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The effects of crude extracts and fractions of *Alchemilla abyssinica* on smooth muscle of Guinea pig ileum

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Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in any other University and that all sources of materials used for the thesis have been duly acknowledged.

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List of Abbreviations

ACh – Acetycholine

ALIP – Aklilu Lemma Institute of Pathobiology

ANOVA – One way analysis of Variance

ATP – Adenosine triphosphate

BMNP – Bale Mountains National Park

CHCl\textsubscript{3} – Chloroform

Conc. – Concentration

Dist. – Distilled

DMSO – Dimethyl Sulfoide

EHNRI – Ethiopian Health and Nutrition Research Institute

EtOAC – Ethyl acetate

g/l – gram per liter

GPCR – G-protein coupled receptor

GPI – Guinea pig ileum

Hst. – Histamine

LC – Light chain
MeOH – Methanol

MLCK – Myosine light chain kinase

MLCP – Myosine light chain phosphatase

NAd – Noradrenaline

NMR – Nuclear magnetic resonance

NO – Nitric oxide

SEM – Standard error of the mean

SPSS – Statistical package for Social Sciences

TLC – Thin layer chromatography

VIP – Vasoactive intestinal peptide

μg/ml – microgram per milliliter

ηg/ml – nanogram per milliliter
Abstract

*Alchemilla abyssinica* is a plant widely used in traditional medicine. Its wide use among the community plus already established scientific evidences for medicinal values of other *Alchemilla* species provided good ground for this investigation. In this research, CHCl$_3$/EtOAC 1:1 extract of dried aerial parts of *Alchemilla abyssinica*, methanolic extract of the CHCl$_3$/EtOAC residue and fractions of the methanolic extract were tested on isolated guinea pig ileum (GPI) for possible presence of spasmogenic (contractile) or spasmolytic (relaxant) effects. Concentrations of each extract and fraction ranging from 20-600 µg/ml final organ bath concentration were tested. The effects of these test samples on the basal rhythmic contractions of the GPI as well as on its contraction elicited using the agonist, histamine, were determined. The antagonist, Papavarine, was also used as a control smooth muscle relaxant. While the CHCl$_3$/EtOAC 1:1 extract showed neither spasmogenic nor spasmolytic result, the methanolic extract showed marked spasmolytic effect. This methanolic extract was fractionated using column chromatography and the fraction eluted using Hexane/EtOAc 1:2 gave greatest spasmolytic result and it was taken as the final test fraction. The final test fraction produced significant (P<0.05) dose-dependent spasmolytic effects on the agonist induced contractions of the GPI to 95.7% at 20 µg/ml, 43.6% at 70 µg/ml and 14.2% at 120 µg/ml in the organ bath. In conclusion, the results of the present study showed that *Alchemilla abyssinica* possesses spasmolytic property. Also, the result of the present oral acute toxicity study showed *Alchemilla abyssinica* exhibited no toxicity up to doses of 1,000 mg/kg body weight in Swiss albino mice.

*Key words*: *Alchemilla abyssinica*, spasmolytic, smooth muscle (guinea pig ileum)
1. INTRODUCTION

1.1 Traditional medicine

Traditional medicine is the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures. Whether explicable or not, it is used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses (WHO, 2000). According to another description, herbalism is a traditional medicinal or folk medicine practice based on the use of plants and plant extracts. It is also known as botanical medicine, herbal medicine and phytotherapy (Manigaunha et al., 2010). Recorded use of natural products for medicinal purpose dates back to at least 4,000 years (Cortés et al., 1998).

During the last decade, use of traditional medicine has expanded globally and has gained popularity. It has not only continued to be used for primary health care of the poor in developing countries, but has also been used in countries where conventional medicine is predominant in the national health care system (WHO, 2000). Even today, many societies rely partly on herbal remedies. In the past 25 years, a return to former practices has gained attention in many countries (Cortés et al., 1998).

Looking back in time, modern medicine has benefited a lot from traditional medicine in that the latter had provided key leads emanating from folkloric uses of medicinal plants. A large array of modern pharmaceutical agents have been derived from such leads, which were eventually traced back to traditional uses of medicinal plants. Consequently, substances such as the antimalarial
quinine, the decongestant pseudoephedrine, the pain killer codeine, just to name a few, were discovered as a result of ethnomedical information obtained from traditional uses of plants, which are the natural "manufacturing houses" of these drugs. Plants continue to play a major role in providing prototype molecules for possible development into conventional drugs by the pharmaceutical industry (Fekadu, 2007).

Considering the current status of traditional medicine's contribution for the modern medicine as well, out of the top 150 prescribed drugs in the U.S. in 1993, 57% contained at least one major active compound that was derived, directly or indirectly, from biological sources (Griffo and Rosenthal, 1997). It is also estimated that three quarters of the plants that provided active ingredients for prescription drugs came to the attention of researchers because of their use in traditional medicine. In addition out of the 120 active compounds currently isolated from the higher plants and widely used in medicine today, 74% show a positive correlation between their modern therapeutic use and the traditional use of the plant from which they were derived (Nijar, 2010).

Traditional knowledge's role in increasing the efficiency of screening plants for medicinal properties is often highlighted, with various calculations – that it increases the efficiency by more than 400% or that it enhances the probability of drug development at the lead discovery stage by as much as 0.5 or a 50% of success. Shaman Pharmaceuticals of the U.S. calculates its rate of scoring a marketable hit as one in every two plants studied with the use of traditional knowledge. The comparable success rate for random bioprospecting in plants, animals, and
microorganisms is one in 10,000 compounds. The current value of the world market for medicinal plants derived from such leads is estimated at US $43 billion (Nijar, 2010).

This is, however, not an easy task as the development of new drugs is a multi-discipline research activity that requires close cooperation between academic investigators, the pharmaceutical industry and regulatory authorities. Since 10 to 15 years of investment is often required before a product reaches the market, and only one out of 8,000 substances initially tested in animals results in a marketable drug. This activity is both time-consuming and expensive (Feagan and Archambault, 2005).

According to WHO traditional medicine has been shown to have intrinsic utility and hence it should be promoted and its potential developed for the wider use and benefit of mankind. It needs to be evaluated, given due recognition and developed so as to improve its efficacy, safety, availability, and wider application at low cost. It is already the people’s own health care system and is well accepted by them. It has certain advantages over imported systems of medicine in any setting because as an integral part of the people’s culture, it is particularly effective in solving certain cultural health problems. It can and does freely contribute to scientific and universal medicine. Its recognition, promotion, and development would secure due respect for a people’s culture and heritage (WHO, 1978). However, despite such a great contribution of the herbal medicine it has been estimated that less than 10% of the large diversity of 250,000-500,000 plant species on the earth have been studied chemically and pharmacologically for their medicinal properties (Manigaunha et al., 2010).
In an attempt to be more specific regarding the site of this investigation on *Alchemilla abyssinica*, Africa is a continent with the oldest known human habitation, unique floras and peoples with diverse culture and religion. Among the plant resources of the continent about 5,000 species are known to have medicinal value (Asfaw and Demissew, 1998). And, no country in Africa enjoys as great a diversification of geology, land forms, soils, and climate as Ethiopia does. There are more than forty five vegetation types where forests, savannas, woodlands, steppes and grasslands comprise 75% of the vegetation cover (Amare, 1976). As a result, the use of herbal medicine in the country has both long history and wide application.

A cursory look at the history of the use of traditional medicine (especially of medicinal plants) in Ethiopia reveals that such use dates back to the time of the Axumite kingdom, if not to earlier periods. Many manuscripts attesting to this fact, and which are now in the custody of the Ethiopian National Traditional Medicine Preparation and Therapy Association, have been recovered. They mention, among other traditional practices, that a large number of medicinal plants were used. The manuscripts claim that during the era of the Axumite kingdom (7th -11th century A.D.), about 8,000 plants were used as medicinal agents. The following eras passed through applicability of the medicinal plants to various degrees and presently, there are anywhere between 650 and 1,000 medicinal plants in Ethiopia (Fekadu, 2007).

