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ERGOT FUNGUS (*Claviceps purpurea*), ERGOT ALKALOIDS AND ERGOTISM IN THE CENTRAL HIGHLANDS OF ARSI, ETHIOPIA

A Thesis submitted to the School of Graduate Studies of the College of Natural and Computational Sciences, Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (PhD) in Biology (Applied Microbiology)

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Dedication

I dedicate this thesis to my family who passed through so many ups and downs in order to pave the way for my dreams to come true.

DECLARATION

I declare that the thesis, hereby submitted by me (Asnake Desalegn) for the Degree of Doctor of Philosophy (PhD) in Biology (Applied Microbiology) to the School of Graduate Studies of Addis Ababa University College of Natural and Computational Sciences is an independent work of my own and has not been submitted by me or anybody elsewhere at another university or other academic institution for the fulfillment of a similar purpose. Any information and materials obtained from other sources are duly acknowledged in the thesis.

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Abstract

Ergot fungi, *Claviceps* species, parasitize several monocot plants and produce a hardened dark to dark-purple structure called the sclerotia. In Ethiopia, the ergot fungus infects only the wild oat plant (*Avena abyssinica*) which is endemic to Ethiopia and Yemen. Ergot alkaloids produced in the sclerotia of the ergot fungus, were responsible for mass poisoning in various areas of the world, with the most recent report of mass poisoning in Arsi, Ethiopia. This study was initiated with the objectives of identifying the ergot fungus (*Claviceps purpurea*) based on morphological and molecular characteristics, ergot alkaloids, and assessing the knowledge of study participants about ergot fungus and ergotism from the previously reported outbreaks areas of Arsi, Ethiopia. Dimensions of the sexual and asexual structures were studied and statistically significant differences ($n = 30$, $P < 0.001$) in the length and width of sclerotia collected from the study sites Kechema Murkicha, Bucho Selassie and Shaldo Jigessa were observed. Dimensions of sclerotia collected from all the study sites significantly differed ($p < 0.001$) from the dimensions of wild oats seeds ($n = 30$). But, statistically significant differences were not observed ($p > 0.05$) for the dimensions of conidia of ergot fungi collected from different study sites. Growth (100%) of the sexual stage occurred only on Petri dishes incubated for 21 days at 5 ^{O}C followed by incubation at 25 ^{O}C. No growth was observed on the Petri dishes incubated under other temperature treatments. The mean length of stromata ranged from 18.5 mm to 19mm and the mean diameter of capitula ranged from 1.8 mm to 2mm. Cylindrical to flask shaped perithecia with mean length and width of 158.8 ± 3.7 µm and 89.2 ± 1.7 µm, and filiform shaped ascospores with mean length and width of 77.1 ± 3.7 µm and 3.3 ± 0.5 µm respectively were observed.

Phylogenetic analysis of the β-tubulin intron 3 region using maximum parsimony placed our isolates in a separate cluster with strong bootstrap value of 94. Qualitative studies of the ergot alkaloids using UPLC-QTOF High Definition Mass Spectrometery revealed the presence of ergometrine, ergocryptine, ergocornine, ergosine, ergovaline, lysergyl alanine, lysergyl valine, valine methyl ester, their respective -innine isomers and an ergopeptam (ergocryptam). A cross-sectional study conducted to assess the awareness of study participants recruited from Tijo, Digelu and Kechema areas, Arsi, Ethiopia, showed lack of awareness about the fungus and the disease it causes. Among the study participants who were shown the coloured picture of ergot fungus, majority 55 (32.7%) described its name as ‘Sinara Guracha’ which is synonymous with “Black wild

oat". A multiple logistic regression model fitted revealed statistically significant association of the study sites with knowledge of ergot ($p < 0.05$). Finally, morphological and molecular characteristics placed the ergot fungi in the current study under *Claviceps purpurea*. The presence of the *Claviceps purpurea* in farmers' field, detection of additional toxic ergot alkaloids and lack of awareness of the study participants about the ergot fungus, *Claviceps purpurea*, are the potential risks for the community.

Key words/Phrases: *Claviceps purpurea*, ergot alkaloids, *Avena abyssinica*, UPLC-QTOF, HDMS

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List of Abbreviations and Acronyms

AAU----------Addis Ababa University

BLAST ---------- Basic Local Alignment

BS----------Bucho Selassie

CSD ----------Cortical Spreading Depression

CTAB ----------Cetyl -Trimethyl Ammonium Bromide

DA ----------Dopamine

DAA ----------Dopamine Agonist

DB ----------Digelu Bora

DDA ----------Data Dependent Acquisition

DHE ----------Dihydroergotamine

DHEC ----------Dihydroergocryptine

DM ----------Diabetes mellitus

EA ----------Ergot Alkaloids

EFSA----------European Food Safety Authority

FFA ----------Free Fatty Acids

HRMS---------- High resolution mass spectrometer

HT----------Hydroxytryptamine

ITS---------- Internal Transcribed Spacer

KM----------Kechema Murkicha

LC----------Liquid chromatography

L-DOPA ---------- Levo Dihdroxyphenylalanine

MEGA----------Molecular Evolutionary Genetic Analysis

MeOH ----------Methanol

MPTP----------Methyl phenyl tetrahydropyridine

MS----------Mass Spectrometer

m/z ----------Mass to charge ratio

NCBI----------National Center for Biotechnology Information

NIDDM----------Non-Insulin Dependent Diabetes Mellitus

NSAIDS----------Non steroidal Anti-inflammatory Drugs

P450 ----------Cytochrome p 450

PCR ----------Polymerase Chain Reaction

PD----------Parkinson's disease

PDA ----------Potato Dextrose Agar

PPG----------Postprandial Blood Glucose

PPH----------Post Partum Hemorrhage

PRLomas----------Prolactinoma

QR----------Quick Release

QTOF----------Quadrupole Time-of-Flight

RAPD----------Random Amplification of polymorphic DNA

SDW ----------Sterile Distilled Water

SJ----------Shaldo Jigessa

SPSS----------Statistical Package for Social Sciences

T2DM----------Type 2 Diabetes Mellitus

TG----------Triglyceride

TIC----------Total Ion Current

TLC----------Thin Layer Chromatography

UPLC----------Ultrahigh performance liquid chromatography

Chapter 1. General introduction

Ergot is a plant parasitic fungus that infects more than 600 monocot plants. Its name is derived from its black horn shaped structure that grows on the flower head of susceptible plants completely replacing the seeds. The black horn shaped structure is called sclerotia, which is used by the fungus to pass the cold winter conditions. The genus *Claviceps* is adapted to variety of environments including marine, desert, tropical as well as subtropical areas (Alderman *et al*., 2004). Based on habitat specialization *Claviceps purpurea*, is generally grouped into three the G1, which is adapted to the dry open fields, G2 which are adapted to moist or shady environments and G3 which is adapted to wet environments (Pazoutova *et al*., 2000).

When the sclerotia of the ergot fungus fall in farm lands during harvesting, it grows club shaped structure with stalk, known as stroma (stromata). *Claviceps* species are known by producing a stipitate, spherical stromata within which perithecia are partially embedded (Alderman, 2003). Unfertilized ovaries are especially susceptible to infection. From the asci imbedded in the perithecia, thin, filiform-shaped ascospores are forcibly ejected out of mature asci and become airborne. Ascospores that land on stigmas of a susceptible host germinate and produce infection hyphae that grow down the element to infect the base of the ovary. Within several days of infection, a sphacelium producing large numbers of conidia develops. Sugary syrup derived from plant sap, along with conidia, ooze from infected florets in what is commonly referred to as the honeydew stage. Insects that visit the infected plant are normally responsible for dissemination of the asexual spores, conidia leading to secondary infection (Alderman, 2003).

