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InVitro study of the Effects of Coffee and Thyme on Isolated Airway Smooth Muscle Tissue of Guinea pigs

By

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Abbreviations

ACh:	Acetylcholine
AHR:	Airway hyper-responsiveness
ANS:	Autonomic Nervous System
ASM:	Airway Smooth Muscle
CDC:	Centers for Disease Control
COPD:	Chronic Obstructive Pulmonary Disorder
COX:	Cyclooxygenase
CTLs:	Cytotoxic T Lymphocytes
ECP:	Eosinophil Cationic Protein
EIB:	Exercise Induced Bronchoconstriction
EMEA:	European Medicines Agency
FDA:	Food and Drug Administration
FEV1:	Forced Expiratory Volume in 1 Second
GAPC:	Good Agricultural and Collection Practices
GDP:	Gross Domestic Product
GM-CSF:	Granulocyte Monocyte Colony Stimulating Factor
HETE-15	Hydroxyeicosateraenoic acid 15
HMPC:	Committee on Herbal Medicinal Products
IBMX:	1-methyl-3-isobutylxanthine
IGF:	Insulin-like growth Factor
IL:	Interleukin
LTC4:	Leukotriene Receptor Subtype 4
M3:	Muscarinic Receptors Subtype 3
NAEPP:	National Asthma Education and Prevention Program
NAFLD:	Non-Alcoholic-Fatty-Liver-Disease
NSAID:	Non-steroidal Anti-inflammatory Drugs
PAF:	Platelet Activating Factor
PD:	Provocative Dose
PDGF:	Platelet Derived Growth Factor
PEFR:	Peak Expiratory Flow Rate
PGE2:	Prostaglandin E2
RANTES:	Regulated upon activation, Normal T cell Expressed and Secreted
SCF:	Stem Cell factor
SPC:	Summary of Product Characteristics
TGF:	Transforming Growth Factor
TNF:	Tumor Necrosis Factor

Abstract

BACKGROUND: *Coffea arabica and Thymus schimperi* are plants claimed in traditional medicine to possess numerous beneficial/healing effects including therapeutics in asthma due to airway smooth muscle relaxant properties. The objective of this study was to investigate the bronchodilator effects of these plants crude extracts on guinea pig trachea smooth muscles.

METHODS: Isolated guinea-pig trachea rings were used to study the effects of hydroalcholic and n-hexane crude extracts of the beans of *Coffea arabica* and the leaves of Thymus *schimperi in vitro* in the presence of acetylcholine by using single dosage method of extract administration. Salbutamol was used as a standard drug.

RESULTS: Investigation of the effects hydroalcholic (methanol 80%) and n-hexane crude extract of *Coffea arabica* L. and *Thymus schimperi* R. on an isolated trachea smooth muscle tissue in the presence of Ach (*in vitro*) yielded an overall contractile response. The contractile responses of the plant crude extracts in the absence of Ach were clearly evident on the isolated tissue to variable degrees depending on type of plant, type of plant extract and amount of dosage administered to the tissue. The tissue contractile response to Ach (160ng) alone, which peaked at 1.75mm (mean peak point) from baseline on the polygraph chart, was used as a positive control for contraction. In the presence of the plant extracts (both plants and types of extracts), the contractile response of the GPT when Ach was added had an amplified (additive) contractile effect except for the 50µg dose group of n- hexane crude extract of *Coffea arabica*, which showed a relaxant effect of 0.22mm in the presence of Ach and was not of statistical significance. Statistical analysis indicated that the hydroalcholic crude extracts of *Coffea arabica* and *Thymus schimperi* showed significant differences in tissue contraction between the 50µg dose and 100 µg, 200 µg, 400 µg group and between the 50µg dose the two dose groups of 150 µg and 200 µg respectively (P-value 0.05).

CONCLUSION: The hydroalcholic (Methanol 80%) crude extracts of both *Coffea arabica* L. and *Thymus schimperi* R. showed a contractile response on the guinea pig trachea rings *in vitro* which was statistically significant between the dose groups in the presence of Ach. The results yielded are not in full agreement to the traditional claim of a relaxant effect on the airways.

Key words: Coffee, Thyme, Smooth Muscle, Guinea Pig Trachea Rings

1. Introduction

1.1 Medicinal Plants

1.1.1. Herbal Medicine Pros and Cons: Treatment Practice, Trends in Use and Regulation

Herbal medicines, which are also referred to as herbal remedies, have been sources of treatment for millennia (Evans, 2001). The uses of herbal medicines are evidence or science based approach for the treatment and/or prevention of diseases. This practice is referred to as*phytotherapy*. This approach in the use of herbal medicines contradicts with traditional medical herbalism which uses herbal medicines in an all-inclusive manner of treatment. Although these two approaches (traditional/holistic and rational/evidence-based) are entirely different, in some instances they use the same terminology. For example, traditional herbalism is also described as 'phytotherapy' and refers to preparations of plant material as 'herbal medicines'. Currently, an associated link is createdbetween these approaches and many herbalists use scientific evidence to support their traditional use of herbal medicines. Plants have been used medicinally for thousands of years by cultures all over the world. According to the World Health Organization, 80% of the world's population uses plant-based remedies as their primary form of healthcare (Evans, 2001). In the developing world, herbal medicines are still a central part of the medical system, such as Ayurvedic medicine in India and traditional Chinese medicine in China. Herbal medicine has a long history and tradition in Europe.

Herbal medicines continue to be a popular healthcare choice with the general public not only for health maintenance and wellbeing, minor ailments (e.g. coughs and colds), chronic conditions (e.g. back pain) and serious chronic diseases (e.g. asthma, cancer, depression, diabetes), but also for 'enhancement' of functions or processes, such as the use of *Ginkgo biloba* products for memory enhancement (European Self-Medication Industry: AESGP, 1999). The general public receives information on herbal medicines through various sources, including popular magazines and newspaper articles and other sources of advertising literature provided by manufacturers and through word of mouth. Much of this information is presented uncritically, and is targeted to the

consumer along with details of substantial price reductions on products, including continuous sales promotions, that often are the main recommendations for the products.

The quality of herbal medicines is also important with regards to their safety, and safety concerns with herbal medicines, including intrinsic toxicity as well as problems due to adulteration and contamination, continue to arise. As an example, the chemistry of German chamomile (*Matricariarecutita* L. Asteraceae/Compositae), especially of the volatile oil component, is well documented and is similar to that of Roman chamomile. Pharmacological activity is said to be associated with the flavonoid and volatile oil fractions. The plant is used as a food additive and chamomile tea is also a common beverage. A range of pharmacological actions has been documented (e.g. anti-inflammatory and antispasmodic activities) and many of these support the reputed herbal uses.

However, Chamomile sequiterpene lactones are known to possess allergenic properties. They occur predominantly in herbs of the Compositae (Asteraceae) family, of which chamomile is a member. Hypersensitivity reactions have been reported for chamomile and other plants from the same family. Cross-sensitivity to other members of the Compositae family is well recognized. The European Medicines Agency-Committee on Herbal Medicinal Products(EMEA-HMPC) issueda public statement proposing the following summary of product characteristics(SPC) for chamomile products: ‘Hypersensitivity reactions to (German) chamomile (e.g. contact dermatitis) are very rare. Cross reactions may occur in people with allergy to Compositae (e.g. Artemisia). Very rarely severe allergic reactions (anaphylactic shock, asthma, facial oedema and urticaria) following internal use have been reported (EMEA, 2006). This rare condition of occurrence however, still exists to incur a severe threat if not considered. Another area of concern is the concurrent use of herbal remedies and other conventional medicines due to the potential of adverse drug interactions (EMEA, 2006).

The World Health Organization (WHO) has conducted a recent global survey on the regulatory control of herbal medicines and has reported findings from 141 countries (WHO, 2005). This work provides a valuable update to the earlier WHO reviews and illustrates the wide differences in the approach to regulation between these countries (WHO, 1998; 2001). The recent survey

confirms that during the past four years many countries have established, or initiated, the process of establishing national policies and regulations regarding herbal medicines. The most important challenges faced by countries were those related to regulatory status, assessment of safety and efficacy, quality control and safety monitoring. In response to requests from Member States, WHO has resolved to provide technical support for the development of methodology to monitor or ensure product safety, efficacy and quality, preparation of guidelines, and promotion of the exchange information. Guidelines have recently been developed in a number of important areas including consumer information, pharmaco-vigilance and good agricultural and collection practices-GACP (WHO, 2003; 2004).

1.1.2 Medicinal values of the Coffee and Thyme

In this study, the aim is to investigate traditional medicinal knowledge with regards to the validity of the claim. The first plant of interest is Coffee(*Coffeaarabica* L.), which originated in the highlands of Ethiopia (Kaffa) and is a widely consumed beverage throughout the world along being a huge contributor to the gross domestic product (GDP) of Ethiopia(Charrier and Berthaud, 1985). Regarding the consumption of coffee many claims and studies have been put forward. According to Boyles (2008) on cancer development, researchers involved in an ongoing 22-year study by the Harvard School of Public Health state that "the overall balance of risks and benefits of coffee consumption are on the side of benefits. In relation to cancer, men who drank six or more cups of coffee a day were found to have a 20% reduction in developing prostate cancer (Wilson *et al*., 2011). Other studies suggest coffee consumption reduces the risk of being affected by Alzheimer's disease, Parkinson's disease, heart disease, diabetes mellitus type 2, cirrhosis of the liver and gout (Katsky *et al*., 2006). A study in 2009 showed that those who consumed a moderate amount of coffee or tea (3–5 cups per day) at midlife were less likely to develop dementia and Alzheimer's disease in late-life compared with those who drank little coffee or avoided it altogether (Eskilinen *et al*., 2009). Most of coffee's beneficial effects against type 2 diabetes are not due to its caffeine content, as the positive effects of consumption are greater in those who drink decaffeinated coffee (Pereira*et al*., 2006). The presence of antioxidants(Fig. 1) and chlorogenic acids in coffee has been shown to prevent free radicals from causing cell damage (Belay and Gholap, 2009; Chu *et al*., 2009).

Excessive amounts of coffee, however, can, in many individuals, cause very unpleasant, exceptionally even life-threatening adverse effects (Zivković, 2000). The benefits of coffee on abnormal liver biochemistry, cirrhosis and hepatocellular carcinoma have been reported, but there is a lack of satisfactory explanation. A possible opposite, if not antagonistic, role of coffee and Mediterranean Diet with regard to being overweight and insulin resistance is envisaged in the natural history of NAFLD (Non-Alcoholic-Fatty-Liver-Disease)(Catalano *et al*., 2010). Coffee consumption can lead to iron deficiency anemia in mothers and infants (Muñoz *et al*., 1988). Coffee also interferes with the absorption of supplemental iron (Dewey, 1997). Interference with iron absorption is due to the polyphenols present in coffee. Four major classes were identified: flavan-3-ols (monomers and procyanidins), hydroxycinnamic acids, flavonols and anthocyanidins (Ramirez-Coronel *et al*., 2004). Although the inhibition of iron absorption can cause an iron deficiency, iron is considered a carcinogen in relation to the liver. Polyphenols contained in coffee are therefore associated with decreasing the risk of liver cancer development (Nkondjock, 2009). Dorri *et al.,* (2007) have suggested that the smell of coffee can restore appetite and refresh olfactory receptors. They suggest that people can regain their appetite after cooking by smelling coffee beans, and that this method can also be used for research animals.

Over 1,000 chemicals have been reported in roasted coffee; more than half of those tested (19/28) are rodent carcinogens (Ames, 1998). Coffee's negative health effects are often blamed on its caffeine content. Instant coffee has a much greater amount of acrylamide than brewed coffee. Research suggests that drinking caffeinated coffee can cause a temporary increase in the stiffening of arterial walls (Mahmud and Feely, 2001).It may aggravate preexisting conditions such as gastroesophageal reflux disease, migraines, arrhythmias, and cause sleep disturbances (Palmer, 2009).

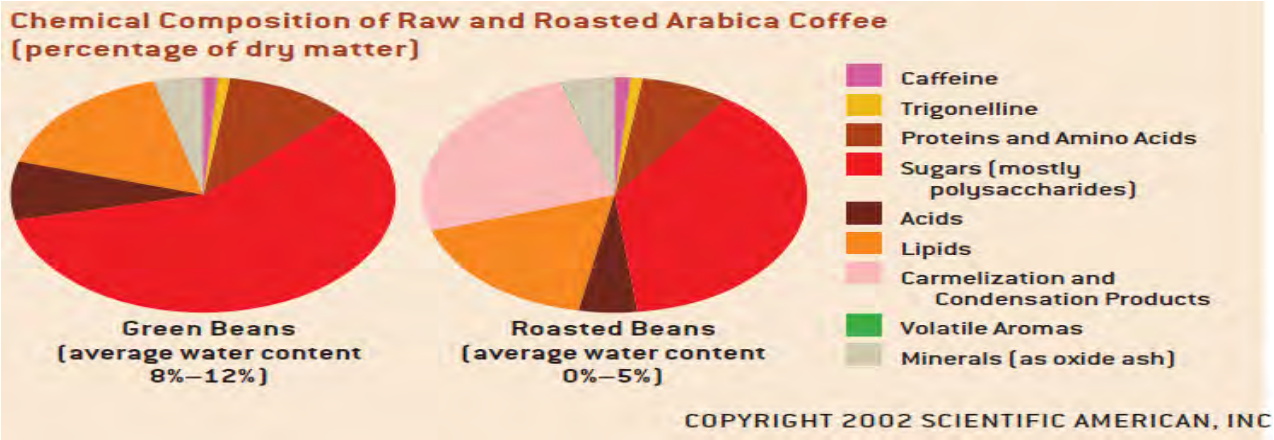

Figure 1: Chemical constituents of green and roasted beans of *Coffea arabica L.* (*Source*: Illy, 2002).

The main constituent or active ingredient in coffee claimed and proven to have a smooth muscle relaxant effect is the alkaloid xanthine (a purine base). This compound is found not only in *Coffea arabica* but also in various human bodily tissues. Purine degradation (the degradation of guanine by the enzyme guanine deaminase) in the human body is the main process in xanthine formation. In addition to this pathway, xanthine is formed from hypoxanthine by xanthine oxidoreductase and from xanthosine by purine nucleoside phosphorylase (PNP). The catabolism of xanthine in the body results in its conversion to uric acid by the action of the xanthine oxidase enzyme (Voet *et al*., 2008). Xanthine derivatives commonly known as xanthines are alkaloid groups used for their effects as mild stimulants and as bronchodilators, notably in treating the symptoms of asthma/ airway constrictions (Walter and Maheswari, 2006).

In addition to caffeine, coffee contains trigonelline (which gives it the scent), proteins and amino acids, polysaccharides, chlorogenic acids, lipids, caramel in case of roasted coffee: heated sugar which turns dark brown, volatile aroma and minerals (Illy, 2002). Methylxanthines such as caffeine increase heart rate induce contraction and cause cardiac arrhythmias at high concentrations. In the CNS they increase alertness, stimulate the respiratory center, and are used for treatment of infantile apnea, which is a brief pause or absence of breathing (Walter and Maheswari, 2006). In high doses, methylxanthines may induce convulsions that are resistant to anticonvulsants.