Approximately similar figure is estimated by the Ethiopian Institute of Biodiversity Conservation and Research as they state; Medicinal plants in Ethiopia comprises more than 800 species of flowering plants (IBCR, 2001). Considering the diversity of modalities, traditional medicine in Ethiopia includes medicinal preparations from plant, animal, and mineral substances, as well as
spiritual healing, traditional midwifery, hydrotherapy, massage, cupping, counter-irritation, surgery, and bonesetting (WHO, 2001).

The use of medicinal plants in the country has been compiled by many contemporary authors (Pankhurst, 1965; Kloos, 1976, Abebe, 1986; Abate, 1989). Worth mentioning is the compilation and documentation of different medicinal plant species with their vernacular and scientific names by Gelahun Abate written in the local language, Amharic (Abate 1989). The medicinal plants of northern Ethiopia are presented by Abebe and Ayehu (1993). A recent book on Ethiopian plants with some of their chemical identifications has also been published (Abebe et al., 2003). The eight volumes of The Flora of Ethiopia, remain the most authoritative accounts of the plants in the nation. Aromatic plants of Ethiopia, is another most recent book that narrates many plants, their synonyms, vernacular names, common English names, description of each, ecology and distribution, uses and essential oils extracted from them (Asfaw and Demissew, 2009).

Over the last decade many of the plants documented for one ailment or another have come to the attention of Ethiopian researchers and their medicinal use has been tested. Worth mentioning are reports by Abegaz and Dagne (1978) on traditional anthelmintic plants, Dagne et al. (1990) on a widely used antipyretic plant *Taverniera abyssinica* and Desta (1995) who documented the antifertility properties of diverse plant species. Among others the work done on *Moringa stenopetala* an herbaceous plant widely used for both food and medicinal use in southern parts of Ethiopia has demonstrated the country’s rich plant resources (Mekonnen and Gessesse, 1998; Mekonnen 1999, Mekonnen and Draeger 2003).
1.2 *Alchemilla abyssinica*

The genus *Alchemilla*, Family Rosaceae is an herbaceous perennial plant widely distributed in cool temperate regions and on high mountains of the tropics. There are about 300 species of *Alchemilla* known to date. *Alchemilla abyssinica* Fresen. (Local name in Oromiffa - Hindrif / Endrif) is a robust herb, see Plate – 1, with decorated basal that grows in moist montane forests as well as on moist places in somewhat overgrazed moorland and on rocky slopes; 2,500-4,400 meters above sea level. This species has been found to grow in Tigray upland, Wollo upland, Gondar, Gojam, Shewa upland, Balle, Harar, Sidama and Kenya (Hedberg and Edwards, 1989; Dagne, 2009). Regarding the medicinal value of the plant the leaves of *Alchemilla abyssinica* are crushed and tied on to open wounds promoting blood clotting and facilitating wound healing (Gashaw, 1991).

Plate 1 – Pictures of *Alchemilla abyssinica*

A – Taken from Balle mountains.
B – Dried specimen just before grinding.
1.3 The smooth muscles

1.3.1 General description

Smooth muscles are responsible for the contractility of the vascular system, respiratory system, gastrointestinal system and the genitourinary systems (Kim et al., 2008). They are composed of fibers usually 1 to 5 micrometers in diameter and only 20 to 500 micrometers in length (Guyton and Hall, 2006).

The smooth muscles unlike the skeletal or the cardiac, lack cross striation and hence their name, smooth. There is considerable variation in the structure and function of smooth muscle in different parts of the body. In general, smooth muscle can be divided into visceral, or unitary smooth muscle and multiunit smooth muscle. Visceral smooth muscle occurs in large sheets, has many low-resistance gap junction bridges between individual muscle cells, and functions in a syncytial fashion. Visceral smooth muscle is found primarily in the walls of hollow viscera. The musculature of the intestine, the uterus, and the ureters are examples. Multiunit smooth muscle is made up of individual units without interconnecting bridges. It is found in structures such as the iris of the eye, in which fine, graded contractions occur. It is not under voluntary control, but it has many functional similarities to skeletal muscle. Each multiunit smooth muscle cell has *en passant* endings of nerve fibers but in visceral smooth muscle there are *en passant* junctions on fewer cells, with excitation spreading to other cells by gap junctions. In addition, these cells respond to hormones and other circulating substances (Ganong, 2005).
1.3.2 Mechanism of action

The smooth muscles have dense bodies which serve like the Z disks of skeletal muscles, exhibit slow cycling of the myosine cross bridges, have low energy requirement to sustain contraction, have slow onset of contraction and relaxation, have higher force of muscle contraction, and exhibit stress relaxation which may be mentioned as their typical features (Guyton and Hall, 2006). Contraction of smooth muscle is regulated by the cytosolic Ca\(^{2+}\) level ([Ca\(^{2+}\)], see (Figures 1 & 2), and the sensitivity to Ca\(^{2+}\) of the contractile elements in response to changes in the environment surrounding the cell (Karaki et al., 1997).

Upon stimulation by an agonist cytosolic Ca\(^{2+}\) concentration rises, partly as a result of extracellular Ca\(^{2+}\), and partly following the mobilization of internal Ca\(^{2+}\) stores (Adebiyi et al., 2004). Smooth muscle contains myosin light chain kinase (MLCK), activated by Ca\(^{2+}\)-calmodulin, the enzyme which transfers the terminal phosphate group of ATP to serine and/or threonine hydroxyl groups of phosphorylatable light chain (LC) according to the following reaction:

\[
\text{LC-OH} + \text{MgATP}^{2-} \rightarrow \text{LC-O-PO}_{3}^{2-} + \text{MgADP}^- + \text{H}^+
\]

Dephosphorylation is brought about by smooth muscle myosin light chain phosphatase (MLCP) according to the following reaction:

\[
\text{LC-O-PO}_{3}^{2-} + \text{H}_2\text{O} \rightarrow \text{LC-OH} + \text{HPO}_4^{2-}
\]
Figure 1.a – Description of the role of Calcium in smooth muscle contraction (Adopted from Silverthorn, 2004).
While phosphorylation of Ser19 on the 20-kDa regulatory light chain of myosin II (MLC20) by Ca$^{2+}$/calmodulin-dependent myosin light-chain kinase (MLCK) is essential for initiation of smooth muscle contraction it is also worth bearing in mind that there is participation of a Ca$^{2+}$-independent MLCK (Murthy, 2006). Moreover, it has been established that the concept that smooth muscle ‘excitation–contraction coupling’ consists of far more than a simple calcium switch. The importance of pathways that regulate plasticity of the cytoskeleton and those that regulate the availability of actin to interact with myosin is only now becoming clear. Similarly,
the relative importance of the mix of pathways that regulate myosin phosphorylation levels in differentiated smooth muscle tissues is just now playing out (Kim et al., 2008).

Figure 2 – Overview of the signals that regulate the activity of the smooth muscle contractile machinery (Adopted from Puetz et al., 2009).

Still another diagrammatic description by Webb (2003) brings in to attention the roles of both voltage-gated and receptor-operated Ca^{2+} channels plus the Rho system in the contraction as well as the various channels that take part in the relaxation of smooth muscles, see (Figures 3.a & 3.b).
Figure 3.a – Regulation of smooth muscle contraction.
(Adopted from Webb, 2003).
1.3.3 Control of smooth muscle activity

Unlike skeletal muscle fibers which are stimulated exclusively by the nervous system, smooth muscle can be stimulated to contract by multiple types of signals: by nervous signals, by hormonal stimulation, by stretch of the muscle, and in several other ways. The principal reason for the difference is that the smooth muscle membrane contains many types of receptor proteins that can initiate the contractile process. Still other receptor proteins inhibit smooth muscle contraction, which is another difference from skeletal muscle (Guyton and Hall, 2006).
The autonomic nervous system (ANS), that controls the activity of the smooth muscles, is largely autonomous (independent) in that its activities are not under direct conscious control. Both endocrine and nervous systems use chemicals for the transmission of information. Chemical transmission in the case of nervous system takes place through the release of small amounts of transmitter substances from the nerve terminals into the synaptic cleft. The transmitter crosses the cleft by diffusion and activates or inhibits the postsynaptic cell by binding to a specialized receptor molecule (Katzung, 1995).

