 g) also yielded 4.35 g of the chloroform layer and 4.12 g of methanol layer. The extraction of *C. coronarium* leaves (36.00 g) yielded 1.23 g of chloroform crude extract and 1.15 g methanol crude extract.

The different plants extracts used in this study are *S. grandiflora* flower chloroform extract (SGF-$CHCl_3$), *S. grandiflora* flower methanol extract (SGF-MeOH), *S. grandiflora* leaves chloroform extract (SGL-$CHCl_3$), *S. grandiflora* leaves methanol extract (SGL-MeOH), *C. coronarium* leaves chloroform extract (CC-$CHCl_3$) and *C. coronarium* leaves methanol extract (CC-MeOH).

3.4.2 Effect of *S. grandiflora* crude extracts on contractile responses of guinea pig ileum to 5-HT

5-HT caused concentration-related constrictor responses on the guinea pig ileum. In the presence of SGF-$CHCl_3$ the maximum contractile response to 5-HT [CRC_1 (2.20±0.38 g), n=4] was significantly ($P<0.01$) inhibited to 1.00±0.24 g (Table 3.2). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed significant ($P<0.05$) reduction of contractions at the maximal response ($3x10^{-6}$ M) after the addition of the SGF-$CHCl_3$ from 89.96±10.04% to 28.49±8.81% (Figure 3.2.a).

The addition of SGF-MeOH (n=4) and SGL-MeOH (n=4) showed non-significant inhibition of the maximal contractile responses to 5-HT (Figure 3.2.b, Figure 3.2.d Table 3.2). For SGL-$CHCl_3$ (n=4) however a non-significant potentiation of the maximum is observed (Figure 3.2.b).

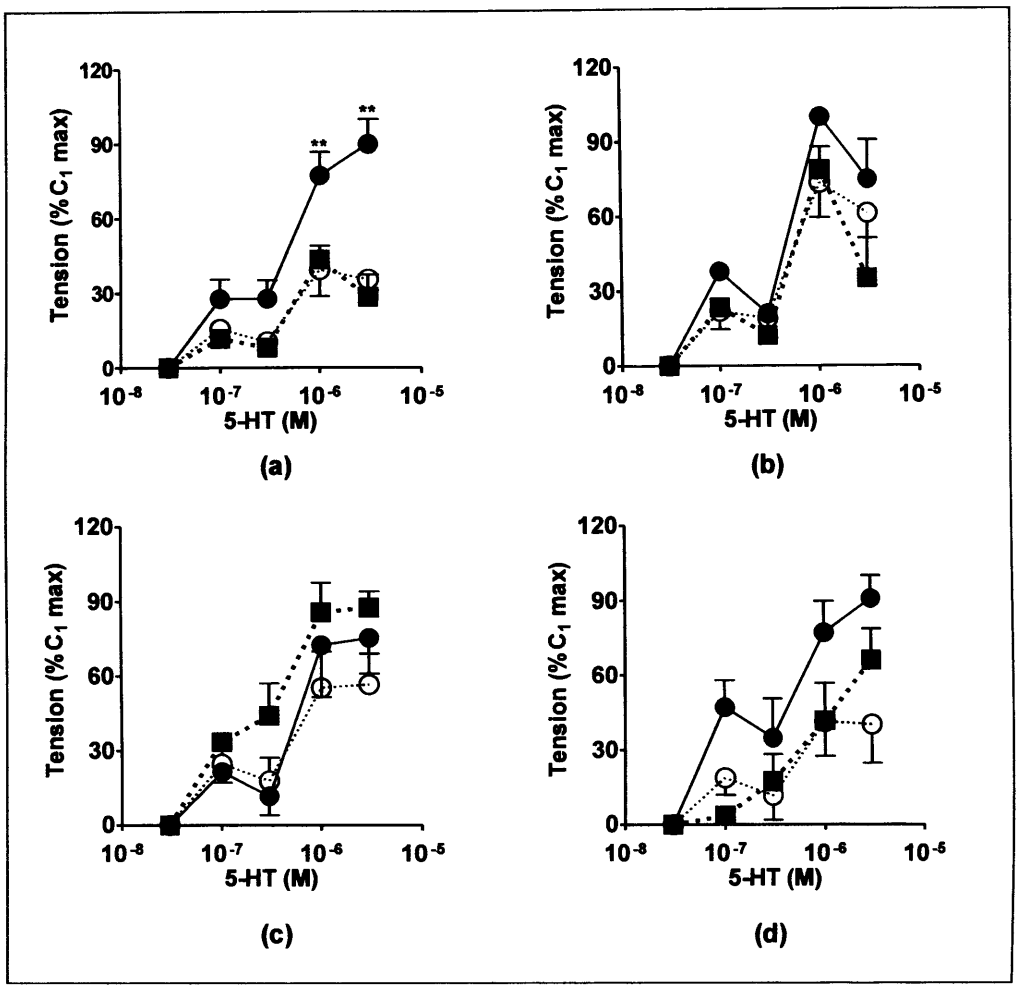


Figure 3.2. Effects of chloroform and methanol extracts of *S. grandiflora* on mean cumulative CRCs of guinea pig ileum for contractions to 5-HT, (a) SGF-$CHCl_3$, n=4, (b) SGF-MeOH, n=4, (c) SGL-$CHCl_3$, n=4, and (d) SGL-MeOH, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant ($P<0.01$, **) differences between CRC_1 and CRC_2. ●—● CRC_1 after 30 min tissue equilibration, ■--■ CRC_2 after 30 min tissue equilibration after the wash out of first curve, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve. 1 mg of *S. grandiflora* crude extract dissolve in 0.1 mL DMSO was added prior to the construction of CRC_2.

Table 3.2. Summary of the effects of S. grandiflora crude extracts on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to 5-HT. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant ($P<0.01$, **) differences between CRC_1 and CRC_2. 1 mg of *S. grandiflora* crude extract dissolve in 0.1 mL DMSO was added prior to the construction of CRC_2.

Plant Extract		CRC_1	CRC_2	CRC_3	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
$SGF\text{-}CHCl_3$	**Max (g)**	2.20±0.38	1.00±0.24**	0.99±0.32		4
	-log EC_{50}	-6.24±0.24	-6.48±0.14	-6.38±0.09	0.76±0.37	
SGF-MeOH	**Max (g)**	1.21±0.30	0.88±0.12	0.95±0.19		4
	-log EC_{50}	-6.56±0.21	-6.79±0.29	-6.46±0.19	0.71±0.28	
$SGL\text{-}CHCl_3$	**Max (g)**	1.68±0.14	1.59±0.09	1.07±0.27		4
	-log EC_{50}	-6.40±0.10	-6.95±0.27	-6.83±0.34	0.38±0.15	
SGL-MeOH	**Max (g)**	1.92±0.59	1.32±0.36	0.83±0.27		4
	-log EC_{50}	-6.07±0.40	-5.94±0.32	-6.40±0.16	4.43±2.48	

3.4.3 Contractile responses in guinea pig ileum to repeated CRCs for methacholine – effect of *S. grandiflora* crude extracts

Methacholine caused concentration-dependent contractile responses on the guinea pig ileum. Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed the addition of SGF-MeOH caused significant ($P<0.01$) inhibition of contractile responses to methacholine at ($3x10^{-7}$ M) [CRC_1 (67.61±6.06%), CRC_2 (23.81±5.42%), n=4] (Figure 3.3.b). In addition SGF-MeOH caused a shift of the curve to higher concentrations indicated by the significant change ($P<0.05$) on the -log EC_{50} to methacholine from -7.03±0.25 to -6.31±0.11 resulting to a dose ratio of 6.31±2.53 (Table 3.3).

The addition of SGL-MeOH causes a non-significant inhibition of the maximum (Figure 3.3.c), and a significant ($P<0.01$) parallel shift of the curves to the right indicated by the true -log EC_{50} to methacholine [CRC_1 (-7.49±0.05), CRC_2 (-7.22±0.05), n=4] but with a very small change indicated by its dose ratio (1.88±0.19) (Table 3.3). Both SGF-$CHCl_3$ (n=4) and SGL- $CHCl_3$ (n=4) showed no significant inhibition of contractile responses induced by methacholine (Figure 3.3.a, Figure 3.3.c).

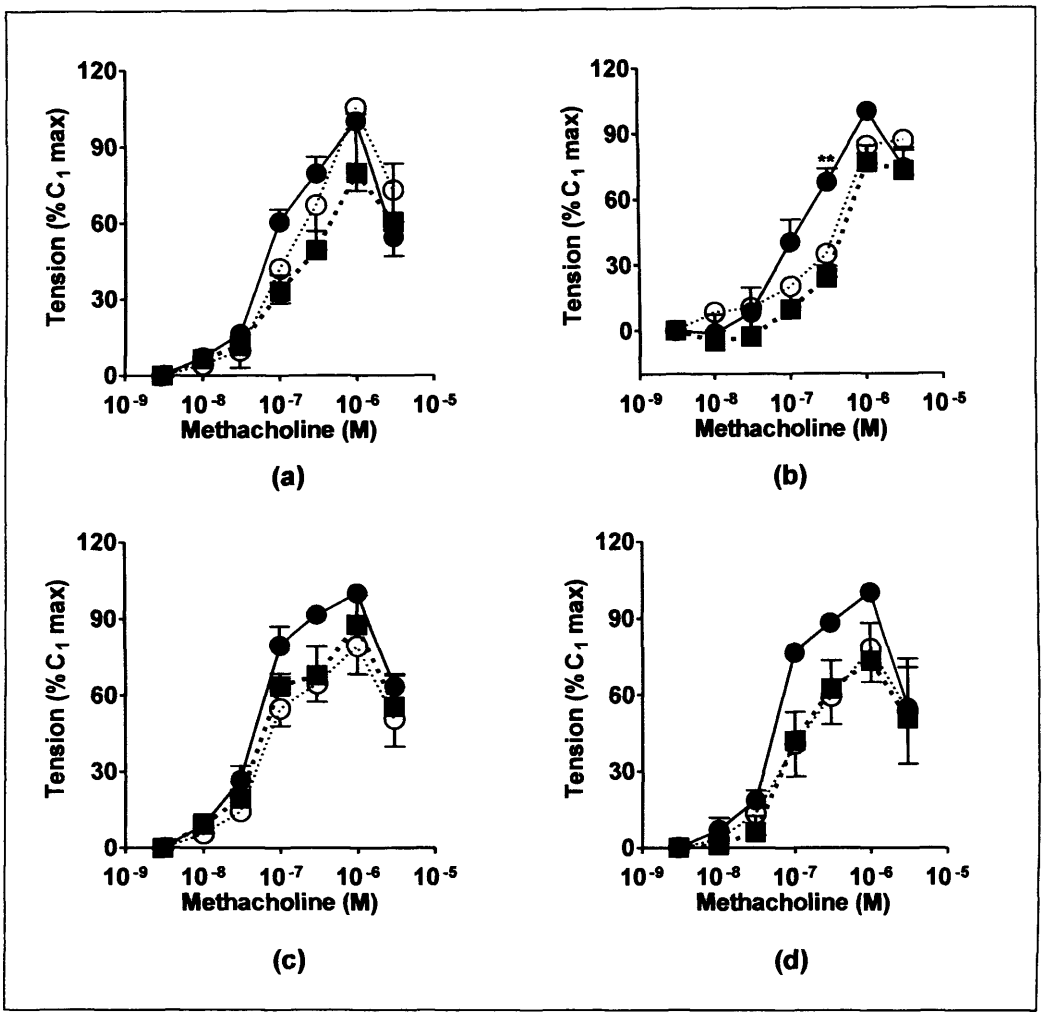


Figure 3.3. Effects of chloroform and methanol extracts of *S. grandiflora* on mean cumulative CRCs of guinea pig ileum for constriction to methacholine (a) SGF-$CHCl_3$, n=4, (b) SGF-MeOH, n=4, (c) SGL-$CHCl_3$, n=4, and (d) SGL-MeOH, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant ($P<0.01$, **) differences between CRC_1 and CRC_2. ●—● CRC_1 after 30 min tissue equilibration, ■--■ CRC_2 after 30 min tissue equilibration after the wash out of first curve, and ○--○ CRC_3 after 30 min tissue equilibration from the washout of second curve. 1 mg of *S. grandiflora* crude extract dissolve in 0.1 mL DMSO was added prior to the construction of CRC_2.

Table 3.3. Summary of the effects of *S. grandiflora* crude extract on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to methacholine. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant (*, $P<0.05$ or **, $P<0.01$) differences between CRC_1 and CRC_2. 1 mg of *S. grandiflora* crude extract dissolve in 0.1 mL DMSO was added prior to the construction of CRC_2.

Plant Extract		CRC_1	CRC_2	CRC_3	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
SGF-$CHCl_3$	**Max (g)**	3.57±1.18	2.15±0.62	3.05±1.23		4
	-log EC_{50}	-7.35±0.08	-7.10±0.24	-7.01±0.19	2.53±1.36	
SGF-MeOH	**Max (g)**	1.94±0.49	1.55±0.38*	1.66±0.31		4
	-log EC_{50}	-7.03±0.25	-6.31±0.11	-6.38±0.22	6.31±2.53	
SGL-$CHCl_3$	**Max (g)**	3.79±0.41	3.39±0.68	3.05±0.65		4
	-log EC_{50}	-7.54±0.10	-7.49±0.09	-7.40±0.11	1.13±0.12	
SGL-MeOH	**Max (g)**	1.66±0.97	1.17±0.70	1.24±0.71		4
	-log EC_{50}	-7.49±0.05	-7.22±0.05**	-7.22±0.10	1.88±0.19	

3.4.4 Effects of *S. grandiflora* crude extracts on the contractile responses of guinea pig ileum to histamine

Histamine caused concentration-related contractile responses on the guinea pig ileum. In the presence of SGF-$CHCl_3$, the maximum contractile response to histamine (CRC_1, 4.55±0.75 g, n=4) was significantly ($P<0.05$) inhibited (CRC_2, 2.10±0.23 g) (Table 3.4). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed significant ($P<0.01$) reduction of the maximum in the presence of SGF-$CHCl_3$. There was also a significant ($P<0.01$) degree of recovery of responses after the plant extract washout at the maximal dose ($1x10^{-5}$ M) to histamine [CRC_1 (100.0±0.0%), CRC_2 (46.5±4.0%), CRC_3 (74.4±6.3%)] (Figure 3.4.a).

Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed significant inhibition ($P<0.01$) of contractile responses in the presence of SGF-MeOH (n=4) at lower doses ($1x10^{-7}$ M to $3x10^{-6}$ M) of histamine (Figure 3.4.b). The contractile responses expressed as percentage of their own CRC maximum set to 100% further showed a significant ($P<0.01$) reduction in the curve to higher concentration indicated by true -log EC_{50} values obtained for histamine (CRC_1, -6.82±0.18, CRC_2, -5.43±0.08) resulting in a dose ratio of 35.19±16.29. Reversibility of contractile responses after plant extract washout was also indicated by the significant ($P<0.05$) reduction in the true -log EC_{50} values obtained in CRC_3 (-6.18±0.12) (Table 3.4).

The maximum contractile responses to histamine CRCs in the presence and after washout of SGL-$CHCl_3$ (n=4) or SGL-MeOH (n=4) showed no significant differences ($P>0.05$) (Table 3.4, Figure 3.4.c, Figure 3.4.d). However, when the contractile responses were expressed as percentage of their own CRC maximum there was a significant shift ($P<0.05$) of the CRC for histamine as shown by the -log EC_{50} values from -6.86±0.23 to -5.96±0.09 in the presence of the SGL-MeOH. After washout significant recovery to -6.38±0.17 was also noted. A dose ratio of 10.19±3.51 was observed (Table 3.4).

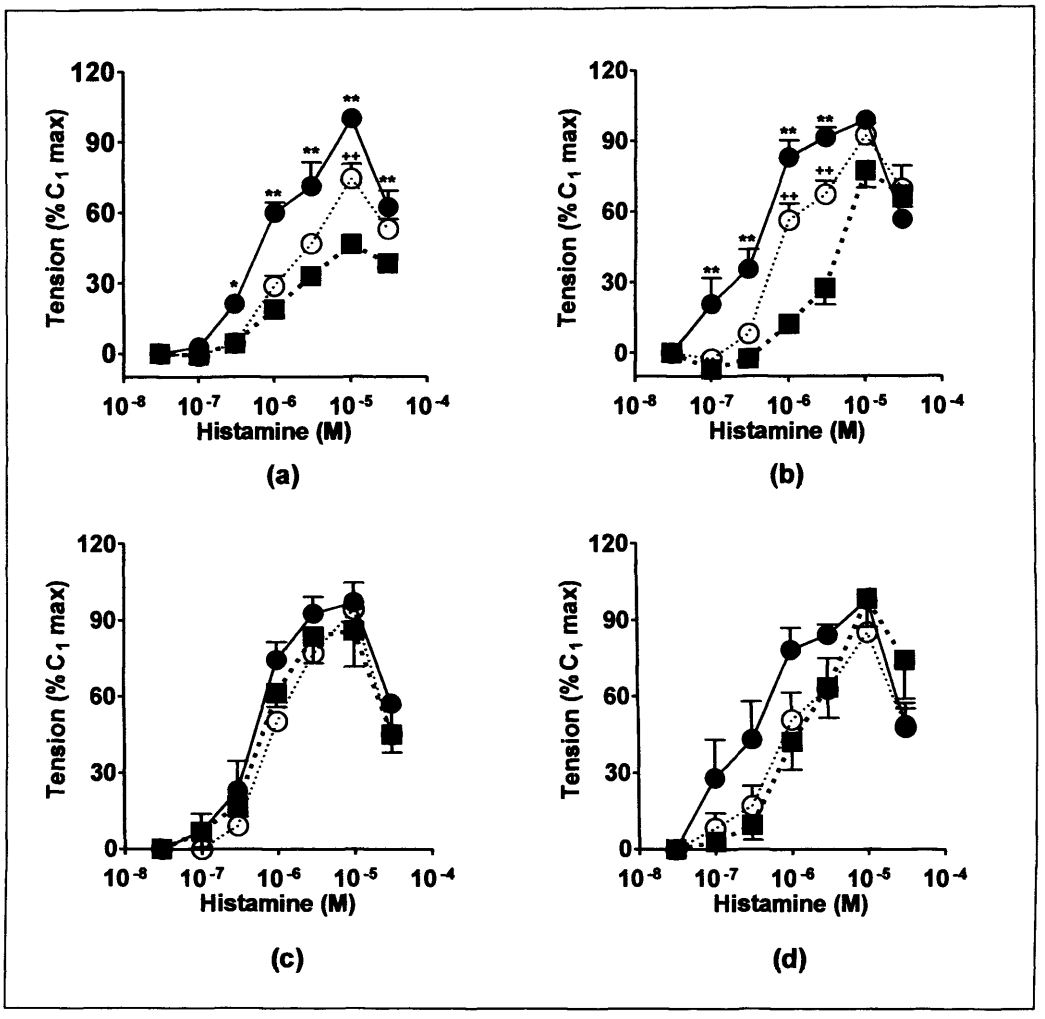


Figure 3.4. Effects of chloroform and methanol extracts of *S. grandiflora* on mean cumulative CRCs of guinea pig ileum for constriction to histamine (a) SGF-$CHCl_3$, n=4, (b) SGF-MeOH, n=4, (c) SGL-$CHCl_3$, n=4, and (d) SGL-MeOH, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant (**, $P<0.01$) differences between CRC_1 and CRC_2 and ($^{++}$, $P<0.01$) between CRC_2 and CRC_3. ●—● CRC_1 after 30 min tissue equilibration, ■--■ CRC_2 after 30 min tissue equilibration after the wash out of first curve, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve. 1 mg of *S. grandiflora* crude extract dissolve in 0.1 mL DMSO was added prior to the construction of CRC_2.

Table 3.4. Summary of the effects of *S. grandiflora* crude extract on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to histamine. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant (*, $P<0.05$ or **, $P<0.01$) differences between CRC_1 and CRC_2 and ($^+$, $P<0.05$) between CRC_2 and CRC_3. 1 mg of *S. grandiflora* crude extract dissolve in 0.1 mL DMSO was added prior to the construction of CRC_2.

Plant Extract		CRC_1	CRC_2	CRC_3	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
SGF-$CHCl_3$	**Max (g)**	4.55±0.75	2.10±0.23	3.49±0.76		4
	-log EC_{50}	-6.29±0.10	-5.94±0.02	-5.94±0.08	2.49±0.56	
SGF-MeOH	**Max (g)**	2.30±0.32	1.75±0.22	2.08±0.19		4
	-log EC_{50}	-6.82±0.18	-5.43±0.08**	-6.18±0.12$^+$	35.19±16.29	
SGL-$CHCl_3$	**Max (g)**	3.82±0.54	3.82±0.24	3.46±0.30		4
	-log EC_{50}	-6.54±0.15	-6.45±0.06	-6.30±0.06	1.47±0.47	
SGL-MeOH	**Max (g)**	2.24±0.76	2.22±0.81	1.99±0.78$^+$		4
	-log EC_{50}	-6.86±0.23	-5.96±0.09*	-6.38±0.17$^+$	10.19±3.51	

3.4.5 Effects of *S. grandiflora* crude extracts on the contractile responses of guinea pig ileum to β-PEA

β-PEA caused concentration-related constrictor responses on the guinea pig ileum. The contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% at the maximal dose ($1x10^{-3}$ M) to β-PEA [CRC_1 (100.00±0.00%)] was significantly ($P<0.01$) inhibited (CRC_2, 36.75±8.91%) by the addition of SGF-$CHCl_3$. After plant extract washout, a significant ($P<0.05$) recovery of the β-PEA maximum [CRC_3 ($3x10^{-3}$ M, 63.87±15.46%)] was also noted (Figure 3.5.a). In the presence of SGL-MeOH significant ($P<0.05$) inhibition was also observed at the maximal dose ($1x10^{-3}$ M) to β-PEA [CRC_1 (99.21±3.04%), CRC_2 (54.70±11.97%)] (Figure 3.5.d). However, no significant change was observed on the true -log EC_{50} values resulting in dose ratio of 1.73±0.85 (Table 3.5).

In the absence, presence and after washout of SGF-MeOH (n=4) or SGL-$CHCl_3$ (n=4) no significant effects were observed on the contractile responses elicited by β-PEA (Figure 3.5.b, Figure 3.5.c, Table 3.5).

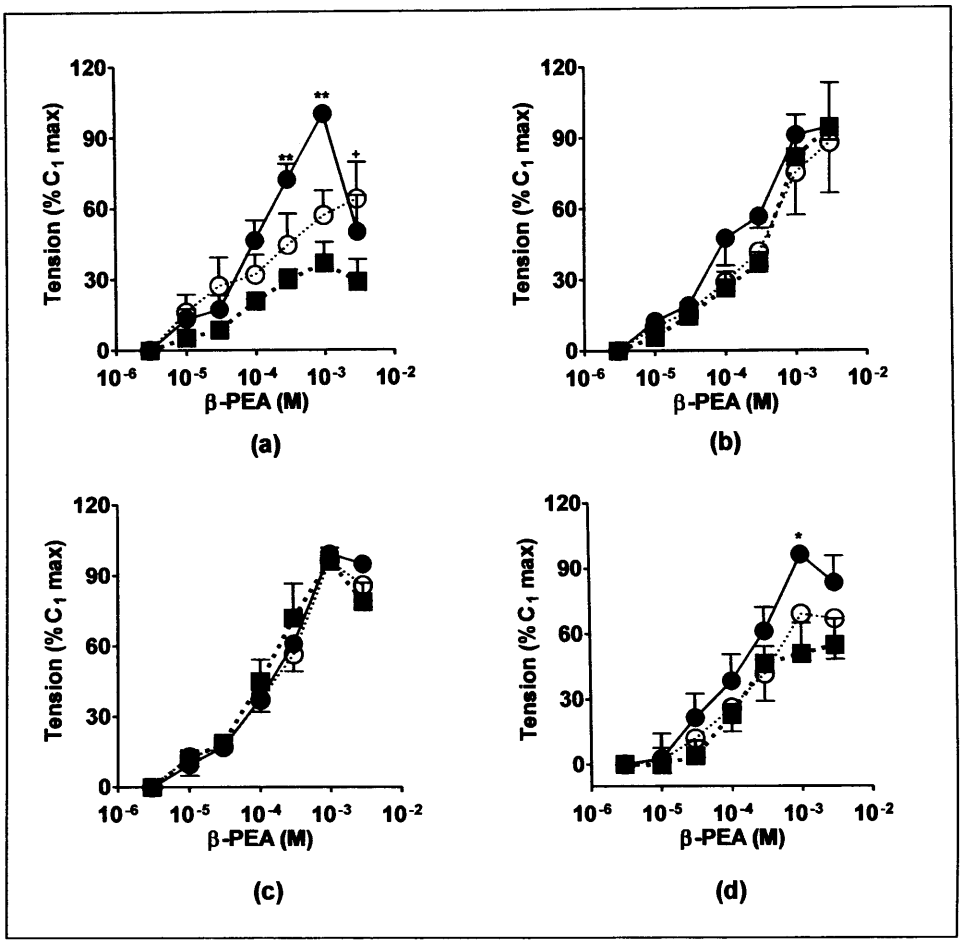


Figure 3.5. Effect of chloroform and methanol extracts of *S. grandiflora* on mean cumulative CRCs of guinea pig ileum for constriction to β-PEA (a) SGF-$CHCl_3$, *n=4*, (b) SGF-MeOH, *n=4*, (c) SGL-$CHCl_3$, *n=5*, and (d) SGL-MeOH, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant differences (*, $P<0.05$ or **, $P<0.01$) between CRC_1 and CRC_2, and ($^+$, $P<0.05$) between CRC_2 and CRC_3. ●—● CRC_1 after 30 min tissue equilibration, ■--■ CRC_2 after 30 min tissue equilibration after the wash out of first curve, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve. 1 mg of *S. grandiflora* crude extract dissolve in 0.1 mL DMSO was added prior to the construction of CRC_2.

Table 3.5. Summary of the effects of *S. grandiflora* crude extract on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant differences (**, $P<0.01$) between CRC_1 and CRC_2. 1 mg of *S. grandiflora* crude extract dissolved in 0.1 mL DMSO was added prior to the construction of CRC_2.

Plant Extract		CRC_1	CRC_2	CRC_3	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
$SGF\text{-}CHCl_3$	**Max (g)**	2.11±0.47	0.79±0.20**	1.22±0.16		4
	-log EC_{50}	4.25±0.16	-4.25±0.19	-4.14±0.37	1.48±0.63	
SGF-MeOH	**Max (g)**	1.02±0.18	0.96±0.28	0.81±0.17		4
	-log EC_{50}	-3.82±0.22	-3.36±0.10	-3.55±0.22	5.36±3.17	
$SGL\text{-}CHCl_3$	**Max (g)**	1.64±0.32	1.58±0.31	1.57±0.28		4
	-log EC_{50}	-3.73±0.06	-3.99±0.12	-3.74±0.09	0.63±0.15	
SGL-MeOH	**Max (g)**	1.39±0.41	0.92±0.27	0.93±0.27		4
	-log EC_{50}	-3.96±0.21	-4.00±0.27	-3.90±0.28	1.73±0.85	

3.4.6 Effects of *C. coronarium* crude extracts on the contractile responses in guinea pig ileum to 5-HT, methacholine, histamine and β-PEA

5-HT, histamine, methacholine and β-PEA caused concentration-related contractile responses on the guinea pig ileum. The presence of CC-$CHCl_3$ significantly ($P<0.05$) inhibited the maximum contractile responses to methacholine from 4.19±0.91 g to 2.21±0.51 g (Table 3.6). Responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed the inhibition of maximum contractions elicited by 5-HT ($1x10^{-6}$ M) [($P<0.05$), CRC_1 (97.19±2.00%) , CRC_2 (63.28±12.12%)], methacholine ($1x10^{-6}$ M) [($P<0.01$), CRC_1 ($1x10^{-6}$ M, 96.113.89%), CRC_2 ($3x10^{-7}$ M, 50.09±5.62%)] and histamine [($P<0.01$), CRC_1 ($1x10^{-5}$ M, 90.84±5.32%), CRC_2 ($1x10^{-5}$ M, 60.69±4.49%)] (Figure 3.6.a-c). The presence of CC-$CHCl_3$ showed non-significant inhibition of the maximal responses CRCs for β-PEA (Figure 3.6.d).

The addition of CC-MeOH to repeated CRCs of 5-HT, methacholine, histamine and β-PEA shows no significant effects on contractile responses (Table 3.7, Figure 3.7).

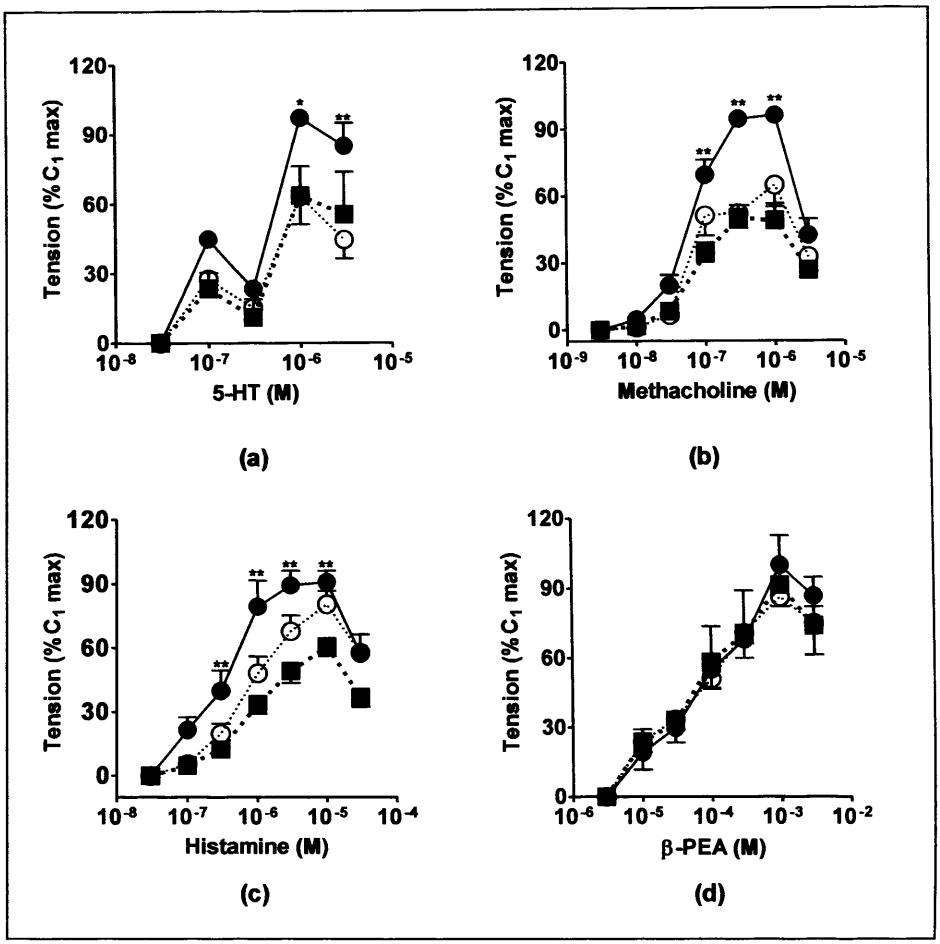


Figure 3.6. Effects of chloroform and methanol extracts of *C. coronarium* chloroform crude extract on mean cumulative CRCs of guinea pig ileum for constriction to (a) 5-HT, n=4, (b) methacholine, n=4, (c) histamine, n=4, and (d) β-PEA, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on each dose between CRC_1 vs. CRC_2, and CRC_2 vs. CRC_3 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant differences (*, $P<0.05$ or **, $P<0.01$) between CRC_1 to CRC_2. ●—● CRC_1 after 30 min tissue equilibration, ■–■ CRC_2 after 30 min tissue equilibration after the wash out of first curve, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve. One milligram of *C. coronarium* chloroform crude extract dissolved in 0.1 mL DMSO was applied prior to the construction of CRC_2.

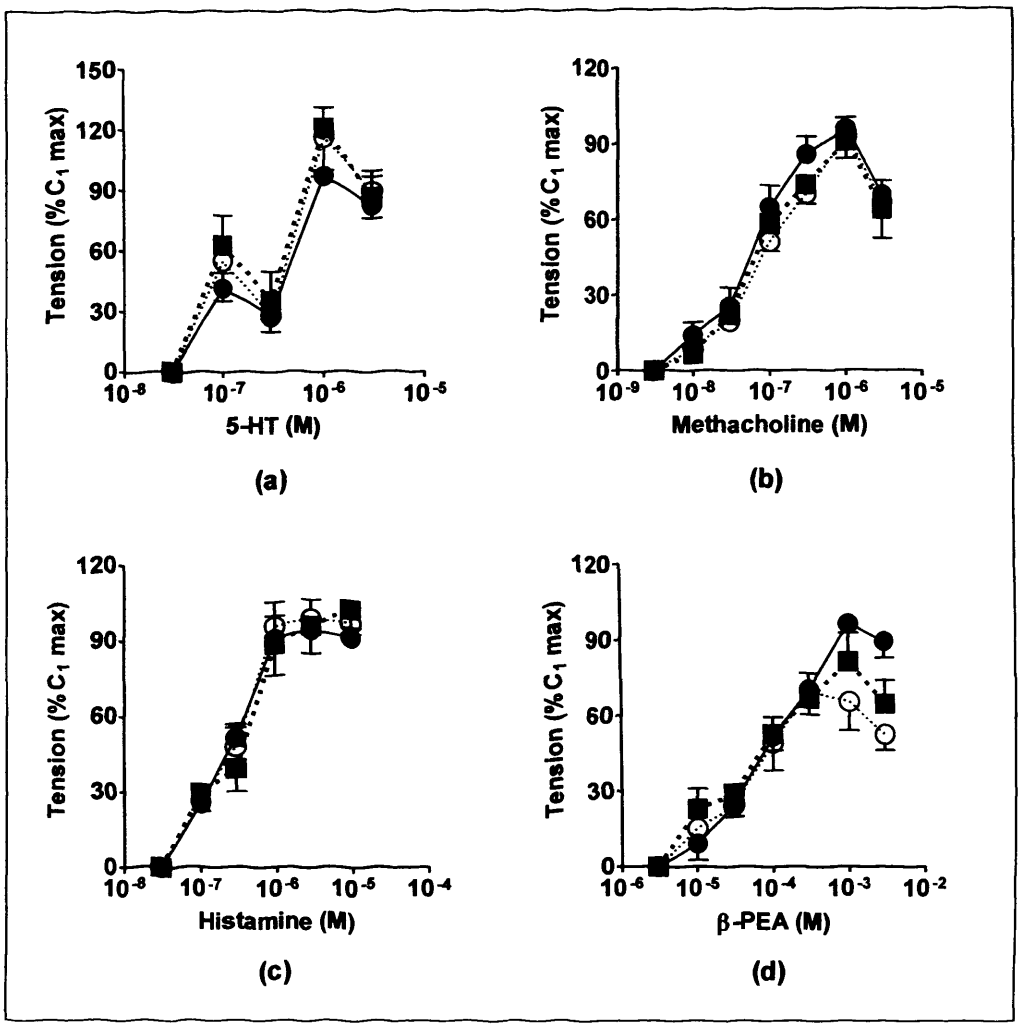


Figure 3.7. Effects of chloroform and methanol extracts of *C. coronarium* methanol crude extract on mean cumulative CRCs of guinea pig ileum for constriction to (a) 5-HT, *n=4*, (b) methacholine, *n=4*, (c) histamine, *n=4*, and (d) β-PEA, *n=4* in the presence of *C. coronarium* methanol crude extract. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on each dose between CRC_1 vs. CRC_2, and CRC_2 vs. CRC_3 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. No significant differences between CRC_1 vs. CRC_2 and CRC_2 vs. CRC_3 were observed. ●—● CRC_1 after 30 min tissue equilibration, ■–■ CRC_2 after 30 min tissue equilibration after the wash out of first curve, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve. One milligram of *C. coronarium* methanol crude extract dissolved in 0.1 mL DMSO was applied prior to the construction of CRC_2.

Table 3.6. Summary of the effects of *C. coronarium* chloroform crude extract on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant differences (*, $P<0.05$) between CRC_1 and CRC_2. One milligram of *C. coronarium* chloroform crude extract dissolved in 0.1 mL DMSO was applied prior to the construction of CRC_2.

Agonist		CRC_1	CRC_2	CRC_3	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
5-HT	**Max (g)**	1.59±0.25	0.99±0.25	0.99±0.17		4
	-log EC_{50}	-6.47±0.15	6.87±0.22	-6.36±0.08	0.84±0.53	
Methacholine	**Max (g)**	4.19±0.91	2.21±0.51*	2.70±0.63		4
	-log EC_{50}	-7.53±0.07	-7.45±0.04	-7.44±0.08	1.23±0.13	
Histamine	**Max (g)**	3.62±1.25	2.18±0.81	2.91±1.10		4
	-log EC_{50}	-6.76±0.25	-6.29±0.12	-6.30±0.15	4.05±1.75	
β-PEA	**Max (g)**	1.05±0.11	0.91±0.14	0.95±0.10		4
	-log EC_{50}	-4.14±0.16	-4.34±0.23	-4.27±0.27	0.94±0.49	

Table 3.7. Summary of the effect of *C. coronarium* methanol crude extract on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. No significant differences ($P<0.05$) between CRC_1 and CRC_2 was observed. One milligram of *C. coronarium* methanol crude extract dissolved in 0.1 mL DMSO was applied prior to the construction of CRC_2.

Agonist		CRC_1	CRC_2	CRC_3	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
5-HT	**Max (g)**	0.91±0.28	1.14±0.35	1.04±0.26		4
	-log EC_{50}	-7.04±0.05	-6.76±0.27	-6.58±0.19	3.53±1.99	
Methacholine	**Max (g)**	1.09±0.37	0.97±0.29	0.98±0.27		4
	-log EC_{50}	-7.42±0.16	-7.34±0.11	-7.25±0.15	1.61±0.81	
Histamine	**Max (g)**	1.24±0.36	1.21±0.27	1.20±0.29		4
	-log EC_{50}	-7.03±0.19	-6.85±0.18	-6.91±0.15	2.70±0.95	
β-PEA	**Max (g)**	0.85±0.27	0.62±0.11	0.61±0.20		4
	-log EC_{50}	-4.08±0.09	-4.41±0.08	4.54±0.11	0.53±0.17	

3.5 Discussion

3.5.1 The antagonistic activity of *Sesbania grandiflora* on contractile responses of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA

In the present study pharmacological screening of the flowering part of *S. grandiflora* revealed that the chloroform extract (SGF-$CHCl_3$) causes a non-competitive irreversible inhibition of 5-HT, non-significant inhibition of methacholine, non-competitive reversible inhibition of the maximal contractile responses to histamine and β-PEA. Thus it was relatively specific and the lack of non-specific activity indicates that this extract does not contain a component with membrane stabilizing or channel blocking properties. The methanol layer (SGF-MeOH) revealed the presence of a competitive reversible histamine H_1 antagonist and to a lesser extent a competitive muscarinic M_3 antagonist by a parallel shift of the contractile response CRC to histamine and methacholine on the guinea pig ileum to the right. In addition, the methanol extracts of the leaves (SGL-MeOH) also showed specific-selective-competitive histamine antagonist actions, and significant non-competitive inhibition to β-PEA. The chloroform extract of the leaves was largely without activity. These suggest that the inhibitory properties of the methanolic flower and leaves extracts of *S. grandiflora* against histamine and methacholine are mediated largely through H_1 receptor antagonism and partly by M_3 receptor antagonism.

Histamine H_1 and muscarinic M_3 receptors, both coupled directly to the G-protein G_q cause smooth muscle contraction mainly through the stimulation of PLC which initiates the formation of PI that leads to intracellular Ca^{2+} release via the stimulation of PI_3 receptor in the sarcoplasmic reticulum (Ehlert, 2003; Nahorski *et al.*, 1997; Notcovich *et al.*, 2010). In the blood vessels, on the other hand both H_1 receptors (Beyak *et al.*, 1995; Kim *et al.*, 2008) and M_3 receptors (Khurana *et al.*, 2005) can also initiate nitric oxide (NO) production in the endothelium resulting to vascular smooth muscle relaxation. However this effect is not relevant here to the ileum.

The traditional belief is that the *S. grandiflora* flowers can lower blood pressure and previous reports on its hypotensive activity (Fojas et al., 1982) indicate that this might be due to the stimulation by the plant extract of H_1 receptors or M_3 receptors which initiates nitric oxide (NO) release that results in hypotension. The histamine-like stimulation which was inhibited by mepyramine, a H_1 receptor inhibitor, in the isolated guinea pig ileum was previously reported also by Fojas et al. (1982). However in the present study it is **ANT**agonistic against H_1 receptors that have been identified rather than agonist activity. In addition the methanolic extracts also revealed anti-inflammatory properties against carrageenan-induce paw edema (Kuhad *et al.*, 2009) which might be mediated by H_1 receptor or M_3 receptor. This further validates the *S. grandiflora* traditional use in the treatment of sprains, contusions and gastrointestinal illnesses such as diarrhoea and dysentery in which the medicinal relevance is probably antagonizing either H_1 receptor or M_3 receptor. This would induce relaxation and inhibition of motility to relieve diarrhoea. Previously it was reported that the root extracts of *S. grandiflora* have anti-diarrhoeal activity (Subramanian et al., 2003). The

anti-diarrhoeal action of the roots extract in the gut has a high probability that derivatives or similar compounds from the leaves are acting also through the H_1 receptor and M_3 receptor.

The various parts of the plants can also cause CNS depression in mice (Fojas et al., 1982) or anticolvulsant profile and anxiolytic (antianxiety) activity (Kasture et al., 2002).

3.5.2 The antagonistic activity of *C. coronarium* on contractile responses of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA

The phytochemical studies carried out on different parts of *C. coronarium* revealed an array of compounds that can be used in various illnesses. Among the *C. coronarium* extracts, CC-$CHCl_3$ showed a significant inhibition of the maximal contractile responses induced by 5-HT, methacholine and histamine but not β-PEA. The lack of specificity of the blocking effects indicates that the compounds present in the plant may block all of the annotated receptors or have a non-specific local anaesthetic relaxation on the tissue. If that was the case, however, it does not explain why β-PEA contractions were not inhibited. The inhibition of contractile responses does not explain the laxative properties of the plant (Gins et al., 2000) since it would favour a constipating action.

Cumambrin A, a sesquiterpene lactone isolated from the plant, was reported to strongly lower blood pressure (Lee et al., 2003c). However the current study does not allow any conclusion on whether the hypotension of cumambrin A is mediated by 5-HT receptors,

muscarinic receptors or histamine receptors since the effects were not examined in the aorta.

The CC-MeOH showed no significant inhibition against 5-HT, methacholine, histamine and β-PEA.

3.6 Conclusion

This chapter further validates the traditional use of *S. grandiflora* in the treatment of diarrhoea, dysentery, inflammation and high blood pressure which might be mediated largely through histamine H_1 and partly by muscarinic M_3 antagonism. Both of the methanol layer of *S. grandiflora* flowers and leaves exhibited histamine H_1 antagonism while muscarinic M_3 antagonism was observed only in the methanol layer of the flowers.

The investigations of *S. grandiflora* and *C. coronarium* showed no potential TAAR antagonist properties.

Chapter 4

Pharmacological effects of *Vitex negundo* and *Moringa oleifera* acid-base extracts on responses of guinea pig ileum, trachea and aorta to 5-HT, methacholine, histamine, phenylephrine and β-PEA

4.1 Introduction

In the present chapter the acid-base extracts of *Vitex negundo* leaves and *Moringa oleifera* bark will be examined on their possible antagonistic activity mediated through the serotonin 5-HT_2 receptors, histamine H_1 receptors, muscarinic M_3 receptors and TAAR in the gut, histamine H_1 receptors and TAAR in the airways, and α_1-adrenoceptor and TAAR in the vascular system. In the phytochemical study of *V. negundo* it revealed the presence of polar bioactive components like phenylnaphthalene-type lignan alkaloid vitedoamine A (Zheng *et al.*, 2009a). While previous work on *M. oleifera* showed the presence of polar components like alkaloids with antihypertensive properties (Dangi *et al.*, 2002).

Traditionally most of the alkaloid containing plants was used for therapeutic and recreational purposes (Dragull *et al.*, 2003). Examples of these substances include the stimulant nicotine and caffeine (Cohen *et al.*, 1991; Rose *et al.*, 1991); psychoactive substances like cocaine (Rudnick *et al.*, 1993), amphetamine and cathinone (Goudie, 1985); and bioactive substances like morphine and its derivatives (Buss *et al.*, 2010; Macht *et al.*, 1917; Mitsui *et al.*, 1995). Although compounds like serotonin and histamine contain basic nitrogen atoms they are usually designated as amines rather than alkaloids (Cseke *et al.*, 2006).