Mature sclerotia of the ergot fungi are composed of toxic secondary metabolites known as ergot alkaloids. The majority of these ergot alakloids are composed of a tetra-cyclic ergoline ring structure synthesized from condensation of L-tryptophan and an isoprene unit. In general, more than 50 different ergot alkaloids are isolated from the sclerotia of ergot fungi. The amount and pattern of ergot alkaloids vary between fungal strains, depending on the host plant and the geographical region (Krska and Crews, 2008). Ergot alkaloids have long been used for treatment of migraine headache, Parkinson disease, and post partum hemorrhage among others. The activity of ergot alkaloids is due to their structural similarity to the neurotransmitters such as serotonin, nor-adrenalin and dopamine (Kobel and Sanglier, 1986).

Despite their long use as therapeutic agents, ergot alkaloids are also well remembered by toxic effects to humans as well as animals. The disease caused as a result of consumption of food contaminated with sclerotia of ergot alkaloids is known as ergotism. The disease manifests in two forms, the gangrenous form also known as St. Anthony's fire and the convulsive form known by the name St. Vitus dance. France and other European countries west of the Rhine River, outbreaks of ergotism were generally of the gangrenous type, where as in Central, Eastern Europe and Scandinavia, outbreaks were of the convulsive type (De Costa, 2002; Edie, 2003).

According to Gabbai *et al.* (1951), epidemics of ergot poisoning occurred in France due to consumption of bread prepared from ergotized rye. Both gangrenous and convulsive symptoms began to appear on August 1951, following a period of 6 - 48 hrs after the consumption of the ergot contaminated rye bread. During this epidemic 25 cases were of severe delirious forms and four cases comprising of three men and one woman died of cardiovascular collapse.

In Ethiopia, gangrenous ergotism occurred in Waro and Gazobelay sub Woredas, Wedla-Delanta and Lasta Awrajas of Wollo and resulted in the death of 47 individuals. Examination of 44 patients out of the 93 registered revealed ongoing dry gangrene of the whole or part of one or more limbs (7.5%), feeble or absent peripheral pulses (36.4%), swelling of limbs (11.2 %), desquamation of the skin (12.8%), and loss of one or more limbs (21.5%). It was noted that 88% of patients had involvement of the lower extremities. The most common general symptoms were weakness, formication, burning sensation, nausea, vomiting and diarrhea. In addition, 50-60 infants and young children died from starvation due to failure of the mothers to lactate (Teshome Demeke *et al*., 1979). In a more recent study by Kelbessa Urga *et al.* (2002), ergotism attributed to the ingestion of barley containing ergotized wild oats was reported in Arsi zone Tijo – Degelu Woreda about 70 km from Assela . According to this report all grain samples collected contained ergot alkaloids with a maximum concentration of ergotamine of 2.51 mg/100 g.

To the knowledge of the researchers, only these two studies were conducted in Ethiopia. The presence of ergotamine and ergometrine in the grain sample was reported by Kelbessa Urga *et al*. (2002). A study by Teshome Demeke *et al*. (1979) also showed the presence of ergometrine in the food samples analyzed. None of them, however, characterized the ergot fungus based on morphological as well as molecular features.

This research was initiated with the following general and specific objectives.

1.1. General Objective

- To characterize ergot fungus (*Claviceps purpurea*), ergot alkaloids and assess the level of awareness of the people living in the highlands of Arsi, Ethiopia about ergot fungus (*Claviceps purpurea*) and ergotism.

1.2 Specific objectives

- To determine the characteristics and diversity of the ergot fungus (*Claviceps purpurea*) in *Avena abyssinica* in Arsi, Ethiopia.
- To determine ergot alkaloids in the sclerotia of the ergot fungus(*Claviceps purpurea*) isolated from *Avena abyssinica*
- To assess the level of awareness of people living in the highlands of Arsi, Ethiopia about the ergot fungus (*Claviceps purpurea*) and ergotism.

Chapter 2. Literature Review

2.1. Diversity, Host range and Distribution of the genus *Claviceps*

Ergot is derived from the old French word argot, meaning the cock´s spur and represents the dark brown, horn-shaped pegs that project from ripening ears of rye in place of rye grains. It is the over wintering sclerotia of the fungus *Claviceps*. The ergot fungus is grouped in the Domain Eukarya; Kingdom *Fungi*; Phylum *Ascomycota*; Class *Sordariomycetes*; Order *Hypocreales*; Family *Clavicipiaceae* and Genus *Claviceps*. The *Clavicipitalean* fungi comprise all sexual and asexual relatives that fall within the phylogenetically defined ascomycete family *Clavicipitaceae* which include well known genera such as *Balansia, Claviceps, Epichloe, Metarhizium and Neotyphodium* (Alderman, 2003).

The order *Clavicipitales* consist of 27 genera and 270 species which are highly evolved and sophisticated parasitic fungi with frequently stalked stromata, long asci without apical rings, but with extremely thickened tips and long thread like ascospores that may fragment during or at release (Kendrick, 1992).

The genus *Claviceps* includes species adapted to a variety of niches in habitats ranging from marine to desert and subtropical to arctic, and most of the species of Claviceps have a host range limited to a genus or several genera of grasses (Alderman *et al*., 2004), sedges (Cyperaceae) and rushes (juncaceae) (Pazoutova, 2000). *Claviceps* species produce stipitate, spherical stromata within which the perithecia are partially embedded and are distributed over the surface resulting in punctate appearance (Alderman, 2003)

Ergot occurs worldwide, most commonly on rye and pearl millet, less often on wheat and certain wild and cultivated grasses, and rarely on barley and oats. An ergot, *C. gigantea*, affecting corn occurs in Mexico and caused 5 – 10 % of the grains in infected heads. Other species of the ergot fungus include *Claviceps paspali* on paspalem, *Claviceps fusiformis* on pearl millets, *Claviceps cyperi* on yellow and purple nut sedge (*Cyperus esculentus L*. and *Cyperus rotundus L.)* (*Cyperus esculentus* and *Cyperus rotundus* of the family Cyperaceae). Ergot disease of sorghum which is caused by the fungus *Claviceps africana* in South Africa (Frederickson *et al*., 1999) and *Claviceps sorghi* in India (Kulkarni *et al*., 1976), recently gained prominence due to sudden expansion in its

geographical distribution from Africa to Asia to America and Australia (Bandyopadhyay *et al.,* 1998)

In order to determine if the pathogen in America and Australia came from the same region Pazoutova *et al.* (2000), evaluated the relatedness of ergot strains from the U. S., Bolivia, Australia, Africa and India. The RAPD banding pattern and other molecular techniques grouped the isolates from the U.S. and Bolivia in one group, and the isolates from Australia and India in another group. Thus, it is believed that isolates from North and South America came from the same clone; whereas, isolates from Australia came from a different clone, related to the Indian isolates.

According to Frederickson *et al*. (1999), the disease affects unfertilized ovaries by replacing them with white sporulating fungal mass, the sphacelium, from which sticky conidia containing honey dew oozes and facilitates rapid infection leading to predisposition of seeds to seedling disease, reduction in quantity and quality of seeds and difficulty harvesting and threshing.

2.2. Life cycle of the ergot fungus

The parasitic life cycle of *C. purpurea* starts with wind borne ascospores shot away from a perithecium. Ascospores germinate on the stigma of the susceptible host to form intracellular mycelium that can grow down to the ovary towards the vascular bundles of the floret stalk (rachilla), and get accesses to photosynthetic products produced by the host plant. Several days after infection, large numbers of different types of spores (conidia), haploid vegetative asexual spores, are produced in a sweet liquid called 'honey dew', which contains glucose, sucrose and fructose, and these spores can spread the infection by direct contact, rain splash or by insect vectors to new grass or cereal flowers during the same season (Alderman, 2003).