The Cambridge physiologist John Newport Langley (1852–1925) first showed that suprarenal extract (adrenaline) contracts and relaxes different smooth muscles, as does stimulation of their sympathetic nerve supply. Then, his student, Elliott, determined that adrenaline most likely acts at the junction between nerves and smooth muscle cells, not on nerve terminals, and made the audacious suggestion that “adrenaline might then be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery”. Langley subsequently developed the concept that a “receptive substance” exists for alkaloids such as nicotine and curare at the junction between motor–nerve and muscle. Thus was developed the concept of transmitter release, the idea of receptors and identification of the first transmitter substance (Bennett, 2004).

Later Dale concluded his classical research period in this area with a comment that was to erect a paradigm that is still taught to this day. To put it as it is “We can then say that postganglionic parasympathetic fibers are predominantly, and perhaps entirely ‘cholinergic’ and that postganglionic sympathetic fibers are predominantly, though not entirely, ‘adrenergic’, while
some, and probably all of the preganglionic fibers of the whole autonomic system are cholinergic.” (Ibid, 2004).

The concept of specific receptors that bind drugs or transmitter substances onto the cell, thereby either initiating biological effects or inhibiting cellular functions, is today a cornerstone of pharmacological research and pharmaceutical development. Yet, while the basic ideas of this concept were first explicitly formulated in 1905 by Langley, drug receptors remained hypothetical entities at least until the end of the 1960s. With the development of receptor-subtype specific pharmaceuticals and advancements in molecular biology to determine the genetic basis of receptor proteins and other vital aspects numerous receptor types and subtypes have since been characterized (Maehle, 2004).

An important traditional classification of autonomic nerves is based on the primary transmitter molecules—acetylcholine or norepinephrine—released from their terminal boutons and varicosities (Katzung, 1995). A search for new transmitters, other than acetylcholine and noradrenaline, was prompted by the discovery in the early 1960s that the paradigm established by Dale more than 30 years earlier was not correct. Nerves that relax some smooth muscles were found that did not involve the release of NAd or ACh. A variety of nonadrenergic, noncholinergic transmitters were subsequently identified, including ATP, VIP, NO, and neuropeptide Y (Bennett, 2004).

It is likely that a given impulse will evoke transmitter release from only some of the varicosities that it encounters. Some substances stored and released from nerves do not act directly on
effector muscle cells but alter the release and/or the actions of other transmitters; these substances are termed neuromodulators. Many other substances (e.g., circulating neurohormones; locally released agents such as prostanoids, bradykinin, Hst., and endothelin; and neurotransmitters from nearby nerves) are also neuromodulators in that they modify the process of neurotransmission both by prejunctional modulation of transmitter release and by postjunctional modulation of transmitter action. Many cotransmitters are also neuromodulators (Burnstock, 2009). See Table 1 for the list.
The effects of histamine are mediated through three pharmacologically distinct subtypes of receptors, *i.e.* the H1, H2, and H3 receptors, which are all members of the G-protein-coupled receptor (GPCR) family (Oda *et al.*, 2000). Recently, a fourth receptor for histamine, the

Table 1 – Putative Neurotransmitters Found in the Enteric Nervous System  
(Adopted from Goyal and Hirano, 1996)

<table>
<thead>
<tr>
<th>Category</th>
<th>Neurotransmitters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amines</strong></td>
<td>Acetylcholine, Norepinephrine, Serotonin (5-hydroxytryptamine)</td>
</tr>
<tr>
<td><strong>Amino acids</strong></td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td><strong>Purines</strong></td>
<td>ATP</td>
</tr>
<tr>
<td><strong>Gases</strong></td>
<td>Nitric oxide, Carbon monoxide</td>
</tr>
<tr>
<td><strong>Peptides</strong></td>
<td>Calcitonin gene–related peptide, Cholecystokinin, Galanin, Gastrin-releasing peptide, Neuromedin U, Neuropeptide Y, Neurotensin, Opioids, Dynorphin, Enkephalins, Endorphins, Peptide YY, Pituitary adenylyl cyclase–activating peptide, Somatostatin, Substance P, Thyrotropin-releasing hormone, Vasoactive intestinal contractor (an endothelin), Vasoactive intestinal polypeptide</td>
</tr>
</tbody>
</table>
histamine H4 receptor (H4R) has been identified as a potential modulator of dendritic cell activation and T cell polarization (Cowden et al., 2010).

Our understanding of these various neurotransmitters and receptors is very essential in that by using drugs that mimic or block the actions of chemical transmitters, we can selectively modify many autonomic functions (Katzung, 1995).

1.3.4 Smooth muscle related disorders and their interventions (modern / traditional)

Smooth muscle contractile activity is a major regulator of functions of the vascular system, respiratory system, gastrointestinal system and the genitourinary systems. Malfunction of contractility in these systems leads to a host of clinical disorders (Kim et al., 2008; Samuel, 2009). Because of this there is a great deal of interest in smooth muscle physiology and the mechanisms that manipulate it. Specific disorders of smooth muscles include: asthma, hypertension, hypotension, premature birth due to uterine related functional defects and intestinal cramps. Their underlying causes and mechanisms of treatment involve smooth muscles.

In certain situations, notably premature labor, the uterine musculature is triggered by various stimuli to contract at a time when it is undesirable or even life-threatening to do so. If the fetus is near term but has not yet produced the surfactant that will enable it to breathe properly after birth, quieting uterine contractions and thereby delaying delivery for a few days may be lifesaving: during those few days, the mother is treated with cortisol to induce immediate
surfactant production by the fetus, which can then be delivered without the threat of developing
the often-fatal condition known as hyaline membrane disease (Warren, 1999). Long-acting
beta2-agonists are also used in preventative (long-term) treatment of asthma. These
bronchodilators help to keep the airways open by relaxing the smooth muscle surrounding the
airways. When used regularly, these long-acting bronchodilators help reduce airway constriction,
reduce lung function, prevent symptoms, and reduce the need for a quick-relief (rescue) inhaler
(Asthma, 2009). Beta-blockers remain to be useful medications in treating hypertension,
especially in patients with a fast heartbeat while resting (tachycardia), cardiac chest pain
(angina), or a recent heart attack (myocardial infarction) (MedicineNet, 2009).

One investigation of this type by Mekonnen (1999) studied the effects of ethanol extract of \textit{M. stenopetala} leaves on guinea pig and mouse smooth muscle. This investigation concluded that
there were significant dose and time dependent reductions of the ACh response with initial
stimulatory effects in both the guinea-pig ileum and the mouse duodenum preparations.
Spontaneous rhythmic contractions greatly reduced, suggesting an antispasmodic property of the
crude leaf extract. The leaf extract showed some oxytocic activity on uterus strips of guinea- pigg
and mice. The results are indicative of the traditional use of the leaves of \textit{Moringa stenopetala}
for stomach pain and to expel retained placentae by women.

Another investigation by Chiwororo and Ojewole (2009) states that globally, primary
dysmenorrhoea is one of the most frequent gynaecological disorders in young women and it is
associated with increased uterine tone, and exaggerated contractility of uterine smooth muscles.
In many rural African communities, a number of medicinal plants, including \textit{Psidium guajava}
Linn. (family: Myrtaceae), are used traditionally for the management, control and/or treatment of
primary dysmenorrhoea. The spasmolytic effect of *Psidium guajava* leaf aqueous extract observed in this study lends pharmacological support to the traditional use of ‘guava’ leaves in the management, control and/or treatment of primary dysmenorrhoea in some rural African communities (Chiwororo and Ojewole, 2009).