4.2 Aims

- To study the antagonistic property of *V. negundo* and *M. oleifera* acid-base crude extracts to 5-HT, methacholine, histamine and β-PEA employing repeated cumulative concentration response curves (CRCs) for the contractile responses of guinea pig isolated ileum.
- To study the antagonistic property of *V. negundo* and *M. oleifera* acid-base crude extracts to histamine and β-PEA employing repeated CRCs for the contractile responses of guinea pig isolated trachea.
- To study the antagonistic property of *V. negundo* and *M. oleifera* acid-base crude extracts to phenylephrine and β-PEA employing repeated CRCs for the contractile responses of guinea pig isolated aorta.

4.3 Material and Methods

The main methods and experimental protocols described in Chapter 2 were retained throughout this study unless otherwise stated.

4.3.1 Collection of *V. negundo* and *M. oleifera*

Vitex negundo and *M. oleifera* were collected from Occapon, Villaverde, Nueva Vizcaya, Philippines in January 2009 for pharmacological evaluation (Table 4.1). Voucher specimens were submitted to the Philippine National Museum for plant identification (See Appendix). Plants samples were purchased from local and commercial sources and sent intact from Nueva Vizcaya, Philippines to Cardiff University, Wales UK.

Table 4.1. Collection of *Vitex negundo* and *Moringa oleifera*

Family	*Verbenaceae*	*Moringaceae*
Species	*Vitex negundo*	*Moringa oleifera*
Place	Occapon, Villaverde, Nueva Vizcaya, Philippines	Occapon, Villaverde, Nueva Vizcaya, Philippines
Date	January 2009	January 2009
Parts used	Leaves	Bark
Drying method	Air drying method (cabinet)	Air drying method (cabinet)
Collected by:	Felix Apolonio, Jr.	Felix Apolonio, Jr.

4.3.2 Protocols for acid-base plant extraction

Air-dried plant parts pulverized to powder were soaked with an excess amount of 0.1 M hydrochloric acid (HCl) overnight to remove most of the polar components. After filtration the pH of the collected filtrate was adjusted to approximately pH=12 using ammonium hydroxide (NH_3OH). The mixture was extracted and partitioned with an equal volume of dichloromethane (DCM). The DCM layer was collected and dried with $MgSO_4$ (Figure 4.1). After filtration and solvent evaporation, the crude extract was collected, weighed and stored in a freezer at -20°C (Hohenschutz et al., 1981; Kam et al., 1999; Mroczek et al., 2006; Silvaa et al., 2007).

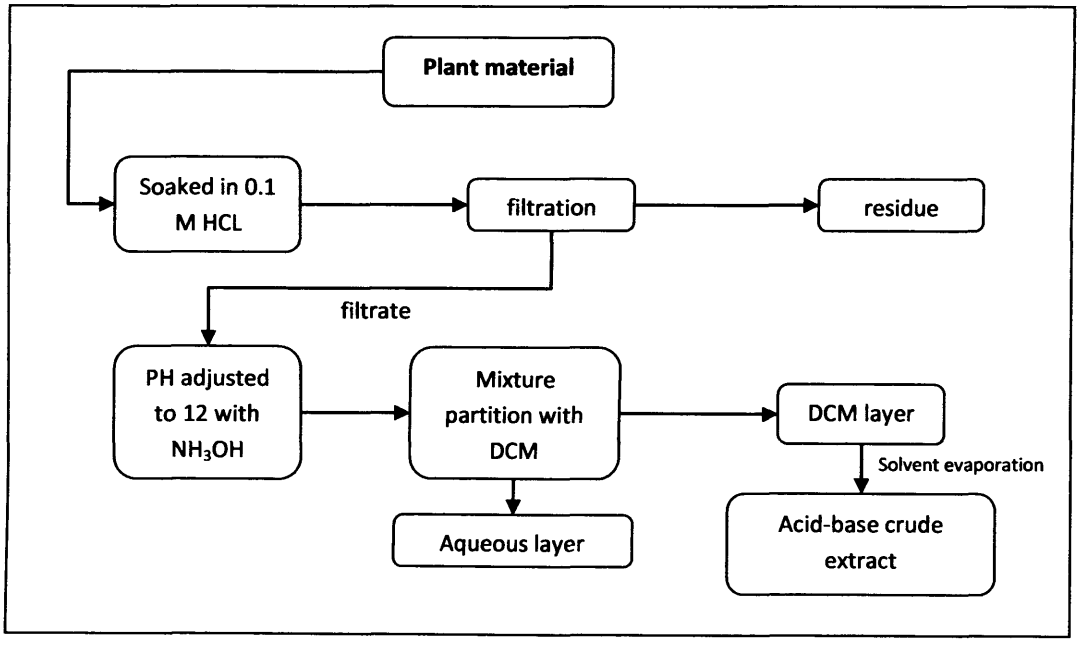


Figure 4.1. Simplified diagram for acid-base plant extraction

4.3.3 Acid-base extraction *V. negundo* and *M. oleifera*

Plant parts of *V. negundo* and *M. oleifera* were subjected to acid-base crude extraction protocols described in section 4.3.2. Air-dried *V. Negundo* (160.00 g) leaves and *M. oleifera* (190.00 g) bark each yielded 0.57 g and 0.09 g respectively.

4.3.4 Experimental protocol

After equilibration, a series of three cumulative CRCs for 5-HT, histamine, methacholine and β-PEA in the guinea pig ileum, repeated CRCs for histamine and β-PEA in the guinea pig trachea, and repeated CRCs for phenylephrine and β-PEA in the guinea pig aorta were obtained in the absence and presence and after washout of *V. negundo* acid-base extracts (VN-E) or *M. oleifera* acid-base extracts (MO-E) (Figure 4.9). Propranolol ($1x10^{-6}$ M) was added 10 minutes before each CRC in tracheal contractions due to β-PEA (refer to section 2.4.13). Preliminary experiments using 1 mg of the VN-E showed the total inhibition of contractile responses induced by 5-HT, methacholine, histamine and β-PEA. Due to this non-selective blocking property of the VN-E the amount was adjusted to 0.33 mg for all subsequent experiments. 1.00 mg of MO-E or 0.33 mg of VN-E was dissolved individually in 0.1 mL DMSO and incubated separately with the tissue for 20 minutes prior to the construction of each CRC_2. Approximate concentration of the plant extract in a 50 mL bath was therefore equivalent to $6.60x10^{-3}$ mg/mL for *V. negundo* and 0.02 mg/mL for *M. oleifera*.

4.4 Results

4.4.1 Effects of *V. negundo* acid-base extract on contractile responses of guinea pig ileum to 5-HT, methacholine, histamine and β-PEA

5-HT, methacholine, histamine and β-PEA caused concentration-dependent constrictor responses on the guinea pig ileum. The maximum contractile responses showed a significant inhibition in the presence of the VN-E to 5-HT ($n=4$, $P<0.01$) from CRC_1 (2.25±0.33 g) to CRC_2 (0.73±0.23 g) and histamine ($n=5$, $P<0.05$) from CRC_1 (1.29±0.28 g) to CRC_2 (0.56±0.11 g) (Table 4.2). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed also significant reduction ($P<0.01$) of the maximal contractile responses to 5-HT ($3x10^{-7}$ M) from 95.87±4.13% to 31.04±6.63% (Figure 4.2.a), histamine ($3x10^{-7}$ M) from 100.00±0.00% to 47.51±10.57% (Figure 4.2.c) and β-PEA ($1x10^{-3}$ M, $n=4$) from 92.62±4.67 to 23.21±11.77% (Figure 4.2.d). A non-significant inhibition of maximal responses to methacholine ($3x10^{-7}$ M, $n=5$) from 100.00% to 66.31±17.90 (Figure 4.2.b) was also observed. The contractile responses expressed as percentage of their own CRC maximum set to 100% showed a significant change to the -log EC_{50} of histamine from -8.10±0.17 to -7.27±0.21. Relatively large and variable dose ratios were obtained for 5-HT (46.7±34.0) and histamine (17.2±11.9) (Table 4.2).

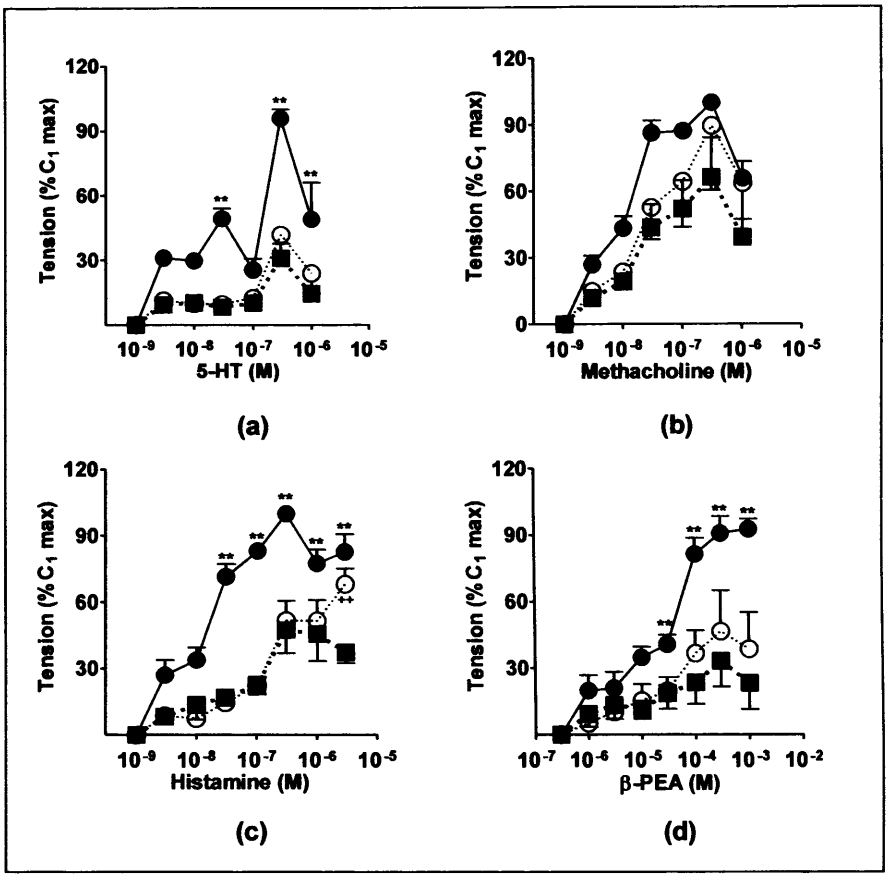


Figure 4.2. Effects of acid-base extracts of *V. negundo* (VN-E) on mean cumulative CRCs of guinea pig ileum for constriction to (a) 5-HT, n=4, (b) methacholine, n=5, (c) histamine, n=5, and (d) β-PEA, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant differences (**, $P<0.01$) between CRC_1 and CRC_2, and ($^{++}$, $P<0.01$) between CRC_2 and CRC_3. ●—● CRC_1 after 30 min tissue equilibration, ■--■ CRC_2 after 30 min tissue equilibration after the wash out of first curve, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve. 0.33 mg of *V. negundo* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2.

Table 4.2. Summary of the effects of *V. negundo* acid-base extracts (VN-E) on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant differences (*, $P<0.05$ or **, $P<0.01$) between CRC_1 and CRC_2, and ($^{++}$, $P<0.01$) between CRC_2 and CRC_3. 0.33 mg of *V. negundo* crude dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2. 0.33 mg of *V. negundo* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2.

Agonist		CRC_1	CRC_2	CRC_3	Dose ratio$\left(\frac{CRC_1}{CRC_2}\right)$	n
5-HT	Max (g)	2.25±0.33	0.73±0.23**	0.97±0.28		4
	-log EC_{50}	-8.22±0.68	-7.22±0.21	-7.17±0.21	46.72±33.96	
Methacholine	Max (g)	1.14±0.22	0.67±0.06	0.93±0.06^{++}		5
	-log EC_{50}	-8.35±0.12	-8.02±1.19	-7.40±0.39	3.59±1.50	
Histamine	Max (g)	1.29±0.28	0.56±0.11*	0.87±0.21		5
	-log EC_{50}	-8.10±0.17	-7.27±0.21*	-6.96±0.04	17.15±11.88	
β-PEA	Max (g)	0.75±0.26	0.24±0.08	0.40±0.19		4
	-log EC_{50}	-4.53±0.20	-4.71±0.15	-4.62±0.09	0.72±0.17	

4.4.2 Effects *V. negundo* acid-base extracts on contractile responses of guinea pig trachea to histamine and β-PEA

Histamine and β-PEA caused concentration-related constrictor responses on the guinea pig trachea. Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed significant effect of the VN-E to histamine (n=4) CRC at lower concentrations ($1x10^{-6}$ to $1x10^{-5}$ M) before the maximum occurred (Figure 4.3.a). In addition, the VN-E caused a significant shift of the curve to the right indicated by the change in -log EC_{50} values obtained for histamine from -5.86±0.14 to -5.21±0.12 resulting in a dose ratio of 4.87±1.37 (Table 4.3). No significant effect of the VN-E to β-PEA (n=4) was observed (Table 4.3, Figure 4.3.b). VN-E also causes a 23.7±1.52% and 4.81±4.93% baseline lowering to phenylephrine and β-PEA from the baselines before plant extract addition (Figure 4.4).

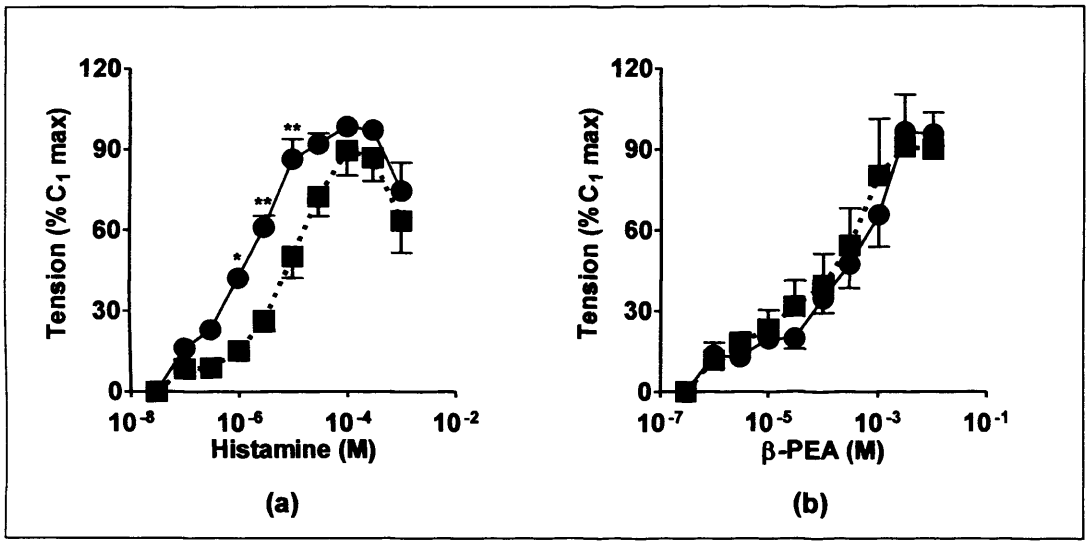


Figure 4.3. Effects of acid-base extracts of *V. negundo* (VN-E) on mean cumulative CRCs of guinea pig trachea for constriction to (a) histamine, *n=4*, (b) β-PEA, *n=4*. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA. Significant differences (*, $P<0.05$ or **, $P<0.01$) between CRC_1 and CRC_2. ●—● CRC_1 after 60 min tissue equilibration and ■–■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1. 0.33 mg of *V. negundo* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2. Propranolol ($1x10^{-6}$ M) was added 10 minutes before the construction of each CRCs in β-PEA.

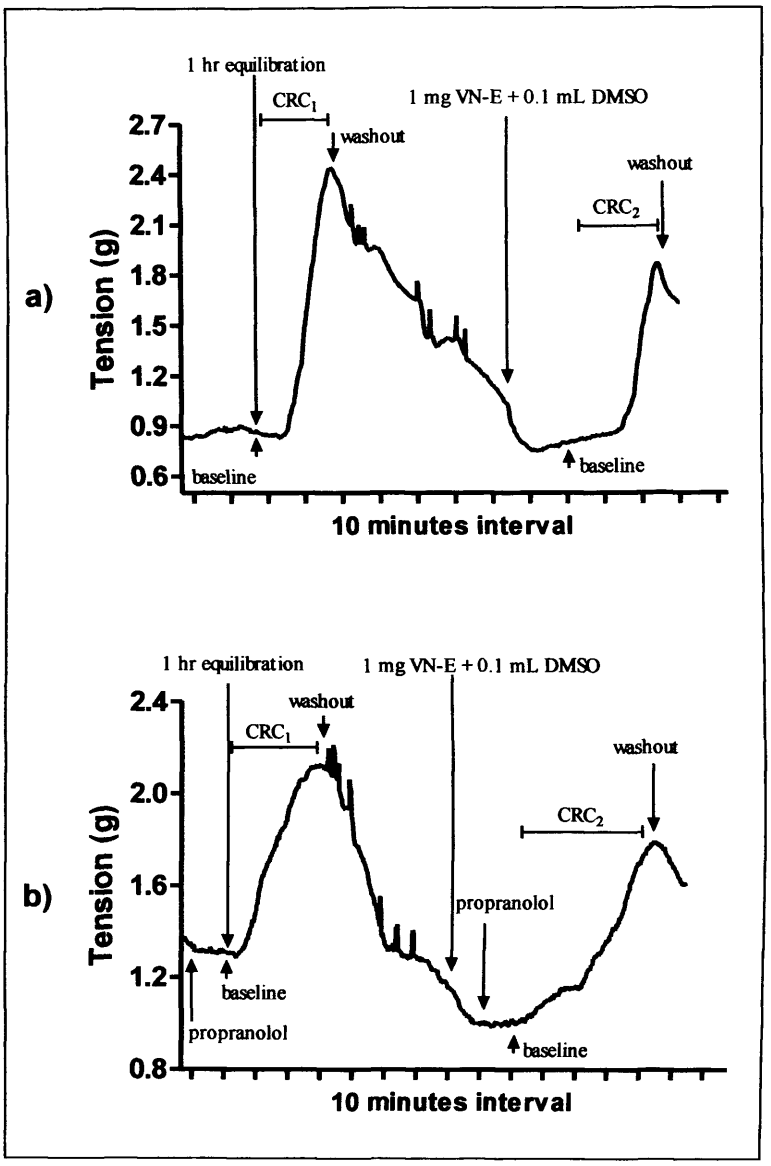


Figure 4.4. Representative chart recording showing the effects of *V. negundo* acid-base extracts in repeated contractile response CRCs of the guinea pig trachea to (a) histamine (refer to section 2.4.9 for histamine concentrations used for each CRC) and (b) β-PEA (refer to section 2.4.12 for β-PEA concentrations used for each CRC).

4.4.3 Effects *V. negundo* acid-base extracts on contractile responses of guinea pig aorta to phenylephrine and β-PEA

Phenylephrine and β-PEA caused concentration-related constrictor responses on the guinea pig aorta. Repeated cumulative CRCs for both phenylephrine and β-PEA previously showed a significant increase in the maximum contractions obtained in the second curve (refer to section 2.5.4 and section 2.5.5). In the presence of VN-E, however, the increase of the maximum in the second CRC was not observed to phenylephrine (n=4, Figure 4.5.a) and β-PEA (n=5, Figure 4.5.b). The -log EC_{50} values on phenylephrine and β-PEA also showed no significant changes in the presence of the plant extract (Table 4.3). VN-E also causes a 10.82±1.43% and 1.27±1.73% baseline lowering to phenylephrine and β-PEA from the baseline before plant extract addition (Figure 4.6).

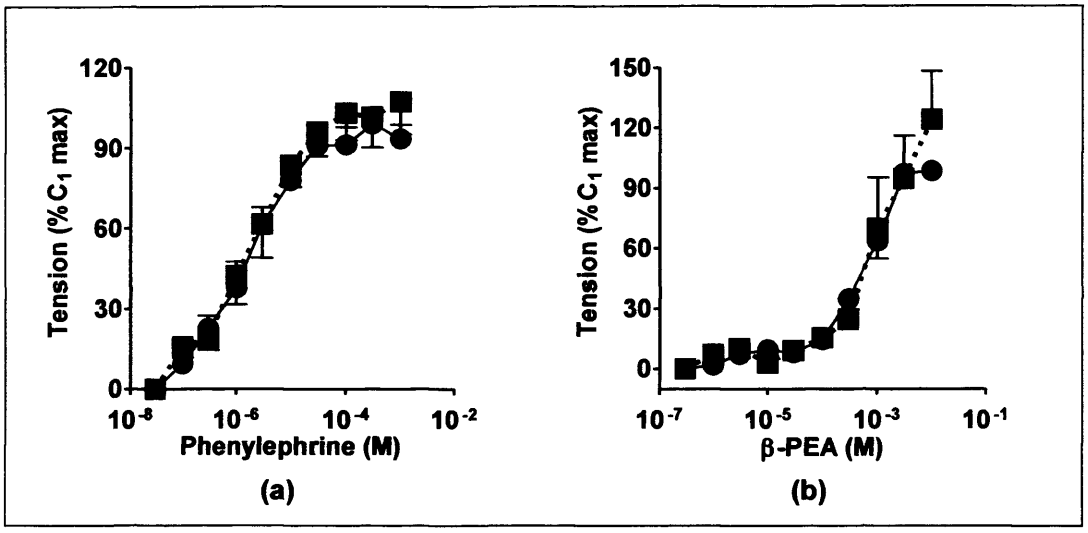


Figure 4.5. Effects of acid-base extracts of *V. negundo* (VN-E) on mean cumulative CRCs of guinea pig aorta for constriction to (a) phenylephrine, n=4, and (b) β-PEA, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA. No significant difference ($P>0.05$) between CRC_1 and CRC_2 was observed. ●—● CRC_1 after 60 min tissue equilibration and ■–■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1. 0.33 mg of *V. negundo* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2. 0.33 mg of *V. negundo* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2.

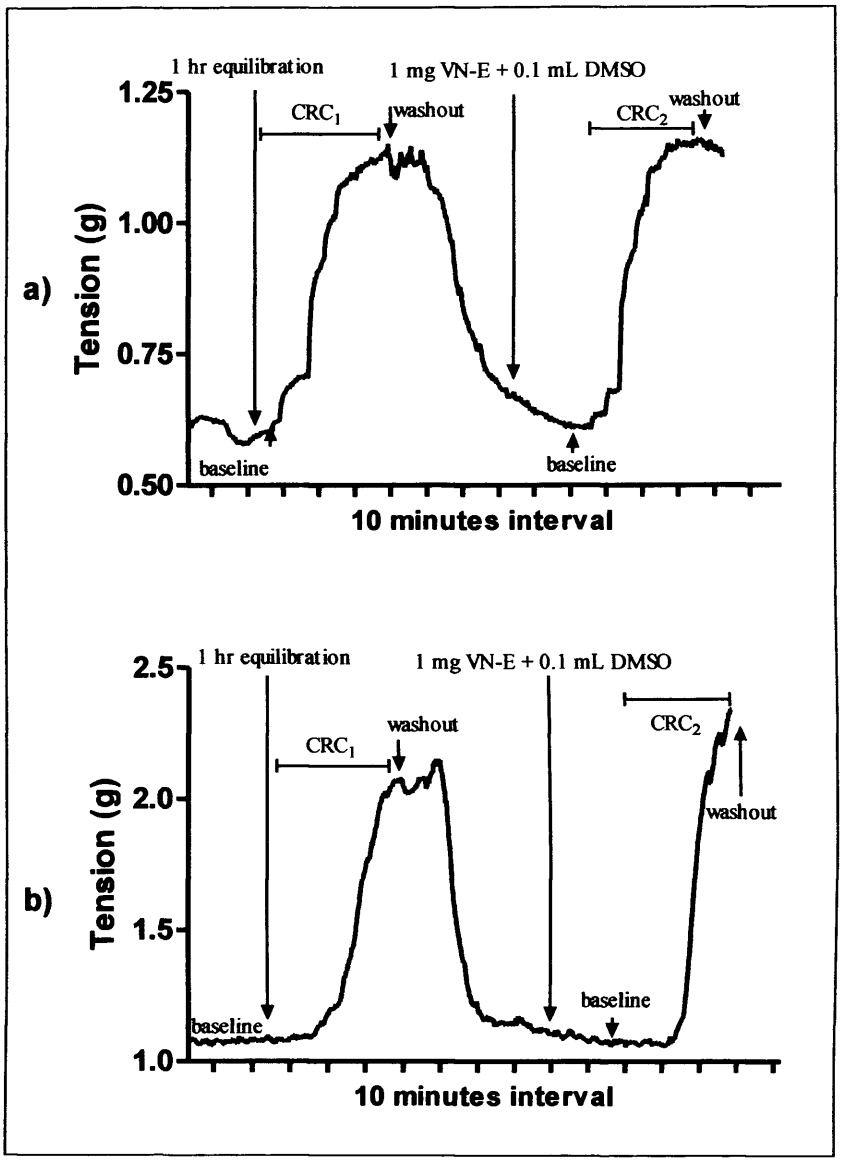


Figure 4.6. Representative chart recording showing the effects of *V. negundo* acid-base extracts in repeated contractile response CRCs of the guinea pig aorta to (a) phenylephrine (refer to section 2.4.15 for phenylephrine concentrations used for each CRC) and (b) β-PEA (refer to section 2.4.17 for β-PEA concentrations used for each CRC).

Table 4.3. Summary of the effects of *V. negundo* acid-base extracts (VN-E) on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig trachea to the agonist histamine and β-PEA, and aorta to phenylephrine and β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant differences **, $P<0.01$) between CRC_1 and CRC_2. 0.33 mg of *V. negundo* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2. Propranolol ($1x10^{-6}$ M) was added 10 minutes before the construction of each CRC in β-PEA.

Tissue	Agonist		CRC_1	CRC_2	Dose ratio$\left(\frac{CRC_1}{CRC_2}\right)$	n
Trachea	Histamine	Max (g)	1.07±0.23	0.93±0.13		4
		-log EC_{50}	-5.86±0.14	-5.21±0.12**	4.87±1.37	
	β-PEA	Max (g)	0.74±0.20	0.62±0.10		4
		-log EC_{50}	-3.34±0.22	-3.50±0.30	2.07±1.42	
Aorta	Phenylephrine	Max (g)	0.93±0.18	0.96±0.15		4
		-log EC_{50}	-5.80±0.16	-5.73±0.23	1.25±0.27	
	β-PEA	Max (g)	0.69±0.13	0.84±0.20		4
		-log EC_{50}	-3.14±0.10	-2.74±0.29	3.82±2.12	

4.4.4 Effects of *M. oleifera* acid-base extract on contractile responses of guinea pig ileum to 5-HT, methacholine, histamine and β-PEA

5-HT, methacholine, histamine and β-PEA caused concentration-related constrictor responses on the guinea pig ileum. The maximum contractile response of 5-HT (n=4), methacholine (n=4) and β-PEA (n=4) showed a significant ($P<0.05$) degree of inhibition in the presence of the MO-E from 2.61±0.31 g, 3.21±0.95 g and 0.68±0.14 g to 1.70±0.54 g, 1.92±0.38 g and 0.33±0.06 g respectively. Significant recovery of responses ($P<0.05$) was observed in β-PEA (0.74±0.12) after the MO-E washout (Table 4.4). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% confirmed the significant reduction of the maximum responses to 5-HT ($1x10^{-6}$ M, $P<0.01$) from 100.00±0.00% to 62.96±14.77%, methacholine ($1x10^{-6}$ M, $P<0.01$) from 93.95±6.05% to 59.41±7.94% and β-PEA ($1x10^{-3}$ M, $P<0.05$) from 89.56±10.10% to 43.04±13.54% (Figure 4.7). The -log EC_{50} values showed no significant change in the presence of the plant extract, however variable shift to the right of the curves was indicated by dose ratio obtained for 5-HT (11.72±9.28) and β-PEA (17.69±17.59) (Table 4.4).

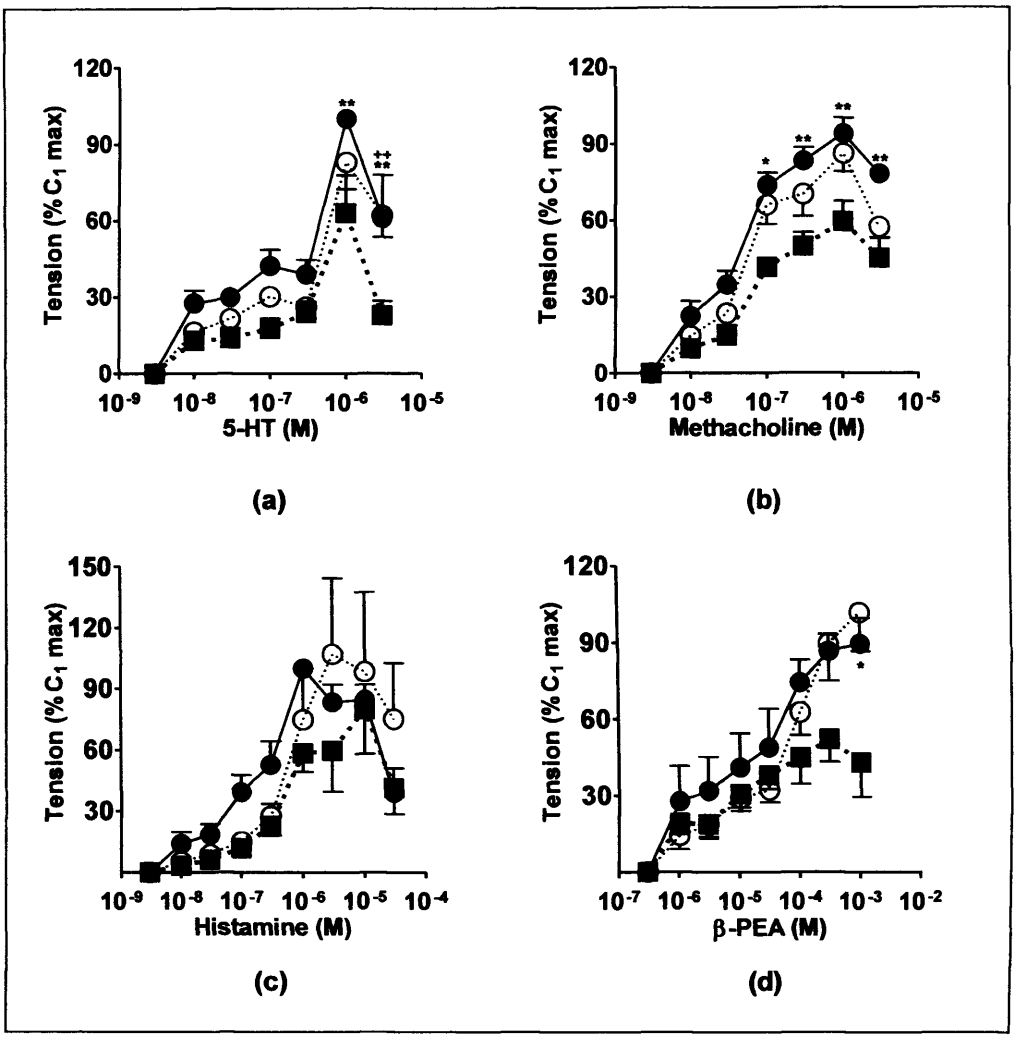


Figure 4.7. Effects of acid-base extracts of *M. Oleifera* (MO-E) on mean cumulative CRCs of guinea pig ileum for constriction to (a) 5-HT, n=4, (b) methacholine, n=4, (c) histamine, n=5, and (d) β-PEA, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant differences (*, $P<0.05$ or **, $P<0.01$) between CRC_1 and CRC_2 or ($^{++}$, $P<0.01$) between CRC_2 and CRC_3. ●—● CRC_1 after 30 min tissue equilibration, ■--■ CRC_2 after 30 min tissue equilibration after the wash out of first curve, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve. 1 mg of *M. oleifera* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2.

Table 4.4. Summary of the effects of *M. oleifera* acid-base extracts (MO-E) on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to the agonist 5-HT, methacholine, histamine and β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant differences (*, $P<0.05$) between CRC_1 and CRC_2 or ($^{+}$, $P<0.05$ or $^{++}$, $P<0.01$) between CRC_2 and CRC_3. 1 mg of *M. oleifera* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2.

Agonist		CRC_1	CRC_2	CRC_3	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
5-HT	**Max (g)**	2.61±0.31	1.70±0.54*	2.17±0.30		4
	-log EC_{50}	-7.38±0.57	-7.01±0.14	-6.48±0.21	11.72±9.28	
Methacholine	**Max (g)**	3.21±0.95	1.92±0.38*	2.67±0.61		4
	-log EC_{50}	-7.44±0.19	-7.16±0.10	-7.35±0.12	2.47±1.13	
Histamine	**Max (g)**	2.25±0.87	1.60±0.44	1.81±0.43		4
	-log EC_{50}	-7.04±0.06	-6.55±0.25	-6.53±0.18	4.39±1.84	
β-PEA	**Max (g)**	0.68±0.14	0.33±0.06*	0.74±0.12^{+}		4
	-log EC_{50}	-5.09±0.71	-5.64±0.35	-4.27±0.35^{++}	17.69±17.59	

4.4.5 Effects *M. oleifera* acid-base extracts on contractile responses in guinea pig trachea to repeated CRCs for histamine and β-PEA

Histamine and β-PEA caused concentration-dependent contractile responses in guinea pig trachea. The maximum contractile response to histamine (0.92±0.11 g, n=4) showed a significant ($P<0.05$) potentiation (1.24±0.14 g) in the presence of the MO-E (Table 4.5). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed a significant ($P<0.05$) increase to histamine ($3x10^{-4}$ M) from 97.60±1.00% to 125.51±9.43% when treated with the plant extract (Figure 4.8.a). No significant changes in the -log EC_{50} values for histamine CRC was observed. The plant extract also showed no significant effects to contractile responses induced by β-PEA (n=4) (Figure 4.8.b, Table 4.5). MO-E causes also a 16.76±5.34% and 6.90±3.90% baseline lowering in the histamine and β-PEA experiments from the resting baseline before plant extract addition (Figure 4.9).

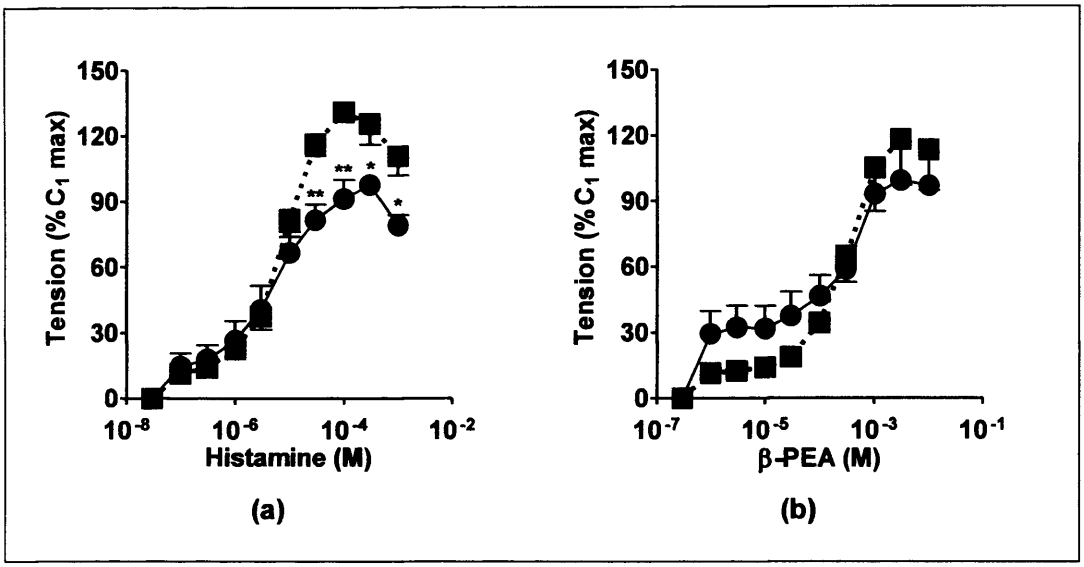


Figure 4.8. Effects of acid-base extracts of *M. Oleifera* (MO-E) on mean cumulative CRCs of guinea pig trachea for constriction to (a) histamine, n=4, (b) β-PEA, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA. Significant difference **, $P<0.01$) were observed on (a). ●—● CRC_1 after 60 min tissue equilibration and ■–■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1. Propranolol ($1x10^{-6}$ M) was added 10 minutes before the construction of each CRC in β-PEA. Significant differences (*, $P<0.05$ or **, $P<0.01$) between CRC_1. 1 mg of *M. oleifera* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2.

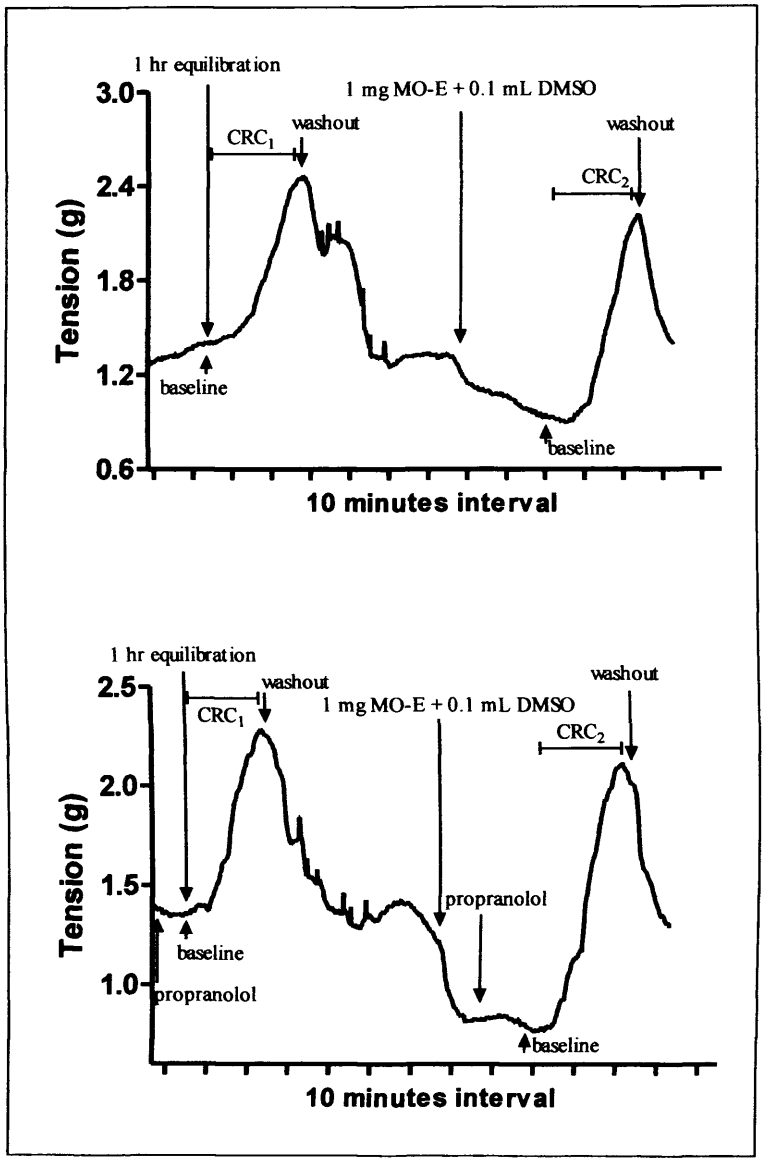


Figure 4.9. Representative chart recording showing the effects of *M. oleifera* acid-base extracts in repeated contractile response CRCs of the guinea pig trachea to (a) histamine (refer to section 2.4.9 for histamine concentrations used for each CRC) and (b) β-PEA (refer to section 2.4.12 for β-PEA concentrations used for each CRC).

4.4.6 Effects *M. oleifera* alkaloid extracts on contractile responses in guinea pig aorta to repeated CRCs for phenylephrine and β-PEA

Phenylephrine and β-PEA caused concentration-dependent constrictor responses to guinea pig aorta. Repeated cumulative CRCs for both phenylephrine and β-PEA without any treatment previously showed significant potentiation in the contractile maximum obtained in the second curve (refer to Figure 2.21, Figure 2.23 and Table 2.4). The maximum contractile responses to phenylephrine (0.96±0.26 g, n=4) and β-PEA (0.62±0.26 g, n=5) in the presence of MO-E were not significantly different ($P>0.05$) from the responses in its absence (1.12±0.31 g and 0.57±0.23 g) (Table 4.5). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed no significant ($P>0.05$) differences obtained for phenylephrine ($1x10^{-3}$ M) from 99.67±0.19% to 96.62±15.01%, and β-PEA ($1x10^{-2}$ M) from 98.64±1.36% to 86.87±13.14% (Figure 4.10). No significant change in the -log EC_{50} values on phenylephrine and β-PEA was observed (Table 4.5). MO-E also causes also a baseline lowering of 13.80±4.71% to phenylephrine and 2.85±3.850% increase to β-PEA baseline from the resting baselines before plant extract addition (Figure 4.11).

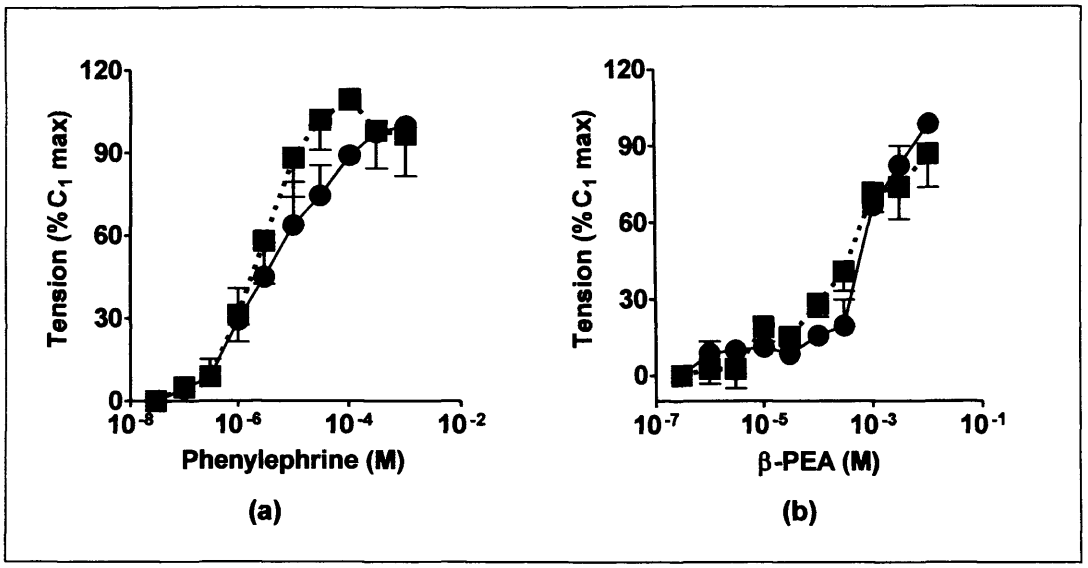


Figure 4.10. Effects of acid-base extracts of *M. Oleifera* (MO-E) on mean cumulative CRCs of guinea pig aorta for constriction to (a) phenylephrine, n=4, and (b) β-PEA, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA. No significant difference ($P<0.05$) were seen on phenylephrine and β-PEA. ●—● CRC_1 after 60 min tissue equilibration and ■--■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1. 1 mg of *M. oleifera* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2.