Caffeine is catabolized into more than 25 metabolites in humans, mainly Paraxanthine, Theobromine, and Theophylline (Etherton and Kochar, 1993;Deree *et al*., 2008). Caffeine metabolism yields paraxanthine as a final product, which represents 72 to 80% of caffeine metabolism. There are five main metabolic pathways which contribute to caffeine metabolism in adults (Miners and Birkett, 1996; Miners and McKinnon, 2000). The first three consist of demethylization of N-3 to form Paraxanthine, N-1 to form Theobromine (vasodilator, increased cerebral and muscular blood flow), and N-7 to form Theophylline (vascular, bronchiole, muscular, and respiratory relaxant). The hepatic cytochrome P-450 (CYP) isoenzyme metabolizes most of the caffeine (95%) by three demethylizations which on average give an *in vivo* metabolism percentage of *85% paraxanthine, 10% theobromine, and 5% theophylline*.These metabolites act as both competitive nonselective phosphodiesterase inhibitors (Essayan, 2001) which raise intracellular cAMP, activate PKA thereby inhibiting TNF-α and leukotriene synthesis (Marques *et al*., 1999; Deree *et al*., 2008). This process is believed to reduce inflammation and innate immunity and also serves as a nonselective adenosine receptor antagonists which inhibit sleepiness-inducing adenosine(Daly *et al.,* 1987; Peters *et al*., 2005).The fourth pathway results in the formation of uracil metabolites, and the fifth consists of renal elimination of the remaining percentage of caffeine that was not able to be degraded in the process. The large inter-individual differences observed in plasmatic concentration of caffeine following the administration of an equal dose are mainly due to variations in metabolism. These variations depend on four factors: genetic polymorphisms, metabolic induction and inhibition of cytochrome P-450, individual (weight, sex), and the presence of hepatic diseases (Miners and McKinnon, 2000). Caffeine is absorbed rapidly and completely from the intestinal tract, making it 100% bioavailable. The time in which maximum plasmatic concentration is obtained (Tmax) is 30 to 45 minutes fasting and is delayed with food ingestion (Nehlig, 1999; Fredholm *et al*., 1999; Miners and McKinnon, 2000; Bispo *et al*., 2002). It has an average metabolic half-life in humans of 2.5 to 4.5 hours (Arnaud, 1993).

The second plant of interest in the current study is Thyme**.***Thymus schimperi* R is mainly composed of thymol (59.3%) and carvacrol (14.9%), which are monoterpenes (Dagne *et al*., 1998). Thymol, an antiseptic, is the main active ingredient in Listerine^{R} mouthwash(Van Den Broucke, 1983). Before the advent of modern antibiotics, it was used to medicate bandages as

antimicrobial agent and its use as an antimicrobial agent has been justified by scientific research (Lattaoui and Tantaoiu-Elaraki, 1994). It can also be found as the active ingredient in all-natural, alcohol-free hand sanitizers (Filoche *et al*., 2005).Traditionally, it has been used for dyspepsia, chronic gastritis, asthma, diarrhoea in children, enuresis in children, laryngitis, tonsillitis (as a gargle), and specifically for pertussis and bronchitis. *In vitro* antispasmodic activity of thyme and related *Thymus* species has been associated with the phenolic components of the volatile oil and with the flavonoid constituents (Van Den Broucke and Lernli, 1981); their mode of action is thought to involve calcium-channel blockage (Blazquez, 1989). It has been stated that the flavonoids thymonin, circilineol and 8-methoxycircilineol have potent spasmolytic activity in guinea pig ileum preparations *in vitro* (Van Den Broucke, 1983).In the advent of modern scientific methods to the practice of traditional medicine, the possibilities of unlocking compounds from plants that have the ability to heal diseases presents a promising opportunity in making the quality of life better for many people all over the world. Issues of efficacy, safety and quality can also be addressed by following an evidence based scientific approach.

1.2 Autonomic Nervous System

Autonomic Nervous System (ANS), in vertebrate anatomy, is one of the two main divisions of the nervous system, supplying impulses to the body's heart muscles, smooth muscles, and glands. It controls the action of the glands, the functions of the respiratory, circulatory, digestive, and urogenital systems; and the involuntary muscles in these systems and in the skin (Gearien and Mede, 1974). Controlled by nerve centers in the lower part of the brain, the system also has a reciprocal effect on the internal secretions, being controlled to some degree by the hormones (neurotransmitters such as ACh) and exercising some control, in turn, on hormone production. The autonomic nervous system is made up of two antagonistic divisions. The sympathetic (thoracicolumbar division) stimulates the heart, dilates the bronchi, contracts the arteries, and inhibits the digestive system, preparing the organism for physical action. On the other hand, the parasympathetic (craniosacral division) has contrasting effects, which prepares the organism for feeding, digestion, and rest (Katzung, 1995).

The sympathetic division consists of a chain of interconnected ganglia (groups of nerve cells) on each side of the vertebral column, which sends nerve fibers to several large ganglia, such as the coeliac ganglion. These give rise to nerves passing to the internal organs. The ganglia of the sympathetic chains are connected to the central nervous system by fine branches connecting each ganglion with the spinal cord (Kasper *et al*., 2005). Fibers of the parasympathetic system arise in the brain and, with the cranial nerves, especially the vagus and accessory nerves, pass to ganglia and plexuses (networks of nerves) within the various organs (Guyton and Hall, 2006). The lower part of the body is innervated by fibers arising from the lowest (sacral) segment of the spinal cord and passing to the pelvic ganglion, which gives rise to nerves for such organs as the rectum, bladder, and genital organs (Fig. 2).

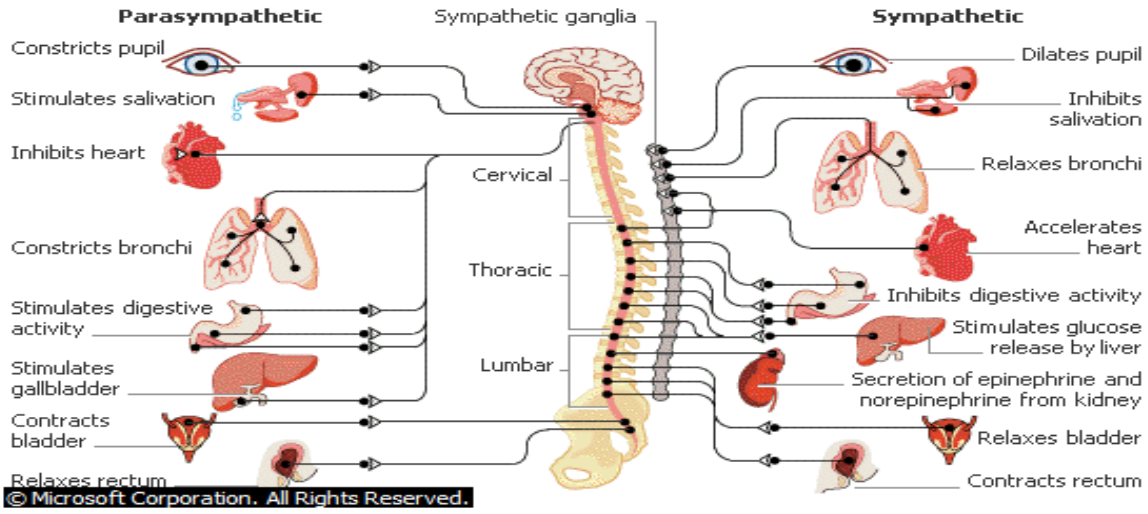

Figure 2:The ANS directs all activities of the body that occur without a person's conscious control, such as breathing and food digestion. It has two parts: the sympathetic division, which is most active in times of stress, and the parasympathetic division, which controls maintenance activities and helps conserve the body's energy. (Source: Microsoft ® Encarta ® 2009.)

1.3 Smooth Muscles

1.3.1 Contraction

Smooth muscle contraction is caused by the sliding of myosin and actin filaments over each other. The cleavage of ATP provides the energy for contraction. Myosin functions as an ATPase utilizing ATP to produce a molecular conformational change of part of the myosin and produces movement. Movement of the filaments over each other happens when the rounded heads protruding from myosin filaments attach and interact with actin filaments to form crossbridges. The myosin heads tilt and drag along the actin filament a small distance (10-12 nm). The heads then release the actin filament and then changes angle to relocate to another site on the actin filament a further distance (10-12 nm) away (Guyton and Hall, 2006). They can then re-bind to the actin molecule and drag it along further. This process is called crossbridge cycling and is the same for all muscles. Unlike cardiac and skeletal muscle, smooth muscle does not contain the calcium-binding protein troponin. Contraction is initiated by a calcium-regulated phosphorylation of myosin, rather than a calcium-activated troponin system (Masaki, 1990).Crossbridge cycling causes contraction of myosin and actin complexes, in turn causing increased tension along the entire chains of tensile structures, ultimately resulting in contraction of the entire smooth muscle tissue (Kasper *et al*., 2005).

1.3.2 Phasic or tonic

Smooth muscle may contract phasically with rapid contraction and relaxation, or tonically with slow and sustained contraction. The reproductive, digestive, respiratory, and urinary tracts, skin, eye, and vasculature all contain this tonic muscle type (Aguilar*et al*., 2010). This type of smooth muscle can maintain force for prolonged time with only little energy utilization. There are differences in the myosin heavy and light chains that also correlate with these differences in contractile patterns and kinetics of contraction between tonic and phasic smooth muscle (Phillippe and Chien, 1998).

1.3.3 Activation of myosin heads

Crossbridge cycling cannot occur until the myosin heads have been activated to allow crossbridges to form. When the light chains are phosphorylated, they become active and will allow contraction to occur. The enzyme that phosphorylates the light chains is called myosinlight-chain kinase (MLCK), also called MLC_{20} kinase(Aguilar*et al*., 2010). In order to control contraction, MLCK will work only when the muscle is stimulated to contract. Stimulation will increase the intracellular concentration of calcium ions (Galvez *et al*., 1996). These bind to a molecule called calmodulin, and form a calcium-calmodulin complex. It is this complex that will bind to MLCK to activate it, allowing the chain of reactions for contraction to occur (Walsh, 1985).

Activation consists of phosphorylation of a serine on position 19 (Ser19) on the MLC_{20} light chain, which causes a conformational change that increases the angle in the neck domain of the myosin heavy chain,which corresponds to the part of the cross-bridge cycle where the myosin head is unattached to the actin filament and relocates to another site on it. After attachment of the myosin head to the actin filament, this serine phosphorylation also activates the ATPase activity of the myosin head region to provide the energy to fuel the subsequent contraction. Phosphorylation of a threonine on position 18 (Thr18) on MLC20 is also possible and may further increase the ATPase activity of the myosin complex(Aguilar*et al*., 2010).

1.3.4 Sustained maintenance

Phosphorylation of the MLC_{20} myosin light chains correlates well with the shortening velocity of smooth muscle. During this period there is a rapid burst of energy utilization as measured by oxygen consumption. Within a few minutes of initiation the calcium level markedly decrease, MLC_{20} myosin light chains phosphorylation decreases, and energy utilization decreases and the muscle can relax (Galvez *et al*., 1996). Still, smooth muscle has the ability of sustained maintenance of force in this situation as well. This sustained phase has been attributed to certain myosin crossbridges, termed latch-bridges that are cycling very slowly, notably at the cycle stage where dephosphorylated myosin complexes detach from the actin, thereby maintaining the force

at low energy cost. This phenomenon is of great value especially for tonically active smooth muscle.

Isolated preparations of vascular and visceral smooth muscle contract with depolarizing high potassium balanced saline generating a certain amount of contractile force. The same preparation stimulated in normal balanced saline with an agonist such as endothelin or serotonin will generate more contractile force (Burkhalter, 1995). This increase in force is termed calcium sensitization. The myosin light chain phosphatase is inhibited to increase the gain or sensitivity of myosin light chain kinase to calcium. There are number of cell signalling pathways believed to regulate this decrease in myosin light chain phosphatase: a RhoA-Rock kinase pathway, a Protein kinase C-Protein kinase C potentiation inhibitor protein 17 (CPI-17) pathway, telokin, and a Zip kinase pathway. Further Rock kinase and Zip kinase have been implicated to directly phosphorylate the 20kd myosin light chains (Kasper *et al*., 2005; Guyton and Hall, 2006).

1.3.5Relaxation

The phosphorylation of the light chains by MLCK is countered by a myosin light-chainphosphatase, which dephosphorylates the MLC_{20} myosin light chains and thereby inhibits contraction. Other signaling pathways have also been implicated in the regulation actin and myosin dynamics. In general, the relaxation of smooth muscle is by cell-signaling pathways that increase the myosin phosphatase activity, decrease the intracellular calcium levels, hyperpolarize the smooth muscle, and/or regulate actin and myosin dynamics (Aguilar*et al*., 2010).

1.3.6Relaxation-inducing factors

The relaxation of smooth muscle can be mediated by the endothelium-derived relaxing factor-nitric oxide, endothelial derived hyperpolarizing factor (either an endogenous cannabinoid, cytochrome P450 metabolite, or hydrogen peroxide), or prostacyclin (PGI2). Nitric oxide and PGI2 stimulate soluble guanylate cyclase and membrane bound adenylate cyclase, respectively. The cyclic nucleotides (cGMP and cAMP) produced by these cyclases activate Protein Kinase G and Protein Kinase A and phosphorylate a number of proteins (Guyton and Hall, 2006). The phosphorylation events lead to a decrease in intracellular calcium. It also causes the inhibition of L type calcium channels, IP3 receptor channels while stimulating sarcoplasmic reticulum

calcium pump ATPase (de Silva *et al*., 1993). In addition, a decrease in the 20kd myosin light chain phosphorylation takes place by altering calcium sensitization and increasing myosin light chain phosphatase activity. A stimulation of calcium sensitive potassium channels hyperpolarizes the cell and the phosphorylation of amino acid residue serine 16 on the small heat shock protein (hsp20)by Protein Kinases A and G inducing relaxation. The phosphorylation of hsp20 alters actin and focal adhesion dynamics and actin-myosin interaction (de Silva *et al*., 1993; Kasper *et al*., 2005).