These 'secondary' infections therefore occur on grasses or cereals flowering several days at least after the initial 'primary' infection (Kobel and Sanglier, 1986). Infection of host plant by Claviceps results in increased translocation of water and sucrose towards the diseased flower. Thus, the infected flowers get more photosynthetic products from the host than uninfected flowers which can facilitate the proliferation of the fungus. The ovaries are then replaced by fungal tissue which is longer than the ovary it replaces and develop in to sclerotium up to 3 cm in length. The sclerotium then falls to the ground and over winters in or on the soil until the suitable period comes for its germination (Alderman, 2003).

Some species of Claviceps such as *C. africana*, *C. fusiformis*, *C. cynodontis* , *C. paspali* and *C. sorghi*, produce two types of anamorphic spores that differ in size and mostly divide into microconidia, measuring about 6 x 2.5 µm, and macroconidia, measuring about 16 x 4µm . Firstly, the honeydew contains macroconidia, often microconidia as well, which are able to germinate in the honeydew just below the syrup surface. Secondly, conidiophores emerge and differentiate "secondary conidia" outside the liquid in a secondary conidiation cycle. Masses of conidia, mostly microconidia, whiten the surfaces of sticky colorless honey dew droplets one day after their exudation. Since both conidia types can initiate infection, these ergot fungi spread in the field by a second airborne inoculum in addition to the transmittance of macroconidia with the honeydew (Tenberge, 1999).

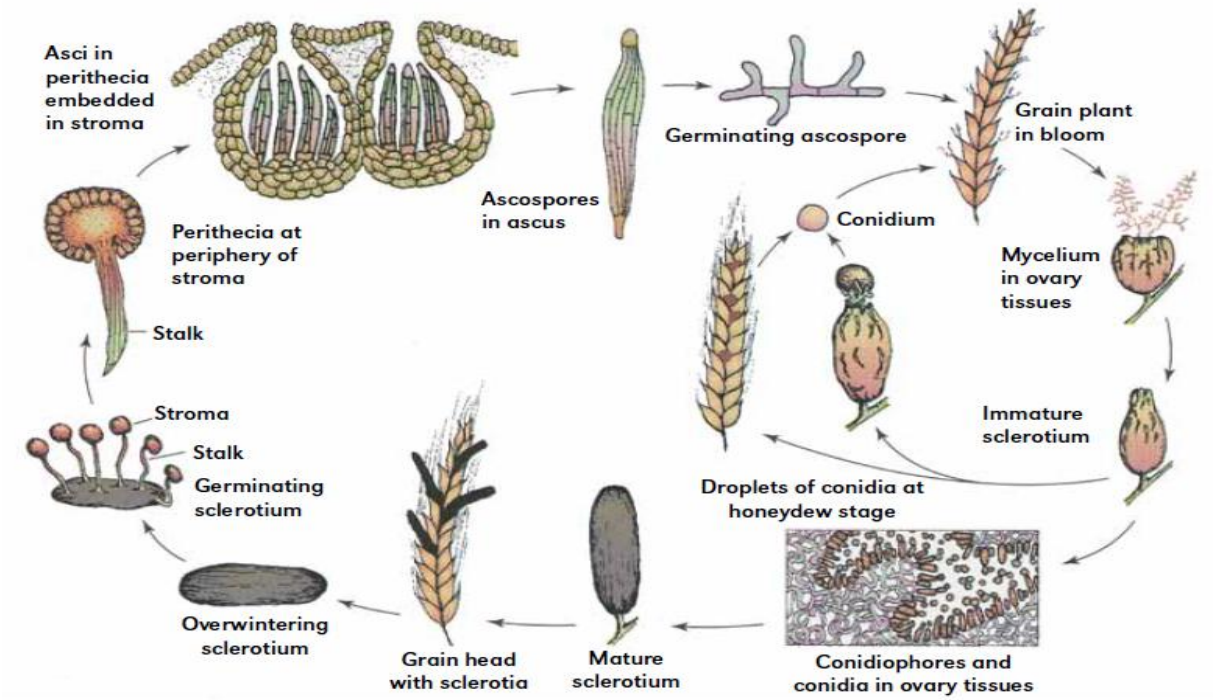

Fig. 2.1: Life cycle of ergot fungus (Agriose, 2005)

2.3. Ergot alkaloids

Ergot alkaloids are secondary metabolites produced by fungi of several species of the genus *Claviceps*, most notably by *Claviceps purpurea*, which parasitize the seed heads of living plants at the time of flowering. Although fungi are the primary source of ergot alkaloids, ergot alkaloids are also synthesized by some plants, mainly of the morning glory family (Wilkinson *et al*., 1988). Other important sources of ergot alkaloids include grasses infected with endophytes, such as *Claviceps spp*. or *Acremonium coenophialum* (Petroski *et al*., 1992).

More than 50 different ergot alkaloids were isolated from the sclerotia of ergot. The amount and pattern of ergot alkaloids vary between fungal strains, depending on the host plant and the geographical region (Krska and Crews, 2008). Their interactions with many receptor sites and the variation of activity and affinity from alkaloids to alkaloids are the main pharmacological features of the ergot compounds. The structural analogy between ergoline and different neurotransmitters (serotonin, nor adrenaline and dopamine) (Fig.2.2) appears to be the basis for their diverse activities (Kobel and Sanglier, 1986).

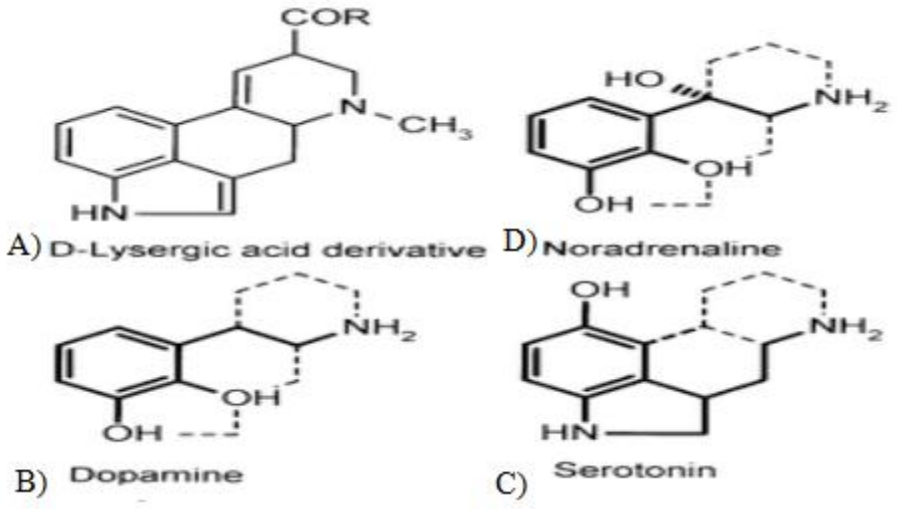

Fig. 2.2: Structural analogy between the ergoline ring system (A) and neurotransmitters (dopamine, B; noradrenalin, D and serotonin, C).

On the other hand, ergot alkaloids also have a long history of animal and human toxicosis. In humans, consumption of ergot contaminated food led to development of limb gangrene and hallucinations (De Costa, 2002). Intoxications with ergot alkaloids have also occurred due to chronic use of ergot alkaloids as medical therapy for Parkinson's disease. Similarly, ergot toxicity has occurred in animals fed on ergotized forage (Kopinski *et al.,* 2007) and endophyte infected grasses (Naude *et al*., 2005)

2.3.1. General characteristics of ergot alkaloids

Ergot alkaloids are groups of biologically active secondary metabolites produced by various members of the Clavicipitaceae, including some members of the genera, *Claviceps, Epicole* and their closely related *Neotyphodium* anamorphs), and *Balansia*, that typically occur as pathogens (Kobel and Sanglier, 1986).