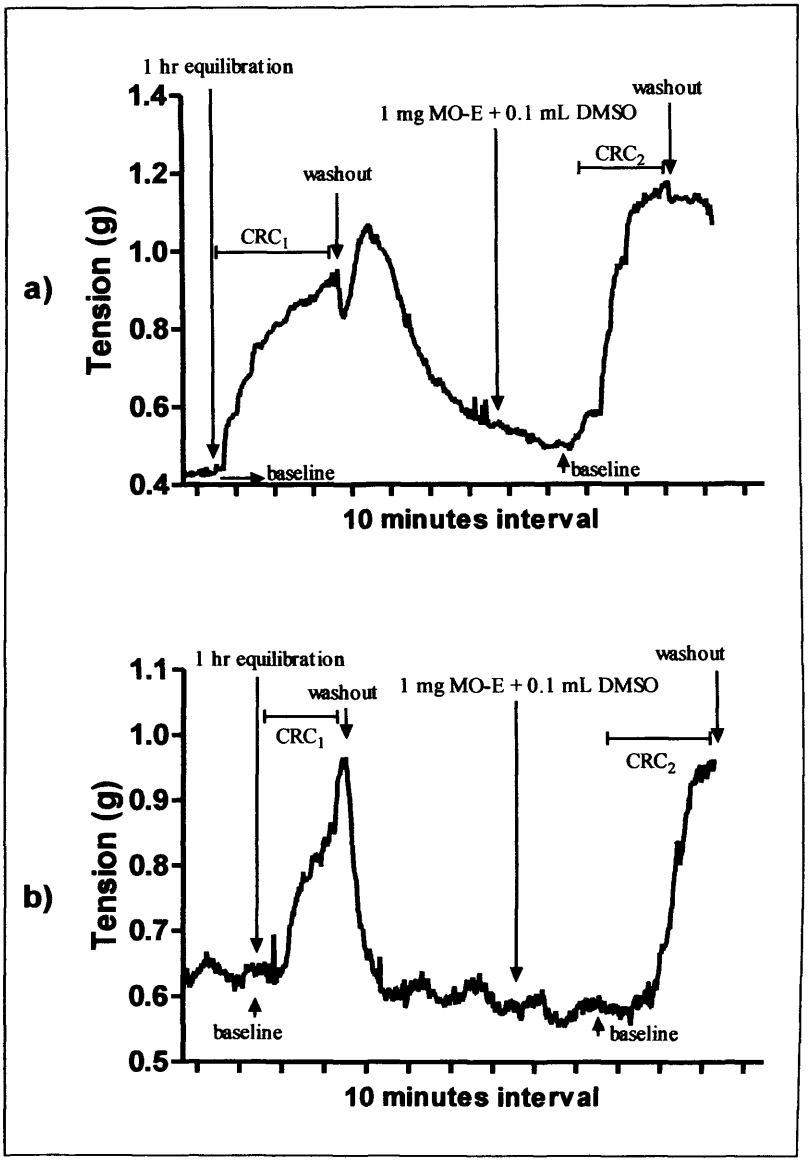


Figure 4.11. Representative chart recording showing the effects of *M. oleifera* acid-base extracts in repeated contractile response CRCs of the guinea pig aorta to (a) phenylephrine (refer to section 2.4.15 for phenylephrine concentrations used for each CRC) and (b) β-PEA (refer to section 2.4.17 for β-PEA concentrations used for each CRC).

Table 4.5. Summary of the effects of *M. oleifera* acid-base extracts (MO-E) on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig trachea to the agonist histamine and β-PEA, and aorta to phenylephrine and β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant difference (**, $P<0.01$) between CRC_1 and CRC_2. 1 mg of *M. oleifera* crude extract dissolved in 0.1 mL DMSO was added 20 min prior to the construction of CRC_2. Propranolol ($1x10^{-6}$ M) was added 10 minutes before the construction of each CRCs in β-PEA.

Tissue	Agonist		CRC_1	CRC_2	Dose ratio$\left(\frac{CRC_1}{CRC_2}\right)$	n
Trachea	Histamine	Max (g)	0.92±0.11	1.24±0.14**		4
		-log EC_{50}	-5.44±0.25	-5.24±0.10	2.72±1.77	
	β-PEA	Max (g)	0.76±0.18	0.85±0.18		4
		-log EC_{50}	-3.78±0.18	-3.55±0.12	2.95±1.95	
Aorta	Phenylephrine	Max (g)	0.96±0.26	1.12±0.31		4
		-log EC_{50}	-5.42±0.37	-5.57±0.29	1.09±0.48	
	β-PEA	Max (g)	0.62±0.26	0.57±0.23		5
		-log EC_{50}	-3.00±0.15	-3.54±0.31	1.01±0.62	

4.5 Discussion

4.5.1 The antagonistic activity of the acid-base extract of *V. negundo* on contractile response CRCs of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA

VN-E (0.33 mg) selectively blocks the maximum contractile responses in the guinea pig ileum induced by 5-HT, histamine and β-PEA. It causes also a non-significant inhibition to methacholine. However, higher concentrations of VN-E totally inhibited 5-HT, methacholine, histamine and β-PEA non-selectively. The lack of specific activity for inhibiting the maximum at high concentrations of VN-E indicates that it contains a membrane stabilizing or channel blocking properties. Similar studies done by Dharmasiri *et al.* (2003) on the water leaf extracts showed the presence of a membrane stabilizing property. The plant extract also showed a high and variable dose ratio for 5-HT but no significant shift of the curve was observed. The presence of a selective-competitive histamine H_1 antagonist was also revealed indicated by curve shift to higher concentrations.

4.5.2 The antagonistic activity of the acid-base extract of *V. negundo* on contractile response CRCs of the guinea trachea to histamine and β-PEA

Traditionally the leaf of *V. negundo* was used to treat asthma (Dayrit *et al.*, 1987) and allergy and itching of the skin (Vishwanathan *et al.*, 2010). Asthma is a chronic inflammatory disease of the respiratory tract that is characterized by airway

hyperresponsiveness and infiltration of inflammatory cells such as mast cells which releases histamine (Deng *et al.*, 2006; Santing *et al.*, 2001; Santing *et al.*, 1994). Allergy and itching of the skin primarily occurs through the stimulatory action of histamine to histamine H_1 receptors on the nerve endings (Minami *et al.*, 2004; Yatsuzuka *et al.*, 2007). In the alleviation of acute allergic reactions, antagonism of histamine H_1 receptors has been known as a therapeutic target (Matsubara *et al.*, 2005).

Histamine causes smooth muscle contraction though the activation of histamine H_1 receptor which is directly linked to G-protein G_q that triggers the stimulation of PLC which leads to the depletion of phosphatidylinositol biphosphate (PIP_2) that subsequently translate to increase of cytoplasmic Ca^{2+} via intracellular Ca^{2+} release (Matsubara *et al.*, 2005; Notcovich *et al.*, 2010; Sohen *et al.*, 2001).

In the present study the presence of a selective-competitive histamine H_1 antagonist in VN-E was further confirmed on the guinea trachea which was indicated by curve shift to the right which was similarly observed in the ileum. Previously it was also shown that the leaf extracts causes bronchodilatation of the cat's trachea (Dayrit *et al.*, 1987). In the report by Dharmasiri *et al.* (2003) the anti-inflammatory and anti-nociceptive activity of *V. negundo* water leaf extracts in rats was shown to be mediated through its antihistamine and membrane stabilising property or through the prostaglandins (PG) synthesis inhibition. For this reasons *V. negundo* traditional use for treatment in diseases related to the airways and allergy of the skin is suggested to be largely mediated through the action of highly polar components present in the plant through the inhibition of the histamine H_1 receptors.

The VN-E showed no significant inhibition against β-PEA.

4.5.3 The antagonistic activity of the acid-base extract of *V. negundo* on contractile response CRCs of the guinea pig aorta to phenylephrine and β-PEA

Previously it was shown that that repeated CRCs of the guinea pig aorta for both phenylephrine and β-PEA causes potentiation of the maximum responses which might be due to repeated uptake and reuptake of transient intracellular Ca^{2+}. In the presence of VN-E this potentiation was totally inhibited for both phenylephrine and β-PEA. This suggests that the plant extract might be interfering with the uptake and reuptake of intracellular Ca^{2+} which is mediated either via the α_1-adrenoceptor or TAAR related mechanisms.

4.5.4 The antagonistic activity of acid-base extract *M. oleifera* on guinea pig ileum to 5-HT, methacholine, histamine and β-PEA, and trachea to histamine and β-PEA

Pharmacological screening of MO-E in the guinea pig ileum showed a selective and non-competitive reversible inhibition to the maximum contractile response to 5-HT, methacholine, and β-PEA. The plant extract showed no significant inhibition to the maximum contractions and showed no significant shift of the curve to histamine but the maximum dose is shifted to higher concentrations.

In the guinea pig trachea, MO-E showed a significant potentiation of the maximum contractions elicited by histamine which implied the presence of components acting as an agonist to histamine H_1 receptors. However, MO-E did not exert contractile effects

when added to the tissue but caused a relaxation. Therefore, the potentiation must be due to either an allosteric interaction at the H_1 receptors or an additive effect of a threshold contraction. Rather than contracting the baseline, MO-E significantly lowers the baseline suggesting the presence of a smooth muscle relaxing component. Since this was also observed before the addition of propranolol (β-adrenoceptor antagonist) in the β-PEA experiments, the relaxation cannot be due to β-adrenoceptor mechanism. No significant effect of MO-E was observed on β-PEA.

In the gut *M. oleifera* is traditionally used in ailments like gastrointestinal motility (Gilani *et al.*, 1994), spasm of the bowels, pain and inflammation (Caceres *et al.*, 1992; Ezeamuzie *et al.*, 1996; Goyal *et al.*, 2009; Mahajan *et al.*, 2007; Sulaiman *et al.*, 2008), diarrhoea (Saralaya *et al.*, 2010), and used for the treatment of cattle dysentery (Anisuzzaman *et al.*, 2007). In the airways the plant is known for treating asthma (Goyal *et al.*, 2009) and also has anti-nociceptive and anti-inflammatory properties (Anwar *et al.*, 2007).

In the present study on the ileum and trachea, MO-E failed to explain previous reported therapeutic uses of the plant due to the lack of antagonism on the selected receptors. This might be due to several reasons like the concentration of the active component is very low in the bark, the active components were not extracted using the current method, the active components of the plant is not in the acid-base extract or the acidic environment used to extract the plant components degraded some of the active constituents.

4.5.5 The antagonistic activity of acid-base extract *M. oleifera* on repeated CRCs of the guinea pig aorta to phenylephrine and β-PEA

Earlier study on *M. oleifera* revealed that it possesses antihypertensive property (Anwar *et al.*, 2007; Dangi *et al.*, 2002). Nitrile glycosides and mustard oil glycosides from the plant was also reported to exhibit hypotension (Faizi *et al.*, 1994; Faizi *et al.*, 1992; Gilani *et al.*, 1994). In addition, the total alkaloid salts of the leaf water extracts was shown to weaken the force of muscular contractions of the frog heart (Dangi *et al.*, 2002). However these reported properties of *M. oleifera* is in disagreement with it folkloric use as cardiotonic (Biswas *et al.*, 1988). Its use as a cardiac stimulant (Oliver-Bever, 1986) can be clarified in the isolation of two alkaloids moringine (Figure 4.12) and moringinine in the root and bark (Bour *et al.*, 2005; Gupta *et al.*, 1999; Karadi *et al.*, 2006). Moringinine causes symphathomimetic effects similar to that of adrenaline which results to vasoconstriction and blood pressure increase (Oliver-Bever, 1986).

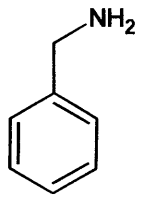


Figure 4.12. Moringine

In the guinea pig aorta, MO-E showed no significant differences on the maximum contractions induced by phenylephrine and β-PEA. Previously it was shown that the repeated CRCs of the aorta to phenylephrine and β-PEA caused potentiation on the

maximum contractile responses. This suggest that MO-E might be blocking the increase of transient Ca^{2+} during Ca^{2+} uptake and reuptake induced by repeated stimulation of the α_1-adrenoceptors or TAARs which might explain the antihypertensive or hypotensive properties or the plant.

4.6 Conclusion

In conclusion, *V. negundo* possesses smooth muscle relaxing properties in the gut and trachea due to the presence of membrane stabilizing components and histamine H_1 receptor antagonist. The selective-competitive inhibition of the plant acid-base extracts due to histamine H_1 receptor provides a sound mechanism for its traditional use in alleviating allergic disorders such as asthma and itching of the skin. Its anti-inflammatory properties might be suggested also to be mediated though histamine H_1 receptor inhibition or through its Ca^{2+} blocking mechanisms mediated either by α_1-adrenoceptors or TARRs.

Moringa oleifera known to cure ailments related to the bowels and airways failed to explain previous reported therapeutic uses due to the lack of antagonism on the selected receptors. Instead the presence of components acting as an agonist to histamine H_1 receptors have been be suggested to be present. The plant antihypertensive and hypotensive property can also be suggested to be due to its Ca^{2+} inhibiting properties mediated either by α_1-adrenoceptors or TARRs.

CHAPTER 5

Pharmacological effects of *Artemisia vulgaris* on responses of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA, and trachea to histamine and β-PEA

5.1 Introduction

Artemisia vulgaris is a herb commonly used in traditional or alternative medicine (Tigno *et al.*, 2000b). An array of medicinal uses of the plant include anti-inflammatory, antiasthma, analgesic, expectorant, antispasmodic, emmenagogue, useful in treatment of abdominal colic, dyspepsia and diarrhoea, (Duke, 1983; Khan *et al.*, 2009; Quisimbing, 1978) and antihypertensive (Tigno *et al.*, 2000a; Tigno *et al.*, 2000b). The pollen of *A. vulgaris* has been also implicated to induce allergy of the airways (Pastorello *et al.*, 2002).

The present chapter was undertaken to examine the possible antagonistic activity of *A. vulgaris* extracts on responses of the guinea pig ileum mediated via 5-HT_2 receptor, muscarinic M_3 receptor, histamine H_1 receptor and Trace amine-associated receptor (TAAR), and trachea mediated via histamine H_1 receptor and TAAR.

5.2 Aims

- To study the antagonistic property of *Artemisia vulgaris* chloroform and methanol crude extracts to 5-HT, methacholine, histamine and β-PEA employing cumulative concentration response curves (CRCs) to the contractile responses of guinea pig isolated ileum.
- To study the antagonistic property of *Artemisia vulgaris* chloroform and methanol crude extracts to histamine and β-PEA employing cumulative concentration response curves (CRCs) to the contractile responses of guinea pig isolated trachea.
- Collection of *Artemisia vulgaris* and preparation of crude extracts
- To isolate and elucidate the histamine H_1 antagonist components of *Artemisia vulgaris* chloroform extract.

5.3 Methods and Materials

The main methods and experimental protocols described in Chapter 2 were retained throughout this study unless otherwise stated

5.3.1 General chemistry

All extractions were carried out under atmospheric conditions. All reagents and solvents employed were of general purpose or analytical grade and purchased from Sigma-Aldrich (Poole, Dorset, UK) or Fisher Scientific (Leicestershire, UK).

For normal phase column chromatography, a glass column was slurry packed in appropriate eluent with silica gel (Fluka Kieselgel 60). For size exclusion column chromatography 50 g of Sephadex LH-20 was swelled with 80:20 methanol-chloroform solvent then eluted with a rate of 1 cm/hr in a 30 mm diameter glass column. Flash chromatography was performed with the aid of a pump. Preparative thin layer chromatography (TLC) (1000 μm) and analytical thin layer chromatography was performed on a pre-coated silica plates (Merck Kieselgel 60) with visualisation via UV light (254 and 365 nm) and/or vanillin stain.

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance DP500 spectrometer at 500 MHz and 125MHz respectively. The NMR solvent was deuterated chloroform ($CDCl_3$) for all cases. Mass spectra were determined under Electron Impact (EI) or Chemical ionization (CI) conditions at the ESPRC National Mass Spectrometry Service Centre, University of Wales, Swansea, Wales, UK. Accurate mass measurements were also performed at the ESPRC National Mass

Spectrometry Service Centre. X-ray crystallography analysis was carried out in the School of Chemistry, Cardiff University, Cardiff, Wales, UK.

5.3.2 *Artemisia vulgaris* collection and crude extraction

Artemisia vulgaris leaves were collected from Aritao, Nueva Vizcaya, Philippines in September 2007 for preliminary pharmacological evaluation (Table 5.1). A voucher specimen was submitted to the Philippine National Museum for plant identification (see appendix). The plant samples were sent intact from Nueva Vizcaya, Philippines to Cardiff University, Wales, UK.

Air-dried *A. vulgaris* was subjected to chloroform and methanol extraction protocols described in section 3.3.2. An initial weight of 243 g of *A. vulgaris* leaves yielded 7.9 g of chloroform crude layer (AV-$CHCl_3$) and 7.52 of methanol crude layer (AV-MeOH) (Figure 5.1).

Table 5.1. Collection of *Artemisia vulgaris*

Family	Asteraceae
Species	*Artemisia vulgaris*
Parts used	Leaves
Drying method	Air drying method (cabinet)
Place	Aritao, Nueva Vizcaya, Philippines
Date:	September 2007
Collected by:	Violeta Abanto and Eric Corpus
Place	Bayombong, Nueva Vizcaya, Philippines
Date:	September 2008-January 2009
Cultivated and harvested by:	Annabelle A. Natividad

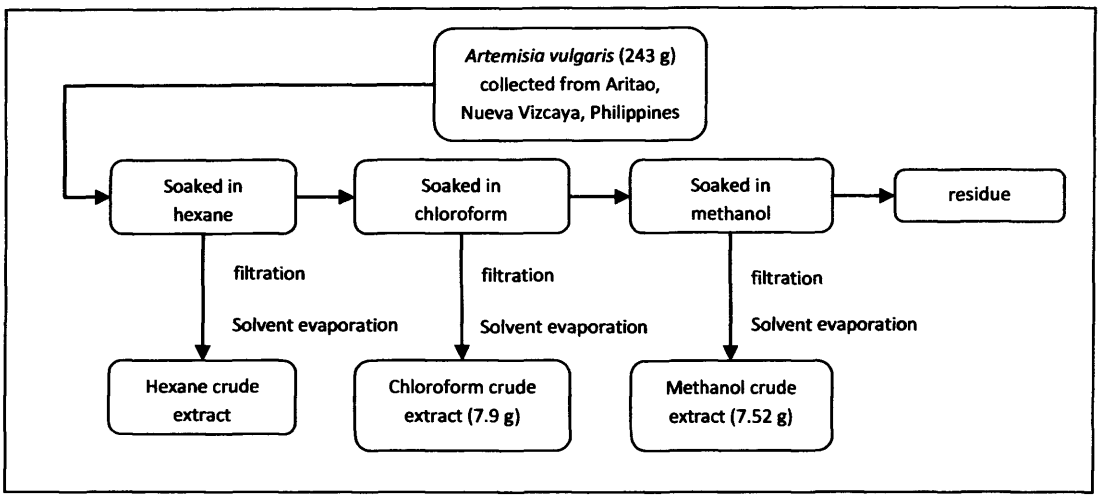


Figure 5.1. Simplified diagram for chloroform and methanol extraction of *A. vulgaris*.

5.3.3 Chloroform extraction of *Artemisia vulgaris*

An additional batch of *Artemisia vulgaris* was cultivated and collected in Bayombong, Nueva Vizcaya, Philippines from September 2008 – January 2009 (Table 5.1). Air dried leaves (1.03 kg) was sent intact to Cardiff University, Cardiff, Wales, UK. The leaves were pulverised to powder and soaked with excess chloroform for at least 24 hours. The resulting mixture was filtered and dried *in vacuo* yielding a syrupy product (53 g) (Figure 5.7). The crude extract were then stored in a freezer at -20°C.

5.3.4 Experimental protocol

After equilibration a series of cumulative CRCs for 5-HT, histamine, methacholine and β-PEA in the guinea pig ileum, and histamine and β-PEA in the guinea pig trachea were obtained in the absence and presence or after washout of different crude extracts of *A. vulgaris* leaves chloroform (AV-$CHCl_3$) or methanol (AV-MeOH) extract (refer to section 5.4.2). Propranolol ($1x10^{-6}$ M) was added 10 minutes before each CRC in tracheal contractions due to β-PEA (refer to section 2.4.13). One milligram of each of the crude extracts were dissolved in 0.1 mL DMSO and incubated separately with the tissue for 20 minutes prior to the construction of each CRC_2. Approximate concentration of the plant extract in a 50 mL bath was therefore equivalent to 0.02 mg/mL.

5.4 Results

5.4.1 Effects of *A. vulgaris* chloroform crude extracts on contractile responses in guinea pig ileum to 5-HT, methacholine, histamine and β-PEA

5-HT, methacholine, histamine and β-PEA caused concentration-dependent contractile responses on the guinea pig ileum. The maximum contractile responses to 5-HT (n=4), histamine (n=4) and β-PEA (n=4) showed significant ($P<0.05$) inhibition in the presence of AV-$CHCl_3$ from 1.17±0.25 g, 1.41±0.26 g and 0.70±0.23 g to 0.10±0.02 g, 0.52±0.13 g and 0.25±0.05 g respectively (Table 5.2). After the plant extract washout only histamine CRC (1.22±0.29 g) showed a significant ($P<0.05$) recovery of responses (Table 5.2). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed significant ($P<0.01$) effect of the plant extract to 5-HT ($1x10^{-6}$ M) from 93.12±6.88% to 13±2.74%, methacholine ($1x10^{-6}$ M, n=4) from 98.17±1.83% to 47.31±6.41%, histamine ($1x10^{-6}$ M) from 88.13±11.87% to 8.62±1.92% and β-PEA ($3x10^{-3}$ M) from 100.00±0.00% to 35.05±9.16% (Figure 5.2). Significant recovery ($P<0.01$) of contractile responses after plant extract washout were observed for 5-HT 29.15±8.22% (Figure 5.2.a) and to the new maximal dose for histamine ($1x10^{-5}$ M), 86.46±14.18% (Figure 5.2.c). The contractile responses expressed as percentage of their own CRC maximum set to 100% showed significant change ($P<0.05$) in the true -log EC_{50} values to histamine from -6.76±0.15 to -5.08±0.36 resulting to a high variable dose ratio of 141.24±105.39 (Table 5.2). In addition the pseudo EC_{25} values obtained for histamine also showed a significant

($P<0.05$) parallel shift of the curves to higher concentration from -6.57±0.12 to -5.13±0.41 resulting in a variable dose ratio of 89.62±73.22 (Figure 5.2.c).

5.4.2 Effects of *A. vulgaris* methanol crude extracts on contractile responses in guinea pig ileum to 5-HT, methacholine, histamine and β-PEA

5-HT, methacholine, histamine and β-PEA caused concentration-dependent contractile responses on the guinea pig ileum. The maximum contractile responses to 5-HT (1.26±0.27 g, n=4) and histamine (2.75±0.83 g, n=4) showed a significant ($P<0.01$) reduction to 0.68±0.21 g and 2.38±0.81 g in the presence of AV-MeOH (Table 5.3). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed significant effect of the plant extract to 5-HT ($P<0.01$, $3x10^{-6}$ M) from 98.41% to 38.18±11.00%, methacholine ($P<0.05$, $1x10^{-6}$ M, n=4) from 100.00±0.00% to 68.57±7.00%, histamine ($P<0.01$, $3x10^{-6}$ M) from 89.59±6.42% to 55.12±5.77% and β-PEA ($P<0.01$, $3x10^{-3}$ M, n=4) from 93.49±4.56% to 62.21±2.07% (Figure 5.3). The contractile responses expressed as percentage of their own CRC maximum set to 100% further showed significant ($P<0.01$) shift of the curve to the right only for histamine indicated by the -log EC_{50} values obtained from -6.72±0.16 to -6.05±0.13 with a dose ratio of 4.85±0.70 (Table 5.3).

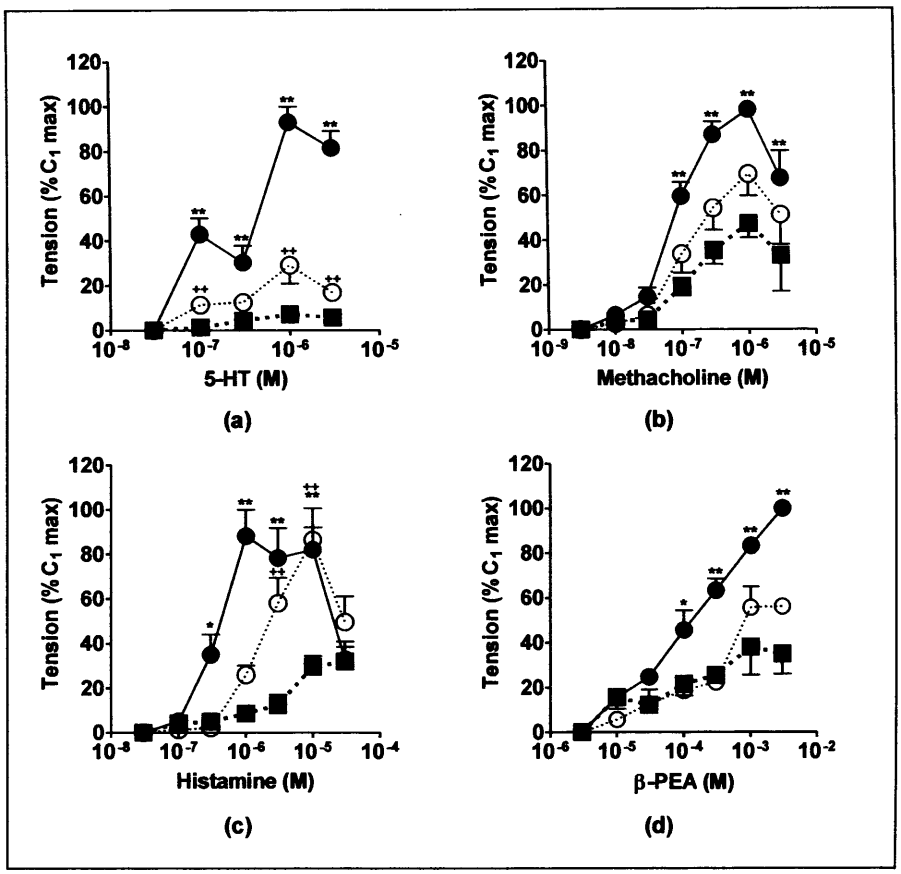


Figure 5.2. Effects of *A. vulgaris* chloroform crude extract on mean cumulative CRCs of guinea pig ileum for constriction to (a) 5-HT, n=5, (b) methacholine, n=4, pseudo EC_{30}: CRC_1 = -7.29±0.07, CRC_2 = -6.90±0.17, dose ratio = 2.90±1.04, (c) histamine, n=4, pseudo EC_{25}: CRC_1 = -6.57±0.12, CRC_2 = -5.13±0.41*, dose ratio = 89.62±73.22, and (d) β-PEA n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant differences (*, $P<0.05$ or **, $P<0.01$) between CRC_1 and CRC_2, and ($^{++}$, $P<0.01$) CRC_2 and CRC_3. ●—● CRC_1 after 30 min tissue equilibration, ■–■ CRC_2 after 30 min tissue equilibration after the wash out of CRC_1, and O--O CRC_3 after 30 min tissue equilibration from the washout of CRC_2. 1 mg of *A. vulgaris* chloroform crude extract dissolved in 0.1 mL DMSO was added 20 minutes prior to the construction of CRC_2.

Table 5.2. Summary of the effects of *A. vulgaris chloroform* crude extract on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant differences (*, $P<0.05$) between CRC_1 and CRC2. 1 mg of *A. vulgaris* chloroform crude extract dissolved in 0.1 mL DMSO was added 20 minutes prior to the construction of CRC_2.

Agonist		CRC_1	CRC_2	CRC_3	Dose ratio$\left(\frac{CRC_1}{CRC_2}\right)$	n
5-HT	Max (g)	1.17+0.25	0.10+0.02*	0.32+0.08		4
	-log EC_{50}	-6.85+0.40	-7.08+0.35	-6.99+0.32	4.87+2.84	
Methacholine	Max (g)	1.83+0.81	0.83+0.26	1.20+0.43		4
	-log EC_{50}	-7.29+0.08	-7.00+0.23	-7.07+0.15	2.82+1.58	
Histamine	Max (g)	1.41+0.26	0.52+0.13*	1.22+0.29^{+}		4
	-log EC_{50}	-6.76+0.15	-5.08+0.36*	-5.96+0.08	141.24+105.39	
β-PEA	Max (g)	0.70+0.23	0.25+0.05	0.42+0.14		4
	-log EC_{50}	-3.87+0.17	-4.11+0.35	-3.43+0.11	0.90+0.46	

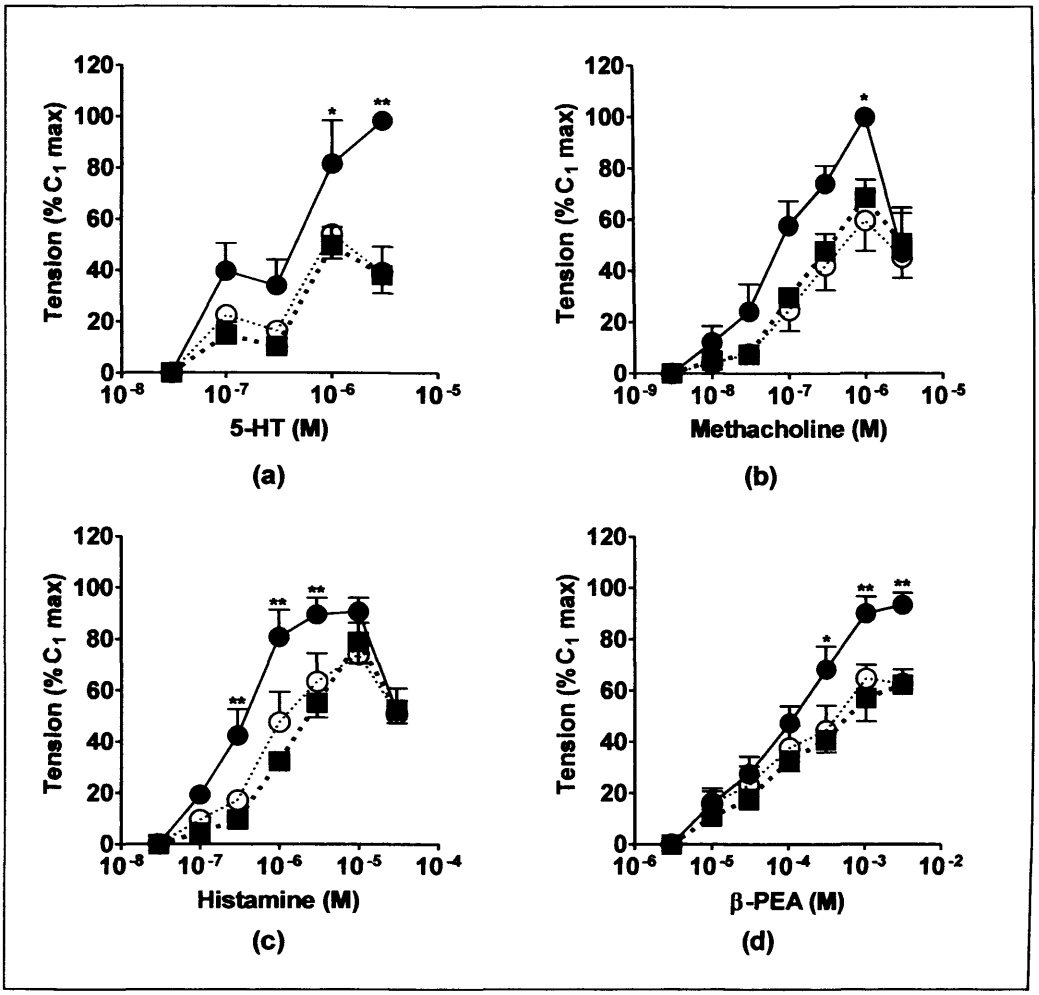


Figure 5.3. Effects of *A. vulgaris* methanol crude extract on mean cumulative CRCs of guinea pig ileum for constriction to (a) 5-HT, n=5, (b) methacholine, n=4, (c) histamine, n=4, and (d) β-PEA, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant differences (*, $P<0.05$ or **, $P<0.01$) between CRC_1 and CRC_2. ●—● CRC_1 after 30 min tissue equilibration, ■--■ CRC_2 after 30 min tissue equilibration after the wash out of CRC_1, and O--O CRC_3 after 30 min tissue equilibration from the washout of CRC_2. . 1 mg of *A. vulgaris* methanol crude dissolved in 0.1 mL DMSO was added 20 minutes prior to the construction of CRC_2.

Table 5.3. Summary of the effects of *A. vulgaris* methanol crude extract on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant differences (**, $P<0.05$) between CRC_1 and CRC2. 1 mg of *A. vulgaris* methanol crude extract dissolved in 0.1 mL DMSO was added 20 minutes prior to the construction of CRC_2.

Agonist		CRC_1	CRC_2	CRC_3	Dose ratio$\left(\frac{CRC_1}{CRC_2}\right)$	n
5-HT	Max (g)	1.26+0.27	0.68+0.21**	0.75+0.26		4
	-log EC_{50}	-6.90+0.32	-6.99+0.12	-7.13+0.22	1.28+0.61	
Methacholine	Max (g)	2.40+0.92	1.66+0.59	1.66+0.71		4
	-log EC_{50}	-7.44+0.33	-6.99+0.16	-6.88+0.21	5.59+3.99	
Histamine	Max (g)	2.75+0.83	2.38+0.81**	2.36+0.91		4
	-log EC_{50}	-6.72+0.16	-6.05+0.13**	-6.41+0.29	4.85+0.70	
β-PEA	Max (g)	0.83+0.24	0.54+0.14	0.58+0.15		4
	-log EC_{50}	-3.95+0.25	-3.88+0.15	-4.08+0.40	1.65+0.55	

5.4.3 Effects of *A. vulgaris* chloroform and methanol crude extracts on contractile responses in guinea pig trachea to histamine and β-PEA

Histamine and β-PEA caused concentration related contractile responses on the guinea pig trachea. In the presence of AV-$CHCl_3$, the maximum contractile response to histamine (0.92±0.11 g, n=4) showed a significant inhibition ($P<0.05$) to 0.59±0.06 g (Table 5.4). Contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% further showed significant inhibition of the plant extract at maximal dose to histamine ($P<0.01$, $1x10^{-4}$ M) from 100.00±0.00% to 42.99±10.79% (Figure 5.4.a) and at $3x10^{-4}$ M β-PEA ($P<0.05$, n=4) from, 63.89±8.32% to 33.43±9.42% (Figure 5.4.b). The contractile responses expressed as percentage of their own CRC maximum set to 100% showed a significant ($P<0.05$) shift of the slope of the curve indicated by the -log EC_{50} values obtained for histamine from -5.80±0.14 to -4.16±0.31 with a dose ratio of 56.42±21.56 (Table 5.4).

In the presence of AV-MeOH, contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed a significant potentiation ($P<0.01$) of contractile responses to β-PEA ($3x10^{-3}$ M, n=4) from 86.45±9.40% to 148.39±27.40% (Figure 5.4.d). No significant effect of AV-MeOH was observed for histamine (n=4) CRC (Table 5.4, Figure 5.4.c).

Both AV-$CHCl_3$ and AV-MeOH showed baseline lowering of 41.80±13.74% and 10.00.0±7.76% in the histamine experiment, and 14.00±1.10% and 4.50±2.85% in the β-PEA experiment from the resting baselines before plant extract addition (Figure 5.4.a, Figure 5.4.d,Figure 5.5 & Figure 5.6).

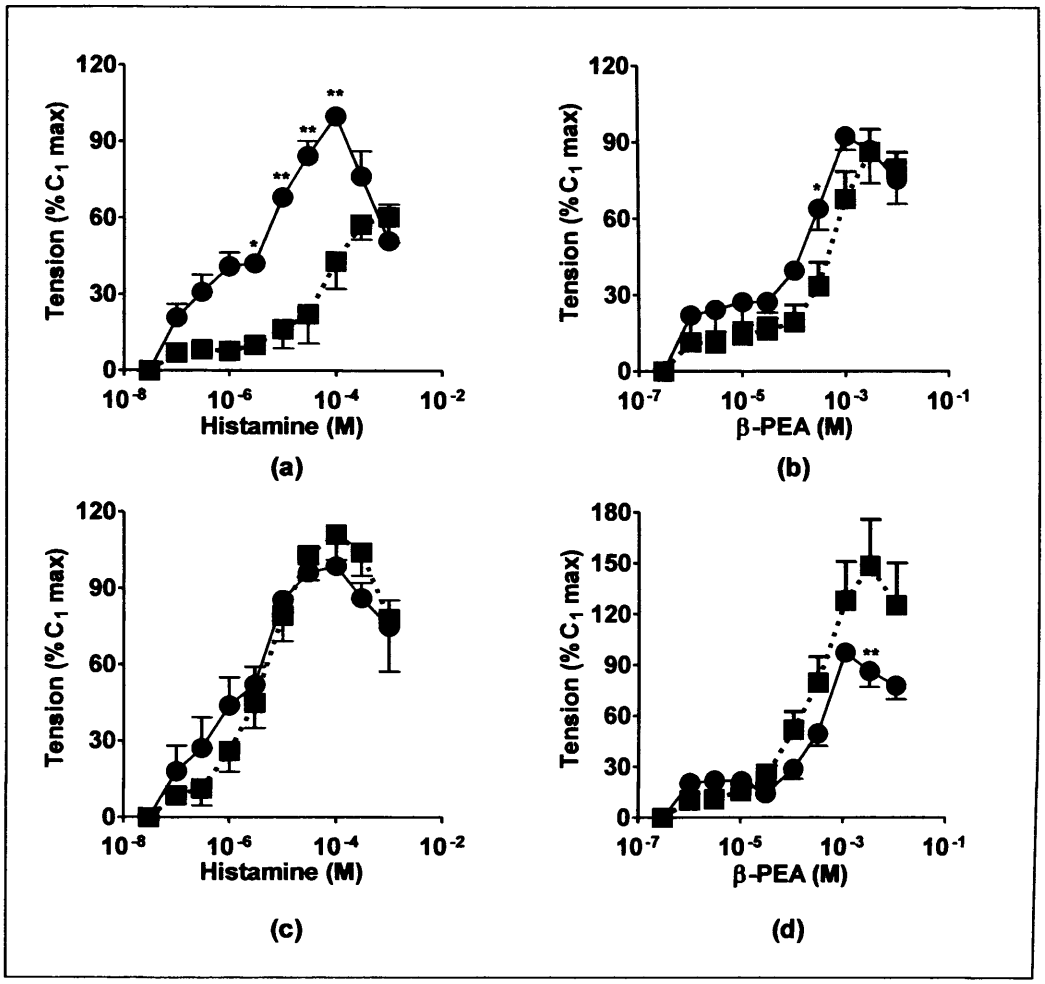


Figure 5.4. Effects of *A. vulgaris* on mean cumulative CRCs of guinea pig trachea for constriction to (a) histamine, n=4, and (b) β-PEA, n=4 in the presence of chloroform crude extract and (c) histamine, n=4, and (d) β-PEA, n=4 in the presence of methanol crude extract. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA. Significant difference (*, $P<0.05$ or **, $P<0.01$) between CRC_1 and CRC_2. ●—● CRC_1 after 60 min tissue equilibration and ■—■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1. 1 mg of *A. vulgaris* crude extracts dissolved in 0.1 mL DMSO was added 20 minutes prior to the construction of CRC_2. Propranolol ($1x10^{-6}$ M) was added 10 minutes before the construction of each CRCs in β-PEA.

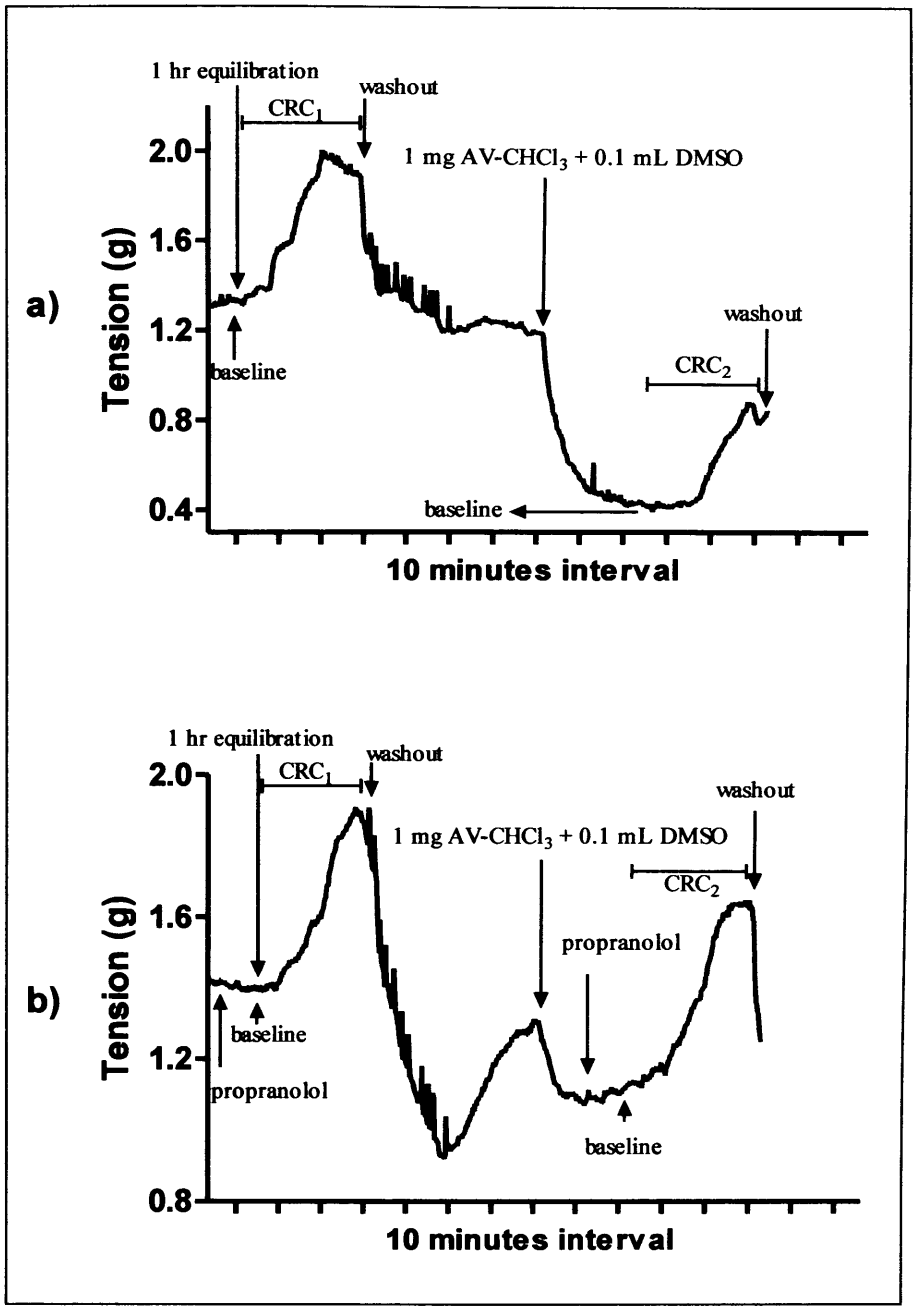


Figure 5.5. Representative chart recording showing the effects of *A. vulgaris* chloroform extract (AV-$CHCl_3$) in repeated contractile response CRCs of the guinea pig trachea to (a) histamine (refer to section 2.4.9 for histamine concentrations used for each CRC) and (b) β-PEA (section 2.4.12 for β-PEA concentrations used for each CRC). $1x10^{-6}$ M propranolol added 10 minutes prior to each CRC of β-PEA.