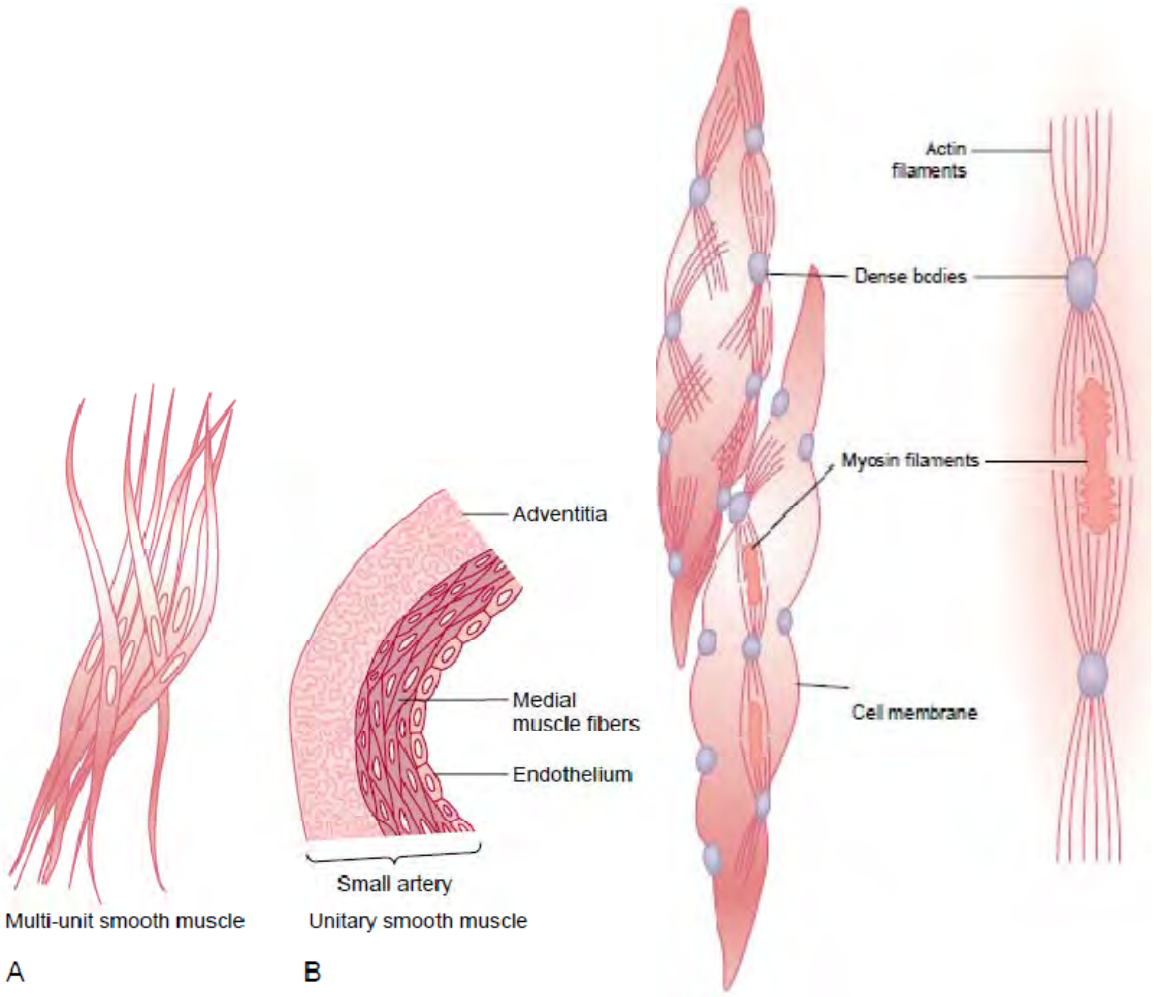

Figure 3: A: Multiunit smooth muscle (e.g. as found in trachea, uterus, and iris) and B: Unitary smooth muscles. Physical structure of smooth muscle. The upper left-hand fibershows actin filaments radiating from dense bodies. The lower left hand fiber and the right-hand diagram demonstrate the relation of myosin filaments to actin filaments (Source: Guyton and Hall, 2006).

1.4 Asthma

Asthma is a constrictive pulmonary disease associated with a persistent inflammatory condition of the lung airways leading to smooth muscle (SM) constriction (tracheobronchial branch), whose cause is partly understood(Kasper *et al*., 2005). The major portion of the volume of the cytoplasm of SM cells of the trachea is taken up by the molecules myosin and actin, which together have the capability to contract. Tracheal SM cells are multi-unit in composition i.e. each muscle cell is innervated independently unlike unitary SMs which act in syncytium during contraction and relaxation (Guyton and Hall, 2006). Inflammation of the airway occurs mainly due to hyper-responsiveness of the tissue to a wide range of stimuli (Kasper *et al*., 2005). It is considered that both hereditary and environmental factors play a role in its onset. Symptoms are commonly accompanied with cough, wheeze, chest tightness and shortness of breath, often worse at night. Reversible airway obstruction and spasm of the airways are characteristic of its complications unlike chronic obstructive pulmonary disorder or COPD (Yawn, 2008).

Clinical classification of asthma considers the frequency of attacks, peak expiratory flow rate (PEFR), which is measured in Liters/minute and forced expiratory volume in one second (FEV1) in determining the mild to severe level of the disease condition (Kumar *et al*.; 2010; Lemanske and Busse, 2010). Treatment of acute symptoms is usually with inhaled short-acting beta-2 agonists such as salbutamol or albuterol (Fanta, 2009).Symptoms can be prevented by avoiding triggers, such as allergensand irritants, and by inhaling corticosteroids. Its diagnosis is usually made based on the pattern of symptoms and/or response to therapy over time (Lemanske and Busse, 2010).

1.4.1 Prevalence

In 1998 a survey conducted regarding the global prevalence of asthma indicated that geographical variation exists with asthma being common in more developed countries, some of the highest rates being in New Zealand, Australia and the United Kingdom (ISAAC, 1998). Asthma prevalence has been much rarer in developing countries such as China, Malaysia, and in Africa and Central and Eastern Europe (Gold and Right, 2005). In many countries the incidence of asthma is on the rise but there isn't a clear cut picture whether this increase is attributed to the

global population boom or due to other factors (Kasper *et al*., 2005). The global asthma prevalence was estimated to be between 7-10% of the global population (Govinda *et al*., 1999 cited in Assefa*et al*., 2008). The number of asthma incidence has increased significantly since the 1970s to 2004 (WHO, 2009). In concert with the WHO report, evidence of asthma incidence increase from the 1960 to 2008 has also been indicated (Grant *et al*., 1999; Anandan *et al*., 2010). As of 2010, 300 million people were affected worldwide. The global mortality in the year 2009 due to asthma was recorded to be 250,000 deaths (Soar *et al*., 2010). Recent evidence in developing countries suggests that the disease is becoming more frequent as developing nations are being more urbanized and westernized (Tippets and Gilbert, 2009).

1.4.2 Etiology

Occupational sensitizersencountered at the workplace give rise to occupational asthma e.g. pesticides, animal waste/dander, detergent enzymes, flour, natural rubber latex (CDC, 2008). Asthma as a result of (or worsened by) workplace exposures is a commonly reported occupational respiratory disease. Still most cases of occupational asthma are not reported or are not recognized as such. It is suggested that 15–23% of new-onset asthma cases in adults are work related (American Thoracic Society 2004 cited inCDC, 2004). Cold air and exercise induce asthma. The inhalation of cold, dry air (non-humid) will precipitate an attack by Exercise-induced wheeze driven by histamine and leukotrienes which are released from mast cells (Helenius and Haahtela, 2000). Many patients with asthma experience worsening of symptoms on contact with cigarette smoke, car exhaust fumes, strong perfumes or high concentrations of dust in the atmosphere (Jeong-Hee *et al*., 2005). Obesity has also been demonstrated to have a strong correlation in the development of asthma (Beuther, 2010). Several factors associated with obesity may play a role in the pathogenesis of asthma, including decreased respiratory function due to a buildup of adipose tissue (fat) and through adipose tissue causing a pro-inflammatory state, which has been associated with non-eosinophilic asthma (Wood and Gibson, 2009). Genetic variation in antioxidant enzymes is associated with more severe asthma (Wood and Gibson, 2009). Emotional factors such as stress may influence asthma by affecting the ANS leading to an immune response related to hyper-responsiveness; however, there is no evidence that patients with the disease are any more psychologically disturbed than non-asthmatic individuals (Chen and Miller, 2005).

1.4.3 Pathogenesis

The pathogenesis of asthma is complex and not fully understood. It involves a number of cells, mediators, nerves and vascular leakage that can be activated by several different mechanisms, of which exposure to allergens is among the most significant. The varying clinical severity and chronicity of asthma is dependent on interplay between airway inflammation and airway wall remodeling (Kasper *et al*., 2005). The inflammatory component is driven by Th2-type T lymphocytes which facilitate IgE synthesis through production of IL-4 and eosinophilic inflammation through IL-5 (Guyton and Hall, 2006). Airway remodeling due to inflammation leads to narrowing of the airways and shortening of the smooth muscle.

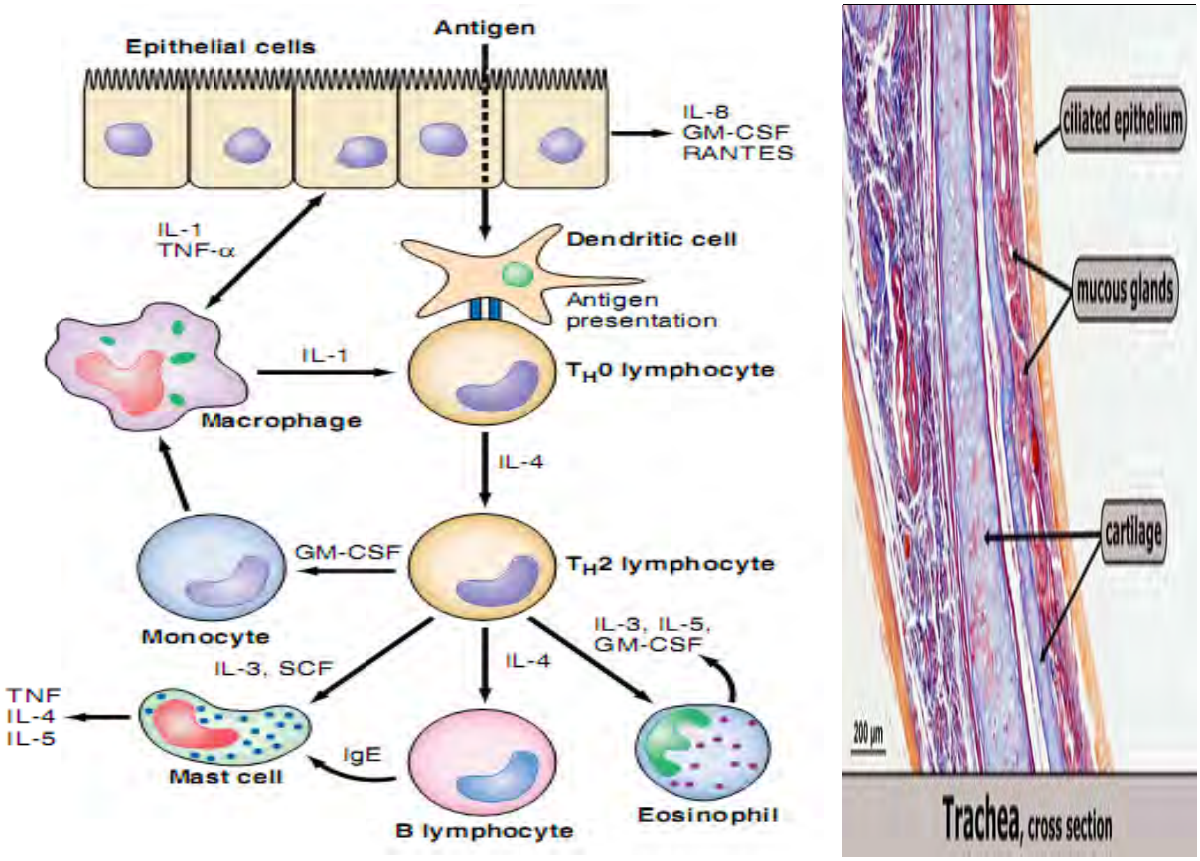

Figure 4: Cytokine network in allergic asthma. ILs GM-CSF, RANTES, TNF, and SCF: Left; and Cross section of the trachea: right. (Source: Barnes in Middleton *et al*., 1998).

1.4.4 Clinical Types and Diagnosis of Asthma

The major symptoms of asthma are wheezing attacks and episodic shortness of breath along with bronchoconstriction and spasm. Symptoms are usually worst during nighttime (Guyton and Hall, 2006). Nocturnal cough can be a common feature. There is variation in the frequency and duration of the attacks. Some patients may only have one or two attacks a year that last for a few hours, while others could have attacks lasting for weeks. Attacks may be precipitated by a wide range of triggers. Asthma is a major cause of impaired quality of life with impact on work, recreation, as well as physical activities and emotions (Kasper *et al*., 2005). Clinically classification is done according to the frequency of symptoms, FEV1 and PEFR (Yawn, 2008) i.e. based on severity of the disease condition and at the moment there is no clear method for classifying different subgroups of asthma beyond this system (Self *et al*., 2003). Within the classifications described below, the cases of asthma response to the same treatment differ. Thus, indicating that cases within a classification show significant differences(Moore and Pascual, 2010).

Diagnosis is mainly based on the severity of the asthma type categorized as shown in Table 1 and by the demonstration of reversible airway obstruction (Kasper *et al*., 2005). Response to treatment of β-adrenergic agonist such as salbutamol (100mcg/puff for adolescents and 200mcg/puff for adults) after administration of 2 puffs with an increase in FEV1 of ≥15% is taken as reversible airway obstruction. Diagnosis can be made after a normal spirometry result is obtained. The patient can then be challenged with allergens such as histamine or metacholine to determine airway hyper-responsiveness (AHR). Once AHR is confirmed, the type of the asthma and the effectiveness of its control under treatment can be determined by measuring PEFR at home or FEV1 in the clinic or laboratory (Allen *et al*., 2008). Other diagnostic tests such as skin prick tests for allergens, measurement of blood and sputum IgE and eosinophil levels are also employed but lack specificity in asthma diagnosis.

1.4.5 Management

Control of extrinsic factors is essential in asthma management. Measures must be taken to avoid causative allergens such as the house-dust mite, pets, and moulds particularly in childhood.

Avoidance of the house-dust mite is possible with effective and comfortable covers for bedding and changes to living accommodation. Active and passive smoking should be avoided, as should beta-blockers in either tablet or eye drop form. Individuals intolerant to aspirin may benefit by avoiding dietary salicylates and should avoid NSAIDs (Kasper *et al*., 2005). Other agents (e.g. preservatives and coloring materials such as tartrazine) should be avoided if shown to be a causative factor.

The mainstay of asthma therapy is the use of therapeutic agents delivered as aerosols or powders directly into the lungs. The advantages of this method of administration are that drugs are delivered direct to the lung and the first-pass metabolism in the liver is avoided; thus lower doses are necessary and systemic unwanted effects are minimized. Medications used to treat asthma are divided into two general classes: quick-relief medications used to treat acute symptoms; and long-term control medications used to prevent further exacerbation (NHLBI, 2007).Short acting β_2-adrenoceptor agonists (SABA), such as salbutamol/albuterolare the first line treatment for asthma symptoms. Salbutamol metered dose inhalers (MDI)are commonly used to treat asthma attacks (Raper and Malta, 1973).Long acting β-adrenoceptor agonists (LABA), such as salmeterol, bambuterol and indacaterol, with a bronchodilation effect lasting up to 12 hours (depending on the frequency of use) has also been promising in the long term control of asthma. They are however not to be used without a steroid due to an increased risk of severe symptoms i.e. cause being slow release and action (Cates *et al*., 2009; Fanta, 2009).Inhaled forms of glucocorticoids (ICS) are the most effective treatment available for long term control (NHLBI, 2007).