There also are a good number of pharmaceutical agents manipulating muscle activity that have been derived from plant sources (Table 2).
<table>
<thead>
<tr>
<th><strong>Drug/Chemical</strong></th>
<th><strong>Action/Clinical Use</strong></th>
<th><strong>Plant Source</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Papavarine</td>
<td>Smooth muscle relaxant</td>
<td><em>Papaver somniferum</em></td>
</tr>
<tr>
<td>Anabesine</td>
<td>Skeletal muscle relaxant</td>
<td><em>Anabasis sphylla</em></td>
</tr>
<tr>
<td>Cissampeline</td>
<td>Skeletal muscle relaxant</td>
<td><em>Cissampelos pareira</em></td>
</tr>
<tr>
<td>Tubocurarine</td>
<td>Skeletal muscle relaxant</td>
<td><em>Chondedendron tomentosum</em></td>
</tr>
<tr>
<td>Pachycarpine</td>
<td>Oxytocic</td>
<td><em>Sophora pschycarpa</em></td>
</tr>
<tr>
<td>Sparteine</td>
<td>Oxytocic</td>
<td><em>Cytisus scoparius</em></td>
</tr>
<tr>
<td>Kheltin</td>
<td>Bronchodilator</td>
<td><em>Ammi visaga</em></td>
</tr>
<tr>
<td>Qulsqualic acid</td>
<td>Anthelmintic</td>
<td><em>Quisqualis indica</em></td>
</tr>
<tr>
<td>Rhomitoxin</td>
<td>Antihypertensive, tranquillizer</td>
<td><em>Rhododendron molle</em></td>
</tr>
<tr>
<td>Tetrandrine</td>
<td>Antihypertensive</td>
<td><em>Stephania tetrandra</em></td>
</tr>
<tr>
<td>Theobromine</td>
<td>Diuretic, vasodilator</td>
<td><em>Theobroma cacao</em></td>
</tr>
<tr>
<td>Theophylline</td>
<td>Diuretic, brochodilator</td>
<td><em>Theobroma cacao</em> and others</td>
</tr>
<tr>
<td>Trichosanthin</td>
<td>Abortifacient</td>
<td><em>Trichosanthes kirilowii</em></td>
</tr>
<tr>
<td>Yuanhuacine</td>
<td>Abortifacient</td>
<td><em>Daphne genkwa</em></td>
</tr>
</tbody>
</table>

Table 2 - Plant based drugs and chemicals (Adopted from Taylor, 2000).

This extensive investigation in medicinal plants plus the complex network of pathways in smooth muscle excitation-contraction mechanism discussed above may inspire great optimism towards the development of new and more effective therapeutic agents in the future.
1.4 Medicinal value of Alchemilla

The leaves of *Alchemilla abyssinica* are crushed and tied on to open wounds promoting blood clotting and facilitating wound healing (Gashaw, 1991). Among the different species of Alchemilla, some species are used in folk medicine either alone or in combination with other plants. Examples of significance are *A. vulgaris* (common name, lady’s mantel), *A. mollis* and *A. xanthochlora*. These species are used as herbal medicines in treating infections of the mouth and throat. Cut and burns are bathed in warm teas prepared from the plant to prevent infection. Infusion of the plant is also used to treat menorrhagia and menstrual cramps. *A. vulgaris* has traditionally been used in oral hygiene and was recently shown to accelerate wound healing when used in combination with glycerine (Shrivastava and John, 2006).

Deeply rooted in Arabic medicine, *Alchemilla vulgaris* L. has been used for treating obesity, gastrointestinal pain, and inflammation (Said et al., 2010). *Alchemilla arvensis* (parsley piert), also referred to as *Aphanes arvensis*, is an uninvestigated plant that has a strong reputation in England. It is particularly noted for its diuretic, demulcent, and antilithic properties (Heron and Yarnell, 1998). Among herbal medicines used for horses for hormone imbalances are leaves of *Alchemilla vulgaris* (Lans et al., 2006).

Different studies have shown that *A. vulgaris* and *A. xanthochlora* contain tannins and flavonoids, mainly quercetin (Jonadet et al., 1986; Geiger cited in Falcher 2009; Hadjieva et al. 2000, D’Agostino et al., 1998). Dried aerial parts of *Alchemilla xanthochlora*, Rothm, *A. glabra* Neygenf, *A. coriacea* Buser and *A. filicaulis* Buser contain flavonoids and tannins (Fraisse et al.,
The water extract of whole plant of *A. diademata* was reported to show an antimicrobial activity against *Staphylococcus aureus* (Barbour *et al.*, 2004). Esculetin, a coumarin isolated from *A. speciosa* was found to be active against certain mutagens (Schimmer and Lindenbaum, 1995; Schimmer and Eschelbach, 1997).

The quercetin group of flavonoid glycosides were isolated from *A. speciosa* (Felser and Schimmer, 1999). Three Icelandic Alchemilla species namely; *A. faeroensis*, *A. alpina* and *A. vulgaris* were found to contain the same pentacyclic triterpenoids as the main secondary metabolites (Olafsdottir *et al.*, 2001). Moreover, the content of a complex biologically active substances including phenolic compounds (9.6%) and polysaccharides (22.67%) in *A. vulgaris* has been established. Paper chromatography, spectrophotometry showed presence of at least 18 phenolic compounds, i.e. flavonoids, coumarines, phenol carboxylic acids, and tannins (Andreeva and Kalinkina, 2000). And plants containing biflavonoids/flavonoids possess inhibitory effects on smooth muscle activity (Udia *et al.*, 2009, Duarte *et al.*, 1993).

### 1.5 Significance of the present study

According to World Bank (2001) report, about 80% of the human population in Ethiopia rely on traditional medicine. However, it is a real concern among the global scientific community that a number of herbal preparations are widely used in traditional system of medicine for the management of different disorders but, many of them have not been investigated for their described effects (Manigaunha *et al.*, 2010). This wide use of traditional medicines in developing countries like Ethiopia plus the already existing medicinal use of this very species *Alchemilla abyssinica* was a good ground to investigate the scientific relevance of the plant. Thus it was
expected to establish the relevance and provide scientific bases for the claimed healing effects of *Alchemilla abyssinica*.

Moreover, the already voluminous work in different parts of the world on closer taxonomic relatives of this species increased the likely hood of the investigation to come up with some worthy findings. So, investigations on such plants could serve as sources of lead compounds for further plant derived drug development. In addition, even if there is large use of traditional medicines worldwide, in many cases the real pharmacological effects of the herbal remedies are unknown, and furthermore the indiscriminate use of them without proper instructions may entail toxic, deleterious effects (Cortés *et al.*, 1998). Therefore, this research project involving bioassay guided fractionations investigated bioactivity and phytochemical study on *Alchemilla abyssinica* Fresen.
2. OBJECTIVES

2.1 General objective

To investigate the effects of crude extracts and fractions from *Alchemilla abyssinica* on smooth muscles of animal models.

2.2 Specific objectives

The specific objectives are:

- Testing the water and hydroalcoholic extracts of *Alchemilla abyssinica* on smooth muscles of Swiss albino mice and guinea-pigs.
- Bioassay guided fractionation of the active crude extracts and testing the fractions on smooth muscle preparations
- Isolation of active principles of *Alchemilla abyssinica*
3. MATERIALS AND METHODS

3.1 Study design:

This investigation involves laboratory based experiments (quantitative and descriptive).

3.2 Study setting:

The bioassay tests on the Polygraph were done at the Biomedical Laboratory in the Faculty of Life Sciences while the extractions and fractionations were done at Organic Chemistry Laboratory in the Faculty of Chemical and Physical Sciences, College of Natural Sciences, Addis Ababa University.

3.3 Plant material collection

Areal part of *A. abyssinica* Fresen was collected from the Bale Mountains National Park near Dinsho town (500 km south of Addis Ababa), see Figure 4, in March, 2007. The collected specimen was transported according to the standard protocol and it was compared with the already existing collection of the same species in the National Herbarium of Addis Ababa University and was authenticated by a taxonomist. The representative plant specimen was kept in the Herbarium of ALIP and was labelled.

3.4 Preparation of extracts and fractionation

*Achelamilla abyssinica* aerial parts were openly dried at room temperature in the medicinal plants laboratory of Faculty of Life Sciences, AAU. The dried parts were ground into fine powder using mortar and pestle.
Figure 4 – Map of Bale Mountains National Park, site from where *Alchemilla abyssinica* was collected (Adopted from Ethio travel, 2011).
Part of this powdered plant specimen, 20 gm was taken and was soaked in 200 ml dist. H₂O, and was shaken for 24 hours using a shaker. After that pressure filtration using Whatmann No. 1 filter paper was conducted to get the filtrate which was lyophilized to get final water extract of *Alchemilla abyssinica* and the extract was kept in refrigerator at -62°C until used for the experiment.