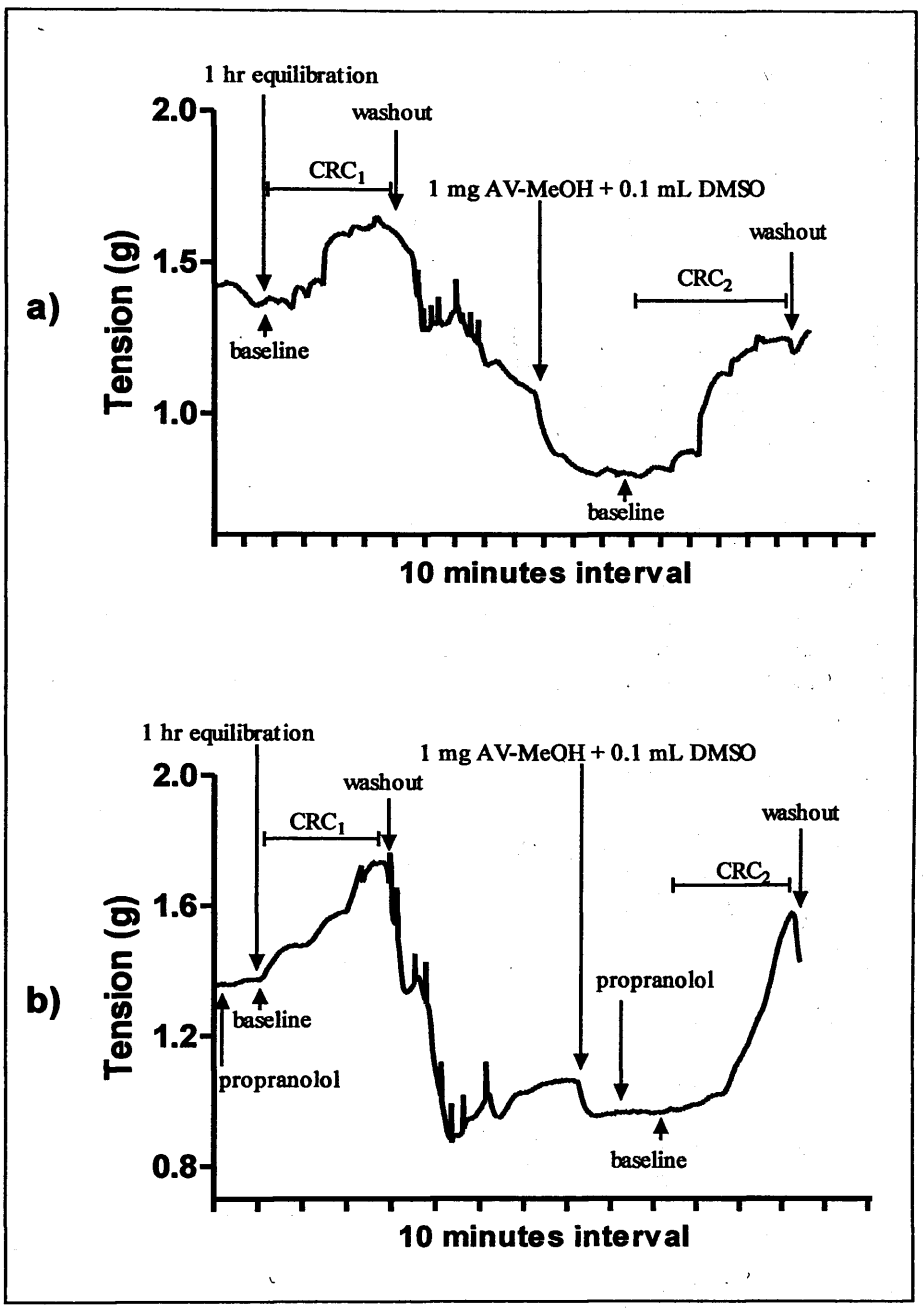


Figure 5.6. Representative chart recording showing the effects of *A. vulgaris* methanol extract (AV-MeOH) in repeated contractile response CRCs of the guinea pig trachea to (a) histamine (refer to section 2.4.9 for histamine concentrations used for each CRC) and (b) β-PEA (section 2.4.12 for β-PEA concentrations used for each CRC). $1x10^{-6}$ M propranolol added 10 minutes prior to each CRC of β-PEA.

Table 5.4. Summary of the effects of *A. vulgaris* chloroform and methanol crude extract on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig trachea to histamine and β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant difference (*, $P<0.05$ or **, $P<0.01$) between CRC_1 and CRC_2. 1 mg of *A. vulgaris* crude extracts dissolved in 0.1 mL DMSO was added 20 minutes prior to the construction of CRC_2. Propranolol ($1x10^{-6}$ M) was added 10 minutes before the construction of each CRCs in β-PEA.

	Agonist		CRC_1	CRC_2	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
Chloroform crude extract	Histamine	Max (g)	0.92+0.11*	0.59+0.06		4
		-log EC_{50}	-5.80+0.14**	-4.16+0.31	56.42+21.56	
	β-PEA	Max (g)	0.64+0.08	0.54+0.05		4
		-log EC_{50}	-3.52+0.28	-3.28+0.13	3.42+2.20	
Methanol crude extract	Histamine	Max (g)	0.57+0.07	0.63+0.08		4
		-log EC_{50}	-6.10+0.36	5.51+0.08	18.24+16.67	
	β-PEA	Max (g)	0.35+0.07	0.48+0.06		4
		-log EC_{50}	-3.61+0.06	-3.67+0.07	0.96+0.23	

5.4.4 Extraction and isolation of yomogin and 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide from AV-$CHCl_3$

The AV-$CHCl_3$ showed a high degree of histamine H_1 receptor antagonism in the guinea pig ileum trachea. Thus further evaluation using fresh samples (refer to section 5.3.3) on the chloroform layer of *A. vulgaris* for its H_1 receptor inhibition was carried out in a bioassay-guided extraction in the guinea pig ileum.

For histamine antagonist evaluation of *A. vulgaris,* 5 g of chloroform crude extract was passed though gel filtration chromatography using Sephadex LH-20 to remove most of the fatty acids, pigments and chlorophylls. Six sub-fractions (F_1-F_6, 50 mL per fraction) were obtained and dried under reduced pressure (Figure 5.7). 1 mg of each sub-fraction was dissolved in DMSO and evaluated individually for histamine antagonist activity in the guinea pig ileum. Fraction 3 and 4 were active against histamine and were then combined and further purified by repeated preparative TLC with EtOAc in DCM (1:9) as an eluent, which finally gave a 9.0 mg of a 1:1 mixture of two compounds based from preliminary ^{1}H-NMR analysis. To increase the amount of this active mixture for further spectral analysis another batch of extraction was carried using normal phase column chromatography.

Twenty five grams of the chloroform extract crude mixture was repeatedly washed with petroleum ether to remove most of the fatty acids, pigments and chlorophylls (Figure 5.7). The defatted mixture was run through silica gel (750 g) column chromatography using gradient elution of ethyl acetate (EtOAc) in dichloromethane (CH_2Cl_2) (250 mL of 10% polarity increment) to yield a mixture (215.0 mg) of two components in Fraction 6 (250 mL per fraction). This mixture was determined to be of the same

constituents that exhibited histamine H_1 antagonism previously. The two components were identified as yomogin and 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide by direct comparison of the ^{1}H-NMR and ^{13}C-NMR spectral data with those in literature (Ryu *et al.*, 1998; Tigno *et al.*, 2000b). Yomogin was further purified through repeated recrystalization with MeOH as white crystalline needles (27.0 mg).

5.4.5 Structure elucidation of yomogin

Yomogin, a colourless needle was purified through recrystalization in MeOH. Previously it was identified and isolated from the herbs *Artemisia princeps* (Ryu *et al.*, 1998) and from the Philippine *Artemisa vulgaris (Tigno et al., 2000a; Tigno et al., 2000b)*.

Inspection of the proton spectrum of yomogin (Figure 5.8) showed 12 group signals. Based on the integration, there are 16 protons, of which 10 are apparently isolated single protons, and 6 are due to a methyl group. The ^{1}H-NMR δ (500 MHz, $CDCl_3$): 1.37 (3H, s,CH_3-14), 1.73 (1H, dd, J=4.8, 15.3 Hz, H-9a), 2.00 (3H, s, CH_3-15), 2.33 (1H, dd, J=12.95, 12.97 Hz, H-6a), 2.48 (1H, dd, J=2.75, 15.3 Hz, H-9b), 3.02 (1H, dd, J=7.1, 14.25 Hz, H-6b), 3.12 (1H, m, H-7), 4.52 (1H, m, H-8), 5.77 (1H, d, J=1.1 Hz) and 6.30 (1H, d, J=1.25 Hz) , H-13], 6.28 (1H, d, J=9.9 Hz, H-2), 6.83 (1H, d, J=9.85 Hz, H-1).

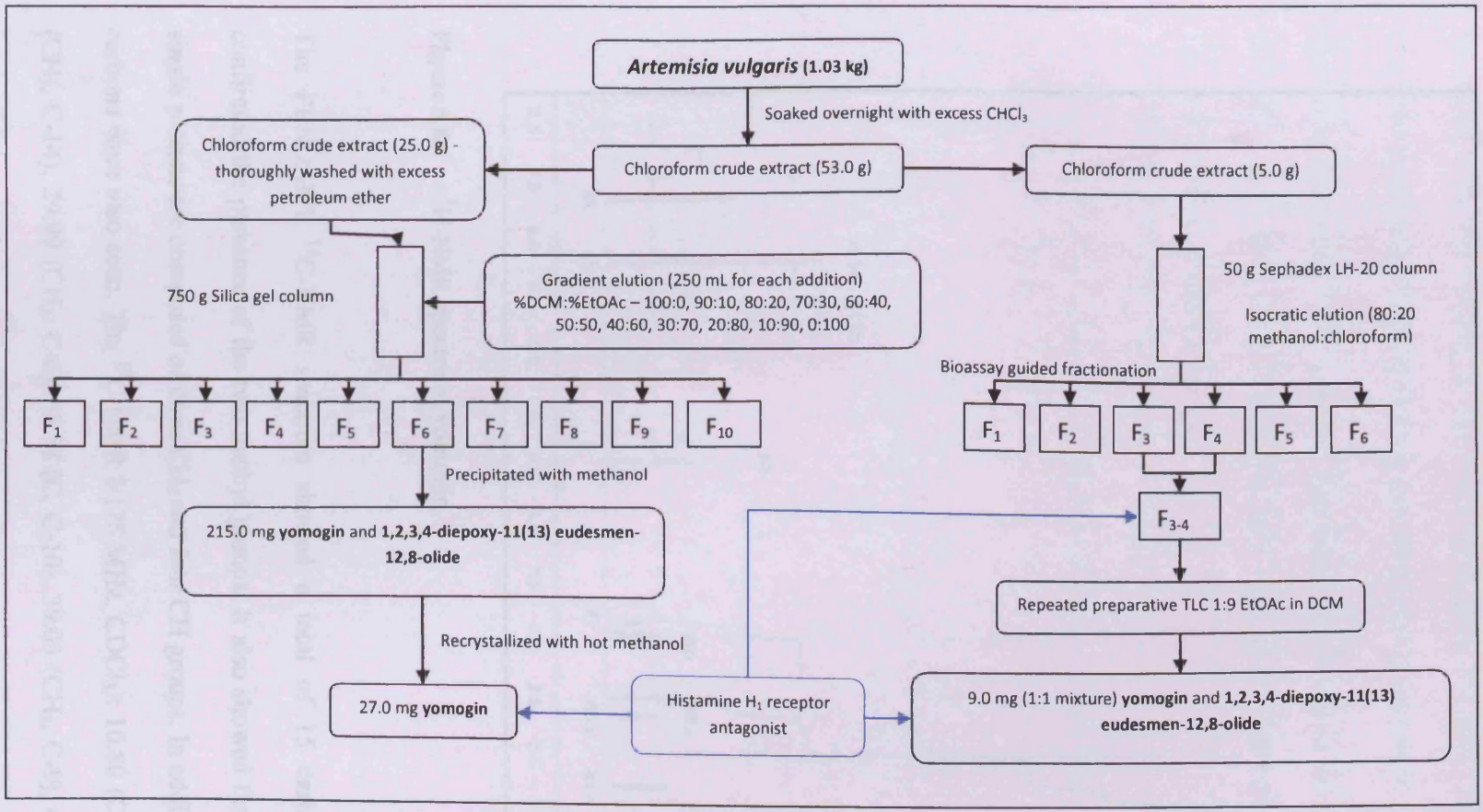


Figure 5.7. Isolation chart of yomogin and 1,2,3,4-diepoxy-11(13)eudesmen-12,8-olide using silica gel column and Sephadex LH-20 column.

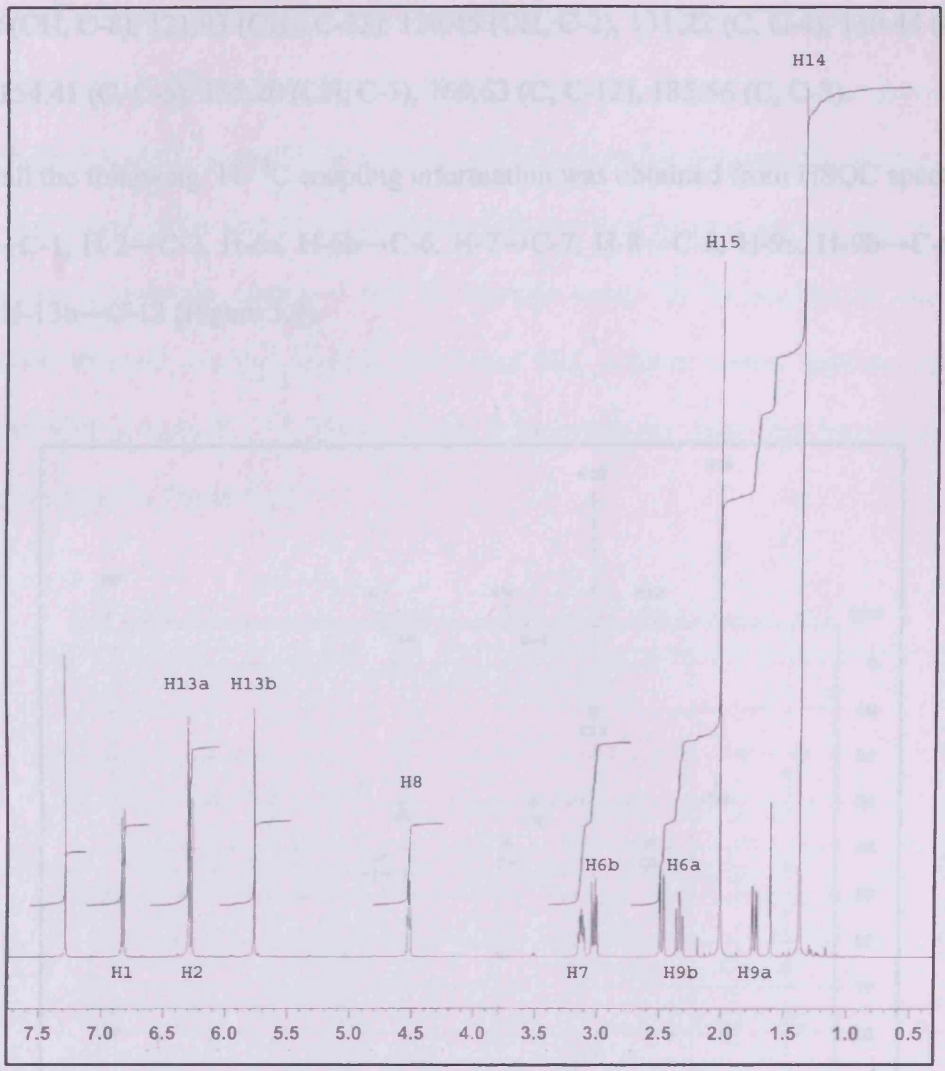


Figure 5.8. ^{1}H-NMR spectra of yomogin

The PENDANT ^{13}C-NMR spectrum showed a total of 15 carbons and further confirmed the presence of the two methyl groups. It also showed that the 10 isolated single protons are composed of three CH_2 and four CH groups. In addition 6 quaternary carbons were also seen. The ^{13}C-NMR δ (75 MHz, $CDCl_3$): 10.80 (CH_3, C-15), 25.72 (CH_3, C-14), 29.99 (CH_2, C-6), 38.58 (C, C-10), 39.03 (CH_2, C-9), 41.94 (CH, C-7),

75.36(CH, C-8), 121.93 (CH_2, C-13), 126.45 (CH, C-2), 131.22 (C, C-4), 140.44 (C, C-11), 154.41 (C, C-5), 155.20 (CH, C-1), 169.63 (C, C-12), 185.56 (C, C-3).

Overall the following ^{1}H-^{13}C coupling information was obtained from HSQC spectrum: H-1→C-1, H-2→C-2, H-6a, H-6b→C-6, H-7→C-7, H-8→C-8, H-9a, H-9b→C-9, H-13a, H-13b→C-13 (Figure 5.9).

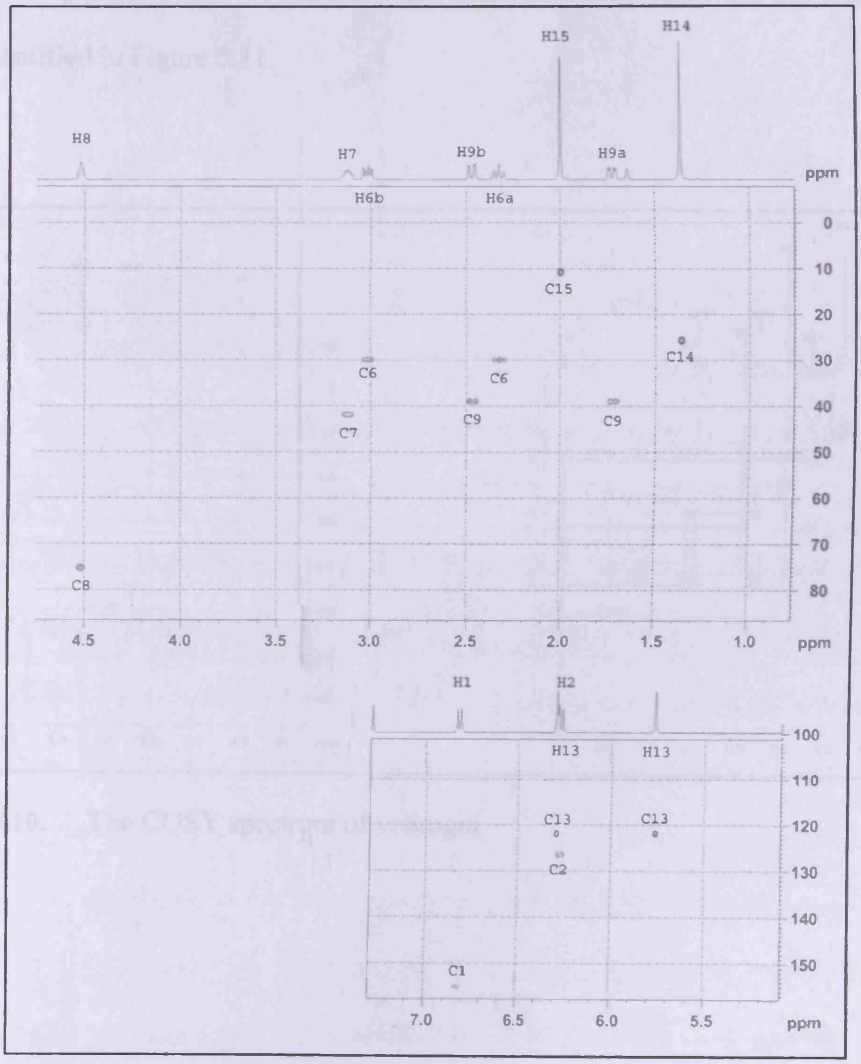


Figure 5.9. HSQC spectra of yomogin

Inspection of the ^{1}H-^{1}H COSY spectrum cross peaks identified the coupling of two aromatic protons (H-1↔H-2) and the coupling path (H-6a, H-6b↔H-7↔H-8↔H-9a, H-9b) (Figure 5.10). In addition, a weak ^{1}H-^{1}H correlation between the protons H-6a and H-15, and H-9a and H-14 indicates that the two methyl groups are in close proximity to position carbon 6 and 9. Another weak ^{1}H-^{1}H correlation was also observed between the two protons H-13 and H-7 protons which indicate that the olefenic CH_2 is close to C-7. Based on this information the following fragments have been identified in Figure 5.11.

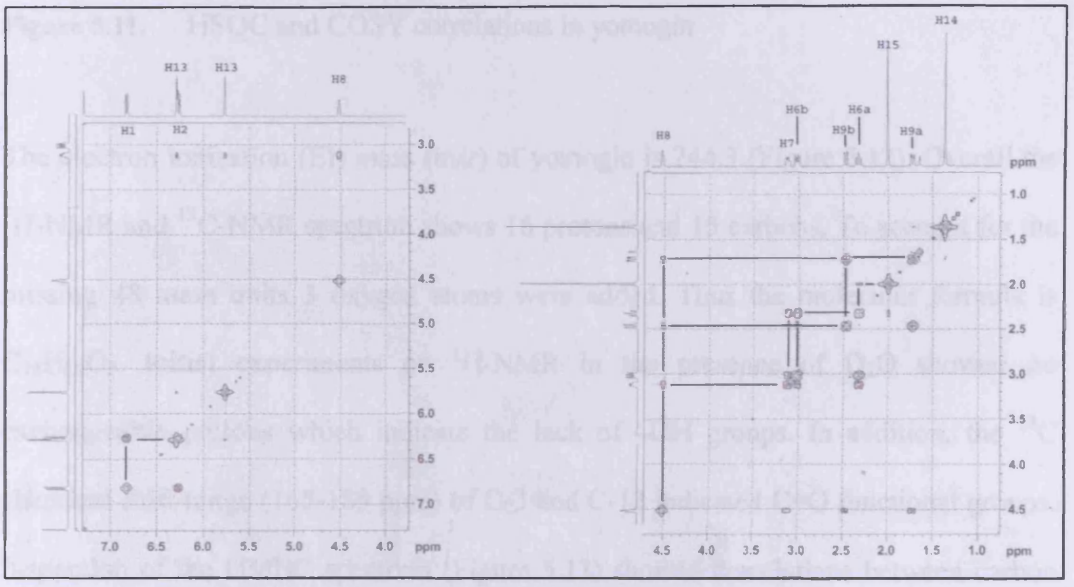


Figure 5.10. The COSY spectrum of yomogin

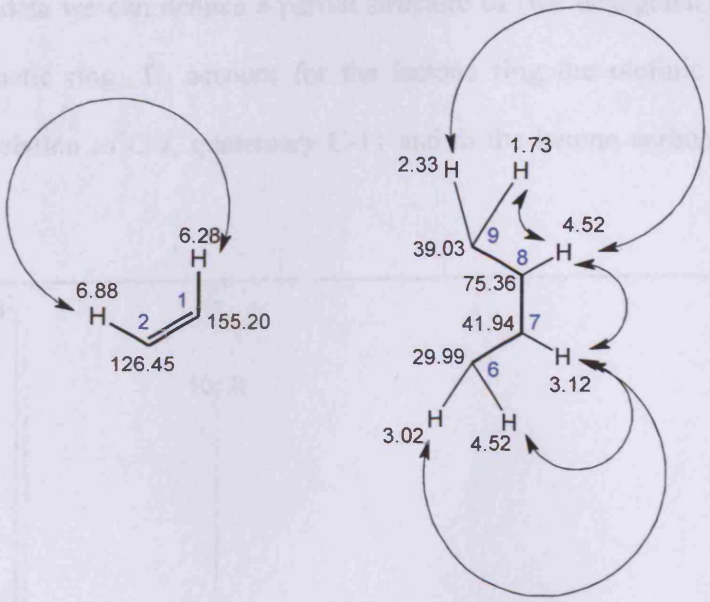


Figure 5.11. HSQC and COSY correlations in yomogin

The electron ionization (EI) mass (m/z) of yomogin is 244.3 (Figure 5.12). Overall the ^{1}H-NMR and ^{13}C-NMR spectrum shows 16 protons and 15 carbons. To account for the missing 48 mass units 3 oxygen atoms were added. Thus the molecular formula is $C_{15}H_{16}O_3$. Initial experiments on ^{1}H-NMR in the presence of D_2O showed no exchangeable protons which indicate the lack of –OH groups. In addition, the ^{13}C chemical shift range (165-185 ppm) of C-3 and C-12 indicated C=O functional groups. Inspection of the HMBC spectrum (Figure 5.13) showed correlations between carbon C-3 to the protons H-2 and H-15. The quaternary carbon C-4 also showed coupling to protons H-15, H-6a and H-6b. The methyl carbon C-15 also showed correlations to protons H-1, H-9a and H-9b. Molecular ion peaks (m/z) at 77.2, 91.2 and 105.2 are fragmentation characteristics of aromatic compounds which corresponds to the formation of phenyl cation ($C_6H_5^+$), tropylium ion ($C_7H_7^+$) and substituted tropylium ion ($C_8H_9^+$), respectively (Figure 5.12). Based on these observations and using the

COSY/HSQC data we can deduce a partial structure of two hexagonal adjacent rings with one aromatic ring. To account for the lactone ring the olefinic protons H-13 showed a correlation to C-7, quaternary C-11 and to the ketone carbon C-12 (Figure 5.14).

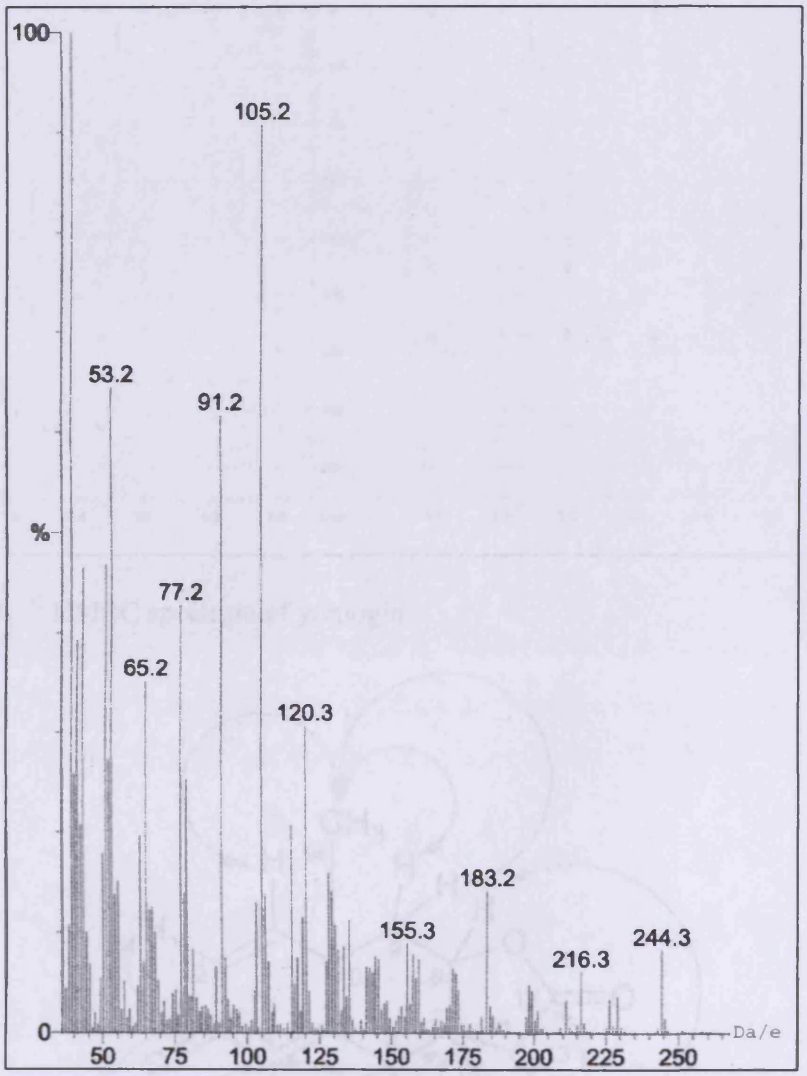


Figure 5.12. Mass spectrum of yomogin.

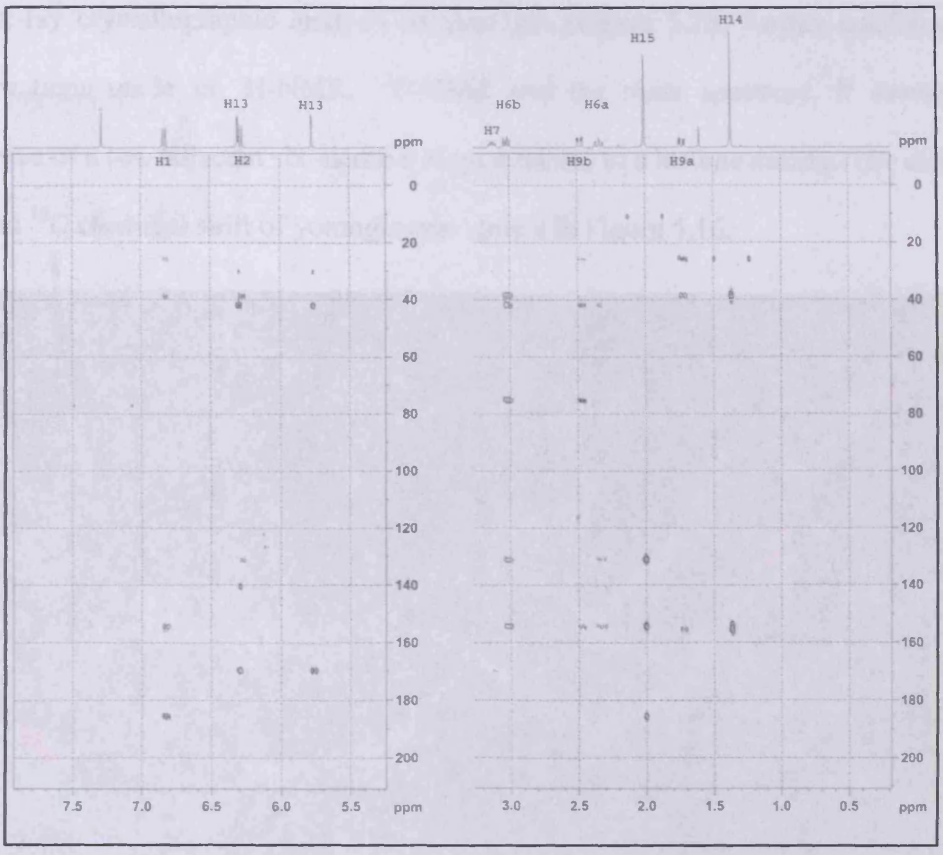


Figure 5.13. HMBC spectrum of yomogin

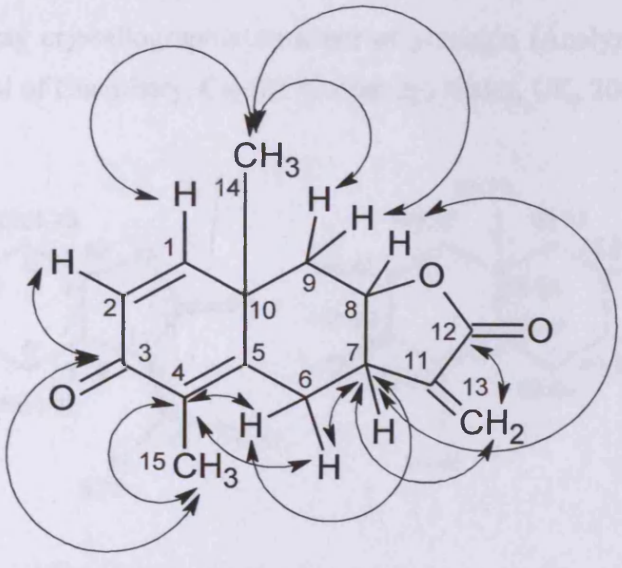


Figure 5.14. HMBC correlations in yomogin

The x-ray crystallographic analysis of yomogin (Figure 5.15) further confirmed the observations made in ^{1}H-NMR, ^{13}C-NMR and the mass spectrum. It showed the presence of a two adjacent six member rings attached to a lactone moiety. The complete ^{1}H and ^{13}C chemical shift of yomogin was shown in Figure 5.16.

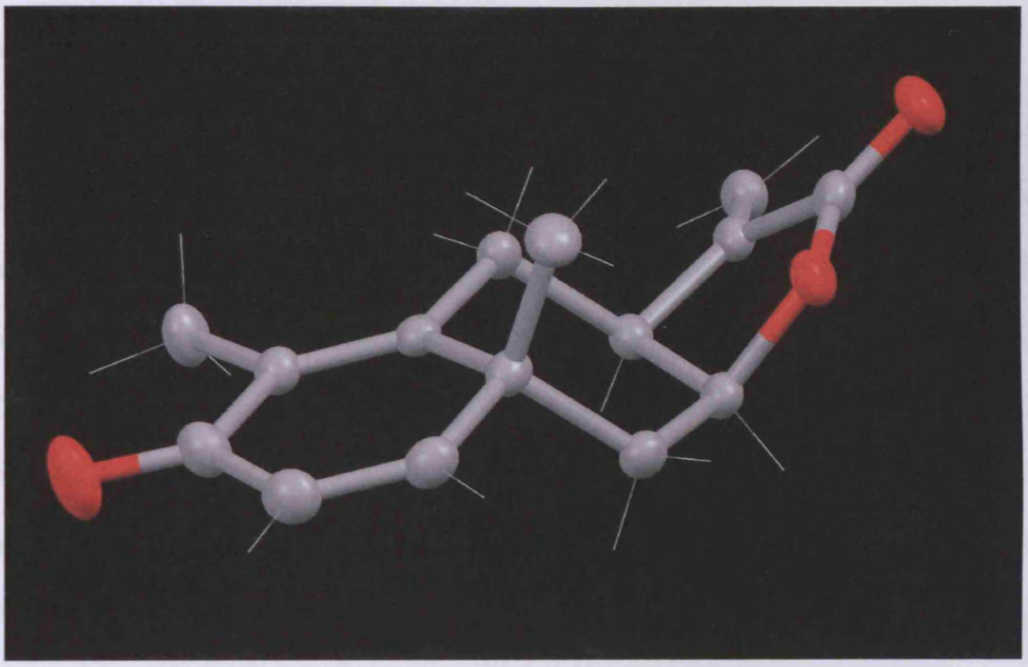


Figure 5.15. X-ray crystallographic structure of yomogin (Analyzed by Dr. Benson Kariuki, School of Chemistry, Cardiff University, Wales, UK, 2009)

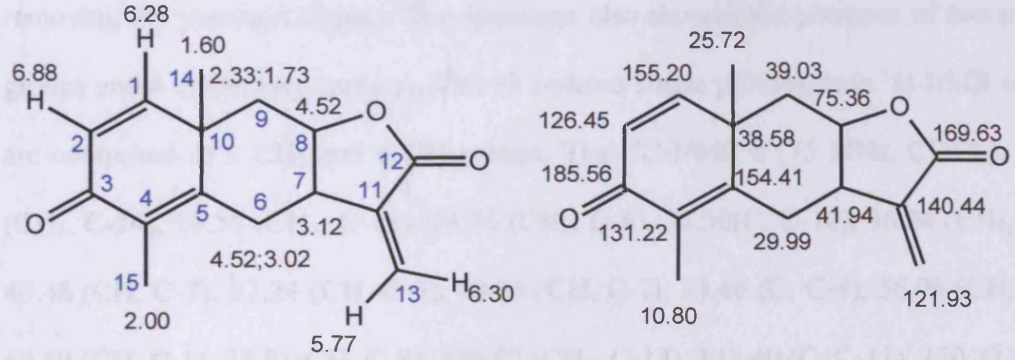


Figure 5.16. ^{1}H and ^{13}C chemical shift of yomogin

5.4.6 Structure elucidation of 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide

1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide was isolated and analysed in a 1:1 mixture with yomogin. Previously the compound is isolated in the *Artemisia vulgaris* of Philippine variety (Tigno *et al.*, 2000b).

The analysis of ^{1}H-NMR spectrum of the mixture revealed two integration patterns. After removing the proton signals from yomogin, 14 group signals have been isolated. Based on the integration, there are 18 protons, of which 12 are apparently isolated single protons, and 6 are due to a methyl group. The ^{1}H-NMR δ (300 MHz, $CDCl_3$): 1.08 (3H, s, CH3-15), 1.22 (3H, s,CH3-14), 1.41 (1H, m, H-6a), 1.81 (dd, 3.15, 14.23 Hz, H-5), 1.88 (1H, dd, J=4.5, 4.55, 15.55 Hz, H-9b), 2.24 (1H, dd, J=1.65, 1.30, 15.55 Hz, H-9a), 2.8 (1H, d, J=3.9 Hz, H-1), 1.97 (1H, m, H-6b), 3.10 (1H, d, J=2.9 Hz, H-3), 3.12 (1H, m, H-7), 3.45 (1H, dd, J=3.15, 2.15 Hz, H-2), 4.52 (1H, m, H-8), 5.65 (1H, d, J=0.8 Hz, H-13a), 6.17 (1H, d, J=0.9 Hz, H-13b) (Figure 5.17).

Inspection of the PENDANT ^{13}C-NMR spectrum showed a total of 15 carbons after removing the yomogin signals. The spectrum also showed the presence of two methyl groups and 4 quaternary carbons. The 12 isolated single protons from ^{1}H-NMR signals are composed of 3 CH_2 and 6 CH groups. The ^{13}C-NMR δ (75 MHz, $CDCl_3$): 17.32 (CH_3, C-14), 19.54 (CH_3, C-15), 24.76 (CH_2, C-6), 33.50(C, C-10), 36.24 (CH_2, C-9), 40.48 (CH, C-7), 37.24 (CH, C-5), 48.04 (CH, C-2), 53.46 (C, C-4), 56.06 (CH, C-3), 60.59 (CH, C-1), 75.91(CH, C-8), 120.87 (CH_2, C-13), 141.40 (C, C-11), 170.22 (C, C-12). Further inspection of the ^{13}C-NMR spectrum revealed that the second compound in the mixture has a lactone moiety because of the close similarity of the carbon signals when compared to yomogin (Figure 5.18).

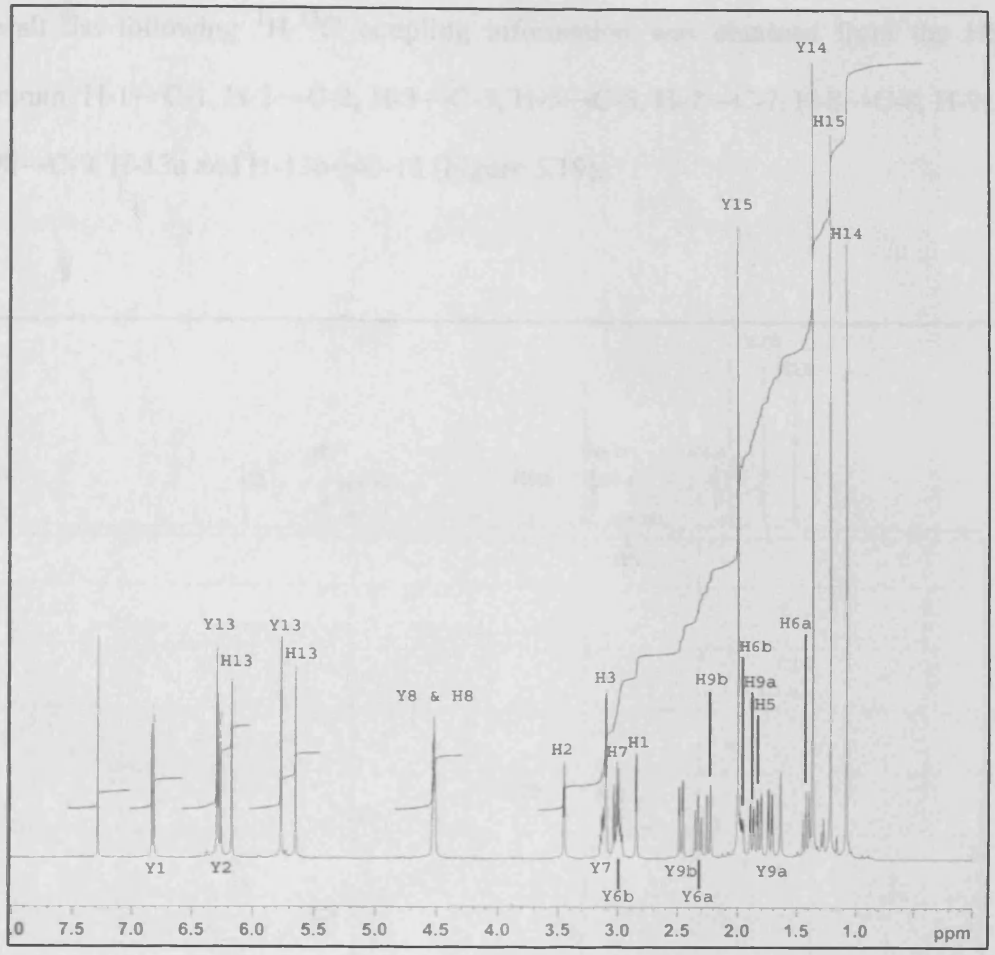
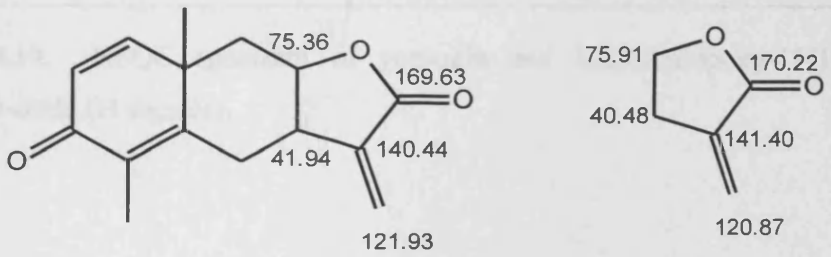


Figure 5.17. ^{1}H-NMR spectrum of yomogin (Y signals) and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide (H signals).



Yomogin fragment 1

Figure 5.18. The lactone moiety of yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide

Overall the following ^{1}H-^{13}C coupling information was obtained from the HSQC spectrum: H-1→C-1, H-2→C-2, H-3→C-3, H-5→C-5, H-7→C-7, H-8→C-8, H-9a and H-9b→C-9, H-13a and H-13b→C-13 (Figure 5.19).

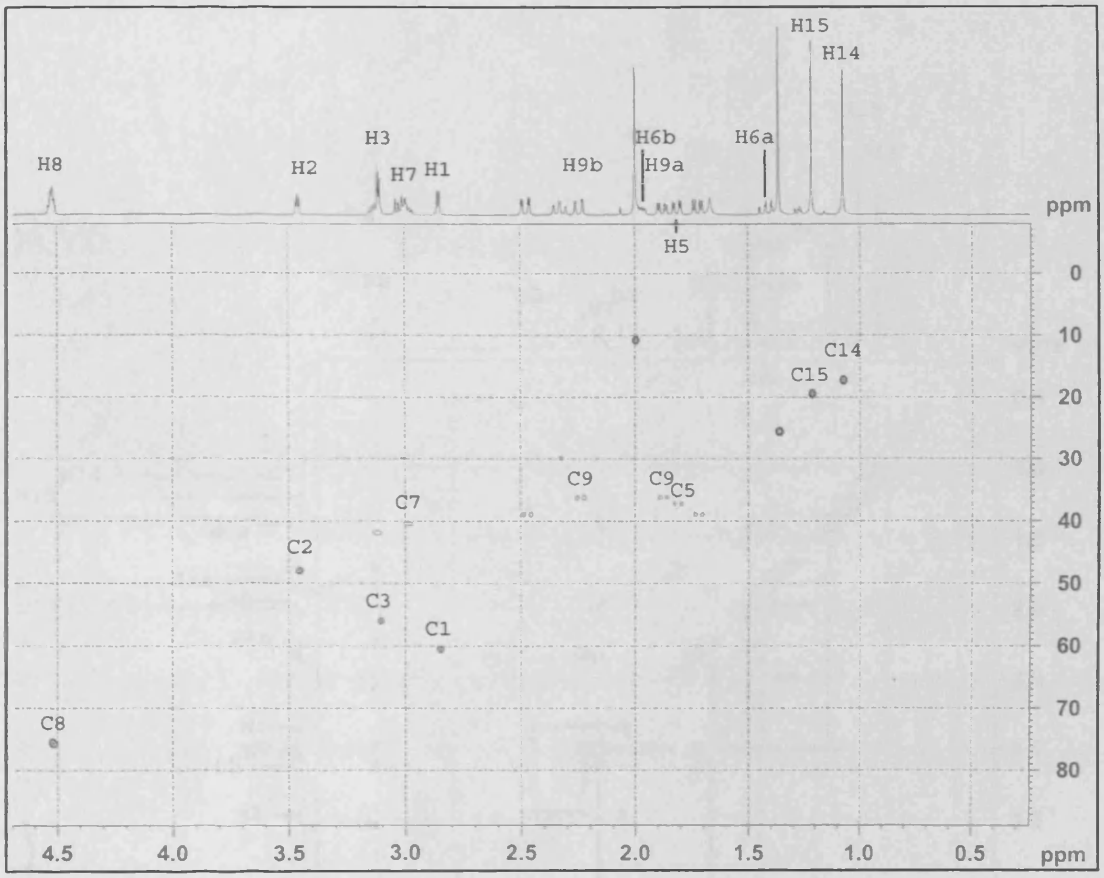


Figure 5.19. HSQC spectrum of yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide (H signals).

Inspection of the ^{1}H-^{1}H COSY spectrum cross peaks identified the coupling of three CH protons (H-1↔H-2↔H-3) and the coupling path H-5↔H-6a, H-6b↔H-7↔H-8↔H-9a, H-9b (Figure 5.20). Combination of this assembly to the lactone moiety resulted to two partial structures in Figure 5.21.