The current study intendsto scientifically examine the indigenous knowledge and practice of two plants, *Coffea arabica* and *Thymus schimperi*, to their claimed anti-asthmatic properties, which is believed to be through bronchodilation. Many conventional drugs have been of plant origin. Numerous plants are responsible for the alleviation of the burden of diseases for a great deal of people throughout the world. The merging of traditional knowledge with modern scientific methods in the use of traditional medicinal plants is capable of providing a well regulated and risk minimized health care service to the public.

2. Objectives

2.1 General Objective

- Evaluation of the claimed traditional knowledge and use of the crude extracts of *Coffea arabica* L. beans and *Thymus schimperi*R. leaves as bronchodilators.

2.2 Specific Objectives

- To investigate the effect of hydroalcholic (Methanol 80%) and n-Hexane crude extracts of *Coffea arabica* L. on isolated guinea pig trachea rings.

- To investigate the effect of hydroalcholic (Methanol 80%) and n-Hexane crude extracts of *Thymus schimperi* R. on isolated guinea pig trachea rings.

- To compare the smooth muscle effects of these plants with a standard drug.

3. Materials and Methods

3.1. Plant Material Collection

Whole plant sample and beans of *Coffeaarabica* were collected in July of 2010 from an organic farmer in Yirgalem, SPNNR (6° 45' 0 N, 38° 25' 0 E) a town located 360 km south of Addis Ababa, Ethiopia. *Thymus schimperi* R. was collected in the same month from the Ras Kassa neighborhood, around the location of the French Embassy in Addis Ababa(9°3'11"N 38°46'11"E), Ethiopia. The plant samples were then identified at the National Herbarium, Department of Biology, Addis Ababa University. A deposit of the plant samples was made at the National Herbarium after identification (Voucher #002 and #003 for coffee and thyme, respectively).

3.2. Description of the plants used in the study

Coffeaarabica L. *(1753)* (Amharic: Bunna; English: Coffee) is a member of the Rubiaceae family. It's a widely cultivated plant around the world serving many consumers for its central nervous system (CNS) stimulating effect (Nehlig *et al*., 1992) and producers/traders for the income it generates. The plant is a shrub or a small tree, which grows up from 4m to 6m in length. It mostly grows in the altitudinal range between 1000-1900 m above sea level (asl).The leaves of *Coffea arabica* L. are elliptic with a length in the range of 6-18 cm and a width of 2-9 cm (Plate 1). Its seeds (beans) are convex in shape dorsally and grooved at the ventral side (Hedberg *et al*.; 2003). Coffee ("The Green Gold") is a tropical cash crop of great economic and social importance in Ethiopia.

Plate 1 *Coffea arabica L* collected from a farmer in Yirgalem, SPNNR July, 2010 (6° 45' 0 N, 38° 25' 0 E)

The plant *Thymus schimperi* R (1932) of the family Lamiaceae is a perennial herb which is woody at its base. The length of the plant ranges from 5cm to 40cm. Its stems are flat (drained or prostrate) and the young parts are pubescent. The leaves of *Thymus schimperi*are blue green in the upper parts of the plant while the color changes to a paler green below (Hedberg *et al*., 2006). The shapes of the leaves are blade like or elliptic being obtuse at the apex with 1 to 4 lateral veins. The size of *Thymus schimperi* leaves range from 3.5-15mm in length by 2.5-6mm in width. It grows on grassland, exposed rocks, top of mountains and ditches (Plate 2). Its habitat is both afroalpine and afromontane vegetation zones in between the altitudinal range of 2250m-4000m above sea level (Demissew,1993).

Plate 2*Thymusschimperi* R collected at the Ras Kassa neighborhood, around the French Embassy in Addis Ababa, Ethiopia (9°3'11"N 38°46'11"E) July, 2010.

3.3. Plant Extraction

Beans of *Coffeaarabica*and leaves of *Thymusschimperi*were washed and set to dry in a shed for 5 days. Once the beans and leaves were dry, they were ground into a powder using a Waring Commercial Blender (USA). The powdered beans and leaves were then mixed with the polar extraction solvent i.e. 80 % methanol in an Erlenmeyer flask, and were placed on an orbital shaker (GFL, Model 3020, Germany) at room temperature for 24 hours. The same procedure was done for the non-polar extract using the organic solvent n-hexane. After 24 hours the extracts were filtered using cotton and a Whatman filter paper. The organic solvents were removed from the filtrate using a rotary evaporator (Buchi RE 121, Switzerland).The filtrates were then placed in a petridish until they were freeze dried (after being placed at -70 degree Celsius in a Sanyo Ultra Low Freezer) by being lyophilized (Christ Alpha1-4, Germany).

3.4. Experimental Animals

Guinea pigs weighing 350-400 grams were acquired from the Ethiopian Health and Nutrition Research Institute(ENHRI) animal house and were placed and acclimatized at the Animal House of the Faculty of Life Science of AAU until the time of experiments. They were maintained under uniform conditions of light illumination and placed at room temperature. They were given the standard vegetable diet and water.

3.5. *In vitro* Experiment

Guinea pigs weighing 350-400 g were gently killedby cervical dislocation. The trachea was rapidly removed and placedin Tyrode/Krebs solution (composition in grams: NaCl 8g, KCl 0.2g,$CaCl_2$ 0.2g,D-glucose1g, $NaHCO_3$ 1g, $MgCl_2$ 0.1g, and NaH_2PO4 0.05g) and kept at 37^0C and continuously gassed with 95% 0_2, 5%CO_2 mixture. Helicoidal strips of five rings were cut from the tracheaaccording to Constantine (1965), and mounted in organbaths filled with Krebs solution maintained at 37^0Cand oxygenated. The lower end of the tracheal strip wasattached to a metal hook at the base of the chamber by aloop of cotton thread (Plate 3). The upper end was

attached in the samemanner to a force transducer (Grass FT.03C) for continuousrecording of isometric tension by a Grass polygraph (model79D, USA). Tissues were stretched at atension of 1.0 g and equilibrated or were let to stabilize for 40-60 minutes.The suspended and stabilized tissues were then treated with Acetylcholine chloride (ACh) 99% (Sigma^{R}) at doses of 80ng, 160ng, 240ng and 320ng to test both for tissue viability and for determination of the suitable dose for contractile response. The tissue treatment with ACh for each concentration was conducted from 30 to 60 seconds after which time the tissue was washed with the Tyrode solution and left to stabilize from 3-5 minutes. In this study, the dose of ACh chosen is 160ng. Treatment dosages of *Coffea arabica* beans were 50, 100, 200 and 400µg of organ bath concentration respectively for the hydroalcholic crude extract; while the treatment dosage n-hexane crude extract for the same plant were 50,100, 150 and 200µg. The vehicle solvent for the hydroalcholic extracts were distilled water, while for the n-hexane extracts were dimethyl sulphoxide (DMSO) and water (1:20 ratio). The same dosages were used for *Thymus schimperi*. The treatment dosages were applied on the tissue for five minutes before the administration of ACh. After the end of the five minutes 160ng of ACh was applied for 30-60 seconds. The tissue was then washed with the physiological solution and left to stabilize for another five minutes. This procedure of application was followed for all crude plant extracts and doses along with ACh throughout the experiment period.

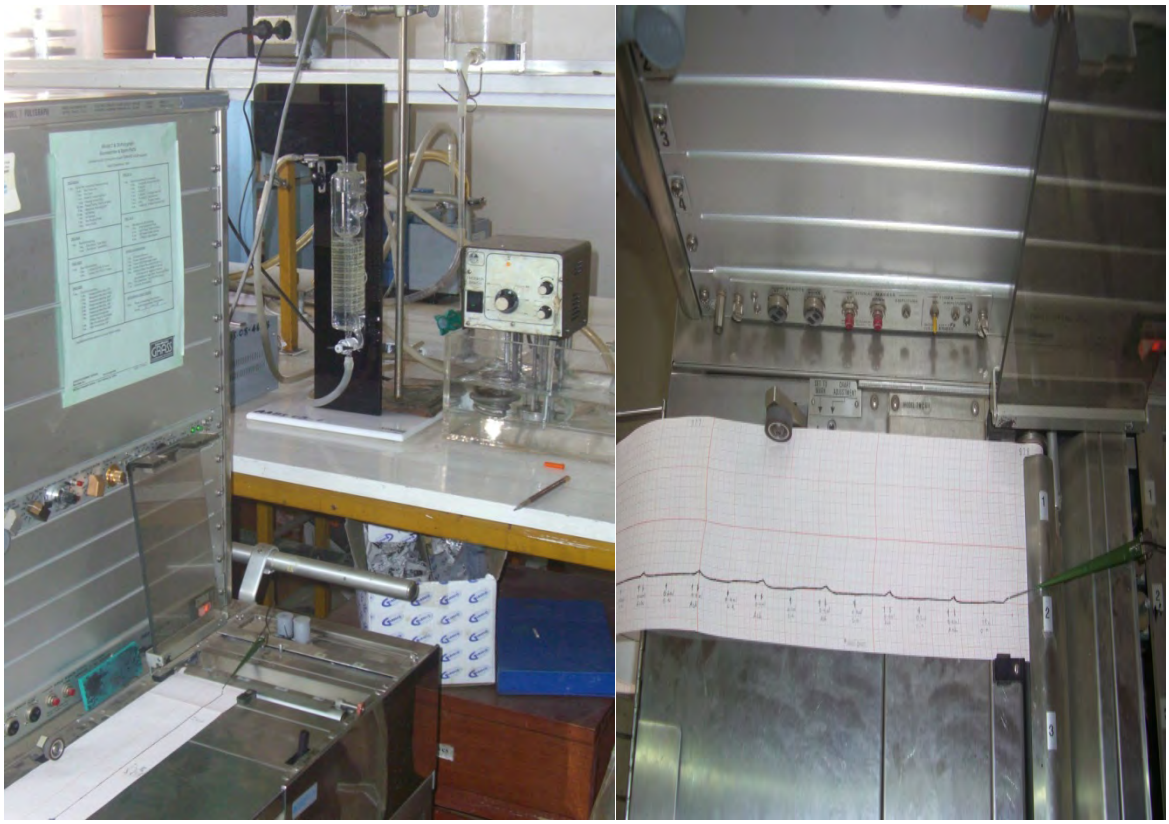

Plate 3 Guinea Pig tracheal rings cut, tied and suspended in 25ml organ bath solution at 1 gm tension (9.8 mm) attached to an isometric force transducer of a Grass polygraph (model79D, USA).

3.6 Statistical Data Analysis

Mean and standard error of the mean (SEM) were calculated for each group of *in vitro* results on guinea pig trachea. Significance of differences between the means was calculated by one way analysis of variance (ANOVA).Tukey and Scheffe post-hoc test (performed by SPSS, version 17) for dose dependent response of the tissue in question was used to determine and compare the effects of the plant extracts (Annexes 5-8). The values were expressed as the mean ± SEM five and three (for hydroalcholic and n-hexane respectively) experiments of each dose of the organ bath concentration. Differences between various doses of the plant extracts on each isolated tissue were considered significant if the p-value was <0.05.

4. Results

4.1 Effect of hydroalcholic extract of *C. arabica* on tracheal rings in the presence of ACh.

The mean contractile response of the trachea SM to the hydroalcholic crude extract of *Coffea arabica* L. in the presence of ACh for all dose groups were greater than the tissue contraction response to ACh (160ng/ml) alone, which gave a mean peak at 1.75 mm from baseline on the polygraph chart. The mean contractile responses for the different dose groups showed an increment in contractile responseas the dosage increased. However, statistical analysis results indicated that there is only a significant difference in tissue contraction between the 50µg dose group and the three remaining dose groups i.e. 100µg, 200µg and 400µg (P-Value 0.05). The intra dose group difference was found to be not significantly different (n=5). In addition, the contractile response difference between the mean dose groups of 100µg, 200µg and 400µg were also found to be statistically not significant. Although there is not a considerable statistical difference between these dose groups, the mean plot does indicate a slight increment in contractile response of the airway smooth muscles as the dose of the plant extract increases. Statistical significance in this case may be evident if the sample size is increased to a larger number (Table 1).

4.2 Effect of n-hexane *C. arabica*extract on tracheal rings in the presence of ACh.

The mean contractile response of tissue to the n-hexane crude extract of *Coffea arabica* L. in the presence of ACh for all dose groups, with the exception of the 50µg dose group, were greater than the tissue contraction response to ACh alone (mean peak at 1.75 mm). The mean peak for the dose group 50µg in the presence of ACh, however, showed a contractile response of 1.53mm above the baseline. This dose group (50µg) had a mean relaxing effect of the tissue smooth muscle in lowering the contraction by 0.22mm. The mean contractile responses for the different dose groups showed an increment in contractile response as the dosage increased from 50µg to 400µg. Statistical analysis of the results showed that there is not a significant difference in tissue relaxation nor contraction between any of the dose groups i.e. 50µg, 100µg, 200µg and 400µg (P-Value >0.05; n=3). Similarly, there was not a considerable statistical difference between

these dose groups, but the mean value indicates an increment in contractile response of the airway smooth muscles as the dose of the plant extract increases (Table 1).

Table 1: The effect of *C. arabica*crude extracts on ACh-induced contraction on isolated tracheal rings.

Type of Extract	**Group**		**Extract concentration(µg/ml)**	**Contractile response (mm)**	**F- value**
Hydroalcholic (Methanol 80%)crude extract of ***Coffeaarabica*** *L*	A	1	50	1.80±0.20*[2a3a4a]	10.6
		2	100	3.20±0.33*[1a]	
		3	200	3.46±0.20*[1a]	
		4	400	3.46±0.20*[1a]	
n-Hexane crude extract of ***Coffeaarabica*** *L*	B	1	50	1.53±0.46	0.28
		2	100	1.83±0.52	
		3	200	2.23±0.84	
		4	400	2.33±0.83	

Control Ach=160ng/ml, n=5 for hydroalcholic extracts while n=3 for n-hexane extracts respectively. Contractile responses are expressed in millimeters of the initial contraction induced by the spasmogen prior to the addition of the plant extracts (Ach contractile response on tracheal rings alone with a mean peak of 1.75mm taken as a positives control for comparison). Data are Mean ±SEM for both sample sizes. Significance: * P-value 0.05.