Another 20 gm of the powdered form of *Alchemilla abyssinica* was taken and was dissolved using 200 ml (4:1) MeOH:dist. H₂O mixture as a solvent. This mixture was kept on the shaker for 24 hours and later pressure filtration using Whatmann No. 1 filter was conducted to get filtrate. This filtrate was first subjected to rotavapor and latter to lyophilizer to get rid of both solvents; alcohol and water respectively. This yielded hydro-alcoholic extract of *Alchemilla abyssinica* which was kept in refrigerator at -62°C until used for the experiment.

The above two extracts were tested on mice duodenum on the Polygraph to serve as preliminary baseline data that would give direction as to where to concentrate regarding the polarity of the solvent that would best isolate the active principle (s).

Later the main extraction and fractionation activity was conducted as follows:

Powdered aerial part of *Alchemilla abyssinica*, 74 gm, was soaked using CHCl₃/EtOAC 1:1 (300 ml) for 8 hours on shaker. This content was filtered using Whatmann No. 1 filter paper and gave 1.5 gm of CHCl₃/EtOAC extract and it was labelled as 90-55 K, a labelling system of the Organic Chemistry laboratory that was utilized. Part of this extract was checked for its effect on
smooth muscles on the Polygraph and the remaining was kept in refrigerator at 4\(^{0}\)C until used for the further experiment.

The residue in the filtration above was soaked using MeOH (300 ml) for 8 hours on a shaker two times. This content was filtered and gave 8 gm of methanol extract and this was labelled 90-55L. Part of this was checked for activity on smooth muscles on the Polygraph and the remaining was kept in refrigerator at 4\(^{0}\)C until used for further experiment.

Almost 8 gm of 90-55 L was taken and was adsorbed on 15 gm of silica gel and was eluted using six solvent systems: Hexane/EtOAc 1:1 and 1:2, EtOAc 100\%, EtOAc/ MeOH 2:1 and 1:1, and MeOH 100\%. See Table 3.
<table>
<thead>
<tr>
<th>Solvent</th>
<th>Ratio</th>
<th>Vol. (ml)</th>
<th>Fraction (label)</th>
<th>Mass (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane/EtOAc</td>
<td>1:1</td>
<td>200</td>
<td>Fr. 1 (90-56B)</td>
<td>0.191</td>
</tr>
<tr>
<td>Hexane/EtOAc</td>
<td>1:2</td>
<td>200</td>
<td>Fr. 2 (90-56C)</td>
<td>0.156</td>
</tr>
<tr>
<td>EtOAc</td>
<td>100%</td>
<td>200</td>
<td>Fr. 3 (90-56D)</td>
<td>0.332</td>
</tr>
<tr>
<td>EtOAc/ MeOH</td>
<td>2:1</td>
<td>200</td>
<td>Fr. 4 (90-56E)</td>
<td>2.633</td>
</tr>
<tr>
<td>EtOAc/ MeOH</td>
<td>1:1</td>
<td>200</td>
<td>Fr. 5 (90-56F)</td>
<td>1.833</td>
</tr>
<tr>
<td>MeOH</td>
<td>100%</td>
<td>200</td>
<td>Fr. 6 (90-56G)</td>
<td>0.742</td>
</tr>
</tbody>
</table>

Table 3 – Column chromatography procedure of Fraction 90-55 L

Here the investigation involving a bioassay guided fractionations, the fractionation proceeded forward as a minimum of two tissue experiments strongly suggested that an extract or a fraction can be regarded to have spasmolytic effect without statistical analysis to get mathematical figures and interpretations. Moreover, whenever two or three of the many fractions shown to have spasmolytic effects, only the one that highly surpasses all have been taken in to consideration and the others that have shown slight spasmolytic effect were omitted at least in this project.
Accordingly, each of the above fractions (1-6) was checked for bioactivity on GPI and based on the results Fraction 2 (90-56C) was found to be the most active and hence 50 mg of it was applied on the Chromatotron. Elutions were collected from the chromatotron using CHCl$_3$/MeOH in 9:1 ratio and the different fractions were collected (see Table 4).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample. 1</th>
<th>Sample. 2</th>
<th>Sample. 3</th>
<th>Sample.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9:1</td>
<td>9:1</td>
<td>9:1</td>
<td>9:1</td>
</tr>
<tr>
<td>Label</td>
<td>(90-57A)</td>
<td>(90-57B)</td>
<td>(90-57C)</td>
<td>(90-57D)</td>
</tr>
</tbody>
</table>

Table 4 – Chromatotron procedure of Fraction 90-56C

This step of using Chromatotron was primarily for purification than fractionation. So, the TLC of all (90-57A, 90-57B, 90-57C, 90-57D) was checked and results were compared to the TLC of 90-56C and sample 2, (90-57B), was found to be the content having high chemical resemblance with 90-56C but much cleaner.

Further isolation was done using Sephadex. Here 23 mg of 90-57B was applied on the sephadex and elution took place using a solvent mixture of CHCl$_3$/MeOH 1:1. Ten fractions were collected and according to the TLC of each, those fractions with the same TLC result were mixed together. The ten fractions were finally grouped in to four putting together those with similar TLC bands (see Table 5).
<table>
<thead>
<tr>
<th>Fraction</th>
<th>1,2,3,4,5</th>
<th>6</th>
<th>7</th>
<th>8-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Label</td>
<td>90-57F</td>
<td>90-57G</td>
<td>90-57H</td>
<td>90-57I</td>
</tr>
<tr>
<td>Mass (mg)</td>
<td>-</td>
<td>1.4</td>
<td>8.8</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Table 5 – Sephadex fractionation of Fraction 90-57B

Each of the above fractions was checked for its effect on smooth muscle activity on the Polygraph. The overall description of extraction and fractionation in the tables above is diagrammatically presented in Figure 5 as follows.
3.5 Laboratory animal preparation

Swiss albino mice (25–30 gm) and guinea-pigs (300–400 gm) were obtained from the animal house of Faculty of Life Science of Addis Ababa University and EHNRI respectively. These animals were housed at a temperature of 24 ± 2°C and maintained under uniform conditions of
12 hrs daylight and 12 hrs dark cycles. The mice were used for preliminary studies on organ bath and for toxicity studies; while the guinea-pigs for the organ bath experiment involving fractions and final test sample. They were given a standard diet and tap water *ad libitum* based on previous works (Mekonnen, 1999; PETCO, 2005).

### 3.6 Acute toxicity test

Swiss albino mice of both sexes (25-30g) were divided into five groups of five mice each and fasted for 4 hrs. The test was performed using increasing doses of *Alchemilla abyssinica* Fraction 90-56 C, the final test sample used for test on the Polygraph was dissolved in DMSO/dist. H$_2$O (1:9) solvent system. This solution was administered orally at concentrations of: 100, 400, 700 and 1,000 mg/kg body weight. To the fifth, control, group the vehicle, DMSO/dist. H$_2$O (1:9), was administered (Jyothi *et al.*, 2006, Gaylord C.C., 2005, OECD, 2001). The mice were allowed food and water *ad libitum* during a 24 hrs test period and kept under regular observation for mortality and any behavioral change during the test period (Ghayur and Gilani, 2006).

### 3.7 Bioassay on Guinea pig ileum

Every time a tissue was required a Guinea pig of either sex was fasted overnight and was sacrificed by a gentle blow on the head and then bled from the neck. The abdomen of each animal was opened, and the ileum was removed and cleaned of attached tissues. A segment of the removed ileum (2–3 cm) from each Guinea-pig, was used. Tyrode’s solution of the following composition (mM), NaCl, 137; KCl, 2.6; MgCl$_2$, 1.05; CaCl$_2$, 0.3; NaH$_2$PO$_4$, 0.04; NaHCO$_3$, 0.9.
11.9; C₆H₁₂O₆, 5.5 was used as previously described by (Mekonnen, 1999; Tolessa et al., 1996). The above chemicals were procured from Sigma Chemical Company, St. Lous, MO, USA. The segments of ileum were tied with threads at both ends in opposite directions and suspended in a thermoregulated 25 mL organ bath containing Tyrode’s solution which was maintained at 37°C and gassed with air. A tension of 1 gm was applied to each tissue and then allowed to equilibrate for at least 30 min before adding Hst. the agonist. The responses were recorded isometrically using a Grass FT.03 strain gauge transducer connected to a Grass Model 7 Polygraph (Grass Instruments Quincy, MA, USA).