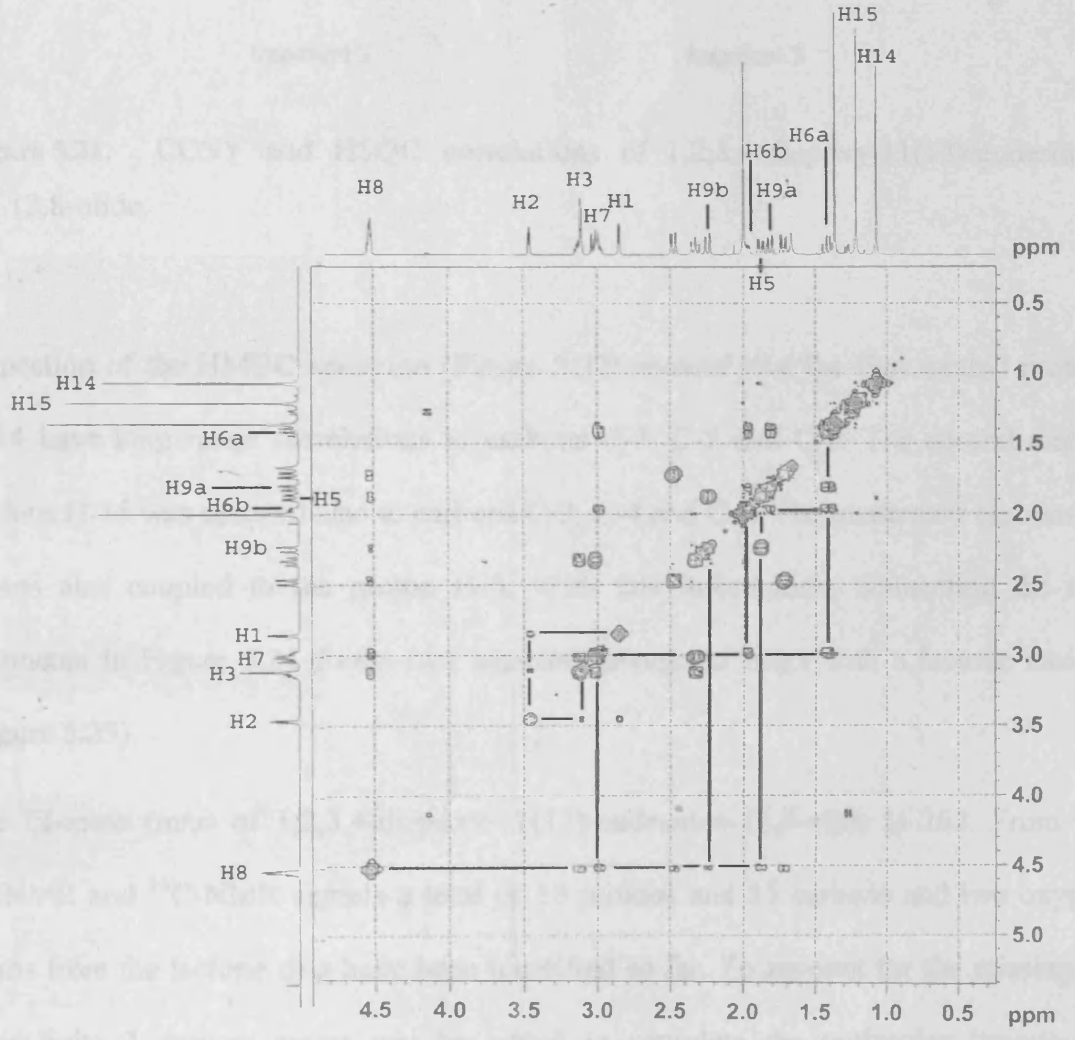
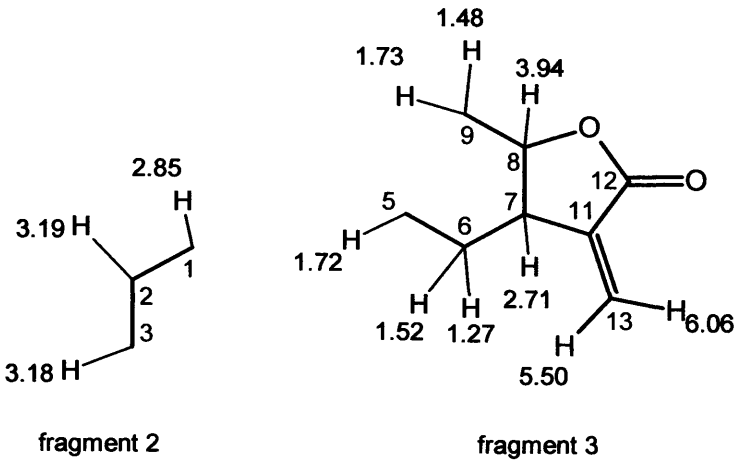


Figure 5.20. COSY spectrum of 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide (H signals).



fragment 2 fragment 3

Figure 5.21. COSY and HSQC correlations of 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide.

Inspection of the HMBC spectrum (Figure 5.22) showed that the first methyl protons H-14 have long range correlations to carbons C-1, C-5 and C-9. The second methyl protons H-15 was coupled also to carbons C-3, C-4 and C-6. The quaternary carbons C-4 was also coupled to the proton H-5. With this information, connecting the two fragments in Figure 5.21 forms two adjacent hexagonal rings with a lactone moiety (Figure 5.23).

The EI-mass (m/z) of 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide is 262. From the ^{1}H-NMR and ^{13}C-NMR signals a total of 18 protons and 15 carbons and two oxygen atoms from the lactone ring have been identified so far. To account for the missing 32 mass units 2 oxygen atoms can be added to complete the molecular formula of $C_{15}H_{18}O_4$. To accommodate the 2 oxygen atoms in the partial structure given in Figure 5.23 the addition of two epoxy groups was necessary at position 1,2 and 3,4 (Figure 5.24).

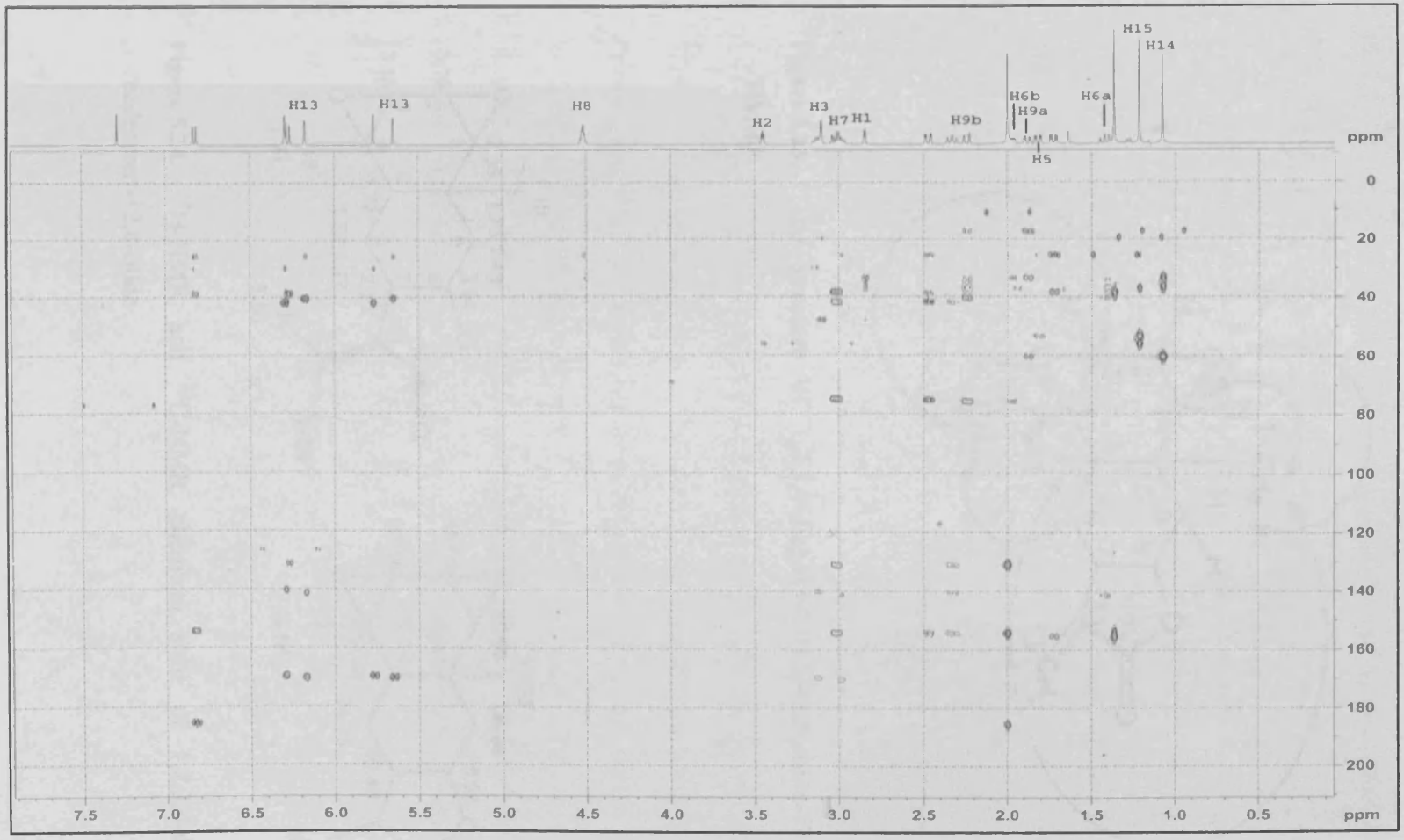


Figure 5.22. HMBC spectrum of 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide (H signals).

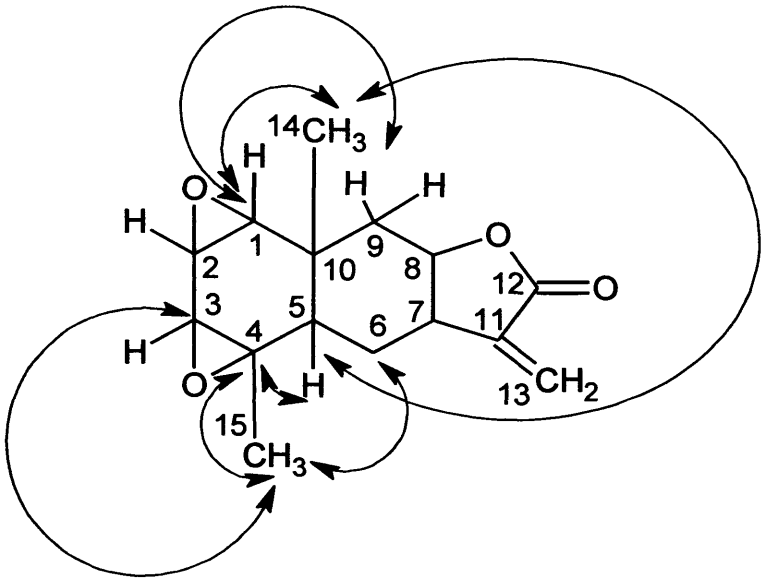


Figure 5.23. The structure of 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide using HMBC.

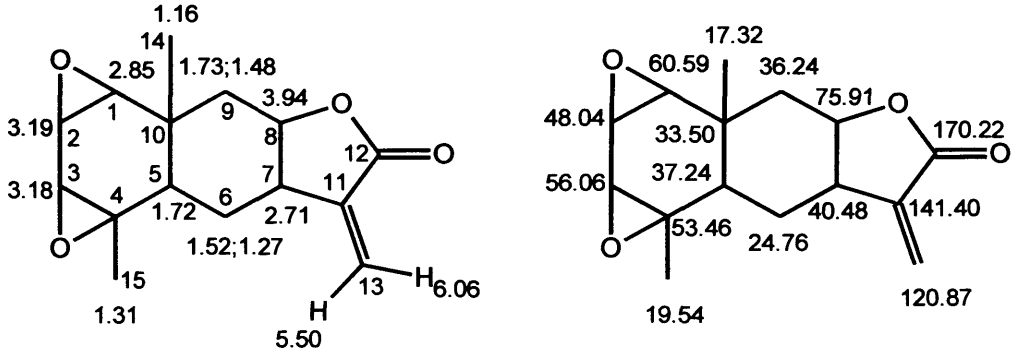


Figure 5.24. ^{1}H-NMR and ^{13}C-NMR chemical shift of 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide

5.4.7 Effects of yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide on contractile responses in guinea pig ileum to histamine

Histamine caused concentration-related contractile responses on the guinea pig ileum. In the presence of yomogin (n=4) contractile responses in the guinea pig ileum expressed as percentage of the maximum obtained in CRC_1 set to 100% showed significant inhibition ($P<0.05$) to histamine ($1x10^{-7}$ M, n=4) before the maximal dose from 72.98±8.98% to 23.32±10.75% (Figure 5.25.a). Yomogin also showed a significant ($P<0.01$) effect by shifting the histamine curve to higher concentrations indicated by -log EC_{50} values obtained from -7.65±0.23 to -6.83±0.22 resulting in a dose ratio of 6.74±0.43 (Table 5.5).

1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide was not purified in pure form because of its close similarity in structure with yomogin. Thus its pharmacological evaluation against histamine was carried out in a mixture with yomogin. In the presence of the mixture (n=4), contractile responses expressed as percentage of the maximum obtained in CRC_1 set to 100% showed significant inhibition ($P<0.01$) of the contractile maximum to histamine ($3x10^{-7}$ M) from 95.48±4.17% to 53.62±4.98% in the guinea pig ileum (Figure 5.25). The -log EC_{50} values obtained showed no significant differences, however, a variable dose ratio of 9.82±6.07 was obtained (Table 5.5).

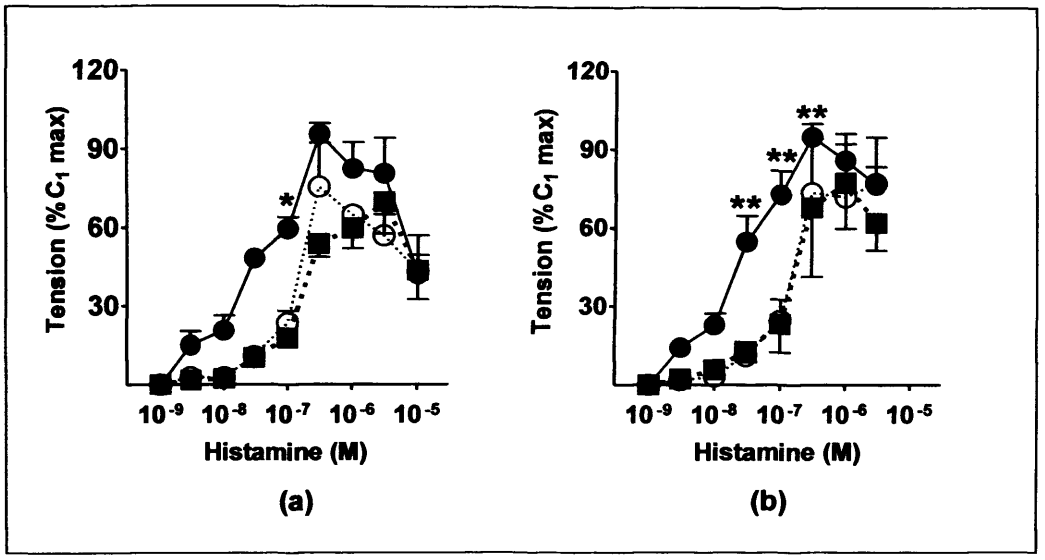


Figure 5.25. Effects of (a) yomogin (n=4) and (b) mixture of yomogin and 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide (n=4) on contractile responses of the guinea pig ileum to histamine. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant (*, $P<0.05$ or **, $P<0.01$) differences were seen between CRC_1 and CRC_2. ●—● CRC_1 after 30 min tissue equilibration, ■--■ CRC_2 after 30 min tissue equilibration after the wash out of first curve, and O--O CRC_3 after 30 min tissue equilibration from the washout of second curve. 1 mg of the plant extracts dissolved in 0.1 mL DMSO was added prior to the construction of CRC_2.

Table 5.5. Summary of the effects of yomogin and 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to histamine. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test. Significant differences (**, $P<0.05$) between CRC_1 and CRC_2. 1 mg of yomogin or a mixture of yomogin and 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide dissolved in 0.1 mL DMSO was added prior to construction of CRC_2.

Plant extract		CRC_1	CRC_2	CRC_3	Dose ratio $\left(\frac{CRC_1}{CRC_2}\right)$	n
Yomogin	Max (g)	2.50±0.85	1.57±0.23	1.82±0.47		4
	-log EC_{50}	-7.65±0.23	-6.83±0.22**	-6.81±0.13	6.74±0.43	
Mixture of yomogin and 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide	Max (g)	1.63±0.30	1.22±0.34	1.42±0.47		4
	-log EC_{50}	-7.70±0.24	-6.98±0.13	-7.01±0.03	9.82±6.07	

5.5 Discussion

5.5.1 The antagonistic activity of the chloroform and methanol extracts of *A. vulgaris* on contractile responses of the guinea pig ileum to 5-HT, methacholine, histamine and β-PEA

The AV-$CHCl_3$ showed a high degree of reducing the maximum contractile responses induced by 5-HT, methacholine, histamine and β-PEA on the guinea pig ileum (Figure 5.2). The degree of inhibition was however different for each agonist with greater effect upon the maxima of 5-HT and histamine. The presence of AV-MeOH produced a similar reduction in the contractile responses induced by 5-HT, methacholine, histamine and β-PEA but to a lesser extent compared to AV-$CHCl_3$. The plant extracts therefore may contain compounds that have non-selective inhibitory effects upon all spasmogens such as smooth muscle relaxant properties.

Histamine can induce smooth muscle contractions in the gut by stimulation of histamine H_1 receptors, which is directly linked to the G-protein Gq that activates PLC which initiates the production of IP_3 and DAG that leads to the increase of cytoplasmic Ca^{2+} through intracellular Ca^{2+} release (Leurs *et al.*, 1995; Mahdy *et al.*, 2008; Timmerman *et al.*, 2009). The addition of AV-$CHCl_3$ showed a parallel shift of the curve to the right to histamine CRC which suggest that plant components are selectively exerting antagonism on the H_1 histamine receptors. A significant recovery of contractile responses after the AV-$CHCl_3$ washout indicates reversibility of this process. AV-MeOH produced a similar parallel shift of the histamine CRC but to a smaller degree compared to AV-$CHCl_3$. In the work of Khan *et al.* (2009) they suggested that the anti-spasmodic and anti-diarrhoeal activity of the methanolic-water

extract of the plant might be mediated through the blockade of the cholinergic receptors and Ca^{2+} mechanisms. The results on the methanol extract however revealed no cholinergic blockade instead histamine H_1 receptor antagonism was observed. Therefore the use of *A. vulgaris* for the treatment of spasm of the bowels and diarrhoea can be suggested to be mediated through histamine H_1 receptor antagonism.

5.5.2 The antagonistic activity of the chloroform and methanol extracts of *A. vulgaris* on contractile responses of the guinea pig trachea to histamine and β-PEA

One of the important traditional use of *A. vulgaris* is for alleviating asthmatic conditions (Quisimbing, 1978; Tigno *et al.*, 2000b). Asthma is a frequent reversible obstruction of the airways in response to an array of physical and chemical stimuli (Fish *et al.*, 1999; O'Byrne, 2008). Characteristics of this disease usually involves airway inflammation and bronchial hyperactivity such as bronchoconstriction that leads to difficulty in breathing (Melillo *et al.*, 2001). Asthma usually occurs as a result of the exposure of allergen which activates the production of IgE antibodies that binds to IgE receptors present in mast cells (Jouvin *et al.*, 1998). This eventually leads to the release of mediators such as histamine that constrict the bronchial smooth muscle (Ackerman *et al.*, 1995), dilates bloods vessels and stimulates mucous glands (Shibano *et al.*, 1998). In the current study, AV-$CHCl_3$ showed significant inhibition to the maximum contractile responses elicited by histamine. In addition, AV-$CHCl_3$ also showed the presence of histamine H_1 antagonist indicated by a curve shift of the histamine CRC to the higher concentration which is similar to histamine antagonism observed in the ileum. AV-$CHCl_3$ showed no significant effects on contractile responses for β-PEA.

The AV-MeOH revealed no inhibition of the maximum contractile response to histamine. It also showed a non-significant shift of the histamine curve to the right but gave a high and variable dose ratio. Previously the methanolic-water extract has been reported to inhibit the carbachol induced contractions in the guinea pig trachea which indicates the presence of a cholinergic antagonist (Khan *et al.*, 2009). It was also reported that the methanolic-water extract causes relaxation on K^+-induced contractions in the trachea (Khan *et al.*, 2009). In addition, AV-MeOH caused a significant potentiation of contractile responses which might suggest the presence of TAAR agonists. Moreover, AV-MeOH did not exert contractile effects when added to the tissue instead a slight lowering of the baseline was observed. This suggests that the potentiation is not due to a direct agonist effect but may be due to an allosteric interaction at the TAAR receptor. The lowering of the baseline suggests the presence of a smooth muscle relaxing component. Whether this was due to a β-adrenoceptor action cannot be concluded since the propranolol (a β-adrenoceptor antagonist) was not added until after the addition of the extract.

Therefore *A. vulgaris* use for treating asthma might be mediated through the cholinergic receptors (Khan *et al.*, 2009) or through the histamine H_1 receptors.

5.5.3 The antagonistic activity of the methanol extracts of yomogin and 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide on contractile responses of the guinea pig ileum and trachea

The bioassay-guided fractionation of the *A. vulgaris* chloroform extracts against histamine H_1 antagonism resulted in the isolation of two sesquiterpene lactones, yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide. Previously both of these compounds were also isolated from the chloroform partition of the aqueous extract from the same plant variety (Tigno *et al.*, 2000b). Yomogin has also been reported to be present in other species of *Artemisia* (Geissman, 1966; Jeong *et al.*, 2004; Nagaki, 1984) and is known for its anti-carcinogenic properties (Zhang *et al.*, 2005). Previous reports have shown that the anti-inflammatory properties of yomogin and similar sesquiterpene lactones from a variety of medicinal plants are due to inhibition of nitric oxide production in LPS-activated macrophages (Dirsch *et al.*, 2000; Ryu *et al.*, 1998; Zhang *et al.*, 2005).

In the present study yomogin exhibited a novel inhibition of histamine H_1 receptors, which was indicated by shifting of the histamine curves to higher concentration in the guinea pig ileum. Because it lacks an amine functional group which most anti-histamine possess it can be suggested that the compound acts indirectly by a new mechanism that specifically inhibits histamine H_1 receptors.

5.6 Conclusion

Artemisia vulgaris has been used traditionally for the treatment of diseases related to the gastrointestinal tract and disorders of the airway, such as spasm of the bowels, diarrhoea and asthma. Its possession of a smooth muscle relaxing property is due to its non-selective inhibitory anaesthetic effects and the presence of histamine receptor antagonism. In the gut and trachea the histamine H_1 receptor mediated-relaxation further validate its traditional medicinal uses. In addition, the sesquiterpene lactone yomogin, a major component, was identified to exhibit the observed histamine H_1 receptor antagonism in the gut. Because yomogin lacks the amine moiety that most antihistamines possess it is thought that it might be working in a novel mechanism. The sesquiterpene lactone 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide was also isolated as one of major components of the chloroform crude extract.

Artemisia vulgaris showed no significant antagonism for TAARs. Instead a TAAR agonist was likely to be present in the methanolic extract as indicated by potentiation of β-PEA contractile responses in the trachea.

CHAPTER 6

Pharmacological effects of *Artemisia vulgaris* on responses of the guinea pig aorta to phenylephrine and β-PEA

6.1 Introduction

In the phytochemical study of *Artemisia vulgaris* several bioactive components have been previously reported to alleviate a number of diseases in the gut, airways (Khan *et al.*, 2009) and cardiovascular system (Tigno *et al.*, 2000a; Tigno *et al.*, 2000b). The phytochemical study of plants also reported various compounds such as polyacetylenes (Wallnofer *et al.*, 1989), coumarins (Murray *et al.*, 1986), flavonoids (Lee *et al.*, 1998; Nikolava *et al.*, 2007) and terpenes (Nagaki, 1984). In the previous chapter two major constituents, yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide, were identified in *A. vulgaris*. In the study of its anti-hypertensive and anti inflammatory properties yomogin a sesquiterpene lactone was previously reported to inhibit iNOS activity (Jeong *et al.*, 2004; Ryu *et al.*, 1998; Tigno *et al.*, 2000a; Tigno *et al.*, 2000b).

In this chapter the possible antagonistic activity of *A. vulgaris* extracts on responses of the guinea pig aorta mediated via α_1-adrenoceptors and TAARs will be examined. In addition the role of extracellular and intracellular Ca^{2+} release in contractions due to α_1-adrenoceptor agonist phenylephrine and the effects of the plant extracts will be looked into. KCl was used as a standard agonist to elicit contractions through extracellular Ca^{2+} influx (Kaczorowski *et al.*, 1999; Shinjoh *et al.*, 1991; Sonkusare *et al.*, 2006). Ryanodine and caffeine, both known to stimulate the ryanodine receptors in the SR were used to study intracellular Ca^{2+} release (Borisova *et al.*, 2007; Ehrlich *et al.*, 1994; Gómez-Viquez *et al.*, 2005).

6.2 Aims

- To study the antagonistic property of *Artemisia vulgaris* chloroform and methanol crude extracts against phenylephrine and β-PEA employing cumulative concentration response curves (CRCs) for the contractile responses of guinea pig isolated aorta.
- To study the effects of *Artemisia vulgaris* chloroform extract on intracellular calcium uptake and reuptake in the guinea-pig aorta using phenylephrine induced contractions and treatment with ryanodine.
- To study the effect of *Artemisia vulgaris* chloroform extract on extracellular Ca^{2+} influx using non-cumulative KCl CRCs in guinea-pig aorta
- To study the effect of *Artemisia vulgaris* chloroform extract on intracellular calcium uptake and reuptake using repeated single dose caffeine challenge.

6.3 Methods and Materials

The main methods and experimental protocols described in Chapter II were retained throughout this study unless otherwise stated. The plant crude extracts used in this study are *A. vulgaris* chloroform extract (AV-$CHCl_3$) and methanol crude extracts (AV-MeOH) (refer to section 5.3.3).

6.3.1 Protocols for repeated agonist cumulative CRCs on guinea pig aorta

After equilibration, repeated cumulative CRCs for phenylephrine and β-PEA on the guinea pig aorta were obtained in the absence and presence of the extracts AV-$CHCl_3$ or AV-MeOH. One milligram of *A. vulgaris* extract was dissolved individually in 0.1 mL DMSO and incubated separately with the tissue for 20 minutes prior to the construction of each CRC_2. Approximate concentration of the plant extract in a 50 mL bath was therefore equivalent to 0.02 mg/mL.

6.3.2 Protocols for repeated phenylephrine cumulative CRCs on guinea pig aorta in the presence of ryanodine

Repeated cumulative CRCs for aortic rings were constructed for contractile responses to phenylephrine. The tissues were left to equilibrate for 60 min before drug addition. After restoring the resting tension by washing the tissues and a total of tissue equilibration of 60 minutes, ryanodine ($1x10^{-5}$ M) was added. When the tension caused by ryanodine had reached the plateau, a second cumulative CRC for phenylephrine was

obtained. One milligram of AV-$CHCl_3$ dissolved in 0.1 mL DMSO was also incubated 20 minutes prior to the addition of ryanodine.

6.3.3 Protocols for repeated single dose curves to caffeine on guinea pig aorta

Repeated single dose curves for aortic rings were prepared for contractile responses to 30 mM caffeine. The tissues were left to equilibrate for 60 min before drug addition. After restoring the resting tension by washing the tissues and equilibration of 60 minutes after the first exposure to caffeine washout the addition of caffeine was repeated. One milligram of AV-$CHCl_3$ dissolved in 0.1 mL DMSO was added 20 minutes before the second caffeine CRC.

6.3.4 Protocols for repeated KCl non-cumulative CRCs on guinea pig aorta

Repeated non-cumulative CRCs for aortic rings were prepared for contractile responses to KCl. To obtain non-cumulative CRCs, successive increasing concentrations of KCl were prepared (4, 25, 40, 60 mM) then added to 50 mL tissue bath after equilibration. Fresh Krebs solution was prepared for each amounts of KCl. To maintain isotonicity equivalent amounts of NaCl was removed for each addition of increasing amounts of KCl. The tissues were left to equilibrate for 60 minutes before drug addition. The tissue bath was drained out after the peak effect has been reach before each preceding concentration. After restoring the resting tension by washing the tissues and equilibration of 60 minutes after the first curve the addition of KCl was repeated. One

milligram of AV-$CHCl_3$ dissolved in 0.1 mL DMSO was added immediately after each new KCl concentration in the second curve.

6.3.5 Drugs and solutions

All chemicals for the Krebs-bicarbonate buffer (analytical grade) were purchased from Fisher Scientific (Leicestershire, UK). Phenylephrine, β-PEA, Caffeine and KCl were obtained from Sigma Aldrich (Poole, Dorset, UK). Ryanodine was obtained from TOCRIS Biosciences (Northpoint, Avonmouth, UK. All reagents were dissolved in distilled water, unless otherwise stated.

6.3.6 Data analysis

The peak responses in grams induced by ryanodine in repeated phenylephrine CRCs were measured from the baseline before its addition. The ryanodine maximum contractions (±S.E.M) were also expressed as a percentage of the maximum contractile response obtained in phenylephrine CRC_1 set to 100%. For CRC_2 the new baseline after the maximum contractions induced by ryanodine stabilised was used.

The maximum (±S.E.M) and minimum (±S.E.M) responses in grams induced by 30 mM caffeine in guinea pig aorta were measured from the baseline before its addition. The maximum (±S.E.M) and minimum (±S.E.M) responses obtained in the second curved were expressed as a percentage of their corresponding maximum and minimum responses obtained in the first curve set to 100% and -100%.

6.4 Results

6.4.1 Effects of *A. vulgaris* chloroform and methanol crude extracts on contractile responses in guinea pig aorta to phenylephrine and β-PEA

Phenylephrine and β-PEA caused concentration-depentent constrictor responses on the guinea pig aorta. Repeated cumulative CRCs for both phenylephrine and β-PEA previously showed a significant potentiation in the contractile maximum obtained in CRC_2 (refer to section 2.5.4 and section 2.5.5). In the presence of the AV-$CHCl_3$ this potentiation for both phenylephrine (n=4) and β-PEA (n=5) were eliminated (Table 6.1, Figure 6.1.a-b). In addition a significant inhibition ($P<0.05$) of contractile responses expressed as a percent of the CRC_1 maximum was also observed in β-PEA ($3x10^{-4}$ M) from 50.46±6.95% to 25.06±3.75% resulting to a dose ratio of 4.55±2.46 (Table 6.1, Figure 6.1.b).

A similar inhibitory property of AV-MeOH against phenylephrine (n=4) was also observed by the inhibition of the potentiation previously described in repeated CRC on guinea pig aorta (Figure 6.1.c). For β-PEA (n=4) however a significant potentiation ($P<0.01$) was still observed in the presence of the AV-MeOH from 98.21±21 to 134.05±15.38 (Figure 6.1.d). This is similar to contractile response values obtained for the controls which suggests that the plant extract did not alter the increase of responses on repeated CRC to β-PEA (refer to section 2.5.5).

AV-$CHCl_3$ causes a baseline lowering of 16.05±3.40% to phenylephrine and 3.06±1.44% to β-PEA, and AV-MeOH causes also a baseline lowering of 19.30±3.50%

to phenylephrine and an increase of 3.55.0±2.00% to β-PEA from the resting baselines before plant extract addition (Figure 6.2, Figure 6.3).

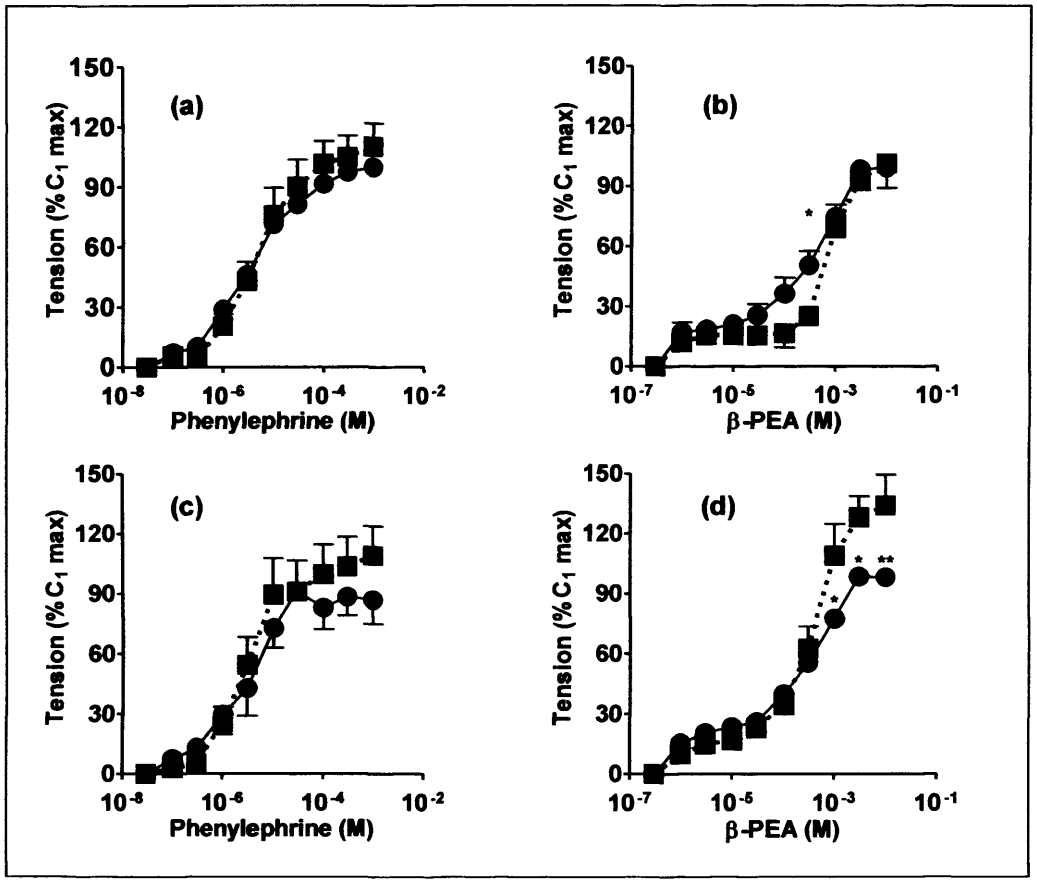


Figure 6.1. Effects of *A. vulgaris* on mean cumulative CRCs of guinea pig aorta for constriction to (a) phenylephrine (n=4) and (b) β-PEA (n=5) treated with chloroform crude extract, and to (c) phenylephrine (n=4) and (d) β-PEA (n=4) treated with methanol crude extract. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant difference (*, $P<0.05$ or **, $P<0.01$) were seen on β-PEA. ●—● CRC_1 after 60 min tissue equilibration and ■—■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1. One milligram of plant extract dissolved in 0.1 mL DMSO was added 20 minutes prior to CRC_2.

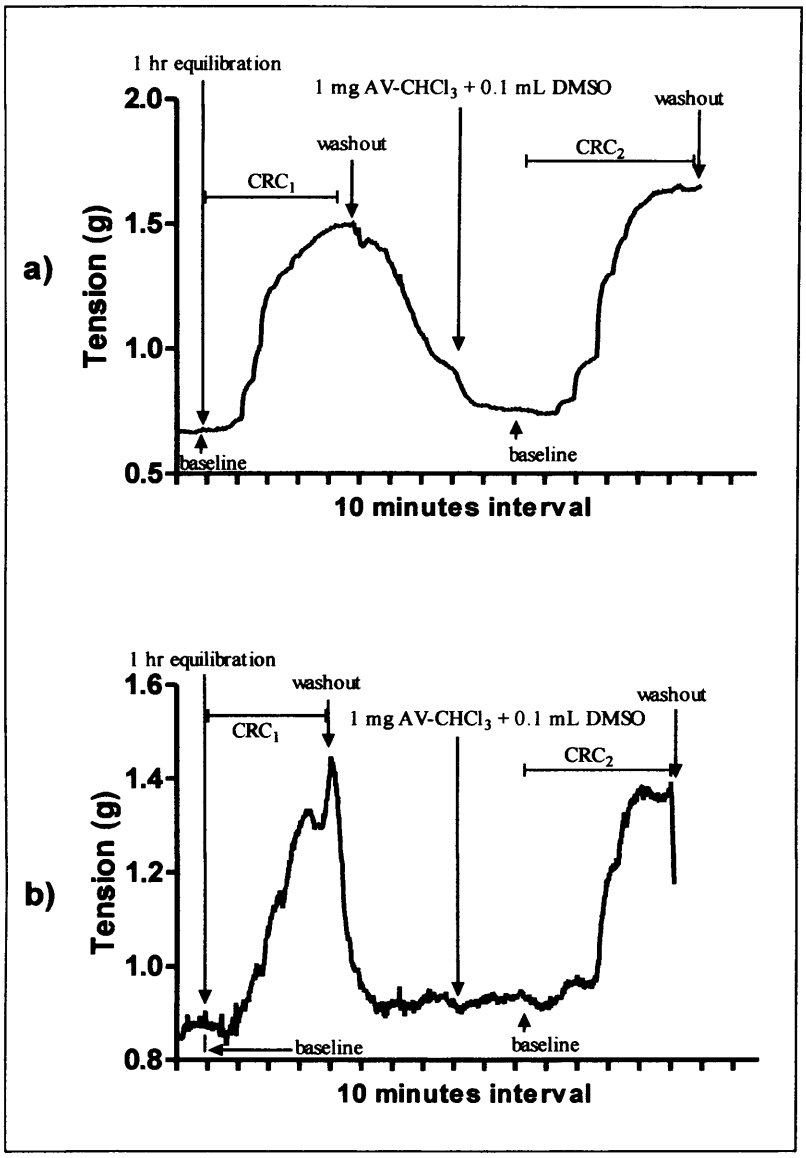


Figure 6.2. Representative chart recordings showing the effects of *A. vulgaris* chloroform extract (AV-$CHCl_3$) in repeated contractile response CRCs of the guinea pig aorta to. (a) Phenylephrine (refer to section 2.4.15 for phenylephrine concentrations used for each CRC) and (b) β-PEA (section 2.4.17 for β-PEA concentrations used for each CRC).

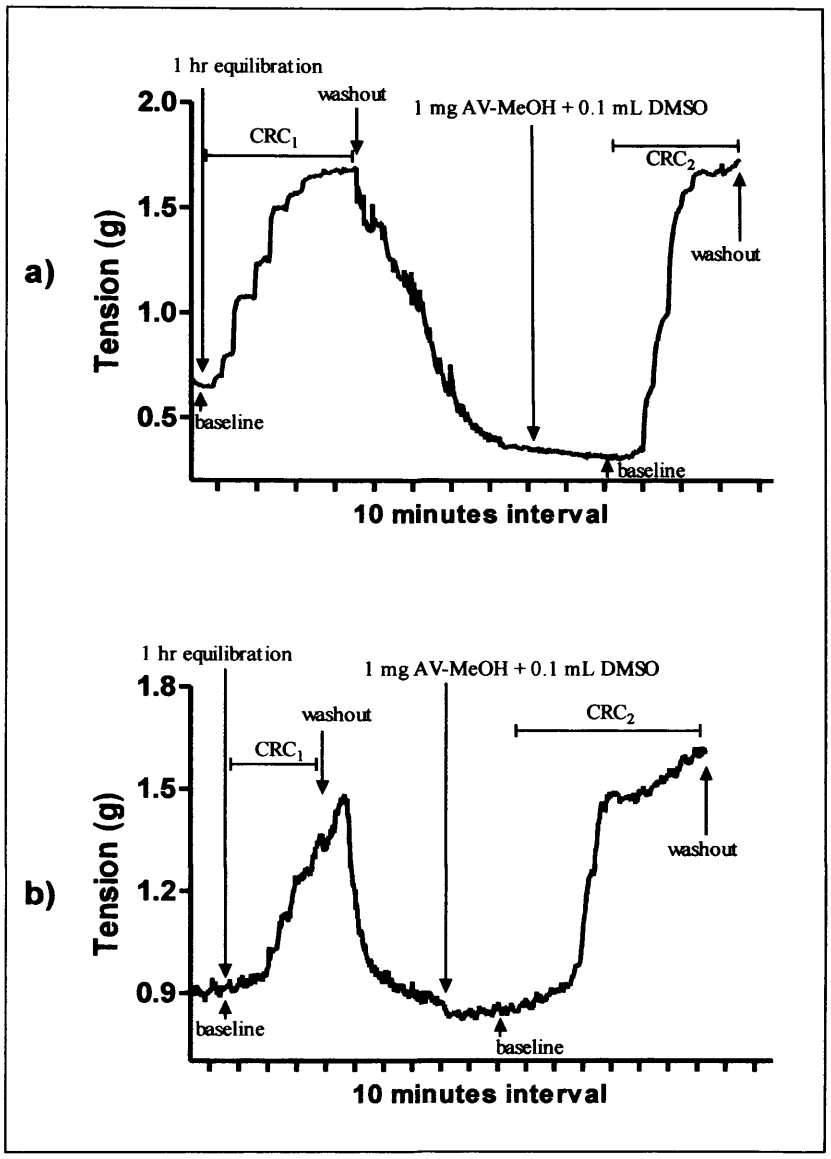


Figure 6.3. Representative chart recordings showing the effects of *A. vulgaris* methanol extract (AV-MeOH) in repeated contractile response CRCs of the guinea pig aorta to (a) Phenylephrine (refer to section 2.4.15 for phenylephrine concentrations used for each CRC) and (b) β-PEA (section 2.4.17 for β-PEA concentrations used for each CRC).

Table 6.1. Summary of the effects of *A. vulgaris* chloroform and methanol crude extract on the maximum and the true -log EC_{50} values of mean cumulative CRC for the constrictor response of the guinea pig ileum to phenylephrine and β-PEA. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test.

A. vulgaris	Agonist		CRC_1	CRC_2	Dose ratio$\left(\frac{CRC_1}{CRC_2}\right)$	n
Chloroform crude extract	Phenylephrine	Max (g)	0.90±0.05	1.00±0.16		4
		-log EC_{50}	-5.47±0.11	-5.32±0.11	1.50±0.28	
	β-PEA	Max (g)	0.67±0.04	0.71±0.10		4
		-log EC_{50}	-3.50±0.19	-3.02±0.07	4.55±2.46	
Methanol crude extract	Phenylephrine	Max (g)	0.66±0.15	0.76±0.25		4
		-log EC_{50}	-5.62±0.13	-5.52±0.14	1.32±0.22	
	β-PEA	Max (g)	0.67±0.11	0.89±0.16		4
		-log EC_{50}	-3.53±0.03	-3.38±0.13	1.60±0.51	

6.4.2 Contractile responses in guinea pig aorta to repeated phenylephrine cumulative CRCs in the presence of ryanodine – Absolute control

The addition of phenylephrine in cumulative CRCs caused concentration-related contractions in the guinea pig aorta. Administration of $1x10^{-5}$ M ryanodine before CRC_2 induced a smaller contraction relative to the maximum response to phenylephrine in CRC_1 (Figure 6.4).

Contractile responses expressed as percentage of the maximum obtained in CRC_1 showed that ryanodine caused non-significant potentiation at $1x10^{-6}$ M from 5.22±4.94% to 50.31±14.54% and maximum potentiation from 97.01±2.93% to 124.73±15.72% to phenylephrine (n=4) (Figure 6.5.a). The contractile response to ryanodine from 1.75±0.98 to 43.98± 16.49 (Figure 6.5.b) was not affected by DMSO but the enhanced maximum response to phenylephrine (n=4) was abolished. Addition of the AV-$CHCl_3$ (n=4) inhibited contractile response to ryanodine. The true -log EC_{50} values for phenylephrine obtained in the presence of AV-$CHCl_3$ showed no significant differences resulting in a dose ratio of 0.90±0.13. -log values of the absolute and DMSO controls were not determined because most of the contractile responses in phenylephrine after the addition of ryanodine were above 50% (Table 6.2). In addition, the contractions induced by ryanodine amounted to 45.50±14.47% without treatment (Figure 6.5.a) and 38.56±17.17% when treated with DMSO (Figure 6.5.b) was totally blocked to 1.39±2.21% (Figure 6.5.c) in the presence of AV-$CHCl_3$.