4.3 Effect of hydroalcholic*T.schimperi* extracton tracheal rings in the presence of ACh.

The mean contractile response of the tissue to the hydroalcholic crude extract of *Thymus schimperi* R. in the presence of Ach for all dose groups were greater than the tissue contraction response to ACh alone. The mean dose group contractile response of the tissue for the dose groups 50µg, 100µg, 150µg and 200µg in the presence of ACh was 2.6mm, 3.3mm, 4.4mm and 4.4mm respectively. The mean contractile responses for the different dose groups showed an increment in contractile response as the dosage increased from 50µg to 150µg and stayed the same at 200µg. Statistical analysis indicated that there was a significant difference in tissue contraction between the 50µg dose group and the two dose groups i.e. 150µg and 200µg (P-Value 0.05), while it indicated an insignificant difference between the 50µg and 100µg dose groups for the same P-value <0.05 (Table 2).

4.4 Tissue response to n-hexane extract of *T.schimperi* in the presence of ACh.

Tissue response to the n-hexane crude extract of *Thymus schimperi*R. in the presence of ACh for all dose groups were greater than the tissue contractile response toACh alone (ACh mean peak=1.75mm). The mean dose group contractile response of the tracheal rings forthe dose groups 50µg, 100µg, 150µg and 200µg in the presence of ACh were 2.0 mm,2.5 mm, 3.0 mm and 3.17 mm respectively. The mean contractile responses for the different dosegroups showed an increment in contractile response as the dosage increased from 50µg to 200µg (Table 2). Sample size here was n=3. Statistical analysis of variance showed that there wasn't a significant inter-dosagegroup difference (P-Value =0.052).

Table 2: The effect *Thymus schimperi*crude extracts of on ACh-induced contraction on isolated tracheal rings.

Type of Extract	**Group**		**Extract concentration (µg/ml)**	**Contractile response (mm)**	**F- value**
Hydroalcholic (Methanol 80%) crude extract of ***Thymusschimperi R***	C	1	50	2.60±0.24*[3c4c]	6.7
		2	100	3.30±0.30	
		3	150	4.40±0.36*[1c]	
		4	200	4.40±0.43*[1c]	
n-Hexane crude extract of ***Thymusschimperi R***	D	1	50	2.00±0 .28	4.0
		2	100	2.50±0.28	
		3	150	3.00±0.00	
		4	200	3.16±0.33	

Control ACh=160ng/ml, n=5 for Hydroalcholic extracts while n=3 for n-Hexane extracts respectively. Contractile responses are expressed in millimeters of the initial contraction induced by the spasmogen prior to the addition of the plant extracts (ACh contractile response on tracheal rings alone with a mean peak of 1.75mm taken as a positives control for comparison). Data are Mean ±SEM for both sample sizes. Significance: * P-value 0.05.

4.5 Tissue response to crude extracts of *C.arabica, T.schimperi* and Salbutamolin the presence of ACh.

The tracheal rings response for both the hydroalcholic crude extracts of *Coffea arabica*and *Thymus schimperi* showed a contractile response which was higher than the n-hexane crude extracts of the two plants. The contractile response due to the hydroalcholic crude extract of *Thymusschimperi*was higher than that of the same crude extract of *Coffeaarabica*. Similarly, the contractile response of the tracheal rings to the n-hexane crude extract of *Thymusschimperi* was greater than the *Coffeaarabica* extract (Fig. 5). The tracheal response toSalbutamol 2mg/5ml (the standard drug used) at 2µg, 4 µg and 8 µg organ bath concentration in the presence of ACh (160 ng) showed a relaxation of -3.00mm below baseline on the polygraph chart (Figs. 6-9) which was maintained (Table 3).

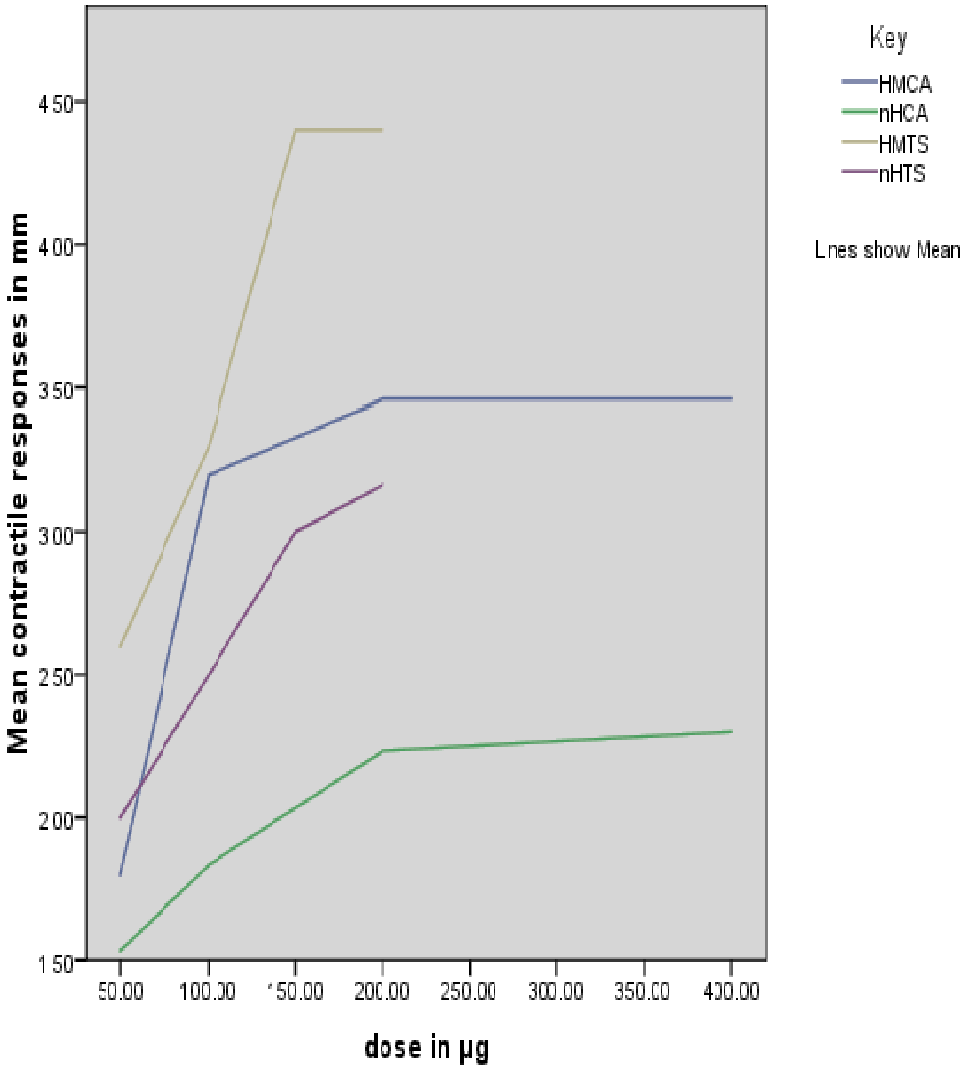

Figure 5: Mean plot graph showing contractile responses of tracheal rings in millimeters (Y-axis) to different doses in µg/ml in organ bath concentration (X-axis) of hydroalcholic crude extract of *Coffeaarabica* (HMCA), n-hexane crude extract of *Coffeaarabica*, (nHCA), hydroalcholic crude extract of *Thymusschimperi* (HMTS) and n-hexane crude extract of *Thymusschimperi* (nHTS).

Table 3: Effect of Salbutamol 2mg/5m on ACh-induced (10µg/ml) contraction on tracheal rings

Salbutamol 2mg/5mlConcentration		Relaxation Response On tracheal rings	Inhibition of ACh induced contraction and sustained relaxation
1	0.125ml	-3.00mm	100%
2	0.25ml	-3.00mm	100%
3	0.5ml	-3.00mm	100%

N.B. Organ bath concentration of the salbutamol doses administered is 2µg/ml, 4µg/ml and 8µg/ml respectively. Organ bath concentration of ACh used is 160ng/ml.

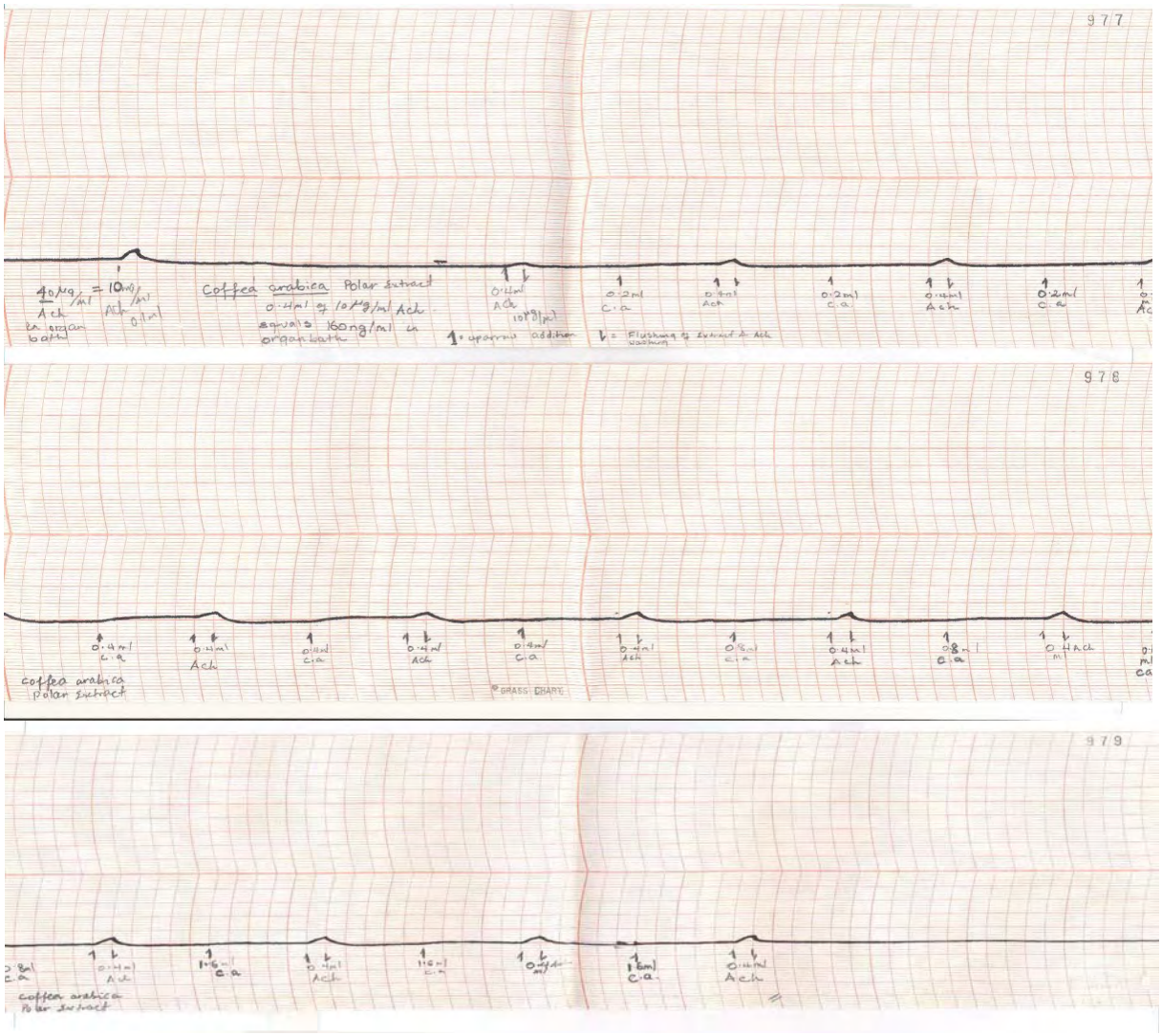

Figure 6: Polygraph traces of hydroalcholic *Coffeaarabica* in the presence of ACh on trachea rings. Upside arrow indicates administration (introduction) while downside arrow indicates washing (flushing) out of administered compounds

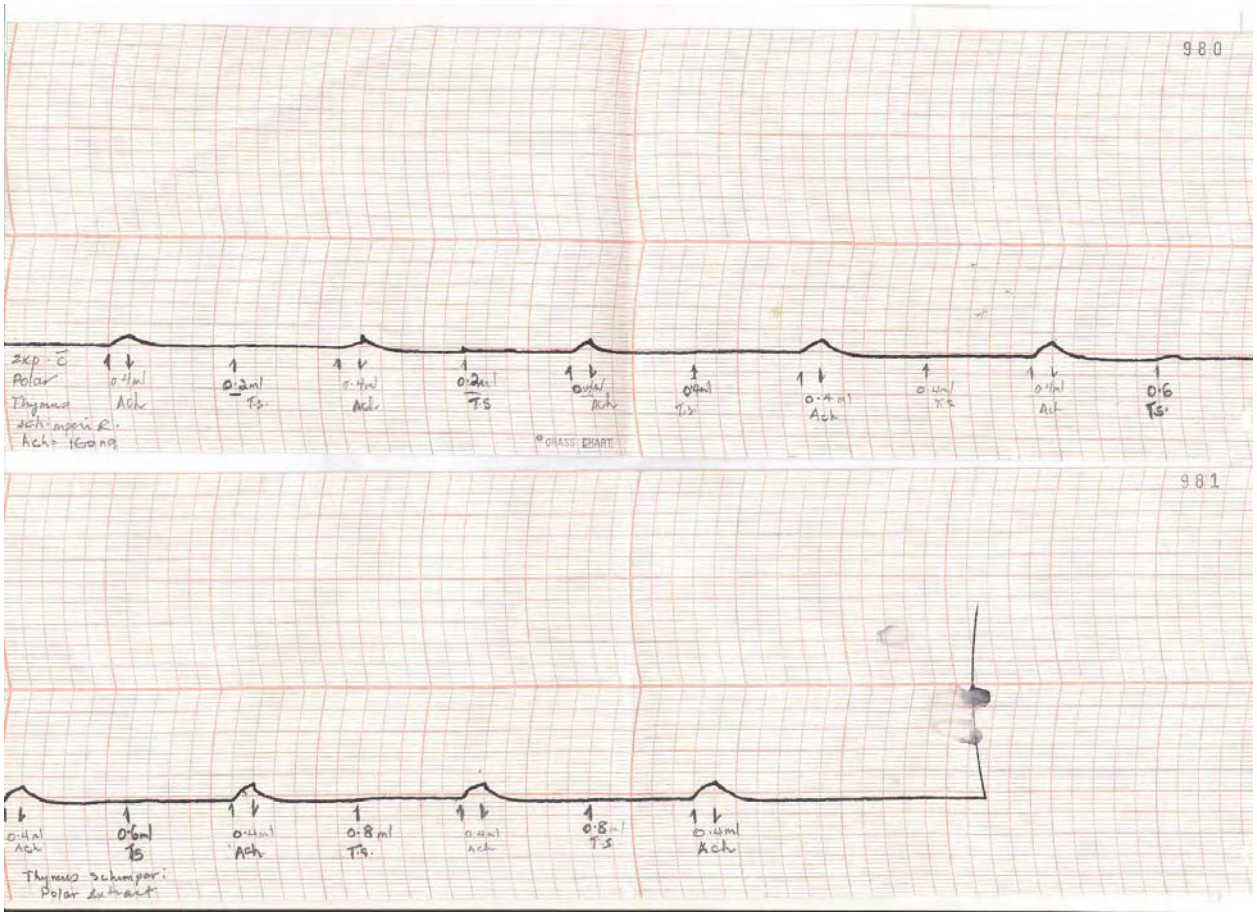

Figure 7: Polygraph traces of hydroalcholic crude extract of *Thymus schimperi* in the presence of ACh on trachea rings. Upside arrow indicates administration (introduction) while downside arrow indicates washing (flushing) out of administered compounds.