The common structural component of most of the ergot alkaloids is a tetracyclic ergoline ring (Fig. 2.3), and based on the structure of ring D in the ergoline nucleus and the types of substituents at C8, all ergot alkaloids can be divided into groups such as clavine ergot alkaloids which are hydroxyl- and dehydro- derivatives of 6, 8-dimethylergoline, (e.g. agroclavine), simple lysergic acid derivatives (eg. ergometrine, Fig. 2.4), peptide ergot alkaloids or ergopeptines (Fig. 2.5) (eg. ergotamine) with an additional peptide moiety linked to the basic tetracyclic ergoline and lactam ergot alkaloids (Fig. 2.5) (eg. ergopeptam) (Kobel and Sanglier, 1986; Flieger *et al*., 1997).

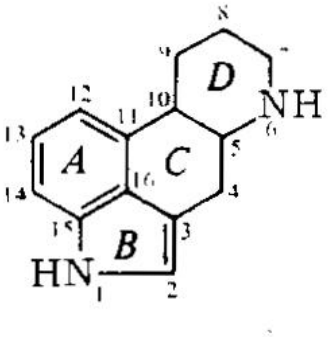

Fig. 2.3 : Chemical structure of ergoline ring

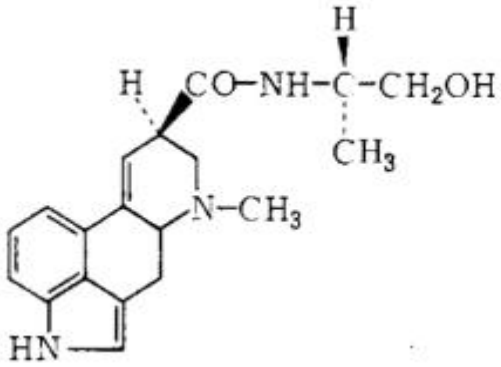

Fig. 2.4 : Chemical structure of ergometrine

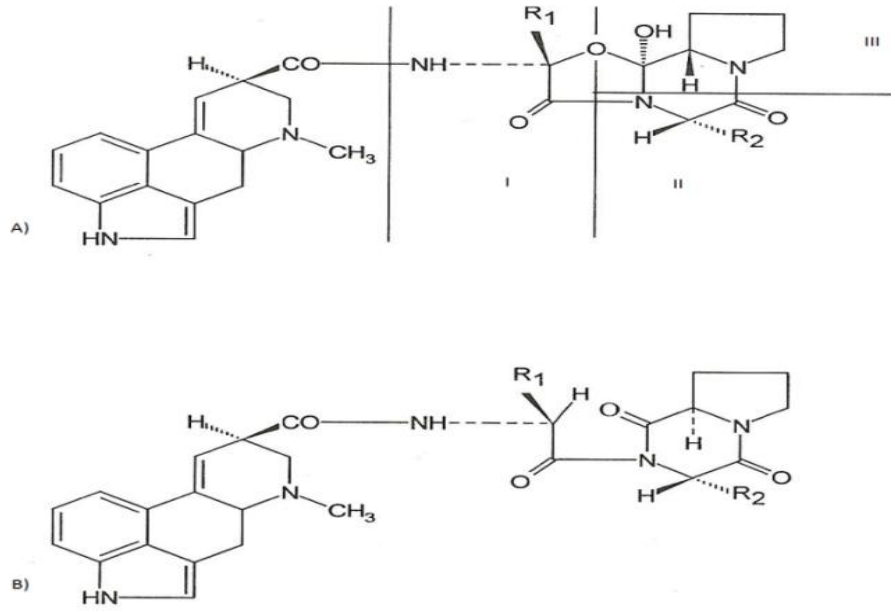

Fig. 2.5 : Chemical structure of ergopeptines (A) and chemical structure of ergopeptam (B)

Ergot alkaloids appear as colorless crystals that are readily soluble in various organic solvents, but insoluble or only slightly soluble in water. Ergot alkaloids containing C9=C10 double bond (ergolenes) readily exhibit epimerisation, especially in the presence of alkalis, with respect to the centre of symmetry at C-8 (Lehner *et al*., 2005).

The nomenclature of ergot alkaloids is very complex and systematic names are used only for the semisynthetic ones. The name for most of the ergot alkaloids is based on the producing organisms or the host plant eg. agroclavine and pyroclavine (Agropyrum), elymoclavine (*Elymus mollis*), setoclavine and penniclavine (Pennisetum), paspalic acid (Paspalum), festuclavine (Festuca), ergosecaline (Secale), however, some of the trivial names of ergot alkaloids are related to the name of a person eg. ergocristine (Cristine), ergoanname (Anna), ergoladinine (Ladislav Cvak), ergogaline (Galena) and the pharmacologic action of the ergot alkaloids eg. ergometrine (*Endometrium uteri*) (Buchta and Cvak, 1999).

2.3.2. Pharmacology of ergot alkaloids

The pharmacological activities of ergot alkaloids are mostly due to the structural similarity between D-lysergic acid derived compounds and neurotransmitters like noradrenalin, dopamine and serotonin (Kobel and Sanglier, 1986). Ergot alkaloids can interact with receptors for these neurotransmitters either as agonist or antagonist, depending on the substituents attached to the carboxyl group of D-lysergic acid (Stadler and Giger, 1984). Major pharmacological activities are described below

2.3.2.1. Ergot alkaloids for the treatment of migraine

Migraine is a common and frequently disabling headache disorder characterized by recurrent episodic attacks of moderate to severe headache variably accompanied by neurological, gastrointestinal and/ or autonomic symptoms. It is usually characterized by severe pain on one or both sides of the head, an upset stomach, and sometimes disturbed vision (Tripathi, 2006)

There are two major forms of migraine: migraine without aura (common migraine) and migraine with aura (classical migraine), migraine with aura consists of focal neurologic symptoms (usually visual symptoms) that precede or accompany headache, and it appears in 15-25% of migraine sufferers (Kelly, 2000).

Headache, and more particularly migraine, is a frequent health problem in children and adolescents. The prevalence peaks are between ages 25 and 55, the most productive years of life (Belvís *et al.,* 2009). Despite its prevalence, migraine remains commonly undiagnosed or misdiagnosed, just as in adults in whom migraine is often attributed to sinus disease (Cady, 2002)**.** Four of every ten women and two of every ten men will contract migraine in their lifetime, most before age 35 years (Stewart *et al.,* 2008), in women migraine is commonly associated with menstruation.

The pathophysiology of migraine is not precisely known, in earliest time it was believed to be due to evil beings within the head; treatment based on this idea included incantations and application to the head of substances intended to drive out the demons and spirits. Traditionally there are two theories about pathophysiology of migraine headache (vascular theory and neurogenic theory).

In the 1940s and 1950s, the vascular theory was proposed to explain the pathophysiology of migraine headache, according to this theory ischemia induced by intracranial vasoconstriction is responsible for the aura of migraine and that the subsequent rebound vasodilation resulted in headache (Nissan and Diamond, 2005)

The neurogenic theory on the other hand offers the hypothesis that migraine originates from neuronal dysfunction. Leao (1944) proposed the theory of cortical spreading depression (CSD) to explain the mechanism of migraine with aura. Cortical spreading depression is a well-defined wave of neuronal excitation in the cortical gray matter that spreads from its site of origin at the rate of 2-6 mm/min. This cellular depolarization causes the primary cortical phenomenon or aura phase; in turn, it activates trigeminal fibers causing the headache phase.

According to more recent theory (neurovascular theory) complex series of neural and vascular events initiate migraine, and migraine with aura is thought to occur due to neuronal hyper excitability (May and Goadsby, 1999).