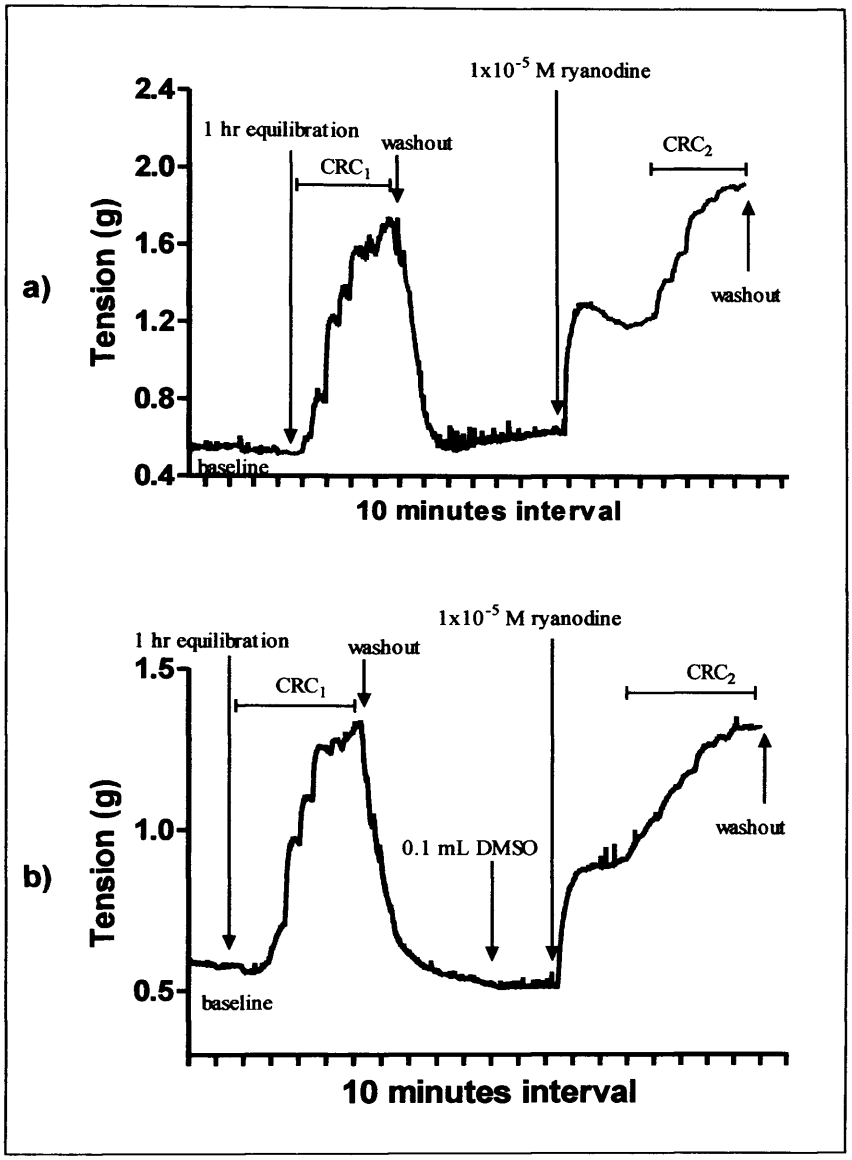


Figure 6.4. Representative chart recordings showing a repeated cumulative concentration-response curve (CRC) for the contractile response of guinea pig trachea to phenylephrine. Ryanodine was added after approximately 60 min after washout of CRC_1. (Refer to section 2.4.15 for phenylephrine concentrations used for each CRC).

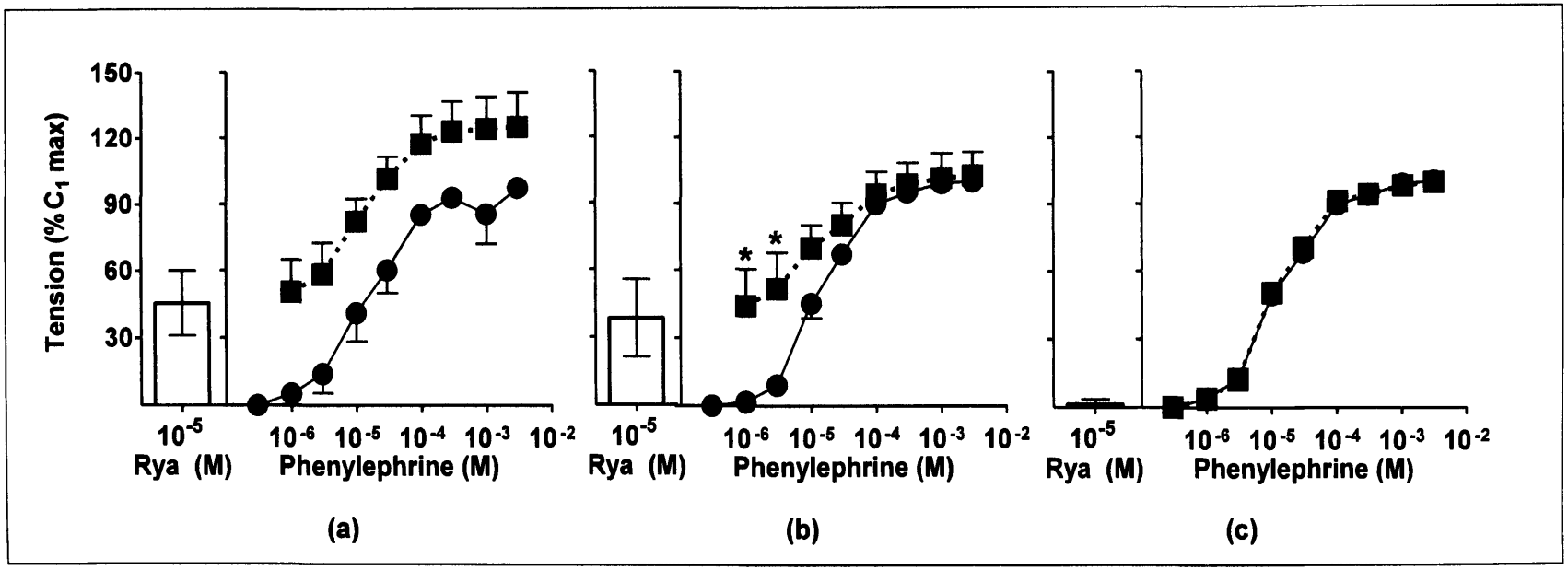


Figure 6.5. The effects of ryanodine on repeated phenylephrine CRCs on guinea pig aorta (a) without treatment, n=4 (b) with DMSO, n=4 and (c) in the presence of *A. vulgaris* chloroform crude extract (n=4). Responses are the mean (±S.E.M.) contractions induced by phenylephrine and ryanodine expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. Significant difference (*, $P<0.05$). ●—● CRC_1 after 60 min tissue equilibration and ■--■ CRC_2 after ryanodine contractions reaches a plateau. Ryanodine ($1x10^{-5}$ M) was added 60 minutes after the washout of CRC_1. 0.1 mL DMSO (b) or 1 mg of plant extract dissolved in 0.1 mL DMSO (c) was added 20 minutes prior to CRC_2.

Table 6.2. Summary of maximum and the true -log EC_{50} of mean cumulative CRC on constrictor response of the guinea pig aorta to phenylephrine CRCs in the presence of ryanodine and treated with *A. vulgaris* chloroform extract. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2 were compared using their corresponding values by paired Student t-test.

Agonist		CRC_1	CRC_2	Dose ratio$\left(\frac{CRC_1}{CRC_2}\right)$	n
Absolute control	Max (g)	1.07±0.14	1.34±0.10		4
	-log EC_{50}	-4.93±0.26	-	-	
DMSO control	Max (g)	0.91±0.06	0.91±0.08		4
	-log EC_{50}	-4.90±0.08	-	-	
AV-$CHCl_3$	Max (g)	1.46±0.19	1.45±0.18		4
	-log EC_{50}	-4.95±0.05	-5.01±0.08	0.90±0.13	

6.4.3 Vasoconstrictions in guinea pig aortic rings to repeated KCl non-cumulative CRCs

The addition of increasing isotonic concentrations of KCl in non-cumulative CRCs caused concentration-related contractions (Figure 6.6).

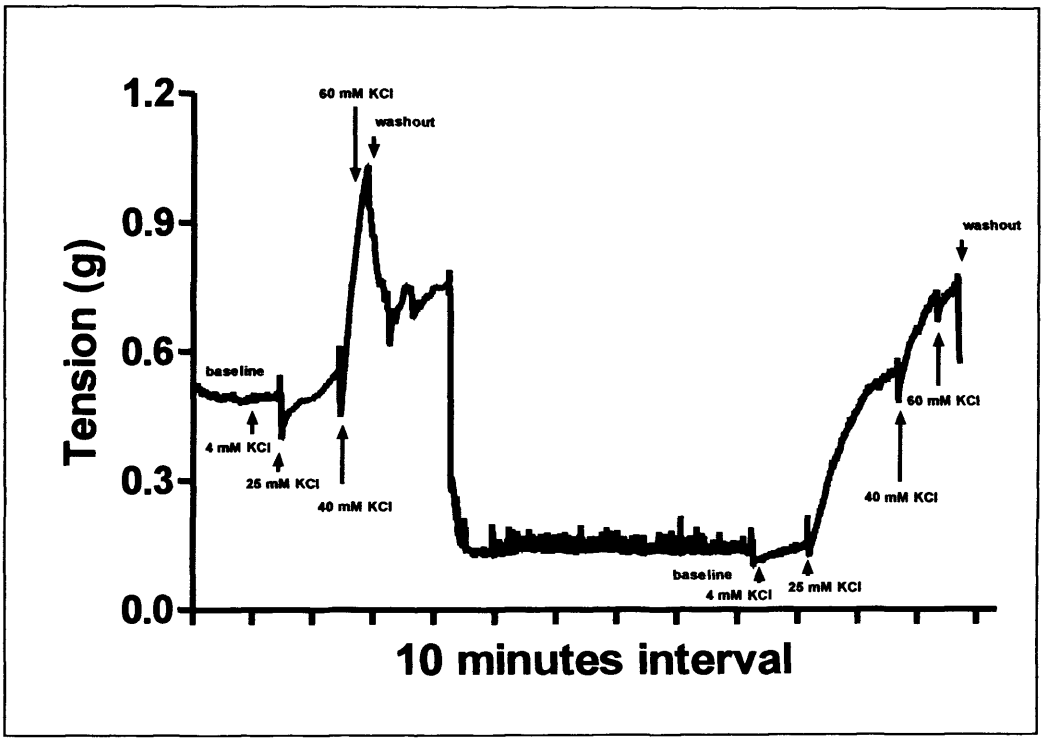


Figure 6.6. Representative chart recording showing a series of cumulative concentration-response curve (CRC) for the contractile response of guinea pig aorta to KCl. To construct non-cumulative CRCs for KCl, successive increasing concentrations of isotonic KCl (4 mM, 25 mM, 40 mM, 60 mM) solutions were added. The 50 mL tissue bath was drained out after the peak effect has been reached for the preceding concentration.

The maximum contractile responses to repeated KCl CRCs showed significant potentiation without treatment ($P<0.05$, n=4), in the presence of DMSO ($P<0.05$, n=5) and in the presence of AV-$CHCl_3$ ($P<0.01$, n=4) from 1.28±0.33g, 1.03±0.13 g and 0.83±0.15 to 1.54±0.38 g, 1.32±0.18 and 1.01±0.15 g (Table 6.3). Contractile responses expressed as a percentage of CRC_1 maximum showed no significant differences at the maxima of the repeated KCl CRCs from 100.00±0.0% for both the absolute control and in the presence of AV-$CHCl_3$ to 123.82±8.24% and 124.125±6.44% Figure 6.7.a-b. In the presence of DMSO a significant increase ($P<0.01$) in repeated KCl CRC from 98.79±1.21% to 128.96±10.45% was observed. No significant differences in EC_{50} values was observed in the absolute control, DMSO control and in the presence of AV-$CHCl_3$ from 30.00±2.97 mM, 33.75±0.75 mM and 31.25±3.09 mM to 30.00±1.96 mM, 34.00±1.68 mM and 32.50±0.29 mM resulting to dose ratios of 1.20±0.08, 1.36±0.07 and 1.30±0.01, respectively.

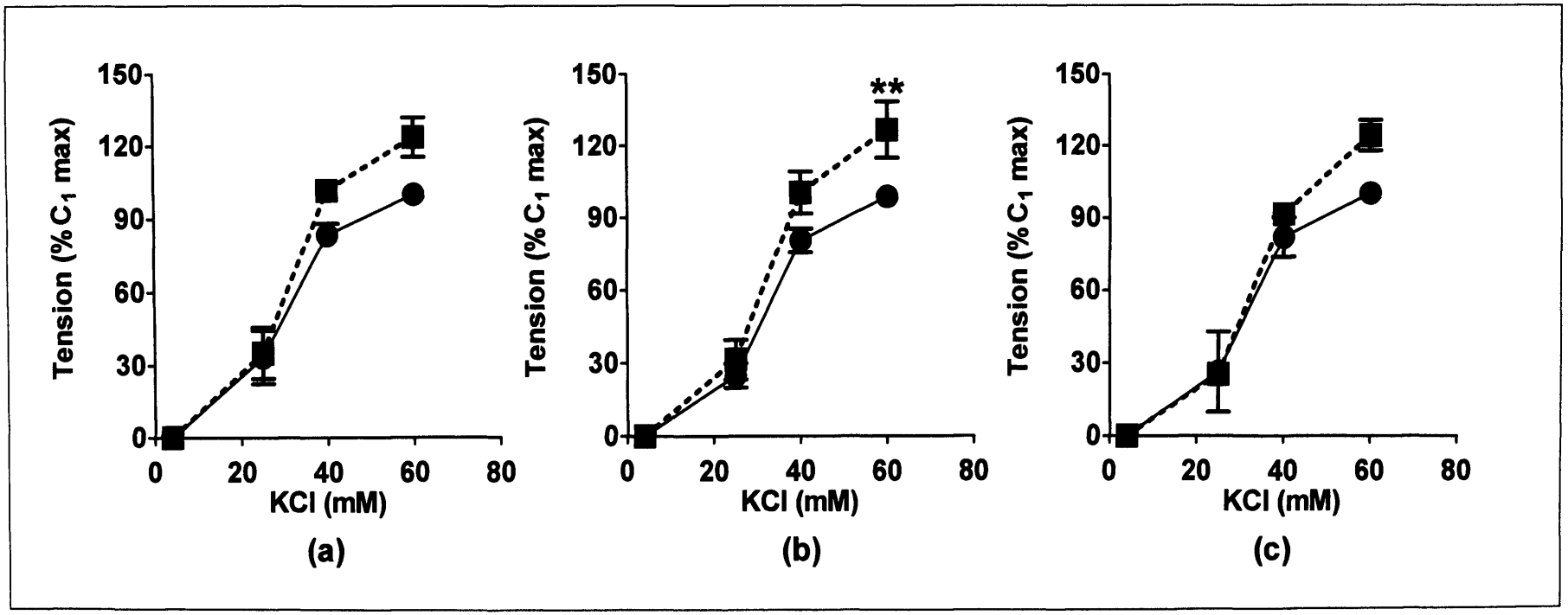


Figure 6.7. Mean cumulative CRCs of guinea pig aorta using the agonists KCl (isotonic solution). (a) Absolute control, *n=4*, (b) DMSO control, n=5 and (c) in the presence of *A. vulgaris* chloroform crude extract, n=4. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the maximum contraction of CRC_1. Mean responses (±S.E.M.) on individual concentrations of CRC_1 and CRC_2 were compared by repeated measures ANOVA followed by Bonferroni pos-hoc test. **Significant differences ($P<0.01$). ●—● CRC_1 after 60 min tissue equilibration and ■--■ CRC_2 after 60 min tissue equilibration after the wash out of CRC_1. One milligram of plant extract dissolved in 0.1 mL DMSO (b) or DMSO (c) was added immediately after each new isotonic concentrations of KCl was made to CRC_2.

Table 6.3. Summary of maximum and the true -log EC_{50} of mean non-cumulative CRC on constrictor response of the guinea pig aorta to KCl – effects of *A. vulgaris* chloroform crude extract. Maximum responses are mean (±S.E.M.) contractions. True EC_{50} (±S.E.M.) were computed based on constrictor responses relative to the maximum of each CRC set to 100%. Mean maximum responses (±S.E.M.) and the true -log EC_{50} (±S.E.M.) of CRC_1 and CRC_2, and CRC_2 and CRC_3 were compared using their corresponding values by paired Student t-test.

Agonist		CRC_1	CRC_2	Dose ratio$\left(\frac{CRC_1}{CRC_2}\right)$	n
Absolute control	**Max (g)**	1.28±0.33	1.54±0.38*		4
	EC_{50} (mM)	30.00±2.97	30.00±1.96	1.20±0.08	
DMSO control	**Max (g)**	1.03±0.13	1.32±0.18*		4
	EC_{50} (mM)	33.75±0.75	34.00±1.68	1.36±0.07	
AV-$CHCl_3$	**Max (g)**	0.83±0.15	1.01±0.15**		4
	EC_{50} (mM)	31.25±3.092	32.50±0.29	1.30±0.01	

6.4.4 The effects of *A. vulgaris* on guinea pig aorta to repeated caffeine contractions

The addition of single dose of 30 mM of caffeine caused contractions followed by relaxation in the guinea pig aorta (Figure 6.8).

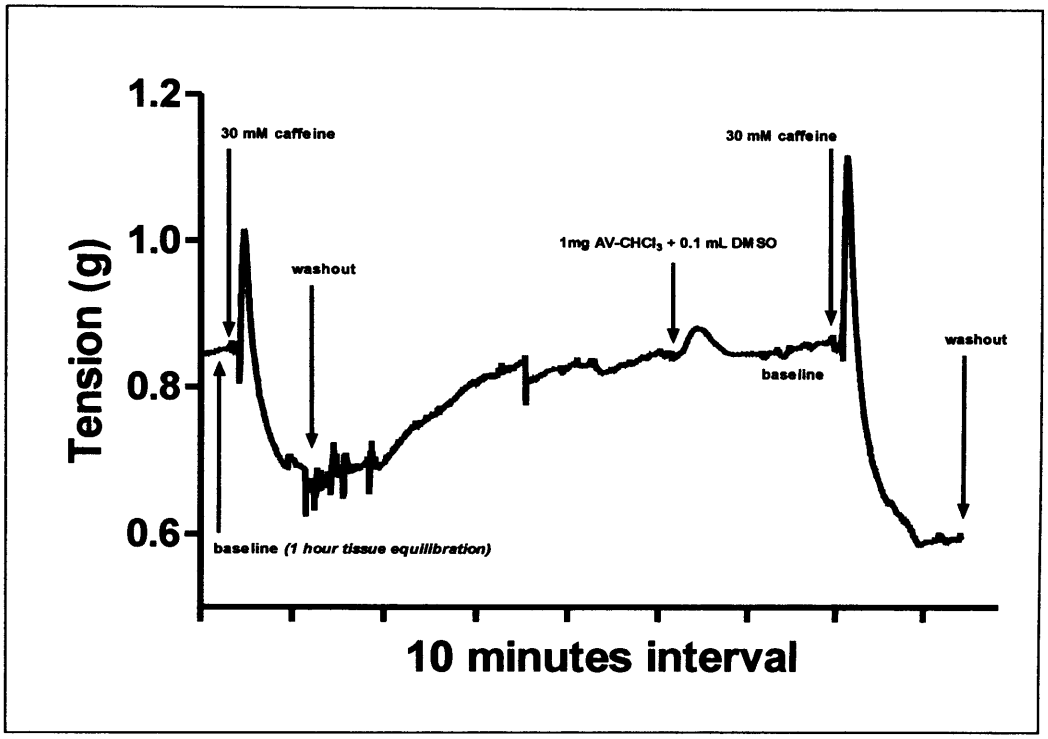


Figure 6.8. Representative chart recording showing repeated single dose concentration-response curve (CRC) for the contractile response of guinea pig aorta to 30 mM caffeine.

The contractile responses induced by repeated 30 mM caffeine challenge (n=4) showed no significant increase on the second challenge from 0.12±0.02 g to 0.16±0.03 g. The relaxation after each contractions induced by caffeine also showed no significant differences in repeated caffeine responses. In the presence of DMSO and AV-$CHCl_3$

the maximum contractions in repeated addition of caffeine showed a significant ($P<0.05$) potentiation from 0.15±0.02 g and 0.18±0.03 g to 0.18±0.04 g and 0.24±0.04 g, followed by non-significant relaxations from 0.07±0.02 g and 0.08±0.03 g to 0.05±0.02 g and 0.11±0.06 g (Table 6.4).

The percentage of maximum contractions induced by repeated caffeine challenge relative to the maximum responses obtained in the first challenge showed no significant differences among the mean contractile maxima of the absolute (134.04±13.114%) and DMSO controls (120.78±7.10%) and with AV-$CHCl_3$ (145.33±19.18%) (Figure 6.9). No significant differences on the relaxations after each contractions induced by caffeine were also observed between the absolute (-70.27±38.00%) and DMSO (-56.02±25.17) controls and in the presence of AV-$CHCl_3$ (-97.83±29.44%) (Figure 6.9).

Table 6.4. Summary of contraction and relaxation responses of the guinea pig aorta to repeated 30 mM caffeine challenge. Maximum and minimum responses are mean (±S.E.M.) contractions. Mean maximum and minimum responses (±S.E.M.) were compared using their corresponding values by paired Student t-test. Significant difference (*, $P<0.05$).

		C_1	C_2	n
Absolute control	**Max (g)**	0.12±0.03	0.16±0.03	4
	Min (g)	0.06±0.02	0.06±0.04	
DMSO control	**Max (g)**	0.15±0.02	0.18±0.03*	4
	Min (g)	0.07±0.02	0.05±0.02	
***A. vulgaris* chloroform crude extract**	**Max (g)**	0.18±0.04	0.24±0.04*	4
	Min (g)	0.08±0.03	0.11±0.06	

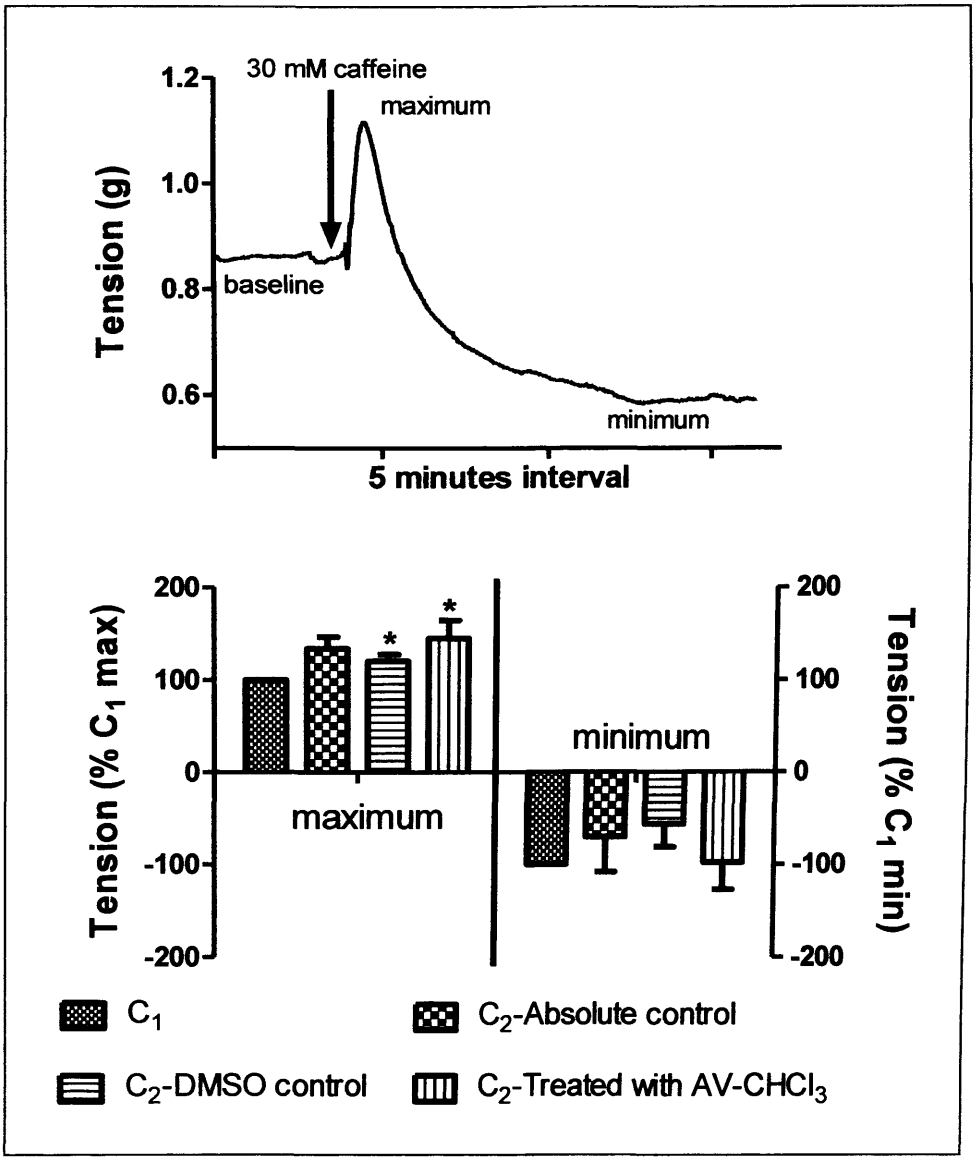


Figure 6.9. The effects of repeated caffeine challenge on contractile responses on guinea pig aorta in the absence (n=4) and presence of DMSO (n=4) or AV-$CHCl_3$ (n=4). Mean maximum and minimum responses (±S.E.M.) were compared using their corresponding values by paired Student t-test. Significant difference (*, $P<0.05$) from C_1. 1 mg of A. *vulgaris* chloroform dissolved in 0.1 mL DMSO was added 20 minutes before the second curve.

6.5 Discussion

6.5.1 Vasoconstrictions in guinea pig aorta to phenylephrine

Vascular smooth muscle contraction like other types of muscles is usually associated with the rise of cytoplasmic concentration of Ca^{2+} (Ford *et al.*, 1999; Karaki *et al.*, 1988; Low *et al.*, 1993). This contraction can either result from intracellular Ca^{2+} release from the sarcoplasmic reticulum (SR) or extracellular Ca^{2+} influx through receptor operated channels (ROCs) (Ford *et al.*, 1999; Low *et al.*, 1993; Rohra *et al.*, 2003; Shinjoh *et al.*, 1991). In the SR two principal pathways have been identified for Ca^{2+} release: one is through the activation of IP_3 receptors, and the second is a Ca^{2+} sensitive pathway regulated by caffeine and ryanodine (Ehrlich *et al.*, 1994; Pan *et al.*, 2000; Wada *et al.*, 1997; Wagenknecht *et al.*, 1997). The increase of cytoplasmic Ca^{2+} facilitates its binding with calmodulin which initiates the activation of myosin light-chain kinase (Houdusse *et al.*, 1996; Silver *et al.*, 1981). The calcium-calmodulin-myosin light kinase complex further undergoes phosphorylation of myosin which initiates contraction and the activation of myosin ATPase (Figure 6.10) (Batchelder *et al.*, 2007; Silver *et al.*, 1981).

Phenylephrine caused concentration-related contractions in the guinea pig aorta. The aortic smooth muscle contractions due to phenylephrine is mediated via the activation of α_1-adrenoceptors that stimulates the IP_3/DAG cascades that leads to the rise in cytoplasmic Ca^{2+} level (Figure 6.10) (Ford *et al.*, 1999). Previously it was shown that repeated exposure of the aortic rings to phenylephrine causes potentiation of the contractile responses.

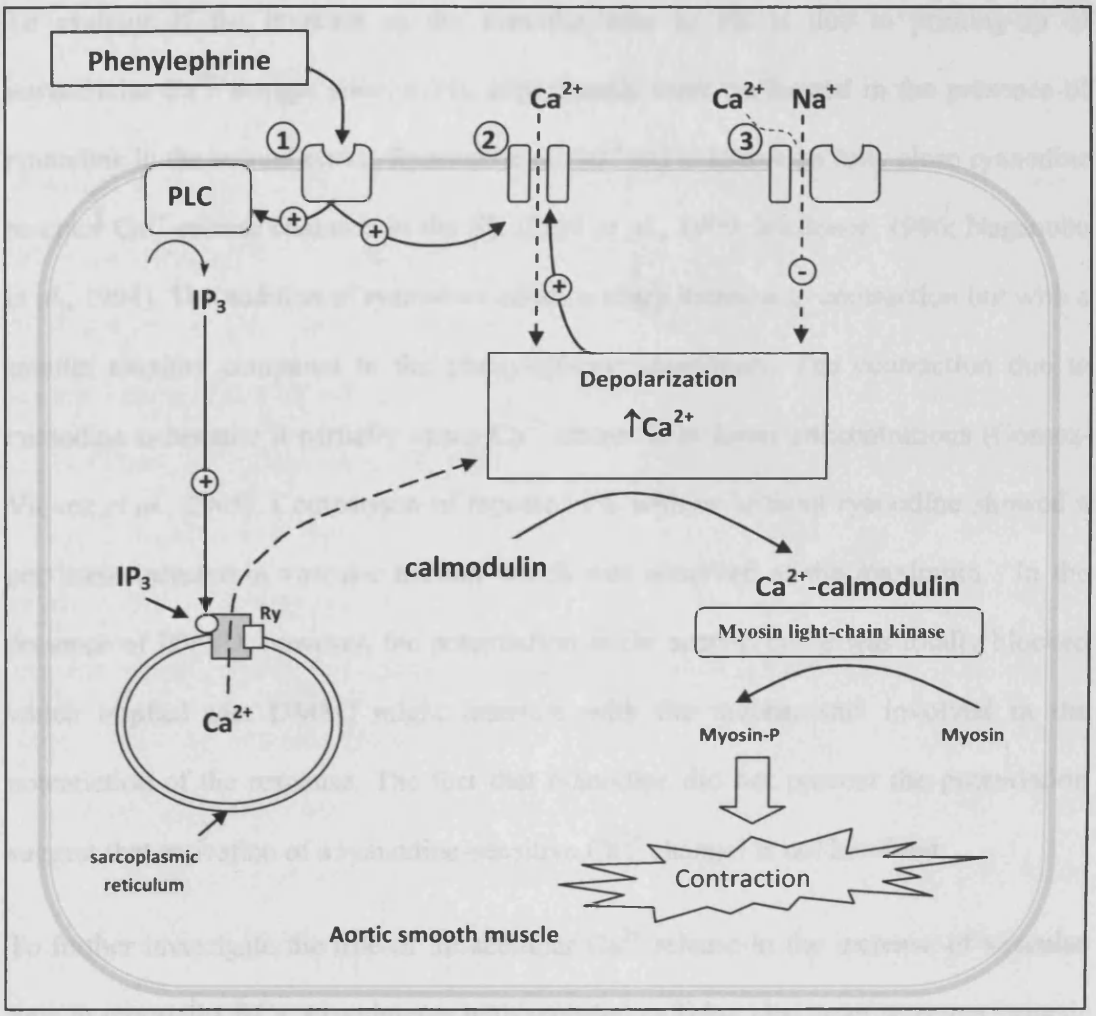


Figure 6.10. The mechanism of smooth muscle contraction due to phenylephrine. 1) α_1-adrenoceptors. 2) Voltage-gated calcium channels. 3) Ligand-gated cation channels. Phospholipase C, PLC; Inositol triphosphate, IP_3; Inositol triphosphate receptor, IP_3R; Ryanodine receptors, Ry. Adapted from (Rang *et al.*, 2007)

To evaluate if the increase in the vascular tone to PE is due to priming-up of intracellular Ca^{2+} storage sites in SR, experiments were performed in the presence of ryanodine in the second curve. Ryanodine ($1x10^{-5}$ M) is known to fully close ryanodine receptor Ca^{2+}-release channels in the SR (Ford *et al.*, 1999; Meissner, 1986; Naganobu *et al.*, 1994). The addition of ryanodine elicits a sharp increase in contraction but with a smaller maxima compared to the phenylephrine maximum. The contraction due to ryanodine is because it partially opens Ca^{2+} channels at lower concentrations (Gómez-Viquez *et al.*, 2005). Comparison of repeated PE with or without ryanodine showed a persistent increase in vascular tension which was observed at the maximum. In the presence of DMSO, however, the potentiation in the second curve was totally blocked which implied that DMSO might interfere with the mechanisms involved in the potentiation of the response. The fact that ryanodine did not prevent the potentiation suggest that activation of a ryanodine-sensitive Ca^{2+} channel is not involved.

To further investigate the role of intracellular Ca^{2+} release in the increase of vascular tone in repeated CRCs, experiments with repeated caffeine challenge were performed. Caffeine is a known agonist of the ryanodine receptors (Borisova *et al.*, 2007; Gómez-Viquez *et al.*, 2005) that fully open Ca^{2+}-release channels in the SR at high concentrations (Ehrlich *et al.*, 1994). With repeated exposures of caffeine, a significant increase of the contractile responses was observed which also occurred in the presence of DMSO. This further suggests that the repeated opening of Ca^{2+}-release channels by caffeine might have similar mechanisms to the rise of contractions due to PE. The secondary relaxation followed after each contraction due to caffeine has been reported

before (Hattori *et al.*, 1994) but no explanation was provided. Neither of the responses was affected by AV-$CHCl_3$ unlike its action on PE in the presence of ryanodine.

To examine the role of the extracellular Ca^{2+} influx component of the contractions in the rise of contractile responses in repeated exposure of aortic ring preparations to PE, experiments of repeated contractions to KCl were also performed. Higher concentrations of extracellular KCl are known to depolarize the cytosol which promotes extracellular Ca^{2+} influx via the voltage-gated calcium channels or ligand gated cation channels (Kaczorowski *et al.*, 1999; Kochegarov, 2003; Shinjoh *et al.*, 1991; Sonkusare *et al.*, 2006). The repeated KCl challenge showed an increase of the low affinity vascular tension similar to contractions obtained to repeated PE challenge. This further showed that the repeated extracellular Ca^{2+} influx at low affinity contractions have similar characteristics to the repeated rise in vascular constriction induced by PE. Thus, it can be suggested that the increase in responses is not due to extracellular Ca^{2+} influx but by intracellular Ca^{2+} release. In the presence of DMSO a potentiation of responses was also observed.

6.5.2 The antagonistic activity of the chloroform and methanol crude extracts of *A. vulgaris* on contractile responses in guinea pig aorta to phenylephrine

Control experiments on the guinea pig aorta showed a significant increase of the maximum of contractile responses on repetition to PE. In the presence of AV-$CHCl_3$ and AV-MeOH the rise in vascular tension was totally inhibited to the pre-extract level. The response however was not blocked further. This suggests that the inhibition is not

merely due to smooth muscle relaxant property or α_1-adrenoceptor blockade. It suggests that the plant components might be blocking the intracellular Ca^{2+} release or extracellular Ca^{2+} influx that controls the enhanced response on repeating exposure.

AV-$CHCl_3$ totally inhibited contractions elicited by ryanodine which suggests that the inhibitory property of the extracts is mediated probably through the intracellular ryanodine receptor Ca^{2+} release mechanism. On the other hand, the inhibition of the increase in vascular tone observed for PE in the presence of AV-$CHCl_3$ cannot be fully explained because DMSO itself inhibited the potentiation of the PE maximum responses. The responses to caffeine exposure and its potentiation on repeated exposure were not affected in the presence of the AV-$CHCl_3$ which also suggests that the plant extract in not inhibiting intracellular Ca^{2+} regulated by caffeine. However, in the vascular smooth muscle ryanodine blocks contractions as a consequence of intracellular Ca^{2+} release induced by caffeine from the SR (Kojima *et al.*, 1994). Therefore it can be suggested that the plant extract is inhibiting the increase in vascular tone similar to the ryanodine action. This might explain the action of *A. vulgaris* extracts of effectively lowering the rat blood pressure only in hypertensive states (Tigno *et al.*, 2000a). In experiments using repeated KCl no alteration of the increase in vascular tension was observed in the presence of AV-$CHCl_3$ which further suggests that the action of AV-$CHCl_3$ in contractions to PE is not through extracellular Ca^{2+} influx.

The previous chapter have shown the isolation of two major components, yomogin and 1,2,3,4-diepoxy-11(13) eudesmen-12,8-olide in AV-$CHCl_3$. Previous study has shown that yomogin inhibits nitric oxide (NO) production (Ryu *et al.*, 1998) and reported for having antihypertensive activity (Tigno *et al.*, 2000a). NO from the endothelium is

known to activate cGMP which can block extracellular Ca^{2+} influx, hyperpolarize the cell through K^+ channels, and decrease intracellular Ca^{2+} release resulting in vascular smooth muscle relaxation (Kansui *et al.*, 2008; Schmidt *et al.*, 1993). However, phenylephrine has been shown to activate α_1-adrenoceptors only but not iNOS (Dora, 2001; López *et al.*, 2009). Thus, an action via NO by yomogin in the extract is unlikely to explain the inhibition of the enhanced vasoconstriction to PE.

6.5.3 The antagonistic activity of the chloroform and methanol crude extracts of *A. vulgaris* on contractile responses in guinea pig aorta to β-PEA

β-PEA caused concentration-related contractions in the guinea pig aorta. Similar studies have shown that β-PEA can induce vasoconstriction in rat aortic rings (Fehler *et al.*, 2010) and porcine isolated coronary artery rings (Herbert *et al.*, 2008). Trace amines are known to induce vasoconstriction through indirect symphathomimetic effect (Broadley, 1996). The progressive exhaustion of stored noradrenaline in repeated release from neuronal storage sites is tachyphylaxis (Broadley, 2010). However, control experiments on the guinea pig aorta showed a significant increase of the vascular tone rather than a decrease with repeated β-PEA exposure which suggests that β-PEA is not acting as an indirect symphathomimetic but is stimulating TAAR.

In the presence of AV-$CHCl_3$ the rise in aortic tension to β-PEA was totally inhibited which suggests that the plant components might be blocking the intracellular Ca^{2+} release similar to its action on ryanodine. This further suggests that TAARs might have a similar mechanism of eliciting contractions via intracellular Ca^{2+} release. However,

like phenylephrine, the presence of the methanolic extract, AV-MeOH, showed no significant reduction on the increase of vascular tension.

6.6 Conclusions

In conclusion, *Artemisia vulgaris* extracts inhibit the increase in vascular tone brought about by repeated activation of intracellular Ca^{2+} release regulated by the ryanodine receptor, resulting from stimulation of α_1-adrenoceptor or TAARs. This mechanism offers a sound mechanism for its effective lowering blood pressure in hypertensive states (Tigno *et al.*, 2000a).

Artemisia vulgaris showed no significant antagonism for TAARs.

Chapter 7

General Discussion

7.1 General discussion

The aim of my thesis was to identify biogenic amine receptor antagonist in the gut, airways and cardiovascular system from selected medicinal plants of Philippine origin. The selected plants were *Artemisia vulgaris, Chrysanthemum coronarium, Moringa oleifera, Sesbania grandiflora* and *Vitex negundo.* This research is particularly relevant in diseases such as hyperactive gut, asthma and allergy, and hypertension. Likewise, this investigation is also equally important in the search for a TAARs antagonist. The lack of specific TAARs antagonist hinders further pharmacological evaluation of its signalling mechanism (Zucchi *et al.*, 2006),

The rationale of this chapter is to present a summary of the most important data obtained and discuss how it validates the traditional beneficial uses of the selected plants. The work in Chapter 2 establishes the controls for preliminary phytochemical and pharmacological work for evaluating the antagonistic activity of the plant extracts. 5-HT, methacholine, histamine, phenylephrine and β-PEA were used as standard agonist for 5-HT_2 receptor, muscarinic M_3 receptor, histamine H_1 receptor, α_1-adrenoceptors and $TAAR_1$, respectively, for contraction of the guinea pig ileum, trachea and aorta. In Chapter 2 it was shown that repeated exposure with concentration-responses curves for the contractions in the presence of the vehicle DMSO produces identical contractile responses. In the aorta however repeated contractions mediated via α_1-adrenoceptors and $TAAR_1$ produces an increase in vascular tone on the second exposure. The preliminary pharmacological screening of the chloroform and methanol extracts of *S. grandiflora* and *C. coronarium* in the guinea pig ileum were examined in

Chapter 3. In Chapter 4, the pharmacological evaluation of the acid-base extract of *V. negundo* and *M. oleifera* in the guinea pig ileum, trachea and aorta were presented. Since most of the biogenic amine receptor antagonists have an amine moiety the acid-base extraction was employed. Chapter 5 and 6 deals with the pharmacological evaluation of *A. vulgaris* chloroform and methanol extracts. The pharmacological evaluation, isolation and structure elucidation of two major components of the extract of *A. vulgaris* yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide were also presented in Chapter 5.

Histamine H_1 receptors and muscarinic M_3 receptors have been known as therapeutic targets for alleviating illnesses related to hyperactive gut, airway hyperresponsiveness, and vascular disease (Brunton *et al.*, 2005). The H_1 receptor and M_3 receptors directly coupled with the G-protein G_q causes direct contractile actions on smooth muscles present in the ileum and trachea through the PLC/IP_3 system (Ehlert, 2003; Leurs *et al.*, 1995; Small, 2005). On the other hand, activation of H_1 receptors and M_3 receptors results also in relaxation of the vascular smooth muscle due to the production and release of an endothelium derived relaxant factor nitric oxide (Beyak *et al.*, 1995; Ehlert, 2003; Hide *et al.*, 1988; Yang *et al.*, 2002).

Sesbania grandiflora is traditionally prescribed for - inflammation resulting from sprain and contusions, diarrhoea and dysentery, and is used also as a laxative (Duke, 1983; Kuhad *et al.*, 2009; Subramanian *et al.*, 2003). Various compounds have been isolated from the different parts of the plant including terpenes (Das *et al.*, 1999), flavonoids (Das *et al.*, 1998; Saxena *et al.*, 1999a), saponins (Tiwari *et al.*, 1964a) and alkaloids (Bhowmick *et al.*, 1988; Fojas *et al.*, 1982). Previous study on the methanolic

leaf extracts revealed the anti-inflammatory properties against carrageenan-induce paw edema (Kuhad *et al.*, 2009).

In the present study the methanolic leaf extracts of *S. grandiflora* revealed histamine H_1 receptor antagonism which further validates the plants traditional use in treatment of inflammation.

The flowers of *S. grandiflora* are considered vegetables in the Philippines (Duke, 1983). The present work on the methanolic flower extracts of *S. grandiflora* revealed the presence of histamine H_1 receptor and muscarinic M_3 receptor antagonism. Inhibition of both of these receptors would induce relaxation and inhibition of gut motility to relieve diarrhoea (Ehlert, 2003; Small, 2005). The traditional belief that the flowers can lower blood pressure (Fojas et al., 1982) might be due also to the activation by the plant extracts of H_1 receptors or M_3 receptors that initiates NO release that results in hypotension (Ehlert, 2003; Small, 2005). However this effect was not determined here in the ileum.

Vitex negundo is traditionally used for its therapeutic effects which include anti-inflammatory, anti-asthma, analgesic, anticonvulsant, and antinociceptive (Dharmasiri *et al.*, 2003; Ismail, 2010; Zaware *et al.*, 2010). The plant was also approved for manufacture and distribution in 1996 in the form of tablets as a remedy for asthma, cough, colds and fever by the Philippine Department of Health (Mendoza, 2010). Phytochemical studies of the plant revealed several groups of bioactive metabolites such as terpenes (Zheng *et al.*, 2010a; Zheng *et al.*, 2010b), flavonoids (Misra *et al.*, 1980; Subramanian *et al.*, 1979), phenols (Rao *et al.*, 1977) and phenylnapthalene-

lignan derivatives which were reported to be potent nitric oxide NO inhibitors (Zheng *et al.*, 2010a). The alkaloid vitedoamine A was also isolated from the plant (Li *et al.*, 2009).

One of the most important uses of *V. negundo* is in the treatment of asthmatic patients (Bansod *et al.*, 2009). In the present study, the plant showed membrane stabilizing components and histamine H_1 receptor antagonism in the ileum and trachea. In asthma, histamine H_1 antagonist becomes important particularly when asthmatic episodes were precipitated by severe histamine release in patients with associated allergy such as rhinitis (runny nose) and urticaria (skin rash) (Gelfand, 2002). Blocking H_1 receptors stops the histamine contribution to allergic rhinitis symptoms such as sneezing, rhinorrhea and nasal ithching and congestion (Buske, 1996; Gelfand, 2002). The plant anti-inflammatory properties can also be validated through its inhibition of the histamine H_1 receptors (Gelfand, 2002).

In the aorta repeated stimulation of α_1-adrenoceptor and $TAAR_1$ by phenylephrine and β-PEA produces an increase in vascular tone. In the presence of *V. negundo* the enhancement of the maximum for both phenylephrine and β-PEA was totally inhibited.

Artemisia vulgaris is a herb commonly used in traditional and alternative medicine (Tigno *et al.*, 2000b). The plant therapeutic uses include anti-inflammatory, anti-asthma, analgesic, antispasmodic (Duke, 1983; Khan *et al.*, 2009; Quisimbing, 1978). It is also useful in treatment of abdominal colic, dyspepsia and diarrhoea, and hypertension (Duke, 1983; Khan *et al.*, 2009; Quisimbing, 1978; Tigno *et al.*, 2000b).

The pollen of the plant has been also implicated to induce allergy of the airways (Pastorello *et al.*, 2002).