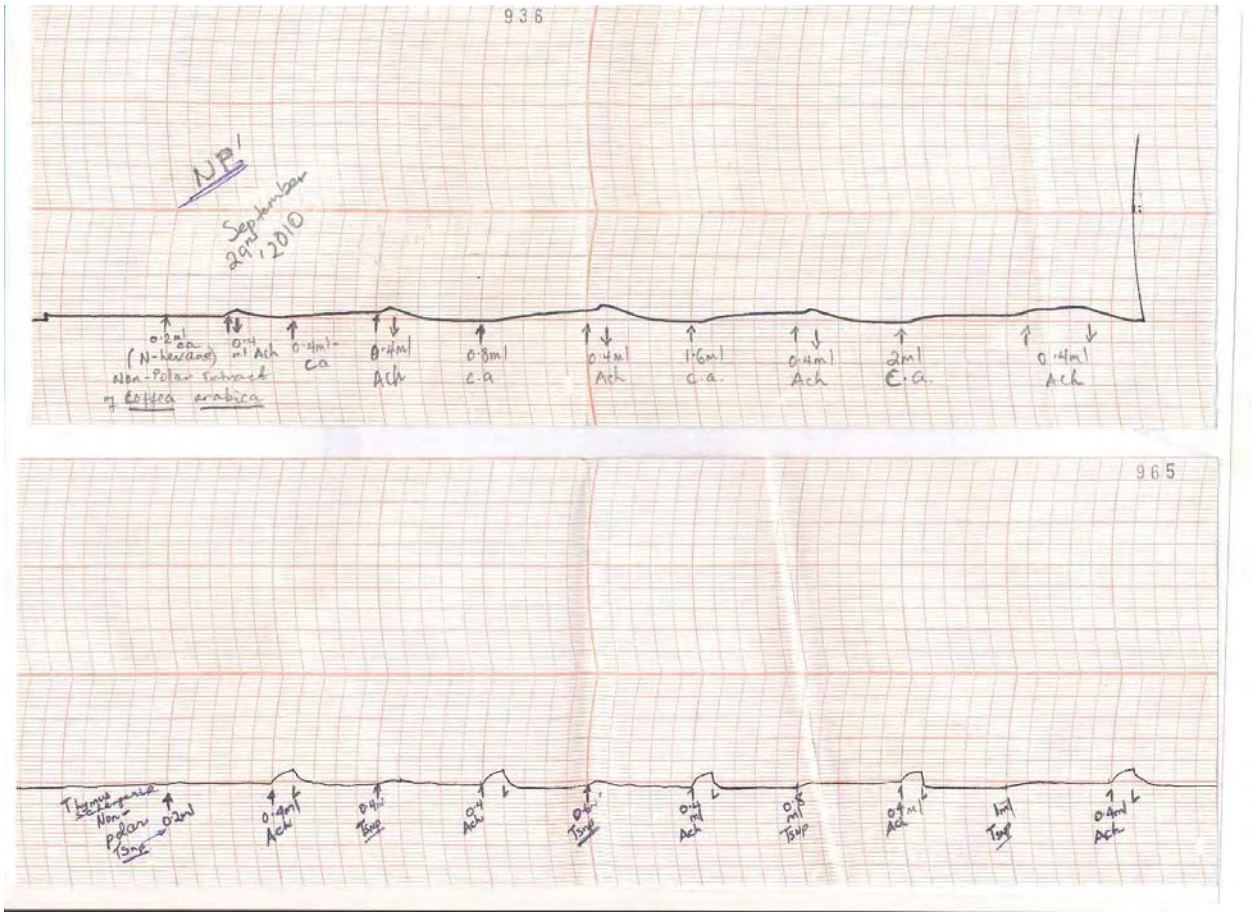

Figure 8: Polygraph trace of n-hexane *Coffea arabica* in the presence of ACh on trachea rings above: Upside arrow indicates administration (introduction) while downside arrow indicates washing (flushing) out of administered compoundsChart # 936. Polygraph trace of n-hexane *Thymus schimperi* in the presence of ACh on trachea rings below: Upside arrow indicates administration (introduction) while downside arrow indicates washing (flushing) out of administered compoundsChart # 965

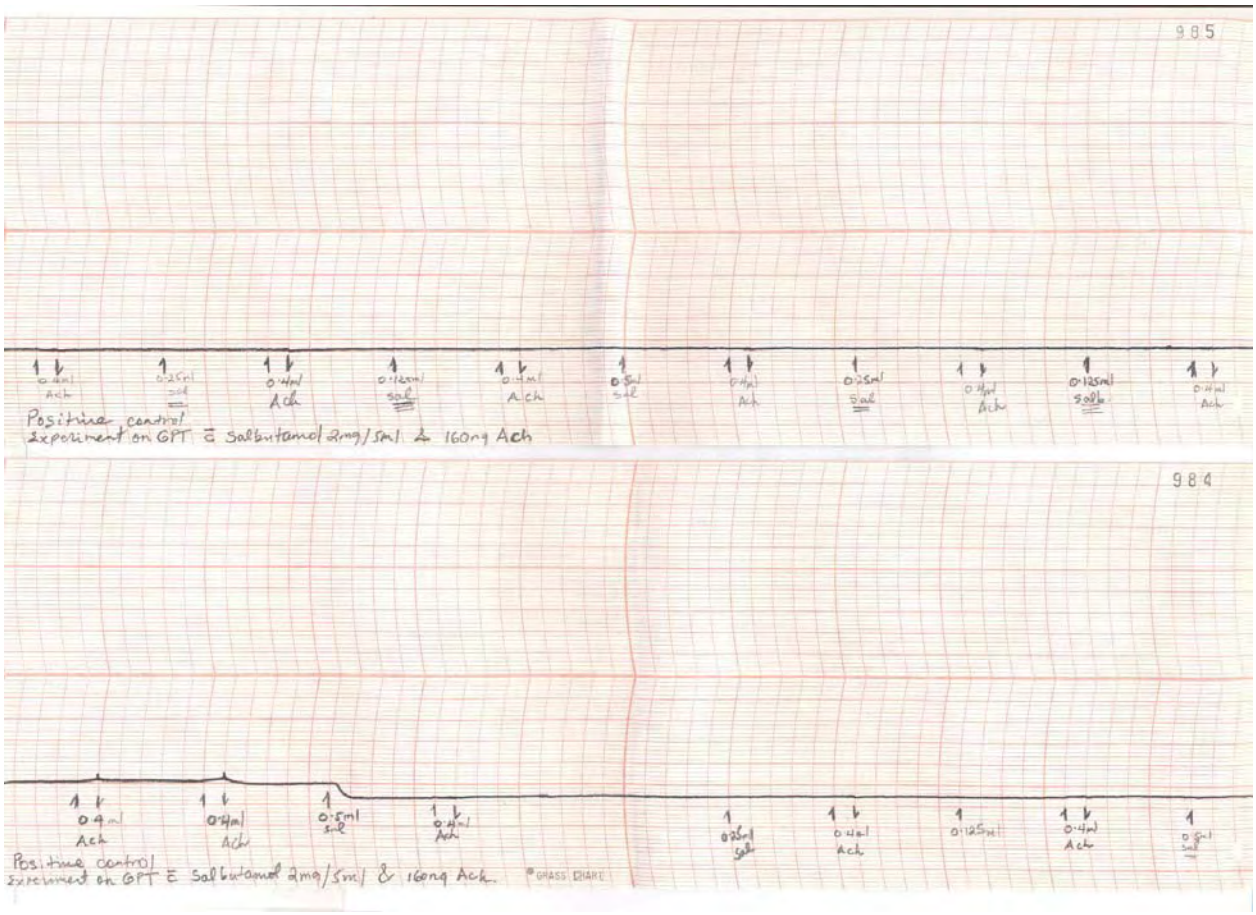

Figure 9: Polygraph traces Salbutamol 2mg/5ml in the presence of ACh (160 ng) on trachea rings showing complete inhibition of contraction (Chart # 985) and indicating SM relaxation and its maintenance in spite the addition of Ach 3mm below baseline(Chart # 984) : Upside arrow indicates administration (introduction) while downside arrow indicates washing (flushing) out of administered compounds Chart # 984-985

5. Discussion

Investigation of the effects hydroalcholic (methanol 80%) and n-hexane crude extract of *Coffeaarabica* L. and *Thymus schimperi* R. on isolated tracheal smooth muscle tissue in the presence of ACh (*invitro*) yielded an overall contractile response. The contractile responses of the plant crude extracts in the absence of ACh were evident on the isolated tissue to variable degrees depending on type of plant, type of plant extract and amount of dosage administered to the tissue. In the presence of the plant extracts (both plants and types of extracts), the contractile response of the isolated tissue when Ach was added had an amplified (additive) contractile effect except for the 50µg dose group of n- hexane crude extract of *Coffeaarabica* L., which showed a relaxant effect of 0.22mm in the presence of Ach and was not of statistical significance. The responses yielded for both plant extracts in the current *in vitro* study conducted were generally not in full agreement to the traditional claim of smooth muscle relaxant effects (Debela *et al*., 1999; Abebe *et al*., 2003).

In the current study, both the hydroalcholic and n-Hexane crude extracts of *Coffea arabica*were administered *in vitro* to the tracheal rings. Hence, the normal metabolic pathway of coffee catabolism, when taken as a beverage via the oral route cannot be considered. The degradation of the methylated xanthine (caffeine) in to paraxanthine, theophylline and theobromine might not have taken place fully, which are all said to be cyclic nucleotide phosphodiesterase inhibitors, which cause SM relaxation (Essayan, 2001; Deree *et al*., 2008).However, of the three major metabolites of caffeine metabolism, theophylline (accounting for only 5 % of the catabolic end products) is the drug used to treat asthma. Therefore, the contraction of the tissue prior to the addition and after the administration of ACh (where contraction was amplified),raises a question whether coffee consumption in general produces bronchodilation. This result is in concert with previous works done by Metha *et al*., (1991) as explained below.

According to a study conducted by Metha *et al*., (1991) the assumption was that xanthine derivatives such as caffeine relax airway smooth muscle (ASM). The study attempted to determine whether caffeine and theophylline relax preterm ASM contracted by acetylcholine and 27 tracheal rings obtained from seven preterm lambs (120-135 day gestation) were studied. The muscle was stretched to the length at which maximum active tension was developed isometrically. The study conducted the administration of multiple doses *in vitro* by usingASM(unlike our current study, which used single dosage method). Theophylline produced a significant decrease ($p<0.001$) in active tension at each dose, whereas caffeine significantly increased ($p< 0.001$) active tension. Addition of caffeine and theophylline to previously uncontracted ASM did not alter tension i.e. the effects were counter active. In contrast to their effect on ASM, the xanthine derivatives caffeine and theophylline had differential effects on prestimulated ASM in preterm lambs. Their findings raisedimportant questions about various aspects of the current therapeutic use of caffeine and theophylline.

The ability of some xanthine derivatives to relax the trachea, contracted by pilocarpine, and to increase the force of contraction of directly stimulated skeletal muscles from the guinea-pig was studied *in vitro*(Jeppsson *et al*., 1982). Relaxation of the trachea occurred at lower concentrations and with a different order of potency as compared with the effects on the slow-contracting soleus muscle (calf or lower leg muscle: skeletal) or on the fast-contracting extensor digitorum longus. One of the compounds, IBMX(1-methyl-3-isobutylxanthine) showed an isoprenaline like effect on the soleus muscle i.e. it depressed the force and fusion of subtetanic contractions. The relaxing effect of theophylline and IBMX on the trachea was additive to that of terbutaline but no clear potentiation was observed. The depression of the contractions of the soleus muscle elicited by terbutaline (β_2-adrenergic receptor agonist) was augmented by IBMX but not by theophylline. Theophylline in concentrations which used alone enhanced the contractions of the soleus muscle inhibited the effect of terbutaline. Again in this study, the investigators concluded that the relative contribution of the various effects of xanthine derivatives differs from compound to compound.

Thence, the general assumption that all methylated xanthines have a smooth muscle relaxant effect could be misleading. The effect of these compounds may be different from one site to anotherdepending on the type of smooth muscle tissue under investigation. In the current study, the hydroalcholic crude extracts of *Coffeaarabica* have demonstrated the opposite to the assumed relaxing effect on airway smooth muscles.

Both the hydroalcholic and n-hexane crude extracts of *Thymusschimperi* R. had an overall contractile response on the tissueboth prior to the addition of ACh and post ACh treatment. The contractile response in the presence of the extracts was amplified when ACh was added i.e. the peak contractile tissue response to Ach alone was doubled in the presence of hydroalcholic crude extracts. These findings, again, are contrary to the traditional claim and other previous work done by investigators.

According to Wienkötter*et al*., (2007), thyme is said to be a bronchospasmolytic and secretomotoric agent. The influence of a thyme extract on β_2-receptors in competition binding experiments and relaxation experiments on rat uteri and trachea has been studied. Furthermore, the influence of the extract as secretomotoric agentwas of interest to the study conducted. Binding experiments were performed using purified rat lung membranes with the β_2-receptor ligand iodocyanopindolol (radio labeled iodine) and fluorescence dye rhodamin 123 for ciliary action in the tracheal area of a rat was investigated using a microdialysis technique. This indicated an evidence for the influence of a thyme extract on β_2-receptors by both bindingstudies and biological effects, thereby acting as an ASM relaxant. Mucociliary clearance improvement was induced*in vivo*, yet the mechanism of action has still to be elucidated (Wienkötter*et al*., 2007).

According to Beer *et al*, (2007) and Beer, (2010), a study on thymol was performed based on prior information on the compound. These prior indications for thyme oil application are/were the antibacterial, the antiphlogistic/anti-inflammatory and the spasmolytic effects. The specific effects of thymol on stomach smooth muscles of a guinea pig were studied. The effect of Thymol from $2x10^{-8}$ to 10^{-6}M induced a clear excitation of the spontaneous contractile activity (SCA) of the smooth-muscle strips (SMS) from guinea pig stomach, which reached about 15% of the maximum contractile activation (10^{-5}M Ach) (Beer *et al*, 2007). The increase of the thymol

concentration led to the inhibition of the excitatory effects. At a concentration of 10^{-4}M thymol, the SCA of SMS was inhibited. These inhibitory processes were said to be manifested more slowly than the excitatory effects registered with the lower thymol doses. The excitatory effects of thymol on the muscle contraction were possible only at lowerdoses and when higher doses were used excitatory effects were completely inhibited by the spasmolytic effect.