Papavarine, a non-selective smooth muscle relaxant and a control drug used for smooth muscle relaxants (Shimizu, 2000) was tested once to standardize the investigation. Dose response curves of the Hst. induced contractions (at 4, 8, 16 and 32 ng/ml concentrations of Hst.) or the first three Hst. concentrations were done for all the tissue preparations and the Hst. concentrations that effect submaximal stimulation was taken as the control in each experiment. The different concentrations were applied with a time interval of 5 minutes.

Each time the added Hst. was left in contact with the tissue for 30 seconds and then the Polygraph paper movement was paused, the solution containing Hst. was discarded and the tissue was rinsed with fresh Tyrode’s solution. The organ bath is then filled with fresh Tyrode’s solution and the Polygraph chart movement was once again turned on. The tissue then was left to resume its normal rhythmic contractions. This was done each time before the addition of the given test material in order to observe its effect. The final organ bath concentration of Hst. that effected the submaximal stimulation of the tissue was 8 ng/ml and it was taken as control conc.
After the rhythmic contractions were established, a given dose of the particular test material in a measured final organ bath concentration were injected to the organ bath and stayed acting its effect on the given tissue for 5 minutes. Control Hst. was then added at the end of the 5 minutes in the presence of the test material. After 30 seconds contact time once again the polygraph paper movement was paused and the content in the organ bath containing Tyrode’s solution, the test sample and Hst. was discarded. The tissue was rinsed once for lower doses and twice for higher doses of the test sample and the organ bath was filled with fresh Tyrode’s solution. This time the movement of the Polygraph paper was started once again. The same procedure was used while testing the different test materials as well as different doses of the given test material.

Each extract or fraction was tested at final organ bath concentrations ranging from 20-600 µg/ml organ bath concentrations while the final test Fraction (90-56C) was tested three times at final organ bath concentrations of 20, 70 and 120 µg/ml. One experiment was made using Papavarine as positive control at final organ bath concentrations 0.4, 0.8 and 1.6 µg/ml.

After one or two dose test material administration, control Hst. was administered to examine the overall performance of the tissue and every test on the Polygraph on GPI ended with final control Hst. thereby proving the reversibility of the rhythmic contraction of the tissue as well as its response to the Hst. compared to the Hst. administered during dose response. The stock solutions of Hst. were prepared using dist. H$_2$O and Tyrode’s solution (10 mg Hst. in 1:9 dist. H$_2$O:Tyrode’s solution). And three serial dilutions were followed using Tyrode’s solution until 1µg/ml Hst. was obtained which finally was used for the organ bath administration.
Papavarine stock solution was prepared by (1:9 dist. H₂O:Tyrode’s solution) and Tyrode’s solution for the serial dilutions. *Alchemilla abyssinica* extract or fractions reconstitution, however, were made using either dist. H₂O or DMSO:dist. H₂O (1:9) as a solvent, the later for those that couldn’t dissolve in dist. H₂O. The safety of the potent solvent DMSO is well established and is widely used in previous investigations as well. (Srivastava *et al.*, 2010; Gaylord C.C., 2005).

Moreover, DMSO:dist. H₂O (1:9) was tested on one tissue preparation and was found to be inactive further assuring its safety on its use for test on the Polygraph. The Tyrode’s solutions, Hst. and Papavarine used were prepared fresh on the day of experiment while the solutions of extract or fraction were mostly prepared on the day of experiment but if some amount remains to be used in the coming few days the prepared solutions were kept well tight in refrigerator at -62°C until used for the next experiment. While preparing the solutions of the given extract or fraction thermo regulated sonicator at 37°C was used to ensure proper dissolving.

The general procedure utilized for preliminary study on the Swiss Albino mice duodenum tests was the same with the procedure for GPI as described above. However, the mice were sacrificed by cerebral dislocation, the organ bath concentrations of ACh used for establishing dose response were 40, 80 and 160 ng/ml, the control ACh utilized was 80 ng/ml, and the final organ bath concentrations of crude *Alchemilla abyssinica* extracts ranged from 50 – 500 µg/ml. Both methanolic and water crude extracts easily dissolved in dist. H₂O, ACh preparation followed the same procedure with Hst. preparation except only 2 serial dilutions were enough to reach the final 10 µg/ml ACh solution used for administering to the organ bath.
3.8 Statistical Analysis

Mean and standard error of the mean (SEM) were calculated for each doses of the final test sample, Fraction (90-56C), in vitro results. The results were analyzed statistically using one-way ANOVA Scheffe post-hoc comparison between the control Hst. contraction and contractions in response for the presence of the fraction using SPSS 14 statistical software package. The values P<0.05 were regarded as statistically significant.

3.9 Ethical considerations

This investigation was conducted after ethical clearance on the use of experimental animals was obtained from the Institutional Review Board of College of Health Sciences, Addis Ababa University.
4. RESULTS

4.1 Results of toxicity test

As observed from the present acute toxicity study, Fraction 90-56 C of *Alchemilla abyssinica* can be considered tolerable in mice when tested up to the oral dose of 1,000 mg/kg body weight with no mortality and behavioral changes within 24 hours.

4.2 Results of organ bath experiments

The experiments done using the different extracts and fractions showed no spasmogenic effect. The numerical data presented both in table and figure form here is only that of the final test fraction while the other polygraph charts were preserved carefully but not included after their reading.

The initial water and hydroalcoholic extracts were tested on mice duodenum and gave no effect for the water and spasmolytic for the hydroalcoholic extracts respectively. This result was obtained twice, in duplicate, but no statistical analysis was conducted. The purpose of these tests was to get the base-line information whether the plant has any spasmogenic or spasmolytic effect on smooth muscles. As already stated marked spasmolytic effect was obtained using the hydroalcoholic extract and there was no effect observed using the water extract.
The CHCl$_3$/EtOAC extract of *Alchemilla abyssinica* (90-55 K), was tested on GPI *in vitro* and was found to have non-spasmolytic effect in the organ bath experiment.

Following this the residue from the above first extraction was further extracted using methanol and this methanol extract (90-55L) was tested on the Polygraph and it was shown to have spasmolytic effect.

Once this spasmolytic effect was established on the methanol extract (90-55L), this extract was fractionated using column chromatography and six fractions: Fr. 1 (90-56B), Fr. 2 (90-56C), Fr. 3 (90-56D), Fr. 4 (90-56E), Fr. 5 (90-56F), Fr. 6 (90-56G) were obtained using the procedure described in detail in the methodology part. Each of these were tested on the Polygraph and the results showed strongly spasmolytic effect only for Fr. 2 (90-56C).

Fr. 2 (90-56C) was further purified and fractioned using Chromatotron and Sephadex and 3 further fractions were obtained. Organ bath experiments were conducted on all the three but as the amount has been extremely minimized at these levels of purification and fractionation, reproducibility couldn’t be established and only one tissue experiment was conducted for each. Based on this then, Fraction 90-57I gave spasmolytic result making it a good candidate for further investigations, Fraction 90-57H may also be worth considering but Fraction 90-57G was the lowest in activity.

Table 6 shows the results of the organ bath investigations of Fraction (90-56C) conducted at 20, 70 and 120 (µg/ml) final organ bath concentrations. The dose-dependent effects of Fraction (90-56C) on GPI have been expressed as the % contraction ± SEM values and results are presented
in tabular as well as graphical forms. According to this finding then, *Alchemilla abyssinica* Fraction (90-56C) caused concentration dependent inhibitions significantly (P<0.05, F= 61.5). It was capable of inhibiting Hst. induced contractions down to 95.7 ± 3.4% at 20 µg/ml, to 43.6 ± 7.1% at 70 µg/ml and 14.2 ± 4.7% at 120 µg/ml organ bath concentrations. That is, Fraction (90-56C) reduced the responses of the GPI tissue preparations to control Hst. by 4%, 53% and 86% for the above three doses respectively (Table 6 and Figure 6).