In ancient time the use of clay effigy of a sacred crocodile with herbs stuffed into its mouth bound to the head of the patient was used as a prescription for migraine headache believing the gods could cure their aliments (Fig. 2. 6) (Villalón , 2003)

Fig. 2.6: Ancient methods attempting to alleviate or cure headache: Egyptian papyrus (2500 BC) which describes bandaging a clay crocodile (with herbs stuffed into its mouth) to the head of the sufferer and praying (Villalón , 2003)

Approaches to treat migraine can be divided into non pharmacologic therapies and pharmacologic therapies. Non-pharmacologic therapies include education of the patient about the disorder, its mechanisms, approaches to treatment, and changes in lifestyle involved in the avoidance of triggers of migraine (Goadsby *et al.,* 2002).

On the other hand, pharmacologic therapies of migraine depend on the severity of the attack, for mild migraine (fewer than one attack per month with tolerable headache that can last for up to 8 hrs) simple analgesics (paracetamol or aspirin), non-steroidal anti-inflammatory drugs (NSAIDS) or anti-emetics can be used. For moderate migraine where the head ache is more intense and one or more attacks that can last 6 – 24 hrs occur per month with prominent nausea/vomiting NSAIDs combinations, ergot alkaloids and sumatriptan can be used. Whereas for severe migraine with 2 – 3 or more attacks per month, severe headache that can last 12 – 48 hours and accompanied by vertigo, vomiting and other symptoms relief specific drugs like ergot alkaloids (ergotamine, dihydroergotamine) or sumatriptan is used (Tripathi, 2006).

Among ergot alkaloids, dihydroergotamine, available as intranasal, subcutaneous, intramuscular or intravenous formulations, has been shown to be effective and well tolerated. It binds to a number of other receptors, including 5-HT1B and 5-HT1F receptors, which may augment its efficacy in migraine. It also binds at 5-HT1A and 5-HT2A receptors, as well as alpha and beta adrenergic receptors and dopaminergic receptors. Binding at these latter sites is responsible for many of the adverse effects observed after DHE administration (Silberstein and Mc Crory 2003; Saper and Silberstein, 2006).

Ergotamine is another ergot alkaloid which is effective for the treatment of migraine. If given at the early stage of attack, relief is often dramatic and lower doses are enough but when pain become severe larger doses are needed and control may be achieved only after few hours. Ergotamine acts by constricting the dilated cranial vessels. Ergotamine is shown to reduce neurogenic inflammation and leakage of plasma in duramater perivascular afferent nerves and this action appears to be through 5 – HT 1B/1D in and around cranial vessels (Tripathi, 2006).

2.3.2.2 Ergot alkaloids for the treatment of Parkinson's disease

Parkinson's disease (PD) is a common neurodegenerative disorder that occurs due to degeneration of the nigrostriatal dopamine tract leading to decreased production and release of dopamine. The disease is characterized by the presence of bradykinesia (slow movement), rigidity, involuntary movements of the upper limbs and postural instability (Hughes *et al.* 1992). The prevalence of Parkinson's disease in industrialized countries is 0.3 % of the general population and it affects older age groups. It occurs throughout the world in all ethnic groups and affects both sexes roughly equally with a little predominance in males. The lowest incidence is reported among Asian and African blacks where as the highest is among European and North Americans (Inmadar *et al.,* 2007). Patients with Parkinson's disease (PD) are troubled not only by motor dysfunctions but also by non-motor dysfunctions such as neuropsychiatric symptoms, sleep disorders, sensory symptoms, and autonomic dysfunctions (Chaudhuri and Schapira, 2009).

Drug therapy of PD involves the use of levodopa (L – DOPA) which can replace endogenous deficient neurotransmitters by undergoing decarboxylation in the presynaptic terminal to form dopamine (DA) (Lim, 2005), and dopamine agonists that act directly on DA receptors mimicking endogenous neurotransmitters (Quinn, 1995). Dopamine agonists (DAA) are classified ergot derivatives (bromocriptine, lisuride, pergolide and cabergoline) and non-ergot derivatives such as apomorphine, pramipexole and ropinirole.

Although L-DOPA is the most effective drug for the treatment of Parkinson's disease (Silva *et al.,* 1999)**,** it is potentially neurotoxic. As a result of which D_2 receptor agonists such as (Bromocriptine, pergolide, ropinirole, pramipexole and cabergoline), have been introduced in to clinical practices for improving symptoms of PD and preventing L – DOPA induced neurotoxicity (Jankovic, 2001).

Dopamine agonists (DAA) are also used as efficacious adjunct treatment to L – DOPA in patients with advanced PD. They are also gaining importance as mono therapy in the early stage of PD. For young – onset patients at high risk of developing motor complications initiating symptomatic treatment with DAA of L – DOPA has been recommended (Olanow *et al.,* 2001).

There are several theoretical advantages of DAA over L-DOPA. First, they usually have a long duration of action that more closely mimics the physiological tonic release of DA from normal nigral neurons and may help to prevent or reduce motor fluctuations (Chase *et al.,* 1996). Therefore, high doses of DA might allow a reduction in L-DOPA daily dose, consequently reducing its adverse effects.

Bromocriptine has been in regular use as adjunct therapy in patients receiving L-DOPA to allow lower doses of L-DOPA to be used and to improve "end of dose" motor fluctuations. Use of bromocriptine as mono therapy in patients starting treatment for the first time has been shown to delay the need for L-DOPA treatment and the occurrence of motor complications (Montrastruc *et al*., 1994).

According to a study conducted by Parkes *et al.* (1976), bromocriptine 2.5-300 mg daily is an effective anti- Parkinsonism drug that causes about a 20-30 % improvement in tremor, rigidity, akinesia, postural deformity, and total functional disability scores. The response to bromocriptine is rapid in onset, occurring within a few days of starting treatment, and tolerance to the anti-Parkinsonism action of bromocriptine does not develop during a year of treatment.

Another ergot alkaloid, α-dihydroergocryptine (DHEC), a well known dopaminergic agent (D_2 agonist and partial D_1 agonist) has been successfully employed in the treatment of PD. It has showed a neuroprotective activity against total cerebral ischemia induced by $MgCl_2$ in mice and histocytic anoxia by NaCN in mice and rats. Moreover, α -DHEC showed a protective activity on neuronal degeneration induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in monkeys, as evaluated through animal's behavior and morphological-cytochemical changes in the substantia nigra, suggesting a preservative effect on neuronal morphology and brain architecture (Coppi, 1995).

Cabergoline, a long acting ergoline DAA with selective affinity for D2-like dopamine receptors and a long plasma half life of 65 hours has also been used for treatment of Parkinson's disease (Rinne *et al*., 1997). It is also effective as adjunct therapy to L-DOPA in patients with advanced PD (Del Dotto *et al*., 1997). Besides, in some patients with complicated PD, high (4 mg), doses of pergolide have been shown to reduce motor fluctuations and achieve good control of Parkinsonian signs and symptoms without the need for L-DOPA treatment.

Despite all these, the use of ergot-derived DAA is now less recommended due to the reported risk of cardiac valvular fibrosis (Horstink *et al*., 2006)

Peralta *et al.* (2006), used serial echocardiographic assessments to investigate the prevalence of heart-valve abnormalities in 75 patients with PD who received pergolide, cabergoline, or non-ergot DAA (such as pramipexole or ropinirole) and 49 age-matched, non-Parkinsonian controls. Significantly greater frequencies of valvular insufficiencies graded moderate or severe were found in people with PD treated with cabergoline or pergolide compared with those on non-ergot derivatives, who had a rate of valvular insufficiencies (10%) similar to that in age-matched, healthy controls.

In another study, 31 new cases of cardiac valve regurgitation were identified on the basis of clinical, echocardiographic, or cardiac catheterization criteria. Among the 31 patients with PD, six were taking cabergoline and six were taking pergolide, whereas 19 had never taken DAA in the year before. Significant cardiac valve regurgitation was observed for pergolide and cabergoline treated groups, but not with the other regimens (Schade *et al*., 2007). Similarly, Yamamoto *et al*. (2006), also reported significantly ($p < 0.05$) increased risk of valvular regurgitation in patients taking cabergoline and positive associations with its cumulative dose and duration of treatment.