In the guinea pig ileum both the *A. vulgaris* chloroform (AV-$CHCl_3$) and methanol (AV-MeOH) extracts showed the presence of histamine H_1 antagonistic properties. Previous work on the methanolic-water extract of the plant suggested that its anti-spasmodic and anti-diarrhoeal activity is possibly mediated through the double blockade of the cholinergic receptors and Ca^{2+} mechanism (Khan *et al.*, 2009). The result on AV-MeOH, however, revealed no cholinergic blockade instead histamine H_1 antagonism was observed. Therefore the use of *A. vulgaris* for the treatment of spasm of the bowels and diarrhoea can be suggested to be mediated through histamine H_1 receptor antagonism.

Bioassay-guided evaluation of AV-$CHCl_3$ for its histamine H_1 receptor antagonism in the ileum resulted to the isolation of two related sesquiterpene lactones, yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide. Further tests have shown that yomogin causes H_1 receptor antagonism. Because yomogin lacks an amine moiety, which most antihistamines posses, it is thought that it is acting indirectly at H_1 receptors by a novel mechanism.

In the current study, AV-$CHCl_3$ further confirmed the presence of histamine H_1 antagonist activity in the trachea. This suggests that the traditional use of *A. vulgaris* for alleviating asthmatic conditions might be mediated through its inhibitory action to histamine H_1 receptors. Its anti-inflammatory properties can also be explained using a similar mechanism.

AV-MeOH showed a non significant shift of the histamine curve to the right indicative of H_1 receptor antagonism but gave a high and variable dose ratio in the guinea pig trachea. Previously the methanolic-water extracts have been reported to inhibit the carbachol-induced contractions in the guinea pig trachea which indicates the presence of chlolinergic antagonist (Khan *et al.*, 2009). It was also suggested that the methanolic water extract causes relaxation on K^+-induced contractions in the trachea (Khan *et al.*, 2009).

In the aorta repeated stimulation of α_1-adrenoceptor and $TAAR_1$ by phenylephrine and β-PEA, respectively, produced an increase in vascular tone on the second exposure. In the presence of AV-$CHCl_3$ the enhancement of the maximum was totally inhibited. Further work in the role of intracellular Ca^{2+} release and extracellular Ca^{2+} influx have shown that that AV-$CHCl_3$ is inhibiting intracellular Ca^{2+} release mechanisms regulated by the ryanodine receptors. This provides a sound mechanism in previous study for the observation that *A. vulgaris* extracts were effective in lowering rat blood pressure only in hypertensive states (Tigno *et al.*, 2000a; Tigno *et al.*, 2000b).

Trace amine associated receptors

Trace amine associated-receptors are a class of GPCRs identified in 2001 (Frascarelli *et al.*, 2008; Zucchi *et al.*, 2006). These receptors are also known as pharmacological targets of trace amines such as β-PEA, tyramine, and tryptamine (Broadley, 2010; Grandy, 2007).

Trace amines are endogenous biogenic amines with close similarity to the structure of major biogenic amines and overlapping functions with the aminergic pathways

(Grandy, 2007; Premont et al., 2001). They are usually regarded as indirectly acting symphathomimetic amines (ISA) (Gilman et al., 1990). Actions of ISAs include inhibition of gut motility (Broadley *et al.*, 2009; Innes *et al.*, 1969), bronchodilatation (Hawthorn *et al.*, 1985) and vasoconstriction (Broadley, 2010).

In the present study, β-PEA was used to stimulate TAARs in the ileum, trachea and aorta. In the guinea pig isolated gut preparations β-PEA caused contraction which agrees with previous reports (Innes *et al.*, 1969) that β-PEA contracts the gut. This is opposite to the expected action of sympathomimetic amines which cause relaxation in the gastrointestinal tract due to the indirect activation by noradrenaline to the α_1- and -β_1 adrenoceptors of the longitudinal smooth muscle (Ahlquist *et al.*, 1959; Broadley *et al.*, 2009; Grassby *et al.*, 1987; Innes *et al.*, 1969). Similar studies have also shown that the contractions due to β-PEA are not inhibited by adrenoceptor, 5-HT_2 receptor and muscarinic receptor antagonist in the gut (Broadley *et al.*, 2009). Thus, it has been concluded that these contractions are mediated via TAARs.

In the guinea pig trachea β-PEA produces a biphasic response. At low concentrations a β-adrenoceptor-mediated relaxation occurs, followed by contraction. The relaxation was attributed to β-adrenoceptor stimulation because this was abolished by the β-adrenoceptor antagonist propranolol. However, the principal response is contraction, indicative of bronchoconstriction at higher β-PEA concentrations (Hawthorn *et al.*, 1985). Previously it was also reported that the depletion of catecholamines by pre-treatment with reserpine produces a small shift of β-PEA to higher concentrations (Hawthorn *et al.*, 1984). However, only a small fraction was attributed to an indirect

effect because it did not affect the maximum contractile response (Hawthorn *et al.*, 1984).

In the guinea pig aorta, β-PEA produced a vasoconstriction. Previous study has shown that the vasoconstrictor action of β-PEA is resistant to adrenoceptor inhibition in guinea pig and rat aorta (Fehler *et al.*, 2010) and pig coronary blood vessels (Baker *et al.*, 2007; Herbert *et al.*, 2008). Therefore β-PEA is not behaving as an ISA. The progressive exhaustion of stored adrenaline in repeated release from neuronal sites by ISA leads to tachyphylaxis, which is a diminishing response with repeated exposure (Broadley, 2010). However, the present study showed that repeated exposure of β-PEA produces an enhancement of the vascular tone which is opposite to the action of ISAs.

The present results have therefore shown that β-PEA owes its pharmacological actions partly due to indirect symphathomimetic effects but predominantly to stimulation of TAAR receptors via unidentified pathways.

7.2 Future work

In the present study, the methanolic extracts of *Sesbania grandiflora* revealed the presence of histamine H_1 receptors and muscarinic M_3 receptors antagonist. Therefore it is of interest to carry out isolation and structure identification of the active components that exhibit this antagonism. Further pharmacological evaluation of *S. grandiflora* in trachea and aorta is also of interest. The traditional belief that the flowers can lower blood pressure (Fojas et al., 1982) which might be due to the activation of H_1 receptors and M_3 receptors that initiates NO release should be investigated further.

In the present study, the alkaloid extracts of *Vitex negundo* revealed the presence of histamine H_1 antagonistic properties in the guinea pig ileum and trachea. Therefore it is of interest to also carry out bioassay-guided isolation to identify the active component that exhibits this antagonism.

The chloroform layer of *A. vulgaris* yielded histamine H_1 antagonist components. The evaluation of the histamine H_1 antagonism in the guinea pig ileum of the plant extract resulted to the isolation of two sesquiterpene lactones, yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide. Therefore, it is of interest to further evaluate these compounds in the trachea and aorta.

In the present study, yomogin was found to have indirect histamine H_1 antagonistic activity in the ileum. Because yomogin lacks the amine moiety that most antihistamine posses it is thought that it might be working in a novel mechanism. Therefore, further studies could focus on the elucidation of this mechanism.

In the present study the methanol layer of *A. vulgaris* also exhibited the presence of histamine H_1 antagonist. Therefore it is of interest to also carry out bioassay-isolation to identify the active components that exhibits this antagonism.

In the aorta repeated stimulation of α_1-adrenoceptor and $TAAR_1$ by phenylephrine and β-PEA, respectively, produced an increase in vascular tone. In the presence of *V. negundo* and *A. vulgaris* extracts the enhancement of the maximum was totally inhibited. Therefore it is of interest to carry out bioassay-guided isolation to identify the active components.

7.3 General Conclusion

The present study describes the preliminary evaluation of Philippine medicinal plants *Artemisia vulgaris, Chrysanthemum coronarium, Moringa oleifera, Sesbania grandiflora* and *Vitex negundo* for their antagonistic activity at selected biogenic amine receptors on smooth muscle of the airways, gastrointestinal tract and vascular system.

The methanolic extracts of *S. grandiflora* (flowers and leaves) revealed the presence of histamine H_1 receptor and muscarinic M_3 receptor antagonist activity in the guinea pig ileum. The *A. vulgaris* chloroform (AV-$CHCl_3$) and methanol extracts (AV-MeOH), and the alkaloid layer of *V. negundo* (VN-E) showed histamine H_1 antagonism in the guinea pig ileum and trachea. Further analysis of AV-$CHCl_3$ isolated two major components, yomogin and 1,2,3,4-diepoxy-11(13)-eudesmen-12,8-olide. Yomogin a sesquiterpene lactone exhibited a novel histamine H_1 receptor antagonism in the ileum.

Repeated exposure of aortic rings to concentration-response curves of phenylephrine and β-PEA resulted in a potentiation of the maximum response on the second exposure. Both the AV-$CHCl_3$ and VN-E inhibited this increase in vascular tension due to the repeated stimulation by phenylephrine and β-PEA. Further analysis of AV-$CHCl_3$ revealed that it is inhibiting the increase of vascular tone mediated via intracellular Ca^{2+} release regulated by ryanodine. This provides a sound mechanism that *A. vulgaris* extracts effectively lowers the rat blood pressure only in hypertensive states (Tigno *et al.*, 2000a; Tigno *et al.*, 2000b). No antagonism of the selected biogenic amine receptors was observed for *C. coronarium* and *M. oleifera* extracts.

My investigations on the selected medicinal plants have expanded the knowledge of their antagonistic properties in isolated tissues. This study also validates some of the traditional uses of the plants and offer insights into their roles in the treatment of diseases related to the gut, airways and vascular system. An aim of this thesis was to identify antagonism for TAARs since there are currently still no selective antagonists available. While a selective potentiation of the response to β-PEA by *A. vulgaris* methanol extracts was observed in trachea, no selective antagonistic activity of β-PEA over the other biogenic amines was found. The selective potentiation of β-PEA may suggest binding to new TAARs which further study may provide a clue to selective manipulation of the receptor.

Chapter 8

BIBLIOGRAPHY

Bibliography

Abourashed, EA, El-Alfy, AT, Khan, IA, Walker, L (2003) Ephedra in Perspective - a Current Review. *Phytotherapy Research* **17:** 703-712.

Aburto, T, Jinsi, A, Zhu, Q, Deth, RC (1995) Involvement of protein kinase C activation in α2-adrenoceptor-mediated contractions of rabbit saphenous vein. *European Journal of Pharmacology* **277**(1): 35-44.

Aburto, T, Lajoie, C, Morgan, K (1993) Mechanisms of signal transduction during alpha 2-adrenergic receptor- mediated contraction of vascular smooth muscle. *Circulation Research* **72:** 778-785.

Achari, B, Chowdhury, US, Dutta, PK, Pakrashi, SC (1984) Studies on Indian medicinal plants. Part 74. Two isomeric flavanones from Vitex negundo. *Phytochemistry* **23**(3): 703-704.

Ackerman, V, Carpi, S, Bellini, A, Vassalli, G, Marini, M, Mattoli, S (1995) Constitutive expression of endothelin in bronchial epithelial cells of patients with symptomatic and asymptomatic asthma and modulation by histamine and interleukin-1. *Journal of Allergy and Clinical Immunology* **5:** 618-627.

Ahlquist, RP, Levy, B (1959) Adrenergic recptive mechanism of canine ileum. *Journal of Pharmac. exp. Ther.* **127:** 146-149.

Akdis, CA, Simons, FER (2006) Histamine receptors are hot in immunopharmacology. *European Journal of Pharmacology* **533**(69-76).

Al-Motarreb, A, Al-Habori, M, Broadley, KJ (2010) Khat chewing, cardiovascular diseases and other internal medical problems: The current situation and directions for future research. *Journal of Ethnopharmacology* **In Press, Corrected Proof**.

Alahuhta, S, Räsänen, J, Jouppila, P, Jouppila, R, Hollmén, AI (1992) Ephedrine and phenylephrine for avoiding maternal hypotension due to spinal anaesthesia for caesarean section : Effects on uteroplacental and fetal haemodynamics. *International Journal of Obstetric Anesthesia* **1**(3): 129-134.

Allen, JA, Yadav, PN, Roth, BL (2008) Insights into the regulation of 5-HT2A serotonin receptors by scaffolding proteins and kinases. *Neuropharmacology* **55:** 961–968.

Andal, KR, Sulochana, N (1986) Chemical examination of the seeds of *Sesbania grandiflora*. *Fitoterapia* **57**(4): 293-294.

Andersen, G, Christrup, L, Sjøgren, P (2003) Relationships Among Morphine Metabolism, Pain and Side Effects During Long-Term Treatment: An Update. *Journal of Pain and Symptom Management* **25**(1): 74-91.

Anisuzzaman, M, Rahman, AHMM, Harun-Or-Rashid, M, Naderuzzaman, ATM, Islam, AKMR (2007) An Ethnobotanical Study of Madhupur, Tangail. *Journal of Applied Sciences Research* **3**(7): 519-530.

Anwar, F, Latif, S, Ashraf, M, Gilani, AH (2007) Moringa oleifera: a food plant with multiple medicinal uses. *Phytotherapy Research* **21**(1): 17-25.

Atawodi, SE, Atawodi, JC, Idakwo, GA, Pfundstein, B, Haubner, R, Wurtele, G, Bartsch, H, Owen, RW (2010) Evaluation of the Polyphenol Content and Antioxidant Properties of Methanol Extracts of the Leaves, Stem, and Root Barks of Moringa oleifera Lam. *Journal of Medicinal Food* **13**(3): 710-716.

Baker, KE, Herbert, AA, Broadley, KJ (2007) Vasoconstriction of porcine left anterior descending coronary artery by ecstasy and cathinone is not an indirect sympathomimetic effect. *Vascular Pharmacology* **47**(1): 10-17.

Baker, RW, Chothia, CH, Pauling, P, Petcher, TJ (1971) Structure and activity of muscarinic stimulants. *Nature* **230:** 439-445.

Banerji, A, Chadha, MS, Malshet, VG (1969) Isolation of 5-hydroxy-3,6,7,3',4'-pentamethoxyflavone from Vitex negundo. *Phytochemistry (Elsevier)* **8**(2): 511-512.

Banerji, J, Das, B, Chakrabarty, R, Jha, H (1988) Isolation of 4,4'-dimethoxy-trans-stilbene and flavonoids from leaves and twigs of Vitex negundo Linn. *Indian Journal of Chemistry, Section B: Organic Chemistry Including Medicinal Chemistry* **27B**(6): 597-599.

Bansod, MS, Harle, UN (2009) Vitex negundo L.: phytochemical constituents, traditional uses and pharmacological properties: comprehensive review. *Pharmacologyonline* **1:** 286-302.

Barnes, NM, Sharp, T (1999) A review of central 5-HT receptors and their function. *Neuropharmacology* **38:** 1083-1152.

Barnes, PJ (1993) β-adrenoceptors on smooth muscle, nerves and inflammatory cells. *Life Sciences* **52**(26): 2101-2109.

Barnes, PJ, Chung, KF, Page, CP (1998) Inflammatory Mediators of Asthma: An Update. *Pharmacological Revies* **50**(4): 515-596.

Barnes, WG, Grinde, E, Crawford, DR, Herrick-Davis, K, Hough, LB (2004) Characterization of a new mRNA species from the human histamine N-methyltransferase gene. *Genomics* **83**(1): 168-171.

Batchelder, EL, Thomas-Virnig, CL, Hardin, JD, White, JG (2007) Cytokinesis is not controlled by calmodulin or myosin light chain kinase in the Caenorhabditis elegans early embryo. *FEBS Letters* **581**(22): 4337-4341.

Berger, M, Gray, JA, Roth, BL (2009) The Expanded Biology of Serotonin. *Annu. Rev. Med.* **60:** 355-366.

Berry, MD (2004) Mammalian central nervous system trace amines. Pharmacologic amphetamines, physiologic neuromodulators. *Journal of Neurochemistry* **90**(2): 257-271.

Berry, MD (2009) Trace Monoamines and Receptors in Mammalian CNS *Encyclopedia of Neuroscience* 1047-1054.

Beubler, E, Schirgi-Degen, A, Gamse, R (1993) Inhibition of 5-hydroxytryptamine- and enterotoxin-induced fluid secretion by 5-HT receptor antagonists in the rat jejunum. *European Journal of Pharmacology: Environmental Toxicology and Pharmacology* **248**(2): 157-162.

Beyak, M, Vanner, S (1995) Histamine H1 and H3 vasodilator mechanisms in the guinea pig ileum. *Gastroenterology* **108**(3): 712-718.

Bhattacharjee, SR, Mullick, DK (1958) Isolation of γ -sitosterol from seeds of *Sesbania grandiflora*. *Science and Culture* **24**: 293-294.

Bhowmick, PK, Basu, PS (1988) Indoleacetic acid production by Rhizobium sp. from a leguminous tree, *Sesbania grandiflora* Pers. *Egyptian Journal of Microbiology* **22**(2): 293-301.

Bischoff, S, Crowe, SE (2005) Gastrointestinal food allergy: New insights into pathophysiology and clinical perspectives *Gastroenterology* **128**(4): 1089-1113.

Biswas, A, Ramaswamy, S, Rajasekaran, M, Bapna, J (1988) Effect of Moringa oleifera bark on the male reproductive functions in rats. *Medical Science Research* **16**(No. 23): 1219-1220.

Blaschko, H (1957) Metabolism and storage of biogenic amines. *Experentia* **15**(1).

Bockaert, J, Pin, JP (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. *The EMBO Journal* **18**: 1723 - 1729

Bodhipadma, K, Noichinda, S, Udomrati, S, Nathalang, G, Kijwijan, B, Leung, DWM (2006) Anthocyanin accumulation in the hypocotyl and petal of Red Agati (*Sesbania grandiflora*), an ornamental legume. *Journal of Applied Horticulture* **8**(2): 143-146.

Boess, FG, Martin, IL (1994) Molecular biology of 5-HT receptors. *Neuropharmacology* **33**(3-4): 275-317.

Borisova, L, Shmygol, A, Wray, S, Burdyga, T (2007) Evidence that a Ca2+ sparks/STOCs coupling mechanism is responsible for the inhibitory effect of caffeine on electro-mechanical coupling in guinea pig ureteric smooth muscle. *Cell Calcium* **42**: 303-311.

Borowsky, B, Adham, N, Jones, KA, Raddatz, R, Artymyshyn, R, Ogozalek, KL, Durkin, MM, Lakhlani, PP, Bonini, JA, Pathirana, S, Boyle, N, Pu, X, Kouranova, E, Lichtblau, H, Ochoa, FY, Branchek, TA, Gerald, C (2001) Trace amines: Identification of a family of mammalian G protein-coupled receptors. *PNAS* **98**(16): 8966-8971

Boulton, AA (1982) Some aspects of basic psychopharmacology: The trace amines. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **6**(4-6): 563-570.

Bour, S, Visentin, V, Prevot, D, Daviaud, D, Saulnier-Blache, JS, Guigne, C, Valet, P, Carpene, C (2005) Effects of oral administration of benzylamine on glucose tolerance and lipid metabolism in rats. *Journal of physiology and biochemistry* **61**(2): 371-379.

Branchek, TA, Blackburn, TP (2003) Trace amine receptors as targets for novel therapeutics: legend, myth and fact. *Current Opinion in Pharmacology* **3**(1): 90-97.

Bratt, K Seconday Plant Metabolites as Defense agiants Hebivores and oxidative Stress, Synthesis, Isolation and Biological Evaluation. Doctor of Philosophy in Organic Chemistry, Uppsala University, UPPSALA, 2000.

Brielmann, HL, Setzer, WN, Kaufman, PB, Kirakosyan, A, Cseke, LJ (2006) Phytochemical: The Chemical Components of Plants. In: *Natural Products from Plants*, Cseke, LJ, Kirakosyan, A, Kaufman, PB, Warber, SL, Duke, JA, Brielmann, HL (eds), 2nd edn, pp 1-49: Taylor & Francis.

Broadley, KJ (1996) *Autonomic Pharmacology*. Taylor & Francis: London.

Broadley, KJ (2010) The vascular effects of trace amines and amphetamines. *Pharmacology & Therapeutics* **125**(3).

Broadley, KJ, Anwar, MA, Herbert, AA, Fehler, M, Jones, EM, Davies, WE, Kidd, EJ, Ford, WR (2009) Effects of dietary amines on the gut and its vasculature. *British Journal of nutrition* **1010**: 1645-1652.

Brody, T, Carner, J, Minneman, K (1998) *Human Pharmacology - Molecular to Clinical*. Third edn. Mosby. A harcourt Health Science Company: London.

Brunton, L, Lazo, J, Parker, K (2005) *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. 11th edition edn. McGraw Hill Publishing.

Burchett, SA, Hicks, TP (2006) The mysterious trace amines: Protean neuromodulators of synaptic transmission in mammalian brain. *Progress in Neurobiology* **79**(5-6): 223-246.

Burns, K, Michalak, M (1993) Interactions of calreticulin with proteins of the endoplasmic and sarcoplasmic reticulum membranes. *Federation of European Biochemical Societies Letters* **318**(2): 181-185.

Buske, LMD (1996) Clinical comparison of histamine H1-receptor antagonist drugs. *Journal of Allergy and Clinical Immunology* **98**(6): s307-s318.

Buss, AD, Butler, MS (2010) *Natural Product Chemistry for Drug Discovery*. The Royal Society of Chemistry 2010: Cambridge.

Caceres, A, Saravia, A, Rizzo, S, Zabala, L, De Leon, E, Nave, F (1992) Pharmacologic properties of Moringa oleifera. 2: Screening for antispasmodic, antiinflammatory and diuretic activity. *Journal of ethnopharmacology* **36**(3): 233-237.

Carnat, A, Heitz, A, Fraisse, D, Carnat, A-p, Lamaison, J-L (2000) Major dicaffeoylquinic acids from *Artemisia vulgaris*. *Fitoterapia* **71**: 587-589.

Caulfield, MP (1993) Muscarinic Receptors—Characterization, coupling and function. *Pharmacology & Therapeutics* **58**(3): 319-379.

Chawla, AS, Sharma, AK, Handa, SS, Dhar, KL (1992) Chemical investigation and anti-inflammatory activity of Vitex negundo seeds. *journal of Natural Products* **55**(2): 163-167.

Chen, W-L, Tsai, T-H, Yang, CCH, Kuo, TBJ (2010) Effects of ephedra on autonomic nervous modulation in healthy young adults. *Journal of Ethnopharmacology* **130**(3): 563-568.

Choi, J-M, Lee, E-O, Lee, H-J, Kim, K-H, Ahn, K-S, Shim, B-S, Kim, N-I, Song, M-C, Baek, N-I, Kim, S-H (2007) Identification of campesterol from *Chrysanthemum coronarium* L. and its antiangiogenic activities. *Phytotherapy Research* **21**(10): 954-959.

Chuang, P-H, Lee, C-W, Chou, J-Y, Murugan, M, Shieh, B-J, Chen, H-M (2006) Anti-fungal activity of crude extracts and essential oil of Moringa oleifera Lam. *Bioresource Technology* **98**(1): 232-236.

Chuda, Y, Ono, H, Ohnishi-Kameyama, M, Nagata, T, Tsushida, T (1996) Structural identification of two antioxidant quinic acid derivatives from garland (*Chrysanthemum coronarium* L.). *Journal of Agricultural and Food Chemistry* **44**(8): 2037-2039.

Clarke, H, Cushley, MJ, Persson, CG, Holgate, ST (1989) The protective effects of intravenous theophylline and enprofylline against histamine- and Adenosine 5'-monophosphate-provoked bronchoconstriction: Implications for the mechanisms of action of xanthine derivatives in asthma. *Pulmonary Pharmacology* **2**(3): 147-154.

Cohen, C, Welzl, H, Bättig, K (1991) Effects of nicotine, caffeine, and their combination on locomotor activity in rats *Pharmacology Biochemistry and Behavior* **40**(1): 121-123.

Cohen, ML, Schenck, KW, Colbert, W, Wittenauer, L (1985) Role of 5-HT2 receptors in serotonin-induced contractions of nonvascular smooth muscle. *The Journal of Pharmacology* **232**(3): 770-774.

Coppin, JP, Xu, Y, Chen, H, Juliani, R, Wu, Q-L, Simon, JE (2008) Determination of flavonoids in Moringa oleifera by LC/UV/MSD. *Abstracts of Papers, 236th ACS National Meeting, Philadelphia, PA, United States, August 17-21, 2008* AGFD-147.

Corboz, MR, Rivelli, MA, Mingo, GG, McLeod, RL, Varty, L, Jia, Y, Hey, JA (2008) Mechanism of decongestant activity of a2-adrenoceptor agonists. *Pulmonary Pharmacology & Therapeutics* **21**: 449–454.

Cotecchia, S, Kobilka, BK, Daniel, KW, Nolan, RD, LapetinaS, EY, Caron, MG, Lefkowitz, RJ, Regang, JW (1990) Multiple Second Messenger Pathways of cu-Adrenergic Receptor Subtypes Expressed in Eukaryotic Cells. *The Journal of Biological Chemistry* **265**(1): 63-69.

Cowan, A, Waton, NG (1971) Distribution of free histamine, and histamine-forming and destroying activities in animal tissues. *Comp. gen. Pharmac.* **3:** 75-82.

Cseke, LJ, Setzer, WN, Vogler, B, Kirakosyan, A, Kaufman, PB (2006) Traditional, Analytical, and Preparative Separations of Natural Products. In: *Natural Products from Plants*, Cseke, LJ, Kirakosyan, A, Kaufman, PB, Warber, SL, Duke, JA, Brielmann, HL (eds), second edn, pp 263-317: Taylor and Francis.

Cushley, MJ, Holgate, ST (1985) Bronchodilator actions of xanthine derivatives administere by inhalation in asthma. *Thorax* **40:** 176-179.

Dahiru, D, Onubiyi, JA, Umaru, HA (2006) Phytochemical screening and antiulcerogenic effect of Moringa oleifera aqueous leaf extract. *African Journal of Traditional, Complementary and Alternative Medicines* **3**(3): 70-75.

Dangi, SY, Jolly, CI, Narayanan, S (2002) Antihypertensive activity of the total alkaloids from the leaves of Moringa oleifera. *Pharmaceutical Biology (Lisse, Netherlands)* **40**(2): 144-148.

Darroch, S, Irving, HR, Mitchelson, FJ (2000) Characterisation of muscarinic receptor subtypes in avian smooth muscle. *European Journal of Pharmacology* **402**(1-2): 161-169.

Das, C, Tripathi, AK (1999) A new triterpenoid of *Sesbania grandiflora*. *Oriental Journal of Chemistry* **15**(3): 561-562.

Das, C, Tripathi, AK (2002) Some potential drugs from *Sesbania grandiflora*. *Oriental Journal of Chemistry* **18**(1): 167-168.

Das, KC, Tripathi, AK (1998) A new flavonol glycoside from *Sesbania grandiflora*. *Fitoterapia* **69**(5): 477-478.

Dayrit, FM, Lagurin, LG (1994) Identification of four iridoids in the pharmacologically-active fraction of Vitex negundo , l. *Philippine Journal of Science* **123**(4): 293-304.

Dayrit, FM, Rosario G. Lapid, M, Cagampang, JV, Lagurin, LG (1987) Phytochemical studies on the leaves of Vitex negundo , L. ("lagundi"). I. Investigations of the bronchial relaxing constituents. *Philippine Journal of Science* **116**(4): 403-410.

Debnath, S, Guha, D (2007) Role of Moringa oleifera on enterochromaffin cell count and serotonin content of experimental ulcer model. *Indian Journal of Experimental Biology* **45**(8): 726-731.

Deng, Y-m, Xie, Q-m, Tang, H-f, Sun, J-g, Deng, J-f, Chen, J-q, Yang, S-y (2006) Effects of ciclamilast, a new PDE 4 PDE4 inhibitor, on airway hyperresponsiveness, PDE4D expression

and airway inflammation in a murine model of asthma. *European Journal of Pharmacology* **547**(1-3): 125-135.

Dharmasiri, MG, Jayakody, JRAC, Galhena, G, Liyanage , SSP, Ratnasooriya, WD (2003) Anti-inflammatory and analgesic activities of mature fresh leaves of Vitex negundo. *Journal of ethnopharmacology* **87**(2-3): 199-206.

Diaz, F, Chavez, D, Lee, D, Mi, Q, Chai, H-B, Tan, GT, Kardono, LBS, Riswan, S, Fairchild, CR, Wild, R, Farnsworth, NR, Cordell, GA, Pezzuto, JM, Kinghorn, AD (2003) Cytotoxic Flavone Analogues of Vitexicarpin, a Constituent of the Leaves of Vitex negundo. *Journal of Natural Products* **66**(6): 865-867.

Dickenson, JM, Bulmer, S, Whittaker, A, Salwey, M, Hawley, J, Hill, SJ (1994) Bradykinin B2- and 5-hydroxytryptamine (5-HT2)-receptor stimulated increases in intracellular calcium in cultured guinea-pig aortic smooth muscle cells. *Biochemical Pharmacology* **47**(6): 947-952.

Dirsch, VM, Stuppner, H, Ellmerer-Muller, EP, Vollmar, AM (2000) Structural Requirements of Sesquiterpene Lactones to Inhibit LPS-Induced Nitric Oxide Synthesis in RAW 264.7 Macrophages. *Bioorganic & Medicinal Chemistry* **8**: 2747-2753.

Dora, KA (2001) Intercellular Ca2+signalling: the artery wall. *Seminars in Cell & Developmental Biology* **12**(1): 27-35.

Dragull, K, Yoshida, WY, Tang, C-S (2003) Piperidine alkaloids from Piper methysticum. *Phytochemistry* **63**(2): 193-198.

Drake, D, Lam, J (1974) Polyacetylenes of *Artemisia vulgaris*. *Phytochemistry* **13**: 455-457.

Duke, JA (1983) Handbook of Energy Crops Vol. 2008: Purdue University, Center for New Crops & Plant Products.

Eglen, RM (2001) Muscarinic receptors and gastrointestinal tract smooth muscle function. *Life Sciences* **68**(22-23): 2573-2578.

Eglen, RM, Choppin, A, Watson, N (2001) Therapeutic opportunities from muscarinic receptor research. *Trends in Pharmacological Sciences* **22**(8): 409-414.

Eglen, RM, Peelle, B, Pulido-Rios, MT, Leung, E (1996) Functional interactions between muscarinic M2 receptors and 5-hydroxytryptamine (5-HT)4 receptors and beta 3-adrenoceptors in isolated oesophageal muscularis mucosae of the rat. *Br. J. Pharmacol.* **119**: 595–601.

Ehlert, FJ (2003) Contractile role of M2 and M3 muscarinic receptors in gastrointestinal, airway and urinary bladder smooth muscle. *Life Sciences* **74**: 355-366.

Ehrlich, BE, Kaftan, E, Bezprozvannaya, S, Bezprozvanny, I (1994) The pharmacology of intracellular Ca2+-release channels. *Trends in Pharmacological Sciences* **15**(5): 145-149.

El-Masry, S, Abou-Donia, AHA, Darwish, FA, Abou-Karam, MA, Grenz, M, Bohlmann, F (1984) Sesquiterpene lactones from *Chrysanthemum coronarium*. *Phytochemistry* **23**(12): 2953-2954.

Eyeson-Annan, ML, Hirst, LW, Battistutta, D, Green, A (1998) Comparative pupil dilation using phenylephrine alone or in combination with tropicamide. *Ophthalmology* **105**(4): 726-732.

Ezeamuzie, IC, Ambakederemo, AW, Shode, FO, Ekwebelem, SC (1996) Antiinflammatory Effects of Moringa oleifera Root Extract. *Pharmaceutical Biology* **34**(No. 3): 207-212.

Fabricant, DS, Farnsworth, NR (2001) The value of plants used in traditional medicine for drug discovery. *Environ Health Perspect* **109**(1): 69-75.

Faizi, S, Siddiqui, BS, Saleem, R, Aftab, K, Shaheen, F, Gilani, AuH (1998) Hypotensive constituents from the pods of Moringa oleifera. *Planta Medica* **64**(3): 225-228.

Faizi, S, Siddiqui, BS, Saleem, R, Noor, F, Husnain, S (1997) Isolation and Structure Elucidation of a Novel Glycoside Niazidin from the Pods of Moringa oleifera. *Journal of Natural Products*(60): 12.

Faizi, S, Siddiqui, BS, Saleem, R, Siddiqui, S, Aftab, K, Gilani, A-uH (1994) Isolation and structure elucidation of new nitrile and mustard oil glycosides from Moringa oleifera and their effect on blood pressure. *Journal of Natural Products* **57**(9): 1256-1261.

Faizi, S, Siddiqui, BS, Saleem, R, Siddiqui, S, Aftab, K, Gilani, AuH (1992) Isolation and structure of elucidation of novel hypotensive agents, niazinin A, niazinin B, niazimicin and niaziminin A + B from Moringa oleifera: the first naturally occurring thiocarbamates. *Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry* **23**.

Fehler, M, Broadley, KJ, Ford, WR, Kidd, EJ (2010) Identification of trace-amine-associated receptors (TAAR) in the rat aorta and their role in vasoconstriction by β-phenylethylamine. *Naunyn-Schmiedeberg's Archives of Pharmacology* **382**(No. 4): 385-398.

Fish, JE, Peters, SP (1999) Airway remodeling and persistent airway obstruction in asthma. *Journal of Allergy and Clinical Immunology* **104**(3): 509-516.

Fitzpatrick, PF (1999) TETRAHYDROPTERIN-DEPENDENT AMINO ACID HYDROXYLASES. *Annual Review of Biochemistry* **68**: 355-381.

Fojas, FR, Barrientos, CM, Capal, TV, Cruzada, SF, Sison, FM, Co, YC, Chua, NG, Gavina, TL (1982) Preliminary phytochemical and pharmacological studies of *Sesbania grandiflora* (L.) Pers. *Philippine Journal of Science* **111**(3-4): 157-181.

Ford, WR, Broadley, KJ (1999) Effects of adenosine receptor agonists on induction of contractions to phenylephrine of guinea-pig aorta mediated via intra- or extracellular calcium. *General Pharmacology* **33**(143-150).

Fox, AW, Abel, PW, Minneman, KP (1985) Activation of α1-adrenoceptors increases [3H]inositol metabolism in rat vas deferens and caudal artery *european Journal of Pharmacology* **116**(1-2): 145-152.

Foxx-Orenstein, A, Kuemmerle, J, Grider, J (1996) Distinct 5-HT receptors mediate the peristaltic reflex induced by mucosal stimuli in human and guinea pig intestine. *Gastroenterology* **111**(5): 1281-1290

Fozard, JR (1984) Neuronal 5-HT receptors in the periphery *Neuropharmacology* **23**(12): 1473-1486.

Frascarelli, S, Ghelardoni, S, Chiellini, G, Vargiu, R, Ronca-Testoni, S, Scanlan, TS, Grandy, DK, Zucchi, R (2008) Cardiac effects of trace amines: Pharmacological characterization of trace amine-associated receptors. *European Journal of Pharmacology* **587**(1-3): 231-236.

Ganong, WF (1991) *Review of Medical Physiology*. 15th edn.

Geissman, TA (1966) Sesquiterpenoid Lactones of Artemisia Species. I. Artemisia princeps Pamp. *Journal of Organic Chemistry* **31**(8): 2523-2526.

Geissman, TA, Ellestad, GA (1961) Vulgarin, a sesquiterpene lactone from *Artemisia vulgaris* L. **27**: 1855-1859.

Gelfand, EW (2002) Role of histamine in the pathophysiology of asthma: immunomodulatory and anti-inflammatory activities of H1-receptor antagonists. *The American Journal of Medicine* **113**(9): 2-7.

Gershon, MD (1999) Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. *Alimentary Pharmacology & Therapeutics* **13**(s2): 15-30.

Gershon, MD (2004) Review article: serotonin receptors and transporters — roles in normal and abnormal gastrointestinal motility. *Alimentary Pharmacology & Therapeutics* **20**(s7): 3-14.

Gershon, MD, Tamir, H (1981) Release of endogenous 5-hydroxytryptamine from resting and stimulated enteric neurons. *Neuroscience* **6**(11): 2277-2286.

Gilani, AH, Aftab, K, Suria, A, Siddiqui, S, Salem, R, Siddiqui, BS, Faizi, S (1994) Pharmacological studies on hypotensive and spasmolytic activities of pure compounds from Moringa oleifera. *Phytotherapy Research* **8**(2): 87-91.

Gilman, AG, Rall, TW, Nies, AS, Taylor, P (1990) The Pharmaocological Basis of Therapeutics, Eighth edn: Pergamos Press.

Gins, VK, Kolesnikov, MP, Kononkov, PF, Trishin, ME, Gins, MS (2000) Hydroxyanthroquinones and flavonoids of garland chrysanthemum. *Prikladnaya Biokhimiya i Mikrobiologiya* **36**(3): 344-353.

Glusa, E, Bevan, J, Heptinstall, S (1989) Verapamil is a potent inhibitor of 5-HT-induced platelet aggregation. *Thrombosis Research* **55**(2): 239-245.

Gómez-Viquez, L, Rueda, A, García, U, Guerrero-Hernández, A (2005) Complex effects of ryanodine on the sarcoplasmic reticulum Ca2+ levels in smooth muscle cells. *Cell Calcium* **38**(2): 121-130.

Gong, H, Simmons, MS, Tashkin, DP, Hui, KK, Lee, EY (1986) Bronchodilator Effects of Caffeine in Coffee: A Dose-Response Study of Asthmatic Subjects. *Chest* **89**(3): 335-342.

Görnemann, T, Villalón, CM, Centurión, D, Pertz, HH (2009) Phenylephrine contracts porcine pulmonary veins via α1B-, α1D-, and α2-adrenoceptors. *European Journal of Pharmacology* **613**(1-3): 86-92.

Goudie, AJ (1985) Comparative effects of cathinone and amphetamine on fixed-interval operant responding: A rate-dependency analysis *Pharmacology Biochemistry and Behavior* **23**(3): 355-365.

Goyal, BR, Goyal, RK, Mehta, AA (2009) Investigation Into the Mechanism of Anti-Asthmatic Action of Moringa oleifera. *Journal of Dietary Supplements* **6**(No. 4): 313-327.

Grandy, DK (2007) Trace amine-associated receptor 1—Family archetype or iconoclast? *Pharmacology & Therapeutics* **116**: 355-390.

Grassby, PF, Broadley, KJ (1987) Partial agonists at guinea-pig atrial β-adrenoceptors display relaxation responses in the guinea-pig ileum independent of β-adrenoceptor stimulation. *General Pharmacology: The Vascular System* **18**(1): 35-31.

Green, S, Turki, J, Bejarano, P, Hall, I, Liggett, S (1995) Influence of beta 2-adrenergic receptor genotypes on signal transduction in human airway smooth muscle cells. *Am J Respir Cell Mol Biol* **13**(1): 23-33.

Guevara, AP, Vargas, C, Sakurai, H, Fujiwara, Y, Hashimoto, K, Maoka, T, Kozuka, M, Ito, Y, Tokuda, H, Nishino, H (1999) An antitumor promoter from Moringa oleifera Lam. *Mutation Research, Genetic Toxicology and Environmental Mutagenesis* **440**(2): 181-188.

Guevara, AP, Vargas, C, Sakurai, H, Hashimoto, K, Fujiwara, Y, Maoka, T (2000) A new rhamnosyl carbamate from the seeds of Moringa oleifera Lam. *ACGC Chemical Research Communications* **10**: 48-51.

Gupta, M, Mazumder, Uk, Chakrabarti, S (1999) CNS activities of methanolic extract of Moringa oleifera root in mice *Fitoterapia* **70**(3): 244-250.

Hameed-Un-Nisa, L, Shehnaz, D, Faizi, S (1998) Measurement of the sympatholytic activity in Moringa oleifera. *New Trends in Natural Product Chemistry, [International Symposium on Natural Product Chemistry], 6th, Karachi, Jan. 4-8, 1996*: 269-277.

Hamm, HE (1998) The Many Faces of G Protein Signaling. *The Journal of Biological Chemistry* **273**(No.2): 669-672.

Hamm, HE, Gilchrist, A (1996) Heterotrimeric G proteins. *Current Opinion in Cell Biology* **8**(2): 189-196.

Hansen, TR, Greenberg, J, Mosnaim, AD (1980) Direct effect of phenylethylamine upon isolated rat aortic strip. *European Journal of Pharmacology* **63**(2-3): 95-101.

Hashiguchi, Y, Nishida, M (2007) Evolution of Trace Amine–Associated Receptor (TAAR) Gene Family in Vertebrates: Lineage-Specific Expansions and Degradations of a Second Class of Vertebrate Chemosensory Receptors Expressed in the Olfactory Epithelium. *Mol. Biol. Evol* **24**(9): 2099-2107.

Hattori, Y, Kawasaki, H, Kanno, M, Gando, S, Fukao, M (1994) Attenuated contractile response of diabetic rat aorta to caffeine but not to noradrenaline in Ca2+-free medium. *European Journal of Pharmacology* **256**(2): 215-219.

Hawthorn, MH, Broadley, KJ (1984) Reserpine-induced supersensitivity occurs for ß-adrenoceptor-mediated responses of heart and trachea but not of the uterus and lung. *European Journal of Pharmacology* **105:** 245-255.

Hawthorn, MH, Broadley, KJ, Gibbon, CJ (1985) Examination of the bronchoconstrictor response of guinea-pig isolated lung to β-phenylethylamine. *General Pharmacology: The Vascular System* **16**(4): 371-378.

Heinrich, M (2010) Ethnopharmacology in the 21st century – grand challenges. *Frontiers in Pharmacology* **1**(Article 8): 1-3.

Herbert, AA, Kidd, EJ, Broadley, KJ (2008) Dietary trace amine-dependent vasoconstriction in porcine coronary artery. *British Journal of Pharmacology* **155**(4): 525–534.

Hide, M, Fukui, H, Watanabe, T, Wada, H, Yamamoto, S (1988) Histamine H1-receptor in endothelial and smooth muscle cells of guinea-pig aorta. *European Journal of Pharmacology* **148**(2): 161-169.

Hohenschutz, LD, Bell, EA, Jewess, PJ, Leworthy, DP, Pryce, RJ, Arnold, E, Clardy, J (1981) CASTANOSPERMINE, A 1,6,7,8-TETRAHYDROXYOCTAHYDROINDOLIZINE ALKALOID, FROM SEEDS OF CASTANOSPERMUM AUSTRALE. *Phytochemistry* **20**(4): 811-814.

Hong, YG, Yang, MS, Park, YB (1999) Effect of cumambrin A treatment on blood-pressure spontaneously hypertensive rats. *Korean J. Pharmacogn* **30:** 226-230.

Houdusse, A, Silver, M, Cohen, C (1996) A model of Ca2+-free calmodulin binding to unconventional myosins reveals how calmodulin acts as a regulatory switch. *Structure* **4**(12): 1475-1490.

Hoyer, D, Hannona, JP, Martin, GR (2002) Molecular, pharmacological and functional diversity of 5-HT receptors *Pharmacology Biochemistry and Behavior* **71**(4): 533-554.

Hulme, EC, Birdsall, NJM, Buckley, NJ (1990) MUSCARINIC RECEPTOR SUBTYPES. *Annu. Rev, Pharmacol. Toxicol.* **30:** 633-673.

Hur, E-M, Kim, K-T (2002) G protein-coupled receptor signalling and cross-talk: Achieving rapidity and specificity *Cellular Signalling* **14**(5): 397-405.

Innes, IR, Kohli, JD (1969) Excitatory action of sympathomimetic amines on 5-hydroxytryptamine receptors of gut. *British Journal of Pharmacology* **35:** 383-393.

Ismail, MYMDoP, Higher College of Technology, Muscat, Oman (2010) Antiasthmatic herbal drugs - a review. *International Journal of Pharmacy and Pharmaceutical Sciences* **2**(3): 28-29.

Itoh, M (1995) The role of brain acetylcholine in phenol-induced tremor in mice. *Archives of Oral Biology* **40**(5): 365-372.

Jacobs, EH, Yamatodani, A, Professor, Timmerman, H (2000) Is histamine the final neurotransmitter in the entrainment of circadian rhythms in mammals? . *Trends in Pharmacological Sciences* **21**(8): 293-298.

Jangwan, JS, Dobhal, M (2008) New flavanone triglycoside from Moringa oleifera. *International Journal of Chemical Sciences* **6**(1): 358-362.

Jayarajah, CN, Skelley, AM, Fortner, AD, Mathies, RA (2007) Analysis of Neuroactive Amines in Fermented Beverages Using a Portable Microchip Capillary Electrophoresis System. *Anal. Chem.* **79:** 8162-8169.

Jeong, S-H, Koo, S-J, Ha, J-H, Ryu, S-Y, Park, H-J, Lee, K-T (2004) Induction of Apoptosis by Yomogin in Human Promyelocytic Leukemic HL-60 Cells. *Biological & Pharmaceutical Bulletin* **27**(No.7): 1106.