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>Group</th>
<th>Extract conc. (µg/ml)</th>
<th>Contractile response (%)</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alchemilla abyssinica</em> Fraction (90-56C)</td>
<td>1</td>
<td>20</td>
<td>95.7 ± 3.4<strong>2,3</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>70</td>
<td>43.6 ± 7.1*1,3</td>
<td>61.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>120</td>
<td>14.2 ± 4.7*1,2</td>
<td></td>
</tr>
</tbody>
</table>

Table 6 – The effect of the final test fraction, Fraction (90-56C), of *Alchemilla abyssinica* on Hst.-induced contraction of GPI.

Control Hst. = 8µg/ml of final organ bath concentration. Responses were expressed as % of initial contractions induced by agonist Hst. prior to the addition of Fraction (90-56C). Data of contractile responses are expressed as mean ± SEM of 3 GPI preparations. The mean difference is significant at *P < 0.05.
A single tissue experiment was also done on Papavarine as a positive control. The dose-dependent spasmolytic effects of Papavarine, at final organ bath concentrations 0.4, 0.8 and 1.6 µg/ml, are expressed as the % contraction values and are presented in Table 7. Papavarine, the smooth muscle relaxant decreased the contractile response of Hst. down to 75.0% at 0.4 µg/ml, to 72.5% at 0.8 µg/ml and 62.5% at 1.6 µg/ml organ bath concentrations. This shows that Fraction (90-56C) of *Alchemilla abyssinica* (at dose 120 µg/ml) may be considered 4.4 folds more potent than the most antagonist Papavarine (1.6 µg/ml) used in the experiment.

<table>
<thead>
<tr>
<th>Dose No.</th>
<th>Papavarine Conc. (µg/ml)</th>
<th>Contractile response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4</td>
<td>75.0</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>72.5</td>
</tr>
<tr>
<td>3</td>
<td>1.6</td>
<td>62.5</td>
</tr>
</tbody>
</table>

Table 7 – The effect of Papavarine on Hst.-induced contraction of GPI.

Control Hst. = 8ng/ml of final organ bath concentration. Responses were expressed as % of initial contraction induced by agonist Hst. prior to the addition of the antagonist papavarine. Data are produced from one GPI preparation.
Figure 6 – Dose-response curve (line graph) showing the mean percentage contraction recorded by Hst. in presence of Fraction (90-56C) of *Alchemilla abyssinica* at different organ bath concentrations as compared to the control Hst. on isolated GPI.

Figure 7 shows one Polygraph tracing of the dose-response of the tissue to Hst. in the absence of any extract or fraction. Figure 8a, shows a Polygraph tracing of the effect of *Alchemilla abyssinica* Fraction (90-56C) on Hst. induced contraction of isolated GPI (all doses) which clearly indicates the dose-dependent spasmolytic effects of the experimental plant. Figures 8b, c and d show three Polygraph tracings of the effect of *Alchemilla abyssinica* Fraction (90-56C) at the three different doses compared with the control which presents their effects, and Figure 8e shows a Polygraph tracing of the normal rhythmic contractions of the GPI in the absence of any test sample.
Figure 7 – Polygraph tracing showing dose-response relation for Hst. in the absence of any test sample. ↑ shows addition and ↓ shows washing out of Hst. Each square represents 5mm and the speed of polygraph chart is 5mm/min.
Figure 8.a – Polygraph tracing showing the effect of *Alchemilla abyssinica* Fraction (90-56C) on Hst. induced contractions of isolated GPI. Contractions are shown both in the absence and presence of Fraction (90-56C) at different organ bath concentrations. ↑ shows addition and ↓ shows washing out of Hst. and Fraction (90-56C). Each square represents 5mm and the speed of polygraph chart is 5mm/min.
Figure 8.b – Polygraph tracing showing the normal rhythmic contractions of isolated GPI. Each square represents 5mm and the speed of polygraph chart is 5mm/min. (This Figure is taken out from Figure 8.a to simplify comparison between the rhythmic contractions in presence and absence of *Alchemilla abyssinica* Fraction (90-56C)).

Figure 8.c – Polygraph tracing showing the effect of *Alchemilla abyssinica* Fraction (90-56C) at 20 µg/ml organ bath concentration on Hst. induced contraction of isolated GPI. ↑ shows addition and ↓ shows washing out of Hst. and Fraction (90-56C). Each square represents 5mm and the speed of polygraph chart is 5mm/min. (This Figure is taken out from Figure 8.a to simplify comparison)
Figure 8.d – Polygraph tracing showing the effect of *Alchemilla abyssinica* Fraction (90-56C) at 70 µg/ml organ bath concentration on Hst. induced contraction of isolated GPI. ↑ shows addition and ↓ shows washing out of Hst. and Fraction (90-56C). Each square represents 5mm and the speed of polygraph chart is 5mm/min. (This Figure is taken out from Figure 8.a to simplify comparison).

Figure 8.e – Polygraph tracing showing the effect of *Alchemilla abyssinica* Fraction (90-56C) at 120 µg/ml organ bath concentration on Hst. induced contraction of isolated GPI. ↑ shows addition and ↓ shows washing out of Hst. and Fraction (90-56C). Each square represents 5mm and the speed of polygraph chart is 5mm/min. (This Figure is taken out from Figure 8.a to simplify comparison).
4.3 Results of the Chemical isolation / identification

The extraction and fractionation of *Alchemilla abyssinica* proceeded as described in the methodology part and Fraction 90-56C was taken as final test fraction. Figures 9 and 10 show the NMR spectra that indicates Fraction 90-56C is a mixture of substances possibly of the flavonoid class.

Figure 9 – The Carbon-13 NMR spectrum of Fraction (90-56C), of *Alchemilla abyssinica* /the final test fraction/
(The tallest three peaks are solvent peaks)
Figure 10 – The Proton NMR spectrum of Fraction (90-56C), of *Alchemilla abyssinica* /the final test fraction/ (The tallest two peaks are solvent peaks)
5. DISCUSSION

The toxicity result obtained in this investigation using Fraction 90-56 C of *Alchemilla abyssinica* can be considered tolerable in mice when tested up to the oral dose of 1,000 mg/kg body weight with no mortality and behavioral changes within 24 hours. This is in agreement with the toxicity study on *Alchemilla vulgaris* by Saad et al., (2006) that states the LD$_{50}$ of *Alchemilla vulgaris* dried leaf extract tested on rats is found to be 17.3 gm/kg body weight. According to Said (2010) also *Alchemilla vulgaris* L. (lady's mantle) is regarded as safe by the German Commission even at large doses without known adverse effects. The LD$_{50}$ reported by Saad, et al. (2006) is within the range of relatively harmless according to Hodge and Sterner Scale (Hodge and Sterner, 1949). It has also been stated Lady’s mantle (*Alchemilla vulgaris* L. or *A. xanthochlora* Rothm.) has no known hazards and/or side effects for proper therapeutic dosages (Duke, 2002).

Regarding the bioassay investigation, this study examined the effects of crude extracts and their bioassay guided fractions of *Alchemilla abyssinica* aerial parts on isolated GPI tissue preparations. In the preliminary pilot study the MeOH:dist. H$_2$O (4:1) crude extract of *Alchemilla abyssinica* gave spasmolytic effects while the water crude extract didn’t. This strongly suggests that the active principle was extracted with methanol with water (Berthod, 2004).

The crude CHCl$_3$/EtOAC (1:1) extract of dried *Alchemilla abyssinica* aerial parts gave neither spasmogenic nor spasmolytic result but the methanol extract of the above residue gave significant spasmolytic effects which suggests that a liquid whose polarity is somewhere above...
CHCl₃/EtOAC (1:1) and less than water is best for extracting the biologically active content from the aerial plant material (Berthod, 2004).

Fraction (90-56C) has shown significant spasmolytic activity at the three doses 20, 70 and 120 (µg/ml). Similar results were reported for closely related species of *Alchemilla abyssinica* by different investigators. *Alchemilla vulgaris* is claimed to have spasmolytic effect (Gladstar as cited in Yarnell and Abascal, 2009). Ivancheva and co-workers (2006) also reported, the infusion of *Alchimella vulgaris* is used as antidiarrheal agent.