Hisayama *et al*. (1986) as cited in Beer, (2010) reported that thymol releases the Ca^{2+}-ions from the intracellular deposits. However, this contravenes the relaxing effect of the muscles. The explanation for this contradiction was through the specific effect of Thymol on the α1- and α2-adrenergic receptors. When lower Thymol doses are used, it is possible to raise the level of the Ca^{2+}-ions minutes after its application in higher doses through activation of Ca^{2+} channels controlled by the receptors.

The study conducted by Beer *et al*, (2007) and Beer, (2010) has shade some light to the current investigation on the tracheal rings and the results produced. The mean dose response curve for the hydroalcholic *Thymusschimperi* crude extract (Fig. 5) does indicate a contractile response at lower doses which tends to plateau as it is increased from 50µg to 200 µg in organ bath concentrations. In addition, the mechanism of contraction may have been a result of calcium release as in the case of the Hisayama *et al*., (1986). Even though hydroalcholic crude extracts were used, *Thymus schimperi*containing 59.3% Thymol (Dagne *et al*., 1998) may have been the cause of tissue contraction in the current study's result.

6. Conclusions

- The hydroalcholic (Methanol 80%) crude extracts of both *Coffeaarabica* L. and *Thymusschimperi* R. induced a contractile response on the tracheal tissue*invitro* which was statistically significant between the dose groups in the presence of ACh.

- The dose dependent contractile response of the tracheal rings in the presence of ACh for both plant crude extracts (Methanol 80%) had a statistically significant difference (P 0.05) as the dose was increased from 50µg to 100µg, 200µg, and 400µg and from 50µg to150µg and 200µg for *Coffeaarabica* and *Thymusschimperi* respectively.

- The n-hexane crude extracts of *Coffeaarabica* and *Thymusschimperi*, even though the results found were not statistically significant, the dose dependent trend of contractile response increment on the tissue could be observed from the dose response curve (Fig.5).

7. Recommendations

- Variable results support and contradict the actions of coffee and thyme as an airway smooth muscle relaxant. The results of the current study were conducted with the crude extracts of the two plants in study. Further*invitro* study by the isolation of the bioactive ingredients of the plants i.e. caffeine and thymol should be conducted to determine a specific response of the airway smooth muscles.

- Retrospective and/or prospective epidemiological studies on the actions of the consumptions of coffee and thyme beverages with respect to their relationship in asthma could also be conducted on human subjects.

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Annexes

Annex 1: Hydroalcholic crude extract effects of*Thymusschimperi* (SPSS Results)

Descriptive

Response

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
50.00	5	2.6000	.54772	.24495	1.9199	3.2801	2.00	3.00
100.00	5	3.3000	.67082	.30000	2.4671	4.1329	2.50	4.00
150.00	5	4.4000	.82158	.36742	3.3799	5.4201	3.00	5.00
200.00	5	4.4000	.96177	.43012	3.2058	5.5942	3.00	5.50
Total	20	3.6750	1.05475	.23585	3.1814	4.1686	2.00	5.50

ANOVA

Response

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.738	3	3.913	6.660	.004
Within Groups	9.400	16	.588		
Total	21.138	19			

Multiple Comparisons

Dependent Variable:Response

	(I) Dose	(J) Dose	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	50.00	100.00	-.70000	.48477	.492	-2.0869	.6869
		150.00	-1.80000*	.48477	.009	-3.1869	-.4131
		200.00	-1.80000*	.48477	.009	-3.1869	-.4131
	100.00	50.00	.70000	.48477	.492	-.6869	2.0869
		150.00	-1.10000	.48477	.147	-2.4869	.2869
		200.00	-1.10000	.48477	.147	-2.4869	.2869
	150.00	50.00	1.80000*	.48477	.009	.4131	3.1869
		100.00	1.10000	.48477	.147	-.2869	2.4869
		200.00	.00000	.48477	1.000	-1.3869	1.3869
	200.00	50.00	1.80000*	.48477	.009	.4131	3.1869
		100.00	1.10000	.48477	.147	-.2869	2.4869
		150.00	.00000	.48477	1.000	-1.3869	1.3869
Scheffe	50.00	100.00	-.70000	.48477	.568	-2.2111	.8111
		150.00	-1.80000*	.48477	.017	-3.3111	-.2889
		200.00	-1.80000*	.48477	.017	-3.3111	-.2889
	100.00	50.00	.70000	.48477	.568	-.8111	2.2111
		150.00	-1.10000	.48477	.204	-2.6111	.4111
		200.00	-1.10000	.48477	.204	-2.6111	.4111
	150.00	50.00	1.80000*	.48477	.017	.2889	3.3111
		100.00	1.10000	.48477	.204	-.4111	2.6111
		200.00	.00000	.48477	1.000	-1.5111	1.5111
	200.00	50.00	1.80000*	.48477	.017	.2889	3.3111
		100.00	1.10000	.48477	.204	-.4111	2.6111
		150.00	.00000	.48477	1.000	-1.5111	1.5111

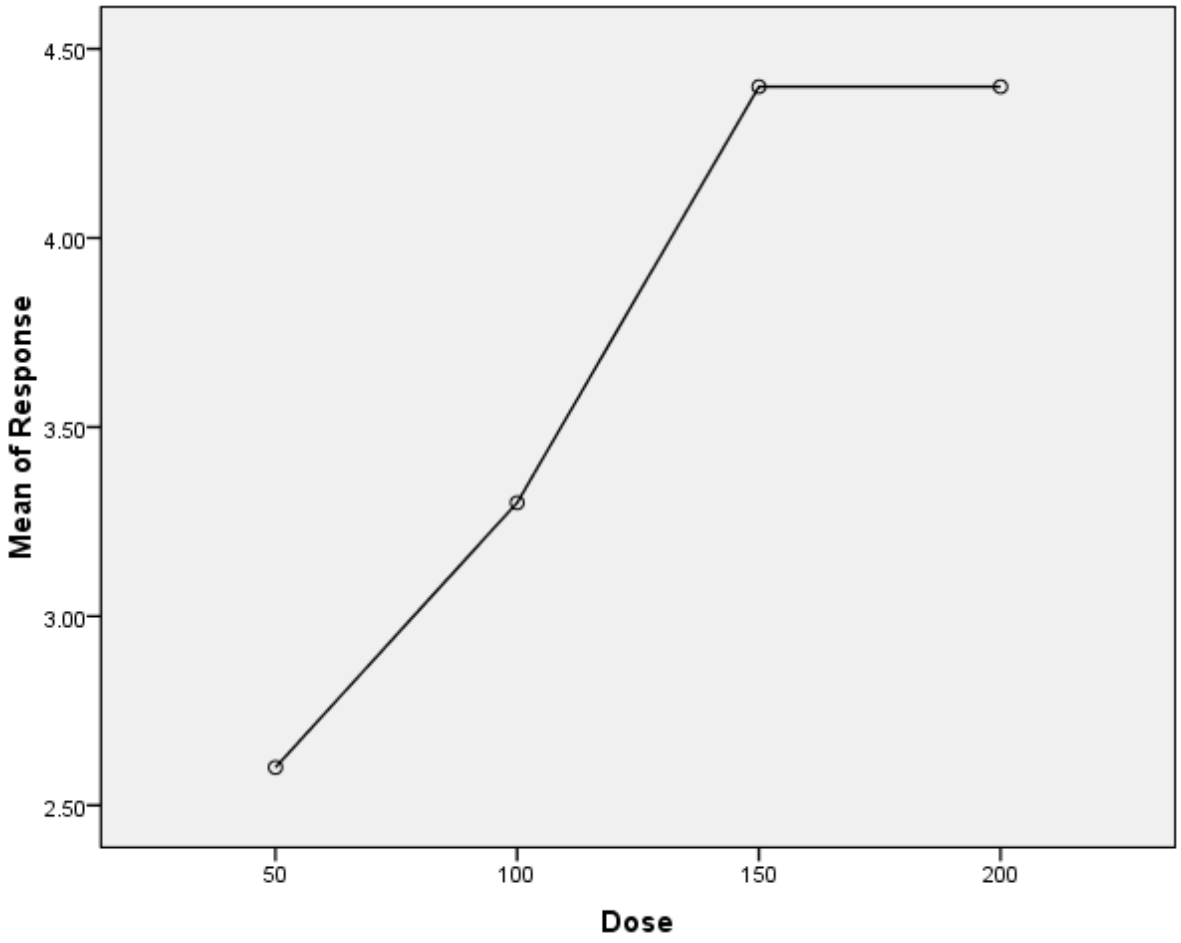
*. The mean difference is significant at the 0.05 level.

Response

	Dose	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	50.00	5	2.6000	
	100.00	5	3.3000	3.3000
	150.00	5		4.4000
	200.00	5		4.4000
	Sig.		.492	.147
Scheffe[a]	50.00	5	2.6000	
	100.00	5	3.3000	3.3000
	150.00	5		4.4000
	200.00	5		4.4000
	Sig.		.568	.204

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.



Control ACh= 10µg/ml preparation with a final organ bath (25ml) concentration of 160ng/ml. Constriction responses are expressed as a percentage of the initial contraction induced by acetylcholine in the absence of plant extracts (160ng/ml: Peak contraction of 1.75mm taken as 100% contractile response). Results of constriction are reported as mean ± SEM of the 5 isolated tracheal rings. Comparative significance to control is taken as P-value <0.05. Doses are expressed in µg/ml and contraction in millimeters.

Annex 2: Hydroalcholic crude extract effects of*Coffeaarabica*on tracheal rings (SPSSResults)

Descriptive

Response

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
50.00	5	1.8000	.44721	.20000	1.2447	2.3553	1.50	2.50
100.00	5	3.2000	.75829	.33912	2.2585	4.1415	2.00	4.00
200.00	5	3.4600	.45607	.20396	2.8937	4.0263	3.00	4.00
400.00	5	3.4600	.45607	.20396	2.8937	4.0263	3.00	4.00
Total	20	2.9800	.86669	.19380	2.5744	3.3856	1.50	4.00

ANOVA

Response

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.508	3	3.169	10.644	.000
Within Groups	4.764	16	.298		
Total	14.272	19			

Multiple Comparisons

Dependent Variable:Response

	(I) Dose	(J) Dose	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	50.00	100.00	-1.40000*	.34511	.005	-2.3874	-.4126
		200.00	-1.66000*	.34511	.001	-2.6474	-.6726
		400.00	-1.66000*	.34511	.001	-2.6474	-.6726
	100.00	50.00	1.40000*	.34511	.005	.4126	2.3874
		200.00	-.26000	.34511	.874	-1.2474	.7274
		400.00	-.26000	.34511	.874	-1.2474	.7274
	200.00	50.00	1.66000*	.34511	.001	.6726	2.6474
		100.00	.26000	.34511	.874	-.7274	1.2474
		400.00	.00000	.34511	1.000	-.9874	.9874
	400.00	50.00	1.66000*	.34511	.001	.6726	2.6474
		100.00	.26000	.34511	.874	-.7274	1.2474
		200.00	.00000	.34511	1.000	-.9874	.9874
Scheffe	50.00	100.00	-1.40000*	.34511	.009	-2.4758	-.3242
		200.00	-1.66000*	.34511	.002	-2.7358	-.5842
		400.00	-1.66000*	.34511	.002	-2.7358	-.5842
	100.00	50.00	1.40000*	.34511	.009	.3242	2.4758
		200.00	-.26000	.34511	.902	-1.3358	.8158
		400.00	-.26000	.34511	.902	-1.3358	.8158
	200.00	50.00	1.66000*	.34511	.002	.5842	2.7358
		100.00	.26000	.34511	.902	-.8158	1.3358
		400.00	.00000	.34511	1.000	-1.0758	1.0758
	400.00	50.00	1.66000*	.34511	.002	.5842	2.7358
		100.00	.26000	.34511	.902	-.8158	1.3358
		200.00	.00000	.34511	1.000	-1.0758	1.0758

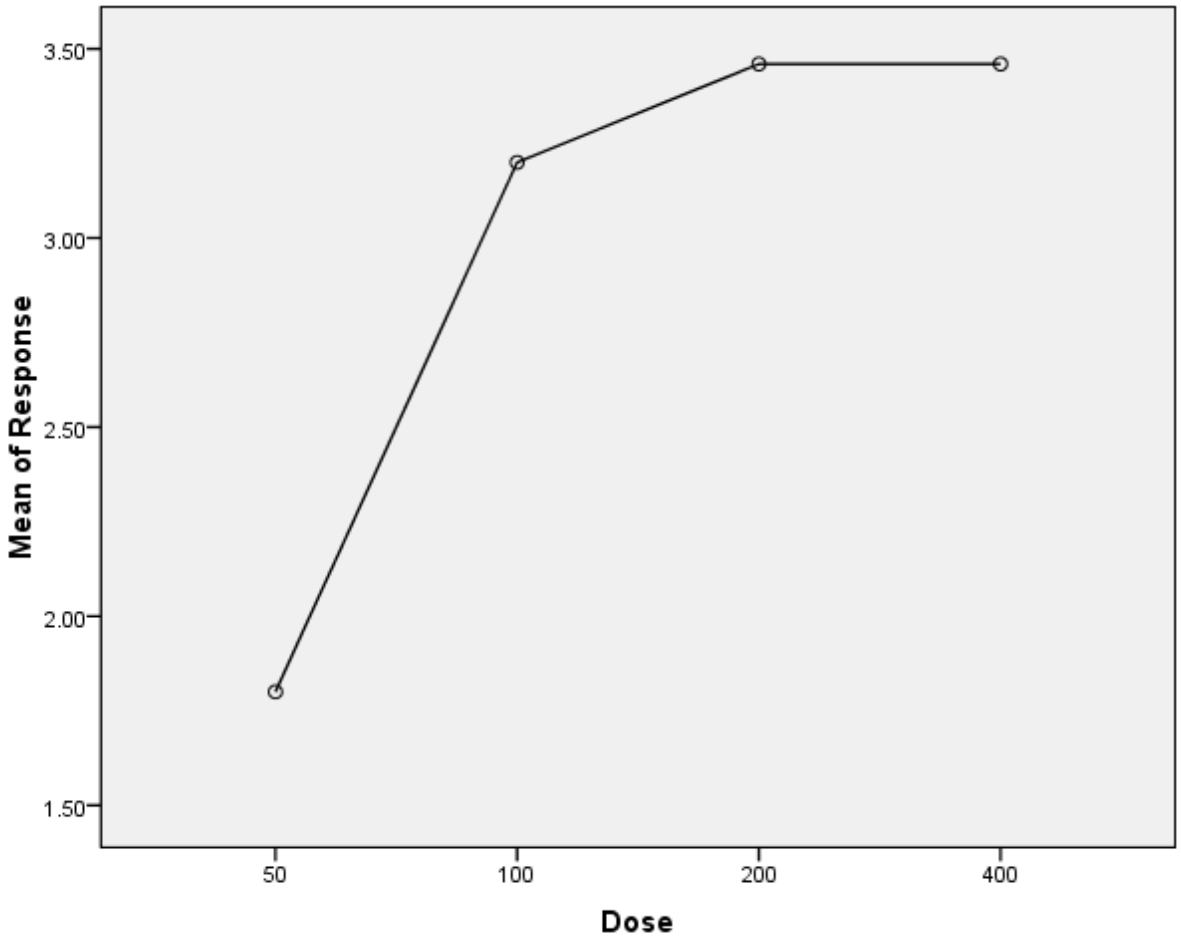
*. The mean difference is significant at the 0.05 level.