2.3.2.3. Ergot alkaloids for the treatment of hyperprolactinomas

Prolactinoma (PRLomas) are the most common subtypes of functioning pituitary tumors that produce and release the prolactin hormone. They are classified based on their size as microadenomas (< 1 cm in diameter) and macroadenomas (> 1 cm in diameter) where more than 90 % of the prolactinomas are classified as microprolactinomas (Molitch, 1997).

Elevation of the level of prolactin due to production by pituitary tumors leads to hyperprolactinemia, which is characterized by gonadal dysfunction, decreased or complete absence of menstruation, increased milk secretion not related to breast feeding, infertility and reduced libido and, if left untreated, it is associated with an increased risk of long-term complications, such as osteoporosis. It is estimated that among women presenting with reproductive disorders, approximately 15 % with anovulation (menstrual cycle in which ovulation fails to occur) and 43 % with anovulation and galactorrhea (milk secretion usually not due to breast feeding).

Pharmacological intervention with DAA is considered the first-line therapy for patients with prolactinomas, because DAA therapy for hyperprolactinemia has become widely accepted as a primary therapy after several studies demonstrated inferior surgical cure rates (Gillam *et al.,* 2006). By mimicking the action of DA, bromocriptine, quinagolide and cabergoline are able to lower prolactin levels, decrease in prolactinoma size and restore ovarian functions. Inhibition of prolactin secretion by binding cell surface D2 receptors located on mammotropes, anterior pituitary cells that produce prolactin in response to signals including DA, estrogen, progesterone and thyrotropin-releasing hormone, and reduction of tumor size is by inducing reduction in cell volume by inhibiting early secretary mechanisms and inhibition of gene transcription (Babkowski and Zacur, 2003).

Bromocriptine, an ergot-derivative that activates the D2 receptor, has been the most widely used drug for treating this type of tumors and is administered daily at doses ranging from 5 to 20 mg. It normalizes the elevated prolactin level in many patients and also exerts an antitumor effect (Colao *et al*., 1997). However, the selective D2 receptor agonist cabergoline is more effective and better tolerated than bromocriptine and is also effective in treatment of tumors resistant to other DAA (Webster *et al*., 1994; Colao *et al*., 1997).

A study conducted by Gruszka *et al*. (2001), pointed out the antitumor activity of octreotide and bromocriptine separately and in a combination. Bromocriptine significantly suppressed the prolactinomas in the experimental rat, and the mechanisms of action of bromocriptine were associated with apoptosis.

In a long term (2 – 12 years) treatment of 51 patients with hyperprolactinemia (23 with macroadenoma, 23 with microadenoma and five with idiopathic hyperprolactinemia), bromocriptine therapy reduced the serum prolactin level to a normal range in all patients except five. In three out of the five patients gonadal functions returned to normal in spite of high circulating serum prolactin level. However, in the other two patients the gonadotropin reserve was impaired even before the therapy (Wang *et al*., 1987).

Although none of the published papers on prolactinoma patients to date have shown an increased risk of clinically significant cardiac valve disease and although most papers have failed to show an association between DAA therapy in prolactinoma patients and echocardiographic evidence of

valvular abnormalities, an increased risk of tricuspid valve regurgitation was found in one report (Colao *et al*., 2008).

2.3.2.5. Use of ergot alkaloids in the third stage of labour and prevention of post partum haemorrhage

The third stage of labour is the time from the birth of the baby to the expulsion of the placenta and membranes. Once the baby is born, the uterus continues to contract and reduces in size. There is a lack of full understanding of the physiology of the third stage of labour, but recent work using ultrasonography has demonstrated that the process of placental separation has three distinct phases. The first, or latent phase, consists of strong uterine contractions, which leads to thickening of the uterine muscle, thus causing a shearing force to occur between the elastic uterine wall and the more rigid placenta, followed by separation of the placenta due to continued contraction and delivery of the placenta in the expulsion phase (Herman *et al*., 2002).

Postpartum haemorrhage (PPH) is defined as blood loss of ≥ 500 ml that encompasses excessive blood loss after delivery and, if untreated, may result in shock and death of the mother. The choice of 500 ml is arbitrary but is a loss that most mothers can tolerate without risk. In countries where many women have severe anemia, maternal blood loss of even 250 ml may be fetal. The clinical consequences of PPH depend on both the amount and the rate of blood loss and whether the mother's health is good, a factor partly included in the definition of PPH (de Groot, 1996). The uterus muscle is composed of a unique interlacing network of muscle fibers, the blood vessels that supply the placenta pass through these networks of muscle fibers. After the delivery of the infant, when the placenta separates from the uterine wall, these fibers contract and constrict the uterine wall. This blood saving mechanism is known as ‘living ligature’ or ‘physiological suture’ of the uterus. This is one of the efficient physiological efficient methods of preventing blood loss (Baskett, 2000).

During the epidemics of ergotism women miscarry, therefore midwives reasoned the ergot must caused uterine contraction and started to use it for prolonged labour with efficient uterine contraction. The use of ergot alkaloids for uterine contraction was documented by Adam Lonicer in his 1582 herbal book. He noted that the ergot spurs in a diseased rye are a special medicine for

women in labour and for the purpose of awakening the pains three of the ergot sclerotia were swallowed (Moir, 1955).

However, frequent uterine ruptures, stillbirth, maternal death and ergotism (gangrene and convulsive forms) from inaccurate doses of the ergot alkaloids occurred. Therefore, after 1828 the ergot alkaloids were no longer used during delivery but only as a measure to prevent postpartum haemorrhage (Van Dongen, 1995; De Groot, 1998).

Mechanisms of preventing PPH vary depending on the uterotonic agents used. It is clearly indicated that methylergometrine which is the most common type of ergot alkaloid increases the muscle tone of the uterus, with superimposed fast rhythmic contractions of the myometrium and tetanic contraction for several hours resulting in compressed myometrial blood vessels. Oxytocin acts through oxytocin receptors in myometrium and leads to fast and long-lasting contractions upon basal tone of the myometrium. Syntometrine, consisting of five units of oxytocin and 0.5 mg of ergometrine, has been designed to take advantage of the rapid onset of action of oxytocin with longer action of ergometrine (De Groot, 1995).

The use of the combination preparation of ergot alkaloid plus oxytocin, syntometrine, is associated with a statistically significant reduction of PPH when compared with oxytocin alone, attributable to the ergometrine effect. However, a statistically significant difference was observed in the presence of maternal side-effects, including elevation of diastolic blood pressure, vomiting and nausea, associated with ergometrine-oxytocin use compared to the use of oxytocin alone (McDonald *et al*., 2004).

2.3.2.6. Ergot alkaloid for the treatment of type 2 Diabetes

Diabetes mellitus (DM) is a chronic metabolic disorder caused by inadequate insulin secretion, insulin resistance (a state in which a normal amount of insulin produces a subnormal biological response), or both, and T2DM is a non-insulin dependent diabetes mellitus (NIDDM) that occurs due to impaired ability of the target cells to respond to insulin, which is usually caused by insulin receptor resistance. It is characterized by elevated postprandial (after meal) plasma glucose concentrations, which result from increased endogenous glucose production (EGP), decreased insulin-mediated muscle glucose disposal and suppression of endogenous glucose release, and

inadequate pancreatic insulin secretion (De Fronzo, 1997). It is estimated that approximately 350 million people will be afflicted by T2DM by the year 2030 (Roglic *et al*., 2005).

In non-diabetic people post meal conditions cause a decline in endogenous glucose production due to suppression of glucagon and inhibition of lipolysis. However, in individuals with T2DM a drop in DA level is thought to lead to an inadequate hypothalamus response, resulting in elevated levels of blood glucose, free fatty acids, and triglycerides, which contribute to insulin resistance, visceral adiposity and beta cell dysfunction (Pal, 2011).