The use of *Alchemilla vulgaris* as traditional medicinal plant to expel retained placenta (Lans *et al.*, 2007) indicates its spasmodenic role in uterine smooth muscles. Its use as antidiahreal agent (Ivancheva *et al.*, 2006) also predicts spasmodelic effect of the plant extract on gastrointestinal smooth muscles. Hence the plant has both spasmodelic and spasmodenic on different types of smooth muscles. The same property, spasmodelic in GPI and oxytocic in rat uterus, is exhibited by ethanolic extract of *Moringa stenoptella* (Mekonnen, 1999). This strongly suggests that the spasmodelic effect of the present plant, *Alchemilla abyssinica* may not be universal to all types of smooth muscles and hence it would be worth investigating the effect of extract on other muscle types.

The mechanism of action of *Alchemilla abyssinica*, could be due to the flavonoids that could possibly exist as also confirmed in *Alchemilla vulgaris* (Andreeva and Kalinkina, 2000). It has already been established that plants containing biflavonoids/flavonoids possess inhibitory effects on smooth muscle activity (Udia *et al.*, 2009, Duarte *et al.*, 1993). In this study the NMR spectra
of the active fraction, Fraction 90-56C, showed the fraction is a mixture of secondary metabolites possibly of the flavonoid class. Though, in depth chemical investigation is required to establish this, it could be stated that the flavonoids in *Alchemilla abyssinica* are among the key contributors for its spasmolytic effect.

There also are reports that indicate ACh and Hst.-induced contractions of intestinal smooth muscle are mainly dependent on extracellular Ca$^{2+}$ (Adebiyi *et al.*, 2004). This is a good ground to predict that the spasmolytic effects observed in this study are also primarily dependent on blocking the entry of extracellular Ca$^{2+}$. Among the four types of Hst. receptors also Hst. induced contraction of GPI is mediated by H$_1$-receptors and this effect (Hst.-induced contraction of GPI) can be antagonized by mepyramine (Rang and Dale, as cited in Adebiyi *et al.*, 2004).

In this investigation the effect of the extracts and fractions could also be mediated through reversible binding of the active substances in the extracts and fractions with the H$_1$-receptors since both the decrease in the Hst. induced contractions and the decrease (abolishment) in the rhythmic contractions have been demonstrated to be completely reversible even using the highest doses tested.

It has been well established that smooth muscle contractile activity is a major regulator of functions of the vascular system, respiratory system, gastrointestinal system and the genito-urinary systems. Malfunction of contractility in these systems leads to a host of clinical disorders (Kim *et al*., 2008; Samuels, 2009). Abdominal pain is one of the most common reasons
why people seek medical care, and is often due to spasm of intra-abdominal visceral organs (Samuels, 2009).

It is a known fact that Smooth-muscle relaxants are beneficial when abdominal pain is the predominant symptom (Jailwala et al., 2000). Other studies reported that myorelaxants are superior to placebo in the management of irritable bowel syndrome (Poynard et al., 2001).

Considering the fact that a good number of pharmaceutical agents manipulating muscle activity have been derived from plant sources (Table 2) the current investigation on Alchemilla abyssinica could also be a source of drug development after complete work on the secondary metabolites.

There are well-documented data on various plants having similar spasmolytic effects in the literature by many authors. Ethanol extract of Helichrysum plicatum (Turkish Helichrysum) flowers induced a relaxant effect on spontaneous rat ileum contractions lending Pharmacological hands to the wide use of the plant in folk medicine (Bigovic et al., 2010). Ethanol extract of the aerial parts of Achyrocline satureioides (Lam.) DC. (Asteraceae) is reported to have significant, dose dependent, relaxant effect on the smooth muscle of corpus cavernosum strips, obtained from Guinea pig (Hnatyszyn et al., 2004).

In an investigation on aqueous and ethanol extracts of the leaves and roots of Asparagus africanus in rats it has been reported that the extract significantly potentiated acetylcholine induced uterine contractions (Tafesse et al., 2006). The effects of an aqueous extract of the leaf
of *Cassia occidentalis* were investigated in rat aortic rings and its relaxant effect has been established (Ajagbonna, et al., 2001). In another investigation the relatively potent relaxant (bronchodialatory) effect of *Adhatoda schimperiana* on the tracheal chain of the guinea pig has been shown (Aseffa, 2008). Relaxant effect of *Thymus vulgaris* on Guinea-pig tracheal chains have been reported (Boskabady, 2006). In another investigation conducted on the abortifacient effect of *Ricinus communis* and *Jatropha curcas* seeds on uterine muscle of guinea pigs has been established (Mekonnen *et al.*, as cited in Tafesse *et al.*, 2006). As previously reported by Desta (1995) a total of 210 extracts/fractions prepared from 70 traditionally used Ethiopian plants studied for effects on uterus, 24% of the samples were found to have uterotonic activity.

The present study is in line with the investigation by Mulatu and Mekonnen (2007) that studied ethanol and aqueous extracts of leaf and root of *Artemisia afra* and leaf of *Artemisia rehan*. These extracts were tested on isolated mouse duodenum and GPI giving marked relaxant effects. The extract of *Taverniera abyssinica* illustrated the ability to antagonize the smooth muscle spasmogenic actions of both acetylcholine and histamine, two of the most important spasmagens responsible for hyperactivity of the gastrointestinal tract (Bogale *et al.*, 1991). Another finding by Assefa *et al.* (2006) reports that the aqueous root extract of *Solanum incanum* inhibited the contractions of isolated GPI in response to acetylcholine in a concentration-dependent manner similar to atropine which indicated that the extract is a relaxant on GPI.
6. CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Acute toxicity study of the fraction tested (Fraction 90-56C of Alchemilla abyssinica) showed that the plant is tolerable in mice up to an oral dose of 1,000 mg/kg. The plant can be regarded safe as shown in the animal model.

In this study the spasmolytic effects of Alchemilla abyssinica aerial part extract and its bioassay guided fractionation results (fractions) were demonstrated. The water extract has shown no effect as a relaxant while the methanolic extract has shown considerable spasmolytic effects. Among the various fractions some have shown higher spasmolytic effects while others low or even no activity as a relaxant.

The spasmolytic activities shown by the extracts and fractions might suggest the presence of active chemical components such as flavonoids or the combined effect of several chemical constituents. Possible mechanism of the spasmolytic effects is also suggested.

The spasmolytic effects of the plant extract or fractions have been shown to be affected by the concentration. The active fraction was easily extracted with moderate polarity.
6.2 Recommendations

Since the spasmolytic effects of the plant and especially the final fraction tested are so encouraging detailed phytochemical screening, further fractionation (finalization) and isolation of active ingredients is recommended to identify the exact chemical compound(s) responsible for the activities observed in the present study of Alchemilla abyssinica.

The present study is focused on the GPI and hence other tissues like uterus, aorta, trachea and others should be checked to investigate whether differences exist in effect depending on differences in tissues.

The existing data on the toxicity of Alchemilla abyssinica is based only on the acute toxicity study. Therefore sub-acute and chronic toxicity studies on the plant extract and final test fraction has to be done. Since medicinal effects of other Alchemilla species have been found in previous investigations e.g. antimicrobial, antitumor and others it is useful to investigate Alchemilla abyssinica also for these effects.

In addition to establishing the medicinal value and protocol following the standard regulations of clinical medicine it is also highly recommended to establish the dosages, contraindications, interactions, and side effects of the herbal preparations as well documented in (Duke, 2002) for Lady’s mantle (Alchemilla vulgaris L. or A. xanthochlora Rothm.)
Finally increased priority should be given by the government, NGOs, researchers and local communities to conserve medicinal plants in order to develop safe, effective, and accessible products as well as mitigating the emerging dangers of bio-piracy. This is tremendously important since a large number of Ethiopians depend on medicinal plants for their primary health care and the unique diversification of geology, land forms, soils, climate and culture Ethiopia enjoys makes the country highly promising source of huge wealth of plant based drugs.

6.3 Limitation of the study

Due to inadequate resources it was not possible to further isolate active principles.
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