Jha, N, Mohanka, R, Azad, R (2009) Antifungal investigation of the constituents of Moringa oleifera lamk. Root bark extract. *Asian Journal of Chemistry* **21**(9): 7437-7439.

Johnson, D, Chandrasekhar, SS, Mautone, AJ (2008) Intranasal phenylephrine-surfactant treatment is not beneficial in otitis media with effusion. *International Journal of Pediatric Otorhinolaryngology* **72**(7): 1085-1089.

Jouvin, M-H, Kinet, J-P (1998) Expression and Function of the High-Affinity Receptor for IgE in Asthma. *Asthma and Allergic Diseases*: 59-74.

Kaczorowski, GJ, Garcia, ML (1999) Pharmacology of voltage-gated and calcium-activated potassium channels. *Current Opinion in Chemical Biology* **3**(4): 448-458.

Kam, T-S, Iek, I-H, Choo, Y-M (1999) Alkaloids from the stem-bark of Alstonia macrophylla. *Phytochemistry* **51:** 839-844.

Kansui, Y, Garland, CJ, Dora, KA (2008) Enhanced spontaneous Ca2+ events in endothelial cells reflect signalling through myoendothelial gap junctions in pressurized mesenteric arteries. *Cell Calcium* **44**(2): 135-146.

Karadi, RV, Gadge, NB, Alagawadi, KR, Savadi, RV (2006) Effect of Moringa oleifera Lam. root-wood on ethylene glycol induced urolithiasis in rats *Journal of Ethnopharmacology* **105**(1-2): 306-311.

Karaki, H, Sato, K, Ozaki, H (1988) Different effects of norepinephrine and KCl on the cytosolic Ca2+-tension relationship in vascular smooth muscle of rat aorta. *European Journal of Pharmacology* **151**(2): 325-328.

Kasture, VS, Deshmukh, VK, Chopde, CT (2002) Anxiolytic and anticonvulsive activity of Sesbania grandiflora leaves in experimental animals. *Phytotherapy research* **16**(5): 455-460.

Kawaguchi, N, Kuwabara, S, Nemoto, Y, Fukutake, T, Satomura, Y, Arimura, K, Osame, M, Hattori, T (2004) Treatment and outcome of myasthenia gravis: retrospective multi-center analysis of 470 Japanese patients, 1999–2000. *Journal of the Neurological Sciences* **224**(1-2): 43-47.

Kazanietz, MG, Enero, MA (1991) Decreased β-adrenoceptor-mediated vasodilation in aorta from aged rats: possible involvement of a stimulatory GTP-binding protean. *European Journal of Pharmacology* **198**(2-3): 177-181.

Khan, A-U, Gilani, AH (2009) Antispasmodic and bronchodilator activities of Artemisia vulgaris are mediated through dual blockade of muscarinic receptors and calcium influx. *Journal of Ethnopharmacology* **126:** 480-486.

Khurana, S, Yamada, M, Wess, J, Kennedy, RH, Raufman, J-P (2005) Deoxycholyltaurine-induced vasodilation of rodent aorta is nitric oxide- and muscarinic M3 receptor-dependent. *European Journal of Pharmacology* **517**(1-2): 103-110.

Kim, D-Y, Camilleri, M (2000) Serotonin: A Mediator of the Brain–Gut Connection. *THE AMERICAN JOURNAL OF GASTROENTEROLOGY* **95**(10): 2698-2709.

Kim, E-Y, Jho, H-K, Kim, D-I, Rhyu, MR (2008) Cirsium japonicum elicits endothelium-dependent relaxation via histamine H1-receptor in rat thoracic aorta. *Journal of Ethnopharmacology* **116**(2): 223-227.

Kitazawa, T, Hashiba, K, Cao, J, Unno, T, Komori, S-i, Yamada, M, Wess, J, Taneike, T (2007) Functional roles of muscarinic M2 and M3 receptors in mouse stomach motility: Studies with muscarinic receptor knockout mice. *European Journal of Pharmacology* **554:** 212–222.

Knoll, J, Miklya, I, Knoll, B, Markó, R, Rácz, D (1996) Phenylethylamine and tyramine are mixed-acting sympathomimetic amines in the brain. *Life Sciences* **58**(23): 2101-2114.

Kochegarov, AA (2003) Pharmacological modulators of voltage-gated calcium channels and their therapeutical application. *Cell Calcium* **33**(3): 145-162

Kojima, M, Dohi, Y, Sato, K (1994) Ryanodine-induced contraction in femoral artery from spontaneously hypertensive rats. *European Journal of Pharmacology* **254**(1-2): 159-165.

Koppen, CJv, Kaiser, B (2003) Regulation of muscarinic acetylcholine receptor signaling. *Pharmacology & Therapeutics,* **98**(2): 197-220.

Krauss, G (2003) *Biochemistry of signal transduction and regulation*. Third edn. Wiley-VCH.

Kristiansen, K (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacology & Therapeutics* **103**(1): 21-80.

Krzanowski, JJ, Polson, JB (1988) Mechanism of action of methylxanthines in asthma *Journal of Allergy and Clinical Immunology* **82**(2): 143-145.

Kuhad, A, Chopra, K (2009) Highhlights from the internation conference on new developments in drug discovery from natural products and traditional medicine. *Drugs of the future* **34**(1): 599-607.

Lam, A, Newhouse, MT (1990) Management of Asthma and Chronic Airflow Limitation: Are methylxanthines Obsolete? *Chest* **98:** 44-52.

Lecci, A, Santicioli, P, Maggi, CA (2002) Pharmacology of transmission to gastrointestinal muscle *Current Opinion in Pharmacology* **2**(6): 630-641.

Lee, K-D, Ha, T-J, Lee, B-W, Lee, J-R, Lee, J, Hwang, S-W, Cho, D-Y, Nam, S-H, Yang, M-S (2003a) Isolation and identification of terpenoids from the leaf of *Chrysanthemum coronarium* L. *Han'guk Nonghwa Hakhoechi* **46**(1): 55-59.

Lee, KD, Ha, TJ, Han, HS, Jang, KC, Jang, DS, Cho, DY, Yang, MS (2003b) Antimicrobial activities of sesquiterpene lactones isolated from the flower of *Chrysanthemum coronarium* L. *Han'guk Nonghwa Hakhoechi* **43**(3): 235-239.

Lee, KD, Park, KH, Ha, TJ, Han, HS, Yang, MS (2003c) Isolation of pyrethrosin derivatives from the flower of *Chrysanthemum coronarium* L. *Agricultural Chemistry and Biotechnology (English Edition)* **46**(2): 72-79.

Lee, KD, Park, KH, Kim, H, Kim, J, Rim, Y-S, Yang, MS (2003d) Cytotoxic activity and structural analogues of guaianolide derivatives from the flower of *Chrysanthemum coronarium* L. *Agricultural Chemistry and Biotechnology (English Edition)* **46**(1): 29-32.

Lee, KD, Yang, MS, Ha, TJ, Park, KM, Park, KH (2002) Isolation and identification of dihydrochrysanolide and its 1-epimer from *Chrysanthemum coronarium* L. *Bioscience, Biotechnology, and Biochemistry* **66**(4): 862-865.

Lee, S-J, Chung, H-Y, Maier, CG-A, R.Wood, A, Dixon, RA, Mabry, TJ (1998) Estrogenic flavonoids from *Artemisia vulgaris* L. *Journal of Agricultural and Food Chemistry* **46**: 3325-3329.

Lehane, L, Olley, J (2000) Histamine fish poisoning revisited *International Journal of Food Microbiology* **58**(1-2): 1-37.

Leijten, PAA, Breemen, Cv (1984) The effects of caffeine on the noradrenaline-sensitive calcium store in the rabbit aorta. *Journal of Physiology* **357**: 327-339.

Leurs, R, Brozius, MM, Jansen, W, Bast, A, Timmerman, H (1991a) Histamine H1-receptor-mediated cyclic GMP production in guinea-pig lung tissue is an l-arginine-dependent process. *Biochemical Pharmacology* **42**(2): 271-277.

Leurs, R, Jansen, W, Bast, A, Timmerman, H (1990) Desensitisation off histamine H1-receptor mediated cyclic-GMP production in guinea-pig lung slices. *European Journal of Pharmacology* **183**(6): 2167.

Leurs, R, Smit, MJ, Bast, A, Timmerman, H (1991b) Homologous histamine H1 receptor desensitization results in reduction of H1 receptor agonist efficacy. *European Journal of Pharmacology* **196**(3): 319-322.

Leurs, R, Smit, MJ, Timmerman, H (1995) Molecular pharmacological aspects of histamine receptors *Pharmacology & Therapeutics* **66**(3): 413-463.

Li, Y, Zeng, G, Zhou, M, Tan, J, Zhou, Y (2009) Chemical constituents from the seeds of Vitex negundo L. *Zhongnan Yaoxue* **7**(1): 24-26.

Lindemann, L, Hoener, MC (2005) A renaissance in trace amines inspired by a novel GPCR family. *TRENDS in Pharmacological Sciences* **26**(5): 274-281.

Linley, PA (2002) *Artemisia vulgaris*. In: *Artemisia*, Wrigth, CW (ed), pp 139-189: CRC Press.

Lodish, h, Berk, A, Kaiser, CA, Krieger, M, Scott, MP, Bretscher, A, Ploegh, H, Matsudaira, p (2008) Chapter 15: "Cell Signaling I: Signal transduction and short-term cellular responses". In: *Molecular Cell Biology*, Sixth edn. New York: W.H. Freeman and Company.

López, RM, Castillo, C, Castillo, EF (2009) Isometric contraction increases endothelial nitric oxide synthase activity via a calmodulin antagonist-sensitive pathway in rat aorta. *Vascular Pharmacology* **50**(1-2): 14-19.

Low, AM, Darby, PJ, Kwan, C-Y, Daniel, EE (1993) Effects of thapsigargin and ryanodine on vascular contractility: cross-talk between sacroplasmic reticulum and plasmalemma. *European Journal of Pharmacology* **230**(1): 53-62.

Lui, AH, McManus, BM, Laher, I (2000) Endothelial and myogenic regulation of coronary artery tone in the mouse. *European Journal of Pharmacology* **410**(1): 25-31.

Macht, DI, Isaacs, S (1917) Action of some opium alkaloids on the psychological reaction time *Psychobiology* **1**(1): 19-32.

Mahajan, SG, Banerjee, A, Chauhan, BF, Padh, H, Nivsarkar, M, Mehta, AA (2009) Inhibitory Effect of n-butanol Fraction of Moringa oleifera Lam. Seeds on Ovalbumin-Induced Airway Inflammation in a Guinea Pig Model of Asthma. *International Journal of Toxicology* **28**(6): 519-527

Mahajan, SG, Mehta, AA (2007) Inhibitory action of ethanolic extract of seeds of Moringa oleifera Lam. on systemic and local anaphylaxis. *Journal of Immunotoxicology* **4**(4): 287-294.

Mahdy, AM, Webster, NR (2008) Histamine and antihistamines. *Anaesthesia & Intensive Care Medicine* **9**(7): 324-328.

Manguro, LOA, Lemmen, P (2007) Phenolics of Moringa oleifera leaves. *Natural Product Research, Part A: Structure and Synthesis* **21**(1): 56-68.

Marco, JA, Sanz, JF, Hierro, Pd (1990) Two eudesmane acids from *Artemisia vulgaris*. *Phytochemistry* **30**(7): 2403-2404.

Marsden, CA, Sleight, AJ, Fone, KCF, Jonson, JV, Crespi, F, Martin, KF, Garrett, JC, Bennett, GW (1989) Functional identification of 5HT receptor subtypes. *ative Biochemistry and Physiology Part A: Physiology* **93**(1): 107-114.

Marsden, CA, Sleight, AJ, Fore, KCF, Jonson, JV, Crespi, F, Martin, KF, Garrett, JC, Bennett, GW (1988) Functional identification of 5HT receptor subtypes. *Comp. Biochem. Physiol.* **93a**(1): 107-111.

Matsubara, M, Tamura, T, Ohmori, K, Hasegawa, K (2005) Histamine H1 receptor antagonist blocks histamine-induced proinflammatory cytokine production through inhibition of Ca2+-dependent protein kinase C, Raf/MEK/ERK and IKK/IκB/NF-κB signal cascades *Biochemical Pharmacology* **69**(3): 433-449.

McCarron, JG, Chalmers, S, Bradley, KN, MacMillan, D, Muir, TC (2006) Ca2+ microdomains in smooth muscle. *Cell Calcium* **40**: 461-493.

McCarty, R, Eisen, G, Bartholow, CL (1991) Plasma catecholamine responses to acute motion stress in laboratory rats. *Physiology & Behavior* **49**(3): 653-656.

McLean, PG, Borman, RA, Lee, K (2007) 5-HT in the enteric nervous system: gut function and neuropharmacology. *Trends in Neurosciences* **30**(1): 9-13.

McLennan, PL, Taylor, DA (1984) Antagonism by ketanserin of 5-HT-induced vasoconstriction unmasks a 5-HT-induced vasodilation. *European Journal of Pharmacology* **104**(3-4): 313-318.

Meissner, G (1986) Ryanodine activation and inhibition of the Ca2+ release channel of sarcoplasmic reticulum. *The Journal of Biological Chemistry* **261:** 6300-6306.

Melillo, G, Melillo, E (2001) The irreversible component of airway obstruction in asthma. *Clinical and Applied Immunology Reviews* **1**(3-4)**:** 235-242.

Mellentin, C, Jahnsen, H, Abraham, WC (2007) Priming of long-term potentiation mediated by ryanodine receptor activation in rat hippocampal slices. *Neuropharmacology* **52:** 118e125.

Mendoza, RL (2010) A Cost-Benefit Analysis of the Legalization of an Informal Health Sector. *Journal of Social Sciences* **6**(1)**:** 74-84.

Middleton, E, Drzewieckia, G (1983) Flavonoid inhibition of human basophil histamine release stimulated by various agents. *Biochemical Pharmacology* **33**(21)**:** 3333-3338

Minami, K, Kamei, C (2004) A chronic model for evaluating the itching associated with allergic conjunctivitis in rats. *International Immunopharmacology* **4**(1)**:** 101-108.

Minneman, KP (2007) Heterodimerization and surface localization of G protein coupled receptors. *Biochemical Pharmacology* **73**(8)**:** 1043-1050.

Minneman, KP, Randall N. Pittman, Molinoff, PB (1981) β-ADRENERGIC RECEPTOR SUBTYPES: PROPERTIES, DISTRIBUTION, AND REGULATION. *Ann. Rev Neurosci* **4:** 419-461.

Miraldia, E, Mastib, A, Ferria, S, Comparinib, IB (2001) Distribution of hyoscyamine and scopolamine in Datura stramonium. *Fitoterapia* **72:** 644-648.

Misra, GS, Subramanian, PM (1980) Three new flavone glycosides from Vitex negundo. *Planta Medica* **38**(2)**:** 155-160.

Mitsui, T, Hida, M, Fujimura, Y (1995) Determination of the total amount of morphine alkaloids in opium by pyrolysis-gas chromatography using principal component analysis *Journal of Analytical and Applied Pyrolysis* **32:** 205-212.

Monczor, F, Fernandez, N, Riveiro, E, Mladovan, A, Baldi, A, Shayo, C, Davio, C (2006) Histamine H2 receptor overexpression induces U937 cell differentiation despite triggered mechanisms to attenuate cAMP signalling. *Biochemical Pharmacology* **71**(8)**:** 1219-1228.

Morissette, G, Bouthillier, J, Marceau, F (2007) Trapping of adrenergic decongestant drugs into cellular endomembrane compartments: Toxicological and pharmacological consequences. *International Immunopharmacology* **7:** 1869–1879.

Moritoki, H, Morita, M, Kanbe, T (1976) Effects of methylxanthines and imidazole on hte contractions of guinea-pig ileum induced by transmural stimulation. *European Journal of Pharmacology* **35**(1)**:** 185-198.

Mroczek, T, Głowniak, K, Kowalska, J (2006) Solid–liquid extraction and cation-exchange solid-phase extraction using a mixed-mode polymeric sorbent of Datura and related alkaloids. *Journal of Chromatography A* **1107:** 9-18.

Mullera, MJ, Priora, T, Hunta, RH, Rangachari, PK (1993) Accelerated communication H1 contractile and H2 relaxant receptors in canine gastric muscularis mucosae. *Life Sciences* **52**(7): PL49-PL53.

Murakami, A, Kitazono, Y, Jiwajinda, S, Koshimizu, K, Ohigashi, H (1998) Niaziminin, a thiocarbamate from the leaves of Moringa oleifera , holds a strict structural requirement for inhibition of tumor promoter-induced Epstein-Barr virus activation. *Planta Medica* **64**(4): 319-323.

Murray, RDH, Stefanovic, M (1986) 6-methoxy-7,8-methylenedioxycoumarin from *Artemisia dracunculoides* and *Artemisia vulgaris*. *Journal of Natural Products* **49**(3): 550-551.

Myers, N, Mittermeier, RA, Mittermeier, CG, Fonseca, GABd, Kent, J (2000) Biodiversity hotspots for conservation priorities. *Nature* **403:** 853-858.

Nagaki, M (1984) Two sesquiterpene lactones from artemisia species. *Phytochemistry* **23**(2): 462-464.

Naganobu, K, Takagi, M, Kawasaki, H, Ito, K (1994) Modification by cyclopiazonic acid and ryanodine of depolarization-induced constriction in rat mesenteric artery. *European Journal of Pharmacology* **251**(2-3): 307-310.

Nagashima, M, Hattori, Y, Tohse, N, Kanno, M (1997) α1-adrenoceptor subtype involved in the positive and negative inotropic responses to phenylephrine in rat papillary muscle. *General Pharmacology: The Vascular System* **28**(5): 721-725

Nahorski, SR, Tobin, AB, Willars, GB (1997) Muscarinic M3 receptor coupling and regulation. *Life Sciences* **60**(1039-1045).

Nelson, DL, Cox, MM (2005) Chapre 12: "Biosignaling". In: *Lehninger: Principles of Biochemistry*, Fourth edn. New York: W.H. Freeman and Company.

Nemeth, PR, Gullikson, GW (1989) Gastrointestinal motility stimulating drugs and 5-HT receptors on myenteric neurons. *European Journal of Pharmacology* **166**(3): 387-391.

Newman, JH, Voelkel, NF, Arroyave, CM, Reeves, JH (1980) Distribution of mast cells and histamine in canine pulmonary arteries. *Respiration Physiology* **40**(2): 191-198.

Nikolava, M, Velickovic, D (2007) Phenological Variations in the surface flavonoids of *Artemisia vulgaris L.* and *Artemisia absinthum L. Turk J Bot* **31:** 459-462.

Notcovich, C, Diez, F, Tubio, MR, Baldi, A, Kazanietz, MG, Davio, C, Shayo, C (2010) Histamine acting on H1 receptor promotes inhibition of proliferation via PLC, RAC, and JNK-dependent pathways *Experimental Cell Research* **316**(3): 401-411.

O'Byrne, PM (2008) Asthma. *International Encyclopedia of Public Health*: Pages 245-255.

Oliveira, MCG, Pelegrini-da-Silva, A, Parada, CA, Tambeli, CH (2007) 5-HT acts on nociceptive primary afferents through an indirect mechanism to induce hyperalgesia in the subcutaneous tissue. *Neuroscience* **145**(2): 708-714.

Oliver-Bever, B (1986) *Medicinal plants in tropical West Africa*. Cambridge ; London ; New York [etc.] : Cambridge university press, cop.

Olsson, C, Holmgren, S (2010) Autonomic control of gut motility: A comparative view. *Autonomic Neuroscience: Basic and clinical*.

Ono, M, Nishida, Y, Masuoka, C, Li, J-C, Okawa, M, Ikeda, T, Nohara, T (2004) Lignan Derivatives and a Norditerpene from the Seeds of Vitex negundo. *Journal of Natural Products* **67**(12): 2073-2075.

Pan, Z, Damron, D, Nieminen, A-L, Bhat, MB, Ma, J (2000) Depletion of Intracellular Ca2+ by Caffeine and Ryanodine Induces Apoptosis of Chinese Hamster Ovary Cells Transfected with Ryanodine Receptor. *The Journal of Biological Chemistry* **275**: 19978-19984.

Pastorello, EA, Pravettoni, V, Farioli, L, Rivolta, F, Conti, A, Ispano, M, Fortunato, D, Bengtsson, A, Bianchi, M (2002) Hypersensitivity to mugwort (Artemisia vulgaris) in patients with peach allergy is due to a common lipid transfer protein allergen and is often without clinical expression. *The Journal of Allergy and Clinical Immunology* **110**(2): 310-317.

Paxon, TL, Powell, PR, Lee, H-G, Han, K-A, Ewing, AG (2005) Microcolumn Separation of Amine Metabolites in the Fruit Fly. *Anal. Chem.* **77**(16): 5349-5355.

Peroutka, SJ (1984) 5-HT1 receptor sites and functional correlates. *Neuropharmacology* **23**(12): 1487-1492.

Perry, SF, Capaldo, A (2010) The autonomic nervous system and chromaffin tissue: Neuroendocrine regulation of catecholamine secretion in non-mammalian vertebrates. *Auton. Neurosci.*

Pletscher, A (1987) The 5-hydroxytryptamine system of blood platelets: physiology and pathophysiology. *International Journal of Cardiology* **14**(2): 117-188.

Poli, E, Pozzoli, C, Coruzzi, G (2001) Role of histamine H3 receptors in the control of gastrointestinal motility. An overview. *Journal of Physiology* **95**: 67-74.

Ponti, FD, Malagelada, J-R (1998) Functional Gut Disorders: From Motility to Sensitivity Disorders. A Review of Current and Investigational Drugs for Their Management. *Pharmacology & Therapeutics* **80**(1): 49-88.

Premont, RT, Gainetdinov, RR, Caron, MG (2001) Following the trace of elusive amines. *PNAS* **98**(17): 9474-9475.

Quisimbing, E (1978) Medicinal Plants of the Philippines. Manila: Katha Publishing Co.

Racké, K, Juergens, UR, Matthiesen, S (2006) Control by cholinergic mechanisms. *European Journal of Pharmacology* **533**(1-3): 57-68.

Racké, K, Reimann, A, Schwörer, H, Kilbinger, H (1995) Regulation of 5-HT release from enterochromaffin cells. *Behavioural Brain Research* **73**(1-2): 83-87.

Ragasa, CY, Morales, E, Rideout, JA (1999) Antimicrobial compounds from Vitex negundo. *Philippine Journal of Science* **128**(1): 21-29.

Ragasa, CY, Natividad, GM, Rideout, JA (1998) An antimicrobial diterpene from *Chrysanthemum coronarium*. *Kimika* **14**(1): 17-20.

Ragasa, CY, Natividad, GM, Rideout, JA (1997) A bioactive thiophene derivative from *Chrysanthemum coronarium*. *Philippine Journal of Science* **126**(4): 275-281.

Rahman, S, Neuman, RS (1993) Multiple mechanisms of serotonin 5-HT 2 receptor desensitization. *European Journal of Pharmacology* **238:** 173-180.

Ramesh, T, Begum, VMH (2006) Hypolipidemic effect of *Sesbania grandiflora* on cigarette smoke exposed rats. *Pharmacologyonline* **3:** 309-323.

Rang, HP, Dale, MM, Ritter, JM, Flower, RJ (2007) *Pharmacology*. Sixt edn. Churchill Livingstone Elsevier.

Rang, HP, Dale, MM, Ritter, JM, Moore, PK (2003) Chapter 3: "How drugs act: molecular aspects". In: *Pharmacology*, Fifth edn: Churchill Livingstone.

Rangachari, PK (1992) Histamine: mercurial messenger in the gut. *Am J Physiol Gastrointest Liver Physiol* **262**(1): 1-13.

Rao, UK, Rao, EV, Rao, DV (1977) Phenolic constituents of the bark of Vitex negundo. *Phenolic constituents of the bark of Vitex negundo* **39**(2): 41.

Raymond, JR, Mukhin, YV, Gelasco, A, Turner, J, Collinsworth, G, Gettys, TW, Grewal, JS, Garnovskaya, MN (2001) Multiplicity of mechanisms of serotonin receptor signal transduction. *Pharmacology & Therapeutics* **92**(2-3): 179-212.

Reimann, A, Racké, K (1994) Regulation of 5-hydroxytryptamine (5HT) release from the enterochromaffin cell: physiological and pathophysiological roles *Pathophysiology* **1**(4): 277-278.

Revilla, A, Javier, E, Vergara, N, Gendrano, O (2000) Quo Vadis, Philippine Forestry: Towards Environmental Disaster or on to Sustainability (Forestry 2050).

Rohra, DK, Saito, S-y, Ohizumi, Y (2003) Functional role of ryanodine-sensitive Ca2+ stores in acidic pH-induced contraction in Wistar Kyoto rat aorta. *Life Sciences* **72**(11): 1259-1269.

Romo de Vivar, A, Montiel, FD, E. (1974) Structure and stereochemistry of sulfur-containing amides isolated from different species of Chrysanthemum. *Revista Latinoamericana de Quimica* **5**(1): 32-40.

Rose, JE, Behm, FM (1991) Psychophysiological interactions between caffeine and nicotine. *Pharmacology Biochemistry and Behavior* **38**(2): 333-337.

Rubinstein, R, Cohen, S (1985) HISTAMINE-MEDIATED ACETYLCHOLINE RELEASE IN THE GUINEA-PIG ILEUM. *European Journal of Pharmacology* **111**: 245-250.

Ruddell, RG, Mann, DA, Ramm, GA (2008) The function of serotonin within the liver. *Journal of Hepatology* **48**(4): 666-675.

Rudnick, G, Clark, J (1993) From synapse to vesicle: The reuptake and storage of biogenic amine neurotransmitters. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1144**(3): 249-263.

Ryu, J-H, Lee, HJ, Jeong, YS, Ryu, SY, Han, YN (1998) Yomogin, an Inhibitor of Nitric Oxide Production in LPS-Activated Macrophages. *Arch. Pharm. Res* **21**(No. 4): 481-484.

Sanger, GJ, Andrews, PLR (2006) Treatment of nausea and vomiting: Gaps in our knowledge. *Autonomic Neuroscience* **129**(1-2): 3-16.

Santing, RE, Boer, Jd, Rohof, A, Zee, NMvd, Zaagsma, J (2001) Bronchodilatory and anti-inflammatory properties of inhaled selective phosphodiesterase inhibitors in a guinea pig model of allergic asthma. *European Journal of Pharmacology* **429**(1-3): 335-344.

Santing, RE, Schraa, EO, Wachters, A, Olymulder, CG, Zaagsma, J, Meurs, H (1994) Role of histamine in allergen-induced asthmatic reactions, bronchial hyperreactivity and inflammation in unrestrained guinea pigs *European Journal of Pharmacology* **254**(1-2): 49-57.

Saralaya, MG, Patel, P, Patel, M, Roy, SP, N.Patel, A (2010) Antidiarrheal Activity of Methanolic Extract of Moringa oleifera Lam Roots in Experimental Animal Models. *International Journal of Pharmaceutical Research* **2**(2): ISSN 0975-2366.

Sathiamoorthy, B, Gupta, P, Kumar, M, Chaturvedi, AK, Shukla, PK, Maurya, R (2007) New antifungal flavonoid glycoside from Vitex negundo. *Bioorganic & Medicinal Chemistry Letters* **17**(1): 239-242.

Sauerberg, P, Kindtler, JW, Nielsen, L, Sheardown, MJ, Honor, T (1991) Muscarinic Cholinergic Agonists and Antagonists of the 3-(3-Alkyl-1,2,4-oxadiazol-5-yl)-1,2,5,6-tetrahydropyridiTnyep e. Synthesis and Structure-Activity Relationships. *J. Med. Chem* **34**: 687-692.

Saxena, VK, Mishra, LN (1999a) Flavone glycoside from *Sesbania grandiflora*. *Journal of the Institution of Chemists (India)* **71**(6): 234-236.

Saxena, VK, Mishra, LN (1999b) A new isoflavone glycoside from the roots of *Sesbania grandiflora*. *Research Journal of Chemistry and Environment* **3**(3): 69-70.

Scaf, A (1971) Effects of the bisquaternary ammonium compound hexaflourenium on the cholinergic system in the jejunum of the rat and the guinea-pig. *European Journal of Pharmacology* **15**(3): 355-362.

Schmid, CL, Bohn, LM (2009) Physiological and pharmacological implications of beta-arrestin regulation. *Pharmacology & Therapeutics* **121**: 285–293.

Schmidt, HHHW, Lohmann, SM, Walter, U (1993) The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1178**(2): 153-175.

Schultheiss, G, Hennig, B, Schunack, W, Prinz, G, Diener, M (2005) Histamine-induced ion secretion across rat distal colon: Involvement of histamine H1 and H2 receptors. *European Journal of Pharmacology* **546**(1-3): 161-170.

Selbiea, LA, Hilla, SJ (1998) G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways *Trends in Pharmacological Sciences* **19**(3): 87-93.

Shafiei, M, Mahmoudian, M (1999) Atypical β-adrenoceptors of rat thoracic aorta. *General Pharmacology* **32**(5): 557-562.

Sharma, RL, Prabhakar, A, Dhar, KL, Sachar, A (2009) A new iridoid glycoside from Vitex negundo Linn (Verbenacea). *Natural Product Research, Part A: Structure and Synthesis* **23**(13): 1201-1209.

Shaul, P, Magness, R, Muntz, K, DeBeltz, D, Buja, L (1990) Alpha 1-adrenergic receptors in pulmonary and systemic vascular smooth muscle. Alterations with development and pregnancy. *Circulation Research* **67**: 1193-1200.

Shen, C, Zeng, G, Tan, J, Li, Y, Tan, G, Zhou, Y (2009) Chemical constitutes of anti-tumor effective extractions of Vitex negundo. *Zhongcaoyao* **40**(1): 33-36.

Shibano, A, Kawai, Y, Senba, E (1998) Histamine-induced internalization of substance P receptors in myoepithelial cells of the guinea pig nasal glands. *Peptides* **19**(8): 1365-1371.

Shinjoh, M, Nakaki, T, Otsuka, Y, Sasakawa, N, Kato, R (1991) Vascular smooth muscle contraction induced by Na+ channel activators, veratridine and batrachotoxin. *European Journal of Pharmacology* **205**(2): 199-202.

Sikander, A, Rana, SV, Prasad, KK (2009) Role of serotonin in gastrointestinal motility and irritable bowel syndrome. *Clinica Chimica Acta* **403**: 47-55.

Silvaa, AMM, Silvaa, AR, Pinheiroa, AM, Freitasa, SRVB, Silvaa, VDA, Souzaa, CS, Hughesa, JB, El-Bacha, RS, Costaa, MFD, Velozob, ES, Tardyc, M, S.L. Costaa (2007) Alkaloids from Prosopis

juliflora leaves induce glial activation, cytotoxicity and stimulate NO production. *Toxicon* **49**(601-614).

Silver, PJ, Holroyde, MJ, Solaro, RJ, Disalvo, J (1981) Ca2+, calmodulin and cycic AMP-dependent modulation of actin-myosin interactions in aorta. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **674**(1): 65-70.

Small, RC (2005) Histamine and antihistamines *Anaesthesia &intensive care medicine* **6**(7): 250-252.

Sohen, S, Ooe, H, Hashima, M, Nonaka, T, Fukuda, K, Hamanishi, C (2001) Activation of histamine H1 receptor results in enhanced proteoglycan synthesis by human articular chondrocyte: involvement of protein kinase C and intracellular Ca2+. *Pathophysiology* **8**(2): 93-98.

Song, M-C, Hong, Y-H, Kim, D-H, Kim, D-K, Chung, I-S, Lee, Y-H, Kim, S-H, Park, M-H, Kim, I-H, Kwon, BM, Baek, N-I (2003a) Development of biologically active compounds from edible plant sources. VI. Isolation of sterol compounds from the aerial parts of garland (*Chrysanthemum coronarium* L.). *Han'guk Nonghwa Hakhoechi* **46**(4): 376-379.

Song, M-C, Kim, D-H, Hong, Y-H, Kim, D-K, Chung, I-S, Kim, S-H, Park, M-H, Kwon, B-M, Lee, Y-H, Baek, N-I (2003b) Search for biologically-active materials from edible plant resources. Part VII. Terpenes from the aerial parts of *Chrysanthemum coronarium* L. *Agricultural Chemistry and Biotechnology (English Edition)* **43**(3): 118-121.

Sonkusare, S, Palade, PT, Marsh, JD, Telemaque, S, Pesic, A, Rusch, NJ (2006) Vascular calcium channels and high blood pressure: Pathophysiology and therapeutic implications. *Vascular Pharmacology* **44**(3): 131-142

Steenwinckel, JV, Noghero, A, Thibault, K, Brisorgueil, M-J, Fischer, J, Conrath, M (2009) The 5-HT2A receptor is mainly expressed in nociceptive sensory neurons in rat lumbar dorsal root ganglia. *Neuroscience* **161**(3): 838-846.

Subramanian, EH, Varghese, S, Rameshkumar, N, Ilavarasan, R, Sridhar, SK (2003) Pharmacological screening of *Sesbania grandiflora* L. poiret extracts. *Natural Product Sciences* **9**(3): 154-157.

Subramanian, PM, Misra, GS (1979) Flavonoids of Vitex negundo. *journal of Natural Products* **42**(5): 540-542.

Subramanian, PM, Misra, GS (1978) Leucoanthocyanidins of Vitex negundo. *Indian Journal of Chemistry, Section B: Organic Chemistry Including Medicinal Chemistry* **16B**(7): 615-616.

Sulaiman, MR, Zakaria, ZA, Bujarimin, AS, Somchit, MN, Israf, DA, Moin, S (2008) Evaluation of Moringa oleifera Aqueous Extract for Antinociceptive and Anti-Inflammatory Activities in Animal Models. *Pharmaceutical Biology* **46**(12): 838-845.

Szarek, JL, Zhang, JZ, Gruetter, CA (1993) 5-HT2 receptors augment cholinergic nerve-mediated contraction of rat bronchi. *Volume 231, Issue 3, 16 February 1993, Pages 339-346* **231**(3): 339-346.

Szarek, JL, Zhang, JZ, Gruetter, CA (1995) Mechanisms of 5-hydroxytryptamine-induced contraction of isolated rat intrapulmonary bronchi. *Pulmonary Pharmacology* **8**(6): 273-281.

Tada, M, Chiba, K (1984) Novel plant growth inhibitors and an insect antifeedant from *Chrysanthemum coronarium* (Japanese name: shungiku). *Agricultural and Biological Chemistry* **48**(5): 1367-1369.

Tapingkaea, W, Tanasupawatb, S, Parkinc, KL, Benjakula, S, Visessanguand, W (2010) Degradation of histamine by extremely halophilic archaea isolated from high salt-fermented fishery products. *Enzyme and Microbial Technology* **46**(92–99).

Taylor, SJ, Chae, HZ, Rhee, SG, Exton, JH (1991) Activation of the β1 isozyme of phospholipase C by a subunits of the Gq class of G proteins. *Nature* **350**: 516-518.

Tewari, A, Bhakuni, RS (2006) Thiocarbamates from Moringa oleifera. *Natural Product Communications* **1**(9): 721-725.

Tigno, XT, Gumila, E (2000a) *In vivo* microvascular actions of *Artemisia vulgaris* L. in a model of ischemia-refurfusion injury in the rat intestinal mesentery. *Clinical Hemorheology and Microcirculation* **23**: 159-165.

Tigno, XT, Guzman, Fd, Flora, AMTV (2000b) Phytochemical analysis and hemodynamic actions of *Artemisia vulgaris* L. *Clinical Hemorheology and Microcirculation* **23**: 167-175.

Timmerman, H, van der Goot, H (2009) Histamine Receptors and their Ligands: Mechanisms and Applications. *Encyclopedia of Neuroscience*: 1149-1166.

Tiwari, RD, Bajpai, RK (1964a) Chemical examination of *Sesbania grandiflora* leaves. II. Isolation and study of a saponin. *Proceedings of the National Academy of Sciences, India, Section A: Physical Sciences* **34**(2): 239-244.

Tiwari, RD, Bajpai, RK, Khana, SS (1964b) Grandiflorol, a tertiary alcohol from leaves of *Sesbania grandiflora*. *Arch. Pharm.* **297**(5): 310-312.

Tiwari, RD, Bajpai, RK, Khana, SS (1964c) On grandiflorol, A tertiatry alcohol from the leaves of *Sesbania grandiflora* (L.) Pers. *Archiv der Pharmazie* **297**: 310-312.

Tjon, GHK, Vries, TJD, Nestby, P, Wardeh, G, Mulder, AH, Schoffelmeer, ANM (1995) Intermittent and chronic morphine treatment induces long-lasting changes in δ-opioid receptor-regulated acetylcholine release in rat striatum and nucleus accumbens. *european Journal of Pharmacology* **283**(1-3): 169-176.

Tobin, G (2002) Presynaptic muscarinic receptor mechanisms and submandibular responses to stimulation of the parasympathetic innervation in bursts in rats. *Autonomic Neuroscience* **99**(2): 111-118.

Turetta, L, Bazzan, E, Bertagno, K, Musacchio, E, Deana, R (2002) Role of Ca2+ and protein kinase C in the serotonin (5-HT) transport in human platelets. *Cell Calcium* **31**(235-244).

Ullmer, C, Schmuck, K, Kalkman, HO, Lübbert, H (1995) Expression of serotonin receptor mRNAs in blood vessels. *FEBS Letters* **370**(3): 215-221.

Valle, JD, Gantz, I (1997) Novel insights into histamine H_2 receptor biology. *Am J Physiol Gastrointest Liver Physiol* **273:** 987-996.

Varshney, IP, Badhwar, G, Khan, AA, Shrivastava, A (1971) Saponins and sapogenins of *Sesbania grandiflora* seeds, *Albizzia lebbek* pods and *Psidium guajava* fruits. *Indian Journal of Applied Chemistry* **54**(5): 214-216.

Verbeuren, TJ, Mennecier, P, Laubie, M (1991) 5-Hydroxytryptamine-induced vasodilatation in the isolated perfused rat kidney: are endothelial 5-HT1A receptors involved? *European Journal of Pharmacology* **201**(1): 17-27.

Vishwanathan, AS, Basavaraju, R (2010) A review on Vitex negundo L. - a medically important plant. *EJBS* **3**(1): 30-42.

Wada, S, Okabe, E (1997) Susceptibility of caffeine- and Ins(1,4,5)P3-induced contractions to oxidants in permeabilized vascular smooth muscle. *European Journal of Pharmacology* **320**(1): 51-59.

Wagenknecht, T, Radermacher, M (1997) Ryanodine receptors: structure and macromolecular interactions. *Current Opinion in Structural Biology* **7**(2): 258-264.

Wallnofer, B, Hofer, O, Greger, H (1989) Polyacetylenes from the *Artemisia 'vulgares'* group. *Phytochemistry* **28**(28): 2687-2691.

Warber, SL, Seymour, M, Kaufman, PB, Kirakosyan, A, Cseke, LJ (2006) Chater 11: " Modes of Action at Target Sites". In: *Natural Products from Plants*, Cseke, LJ, Kirakosyan, A., Kaufman, P. B., Warber, S. L., Duke, J. A., Brielmann, H. L. (ed), second edition edn, pp 415-440. London: Taylor & Francis.

Wassall, RD, Teramoto, N, Cunnane, TC (2009) Noradrenaline. *Encyclopedia of Neuroscience*: 1221-1230.

Wijngaarden, IV, Tulp, MTM, Soudijnc, W (1990) The concept of selectivity in 5-HT receptor research. *European Journal of Pharmacology: Molecular Pharmacology* **188**(6): 301-312.

Wu, Z-Y, Benoit, JN (1995) Effects of propranolol on intestinal microcirculation of normal and portal hypertensive rats. *Journal of Hepatology* **22**(6): 677-684.

Xie, Z, Miller, GM (2009) Trace amine-associated receptor 1 as a monoaminergic modulator in brain. *Biochemical Pharmacology* **78**(9): 1095-1104.

Yanai, K, Tashiro, M (2007) The physiological and pathophysiological roles of neuronal histamine: An insight from human positron emission tomography studies. *Pharmacology & Therapeutics* **113:** 1-15.

Yang, QZ, Hatton, GI (2002) Histamine H1-receptor modulation of inter-neuronal coupling among vasopressinergic neurons depends on nitric oxide synthase activation. *Brain Research* **955**(1-2): 115-122.

Yatsuzuka, R, Inoue, T, Jiang, S, Nakano, Y, Kamei, C (2007) Development of new atopic dermatitis models characterized by not only itching but also inflammatory skin in mice. *European Journal of Pharmacology* **565**(1-3): 225-231.

Yokotani, K, Murakami, Y, Okada, S, Wang, M, Nakamura, K (2000) Histamine H3 receptor-mediated inhibition of endogenous acetylcholine release from the isolated, vascularly perfused rat stomach. *European Journal of Pharmacology* **392**(1-2): 23-29.

Young, SN, Teffa, KL (1989) Tryptophan availability, 5ht synthesis and 5HT function *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **13**(3-4): 373-379.

Yun, D-J, Hashinamoto, T, Yamada, Y (1992) Metabolic engineering of medicinal plants: Transgenic *Atropa belladonna* with an improved alkaloid composition. *Proc. Natl. Acad. Sci.* **89**(24): 11799-11803.

Zaware, BB, Nirmal, SA (2010) An overview of Vitex negundo Linn.: chemistry and pharmacological profile. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* **1**(1): 104-114.

Zhang, S, Won, Y-K, Ong, C-N, Shen, H-M (2005) Anti-Cancer Potential of Sesquiterpene Lactones: Bioactivity and Molecular Mechanisms. *Current Medicinal Chemistry - Anti-Cancer Agents* **5**(No.3): 239-249.

Zheng, C-J, Huang, B-K, Han, T, Zhang, Q-Y, Zhang, H, Rahman, K, Qin, L-P (2009a) Nitric Oxide Scavenging Lignans from Vitex negundo Seeds. *Journal of Natural Products* **72**(9): 1627-1630.

Zheng, C-J, Huang, B-K, Wang, Y, Ye, Q, Han, T, Zhang, Q-Y, Zhang, H, Qin, L-P (2010a) Anti-inflammatory diterpenes from the seeds of Vitex negundo. *Bioorganic & Medicinal Chemistry* **18**(1): 175-181.

Zheng, C-J, Huang, B-K, Wu, Y-B, Han, T, Zhang, Q-Y, Zhang, H, Qin, L-P (2010b) Terpenoids from Vitex negundo seeds. *Biochemical Systematics and Ecology* **38**(2): 247-249.

Zheng, C-J, Tang, W-Z, Huang, B-K, Han, T, Zhang, Q-Y, Zhang, H, Qin, L-P (2009b) Bioactivity-guided fractionation for analgesic properties and constituents of Vitex negundo L. seeds. *Phytomedicine* **16**(6-7): 560-567.

Zucchi, R, Chiellini, G, Grandy, DK (2006) Trace amine-associated receptors and their ligands. *British journal of Pharmacology* **149:** 967-978.

APPENDIX

Certification of plant identification for *Artemisia vulgaris* L.

Republic of the Philippines

NATIONAL MUSEUM
National Art Gallery
Museum of the Filipino People

October 9, 2008

CERTIFICATION

This is to certify that the specimen/s herein listed and presented by the person/s herein noted was\were verified by the office.

NAME : **Gaudencio M. Natividad**

SCHOOL/ OFFICE/ INSTITUTION : **Cardiff University**
ADDRESS : Cardiff, Wales, United Kingdom
PURPOSE : Research

Specimen No.	Family	Scientific Name
One (1)	COMPOSITAE/ASTERACEAE	*Artemisia vulgaris* L.

Determined by:

DR. WILFREDO F. VENDIVIL
Botany Division
National Museum
MANILA

Certification of plant identification for *Vitex negundo, Moringa oleifera, Sesbania grandiflora and Chrysanthemum coronarium.*

Republic of the Philippines

NATIONAL MUSEUM
National Art Gallery
Museum of the Filipino People

February 17, 2009

CERTIFICATION

This is to certify that the specimen/s herein listed and presented by the person/s herein noted was\were verified by the office.

NAME : **Gaudencio M. Natividad**

SCHOOL/ OFFICE/ INSTITUTION : **Philippine Science High School - CVC**
ADDRESS : Bayombong, Nueva Vizcaya
PURPOSE : Research

Specimen No.	Family	Scientific Name
One (1)	VERBENACEAE	*Vitex negundo* L.
One (1)	ASTERACEAE	*Tagetes erecta* L.
One (1)	MORINGACEAE	*Moringa oleifera* L.
One (1)	FABACEAE	*Sesbania grandiflora* (L.) Pers
One (1)	ASTERACEAE	*Chrysanthemum coronarium* L. (Crown daisy)

Determined by:

DR. WILFREDO F. VENDIVIL
Senior Researcher
Botany Division