Obesity, sedentary lifestyles and diets that are rich in fats are known risk factors for diabetes. Both central obesity and high fat diets induce insulin resistance, which leads to hyperinsulinemia, the compensatory response to insulin resistance. Certain ethnic groups such as those of African, Hispanic, Native American and Asian descent are particularly vulnerable to diabetes and its complications. Hyperinsulinemia is often associated with further weight gain, which exacerbates hyperglycemia and leads to chronic over-production of insulin (Lenhard and Gottschalk, 2002).

An ergot derivative such as bromocriptine – QR (quick release formulation of bromocriptine, cycloset) formulation with a well known dopamine agonist activity has been used for the treatment of type 2 diabetes mellitus. It was designed to provide a short duration pulse of this dopamine agonist to centers in the brain (Luo *et al*., 1997; Luo *et al*., 1999). The addition of bromocriptine to routine standard therapies was shown to be safe and associated with fewer cardiovascular outcomes.

A clinical trial revealed improvement of HbA1C (glycated hemoglobin) level, postprandial blood glucose (PPG) and fasting plasma glucose levels after the use of bromocriptine-QR as monotherapy and as an adjunct to other oral diabetes medications, such as sulfonylurea and metformin (Cincotta *et al.*, 2010). A significant decrease in post-meal glucose concentration (excursions) throughout the day after bromocriptine treatment was observed in obese diabetic subjects (Cincotta *et al.,* 1997).

In a double-blind, placebo-controlled trials by Pijl *et al.* (2000), significant metabolic improvements were observed in obese patients with T2DM. At the end of this study, the fasting plasma glucose level decreased by 17 mg/dL and the mean change in plasma glucose after an oral glucose tolerance test decreased by 22 mg/dL and glycosylated hemoglobin (HbA1c) was reduced by 0.6% compared to placebo.

In another double-blind, placebo-controlled trial that involved 40 subjects followed for three months, the fasting glucose level decreased by 27 mg/dL and glycated hemoglobin (Hb A1C) remained unchanged (Aminorroaya, *et al*., 2004). In addition, bromocriptine- QR in a daily dose ranging from 1.6 to 2.4 mg produced a significant weight loss in 17 obese subjects with impaired glucose tolerance (Cincotta *et al.,* 1999). Bromocriptine-QR did not increase the level of insulin; rather it reduces postprandial glucose by improving the body's responsiveness to insulin.

Finally, in an open-label study, bromocriptine reduced body fat stores, improved glycemic control, and diminished the need for oral hypoglycemic agents in obese patients with T2DM (Cincotta and Meier, 1996).

2.3.2.7 Pharmacokinetics of ergot alkaloids

Bioavailability of ergot alkaloids following oral, rectal, sublingual, and intramuscular or aerosol administration depends on the type of ergot alkaloids. For instance, hydrophilic amides like the ergometrine group are rapidly absorbed from the oral route while the less water soluble ergotamine groups have a lower oral bioavailability (Aelling and Nuesch, 1977). On the other hand, ergotamine suppositories increase bioavailability 20 times compared to oral administered doses.

Human and animal experiments suggest that ergot alkaloids are rapidly absorbed and disappear from blood and tissues with a high first pass clearance by the liver. The cytochrome P 450 enzyme system (particularly CYP3A4) plays a major role in elimination through biotransformation (Moubarak *et al.,* 2002).

Ergot alkaloids are excreted primarily via the urinary system as lysergic acid amide or biotransformed ergopeptine alkaloids in cattle (Stuedemann *et al.,* 1998). In horses, ergovaline is excreted solely via the fecal route, whereas lysergic acid is excreted via both fecal and urinary routes (Schultz *et al*., 2006). The major excretory route of dihydroergotamine is through the feces, and only 6% - 7% of unchanged dihydroergotamine is excreted in the feces after intramuscular injection.

2.3.3 Toxicology of ergot alkaloids

2.3.3.1. Toxicity of ergot alkaloids in humans

Sclerotia of many of the *Claviceps* spp. contain toxic alkaloids, and their presence in feed, pasture grasses or food can cause poisoning, ergotism, when ingested by animals, poultry and humans. As noted on an Assyrian cuneiform tablet of around 600 BC ergot was considered as "noxious pustule in the ear of grain", and also mentioned in one of the sacred books of Parees (400 BC to 300BC) as "grasses that cause pregnant women to drop the womb and die in child birth" (De Costa, 2002).

Intoxications induced by *C. purpurea* have been known in Europe for many centuries. The most severe effects of ergot contaminated grains are described in the medieval literature as St. Anthony´s Fire or Holy Fire, with respect to the intense pain resulting from vasoconstriction and subsequent gangrene with loss of fingers, hands, feet and even entire limbs. Other symptoms of ergot alkaloid intoxication include abdominal pains, vomiting, burning sensations of the skin, insomnia and hallucinations (Gabbai, 1951).

The symptoms of ergot alkaloid poisoning vary, probably depending on the particular profiles of alkaloids present in the contaminated flour. Two syndromes have been described as convulsive and gangrenous ergotism. Gangrenous ergotism results from the extreme vasoconstrictive properties of certain ergot alkaloids (ergopeptines), resulting in ischaemia (restricted blood-flow to parts of the body). Limbs may become hypoxic, develop dry gangrene, and self-amputate or require amputation. On the other hand, the symptoms of convulsive ergotism are involuntary muscle contractions, painful flexion or extension of the fingers, wrists, and ankles, involuntary twisting (such as wryneck), paresthesia (skin-crawling and tingling), vertigo, headaches, double-vision, profuse sweating, fever, hallucinations and mania (Eadie, 2001).

In Pont Saint Espirit (Bridge of the Holy Spirit) in France, there was an outbreak in which 200 people in a village of 4000 were affected. Victims suffered hallucinations and were described as running crazed in the street. Those afflicted were described as writhing in agony. Vasoconstriction led to the loss of fingers, toes, and in extreme cases hands and feet. The outbreak was attributed to the ingestion of rye flour contaminated with ergot (Meggs, 2009).

According to De Costa (2002), when large numbers of people came down with the symptoms of ergotism, especially convulsions and hallucinations, many in the 16th and 17th centuries concluded that they must have been victims of witchcraft and witches were blamed for the symptoms, and researchers examined the possible role of ergotism in the Salem, Massachusetts witch trials of 1692.

Moreover, convulsive ergotism has been suggested as a possible scientific explanation for some of the outbreaks of dancing mania, a phenomenon that occurred primarily in mainland Europe from the 14th to the 17th centuries and that was characterized by mass hysteria, uncontrolled ecstatic body movements, convulsions, and hallucinations. An Italian variant was known as tarantism because the sick were believed to have been bitten by the tarantula spider, for which the only cure was thought to be frenetic dancing to certain music, which supposedly dissipated the "poison" from their blood (Lapinskas, 2007).

In Ethiopia, ergotism occurred in Waro and Gazobelay sub Woredas, Wedla-Delanta and Lasta Awrajas of Wollo administrative regions, following two years of drought. During this time, the locally grown barley, the staple food, had become dominated by wild oats heavily contaminated with *C. purpurea* sclerotia. A total of 93 cases of ergotism were reported during the spring of 1978. More than 80% of affected persons were between 5 and 34 years of age. In addition to the 93 cases, 47 deaths were reported as having been due to ergotism. Examination of 44 patients out of the 93 registered revealed ongoing dry gangrene of the whole or part of one or more limbs (7.5%), feeble or absent peripheral pulses (36.4%), swelling of limbs (11.2 %), desquamation of the skin (12.8%), and loss of one or more limbs (21.5%). It was noted that 88% of patients had involvement of the lower extremities. The most common general symptoms were weakness, formication, burning sensation, nausea, vomiting and diarrhea. In addition, 50-60 infants and young children died from starvation due to failure of the mothers to lactate (Teshome Demeke *et al*., 1979)

In a more recent study by Kelbessa Urga *et al.* (2002), ergotism attributed to the ingestion of barley containing ergotized wild oats was reported in Arsi zone Tijo – Degelu Woreda about 70 km from Assela. According to this report all grain samples collected contained ergot alkaloids with a maximum concentration of ergotamine of 2.51 mg/100 g. Acute toxicity studies were also conducted by feeding male, non-pregnant and pregnant Swiss albino mice, with the collected grain

samples. A high mortality rate among mice was observed (55%), and cases of abortion were noted after 3 days of feeding, in all pregnant mice.