Response

	Dose	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	50.00	5	1.8000	
	100.00	5		3.2000
	200.00	5		3.4600
	400.00	5		3.4600
	Sig.		1.000	.874
Scheffe[a]	50.00	5	1.8000	
	100.00	5		3.2000
	200.00	5		3.4600
	400.00	5		3.4600
	Sig.		1.000	.902

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.



Control ACh= 10µg/ml preparation with a final organ bath concentration of 160ng/ml. Constriction responses are expressed as a percentage of the initial contraction induced by acetylcholine in the absence of plant extracts (160ng/ml: Peak contraction of 1.75mm taken as 100% contractile response). Results of constriction are reported as mean ± SEM of the 5 isolated tracheal rings. Comparative significance to control is taken as P-value <0.05. Doses are expressed in µg/ml and contraction in millimeters.

Annex 3: n-Hexane crude extract effects of *Coffea arabica* on tracheal rings (SPSS Result)

Descriptive

response

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
50.00	3	1.5333	.80829	.46667	-.4746	3.5412	.60	2.00
100.00	3	1.8333	.90738	.52387	-.4207	4.0874	.80	2.50
200.00	3	2.2333	1.46401	.84525	-1.4035	5.8701	.90	3.80
400.00	3	2.3333	1.44338	.83333	-1.2522	5.9189	1.50	4.00
Total	12	1.9833	1.07182	.30941	1.3023	2.6643	.60	4.00

ANOVA

response

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.230	3	.410	.288	.833
Within Groups	11.407	8	1.426		
Total	12.637	11			

Multiple Comparisons

Dependent Variable:Response

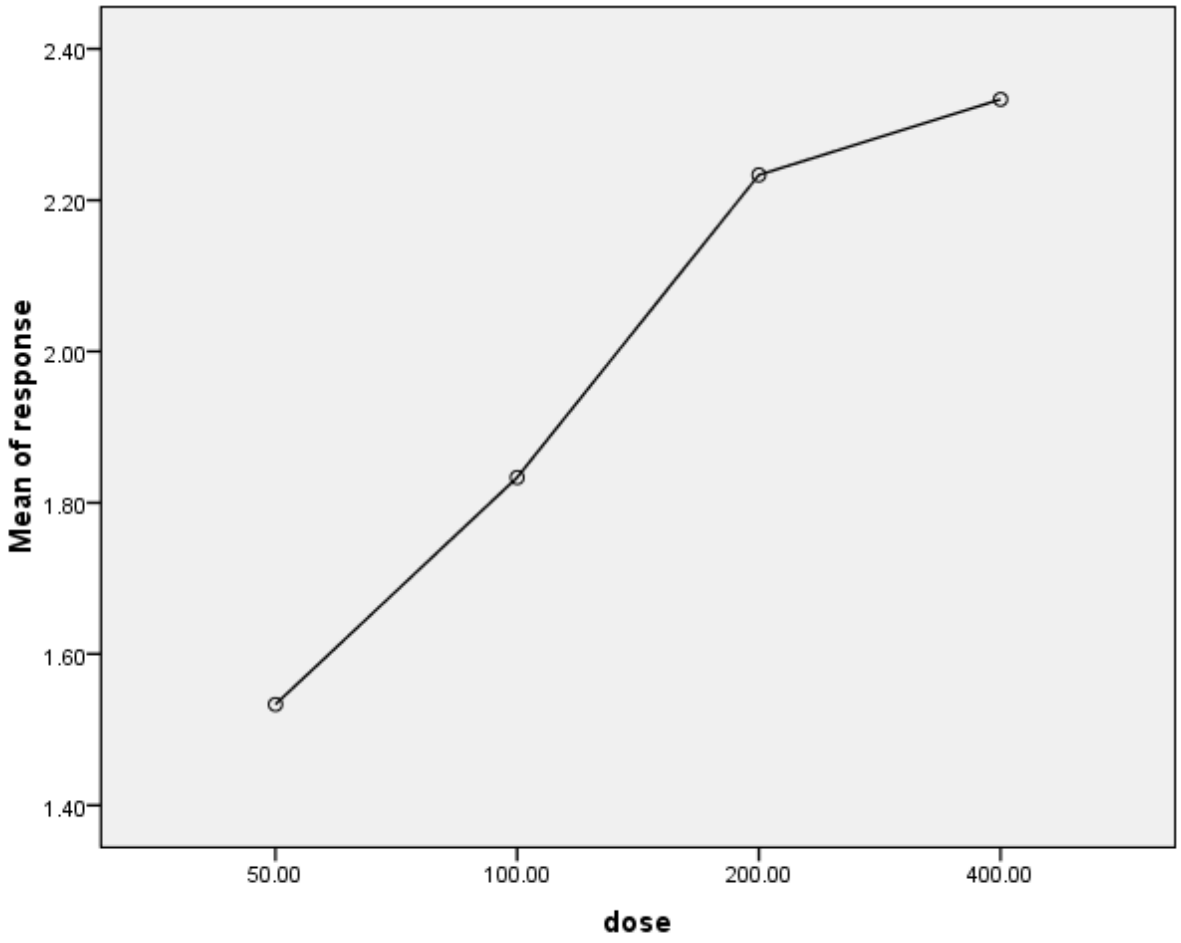
			Mean Difference			95% Confidence Interval	
	(I) dose	(J) dose	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Tukey HSD	50.00	100.00	-.30000	.97496	.989	-3.4222	2.8222
		200.00	-.70000	.97496	.887	-3.8222	2.4222
		400.00	-.80000	.97496	.843	-3.9222	2.3222
	100.00	50.00	.30000	.97496	.989	-2.8222	3.4222
		200.00	-.40000	.97496	.975	-3.5222	2.7222
		400.00	-.50000	.97496	.954	-3.6222	2.6222
	200.00	50.00	.70000	.97496	.887	-2.4222	3.8222
		100.00	.40000	.97496	.975	-2.7222	3.5222
		400.00	-.10000	.97496	1.000	-3.2222	3.0222
	400.00	50.00	.80000	.97496	.843	-2.3222	3.9222
		100.00	.50000	.97496	.954	-2.6222	3.6222
		200.00	.10000	.97496	1.000	-3.0222	3.2222
Scheffe	50.00	100.00	-.30000	.97496	.992	-3.7052	3.1052
		200.00	-.70000	.97496	.912	-4.1052	2.7052
		400.00	-.80000	.97496	.877	-4.2052	2.6052
	100.00	50.00	.30000	.97496	.992	-3.1052	3.7052
		200.00	-.40000	.97496	.981	-3.8052	3.0052
		400.00	-.50000	.97496	.965	-3.9052	2.9052
	200.00	50.00	.70000	.97496	.912	-2.7052	4.1052
		100.00	.40000	.97496	.981	-3.0052	3.8052
		400.00	-.10000	.97496	1.000	-3.5052	3.3052
	400.00	50.00	.80000	.97496	.877	-2.6052	4.2052
		100.00	.50000	.97496	.965	-2.9052	3.9052
		200.00	.10000	.97496	1.000	-3.3052	3.5052

response

	dose	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	50.00	3	1.5333
	100.00	3	1.8333
	200.00	3	2.2333
	400.00	3	2.3333
	Sig.		.843
Scheffe[a]	50.00	3	1.5333
	100.00	3	1.8333
	200.00	3	2.2333
	400.00	3	2.3333
	Sig.		.877

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.



Control ACh= 10µg/ml preparation with a final organ bath concentration of 160ng/ml. Constriction responses are expressed as a percentage of the initial contraction induced by acetylcholine in the absence of plant extracts (160ng/ml: Peak contraction of 1.75mm taken as 100% contractile response). Results of constriction are reported as mean ± SEM of the 5 isolated tracheal rings. Comparative significance to control is taken as P-value <0.05. Doses are expressed in µg/ml and contraction in millimeters.

Annex 4: n-Hexane crude extract effects of *Thymusschimperi*on tracheal rings (SPSS Results)

Descriptive

response

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
50.00	3	2.0000	.50000	.28868	.7579	3.2421	1.50	2.50
100.00	3	2.5000	.50000	.28868	1.2579	3.7421	2.00	3.00
150.00	3	3.0000	.00000	.00000	3.0000	3.0000	3.00	3.00
200.00	3	3.1667	.57735	.33333	1.7324	4.6009	2.50	3.50
Total	12	2.6667	.61546	.17767	2.2756	3.0577	1.50	3.50

ANOVA

response

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.500	3	.833	4.000	.052
Within Groups	1.667	8	.208		
Total	4.167	11			

Multiple Comparisons

Dependent Variable :Response

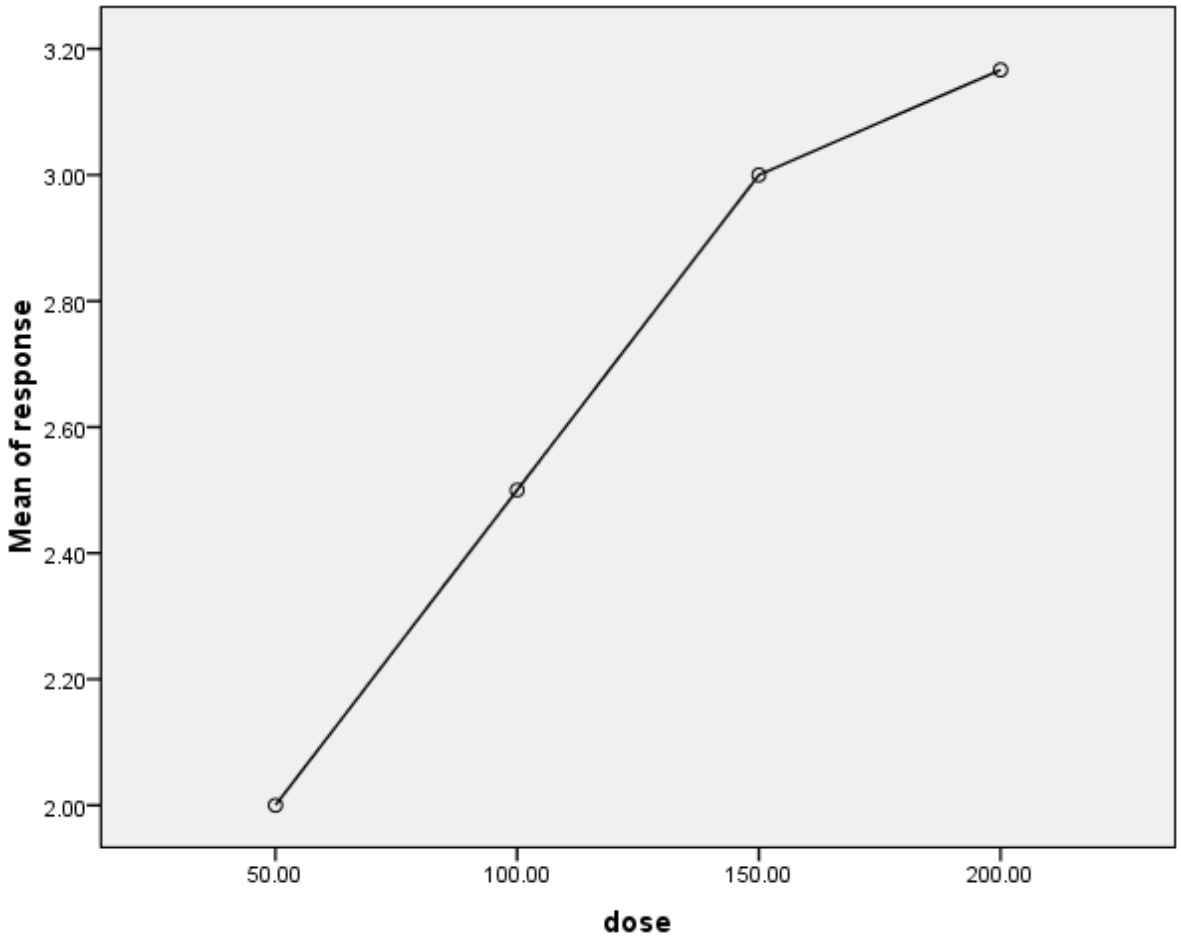
			Mean Difference			95% Confidence Interval	
	(I) dose	(J) dose	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Tukey HSD	50.00	100.00	-.50000	.37268	.565	-1.6934	.6934
		150.00	-1.00000	.37268	.104	-2.1934	.1934
		200.00	-1.16667	.37268	.055	-2.3601	.0268
	100.00	50.00	.50000	.37268	.565	-.6934	1.6934
		150.00	-.50000	.37268	.565	-1.6934	.6934
		200.00	-.66667	.37268	.344	-1.8601	.5268
	150.00	50.00	1.00000	.37268	.104	-.1934	2.1934
		100.00	.50000	.37268	.565	-.6934	1.6934
		200.00	-.16667	.37268	.968	-1.3601	1.0268
	200.00	50.00	1.16667	.37268	.055	-.0268	2.3601
		100.00	.66667	.37268	.344	-.5268	1.8601
		150.00	.16667	.37268	.968	-1.0268	1.3601
Scheffe	50.00	100.00	-.50000	.37268	.633	-1.8016	.8016
		150.00	-1.00000	.37268	.143	-2.3016	.3016
		200.00	-1.16667	.37268	.080	-2.4683	.1350
	100.00	50.00	.50000	.37268	.633	-.8016	1.8016
		150.00	-.50000	.37268	.633	-1.8016	.8016
		200.00	-.66667	.37268	.416	-1.9683	.6350
	150.00	50.00	1.00000	.37268	.143	-.3016	2.3016
		100.00	.50000	.37268	.633	-.8016	1.8016
		200.00	-.16667	.37268	.976	-1.4683	1.1350
	200.00	50.00	1.16667	.37268	.080	-.1350	2.4683
		100.00	.66667	.37268	.416	-.6350	1.9683
		150.00	.16667	.37268	.976	-1.1350	1.4683

response

	dose	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	50.00	3	2.0000
	100.00	3	2.5000
	150.00	3	3.0000
	200.00	3	3.1667
	Sig.		.055
Scheffe[a]	50.00	3	2.0000
	100.00	3	2.5000
	150.00	3	3.0000
	200.00	3	3.1667
	Sig.		.080

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.



Control ACh= 10µg/ml preparation with a final organ bath concentration of 160ng/ml. Constriction responses are expressed as a percentage of the initial contraction induced by acetylcholine in the absence of plant extracts (160ng/ml: Peak contraction of 1.75mm taken as 100% contractile response). Results of constriction are reported as mean ± SEM of the 5 isolated tracheal rings. Comparative significance to control is taken as P-value <0.05. Doses are expressed in µg/ml and contraction in millimeters.