2.3.3.2. Toxicity due to misuse of ergot alkaloid drugs

Drug induced toxicity of the ergot alkaloids could be due to inappropriate dosage of the drug or due to drug interactions. Ritonavir, a competitive inhibitor of cytochrome P450 3A4 (CYP3A4), increases the concentration of ergotamine by inhibiting its metabolism, an effect that can produce symptoms of ergotism at low ergotamine doses (Dresser, 2000; Edie, 2001). Drug interactions may be most apparent when patients are stabilized on the affected drugs (ergotamine, triptans) and the CYP3A4 inhibitor is then added to the treatment regimen. Also, ergot concentrations are probably increased to toxic amounts because HIV-protease inhibitors and macrolides block ergotamine the metabolism (Liaudet, 1999).

The use of high doses of the ergot - derived dopamine agonist cabergoline (> 3 mg/day), especially with cumulative doses > 4000 mg, has been associated with an increase in cardiac vulvular thickening and significant (moderate to severe) regurgitation (Herring *et al*., 2009). Similarly, Ling *et al.* (1999), diagnosed constrictive pericarditis in a patient with PD receiving cabergoline therapy (10 mg) daily, who had symptoms and signs of congestive heart failures.

Garcia *et al*. (2000), presented a case of long-term ergot use for migraine headaches in a woman who had severe chronic lower extremity claudication. This case demonstrates the unique features associated with the diagnosis and management of chronic ergot toxicity.

2.3.3.3. Toxicity of ergot alkaloids in animals

Ergotism is an important veterinary problem particularly in cattle, horses, sheep, pigs and chicken (Benneth and Klich, 2003)**.** It can occur due to consumption of ergot contaminated feed (Hogg, 1991) and/ or endophyte infected grasses.

Some of the symptoms of ergot alkaloids poisoning include increased respiration rate, rectal temperature, salivation, nervousness, decreased weight gains, necrosis of the ears, gangrene of the limbs and decrease in overall performance in beef steers (Studemann and Hoveland, 1988; Haldeland and Vikoren, 2005).

A study conducted by Oresanya *et al.* (2003) investigated the effect of ergot alkaloids on the performance and clinical symptoms in weaned pigs. In this study wheat ergot sclerotia (1880 mg alkaloid kg^{-1}; ergocristine, ergotamine, ergosine, ergocryptine, and ergocornine constituting 40, 36, 11, 7, and 6% of the total, respectively were added on weight basis to the basal diet and 192 weaned pigs were fed the basal diet containing the ergot alkaloid mixture and results were compared with the control group fed diet with no ergot alkaloids for 28 days. The weight gain of pigs fed 1% ergot alkaloids was 38% less than the control group. Moreover, average daily feed intake over the entire period, serum prolactin level and urea nitrogen concentration were significantly decreased by ergot alkaloids.

According to Kanoral and Maes (2009), a concentration of 0.3% sclerotia in the lactation feed is sufficient to cause agalactia in 50% of sows. Newborn piglets of affected sows develop diarrhea within the first eight days. Some sows or gilts can show lameness, in particular lameness of the hindquarters and often necrosis can develop on the tail, ears and hooves.

In one of their trials, Barry *et al*. (1997) used thirty-six pigs of both sexes. Each group was randomly allocated to one of six diets containing up to the equivalent of 5% sclerotia by weight and fed these diets for 28 days in a growth trial. Feed consumption and live weight were individually monitored. There were no clinical signs of illness in the pigs, apart from one pig with a mild abrasion of the footpad. Reduction of feed intake and poor feed conversion was observed over the first 7 days with diets containing more than 0.6% by weight. Over the full period of the trial, growth was reduced by 30% in pigs receiving 5% sclerotia largely as a result of poor feed intake and poor feed conversion.

Kopinski *et al*. (2007) observed that feeding diets containing 1.5% sorghum ergot (7 mg alkaloid kg^{-1}) to sows for 6–10 days prior to farrowing, caused a substantial decline in plasma prolactin level leading to a failure of the sows to produce any milk

Moreover, several cases of ergotism were diagnosed in dairy herds in the eastern Highveld Region of South Africa since 1996, implicated ergotized nut sedge as the possible cause. Chemical analysis revealed the presence of ergopeptine alkaloids, particularly ergocryptine and traces of ergosine, ergocornine, ergocristine and ergotamine in silage fed to the cows. Inspection of the silage showed

it to be extensively contaminated with ergotized yellow nut sedge (Naude´ *et al.,* 2005). More recently, gangrenous ergotism (Fig.2.7) has also been reported among free-living moose and roe deer in Norway (Uhlig *et al*., 2007).

Fig. 2.7 Bilateral gangrene of the hind limbs extending up to the distal third of the metatarsus (Haldeland and Vikoren, 2005)

The other major cause of animal intoxication is consumption of grasses infected with endophytes. For example, tall fescue (*Festuca arundinacea*), which is the major component of pasture systems in the US (over 14 million ha), is frequently infected with the endophyte fungus *Neotyphodium coenophialum*. The fungal endophyte shares a symbiotic relationship with tall fescue, protecting the host plant against disease, insects, and drought. At the same time, it has been identified as the causative agent of fescue toxicosis (Realini *et al*., 2005).

Fescue toxicosis is characterized by a condition called summer syndrome which is a complex disease syndrome that occurs during heat stress periods, when animals are consuming infected fescue forage, and is characterized by hyperthermia, with an accompanying decrease in feed intake and growth. Open mouth breathing (Fig. 2.8) is also one of the characteristics of fescue toxicosis that results from the inability of the animals to dissipate heat due to cutaneous vasoconstriction (Naude *et al*., 2005).

Fig. 2.8 Open mouthed breathing as seen in “summer syndrome” (Naude *et al*., 2005).

Realini *et al.* (2005), compared carcass traits, meat quality and fatty acid composition of beef from cattle grazing tall fescue infected with either wild type or nil ergot alkaloid (AR542) endophyte for 209 days. Differences were observed in fatty acid profile of adipose tissues from cattle fed tall fescue with wild type endophyte and AR542, with a higher proportion of saturated fatty acids and a lower proportion of mono unsaturated fatty acids in adipose tissues of cattle fed on tall fescue with wild type. In addition, a significant difference in average daily weight gain, live weight gain, hot carcass weight was observed with greater values for cattle fed tall fescue with the AR542 endophyte.

Chapter 3: Morphological and Molecular Characteristics of the Ergot fungus, collected from Arsi, Ethiopia.

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Abstract

Ergot is a plant parasitic fungus that infects the ovary of several monocot plants and produces dark horn shaped structures in place of the seeds. This research was initiated with the objective of morphological and molecular characterization of the ergot fungal isolates from wild oat plants (*Avena abyssinica*) from Arsi, Ethiopia. Analyses of the sclerotial dimensions were done for sclerotia collected from four sites (Kechema Murkicha 2011, Bucho Selassie 2014, Bucho Selassie 2015 and Shaldo Jigessa, 2015). Statistically significant differences in the length and width ($n = 30$, $p < 0.001$) was observed with the dimension of sclerotia from Kechema Murkicha 2011 significantly differing from the rest. The dimensions of sclerotia from all the four sites significantly differed from the dimensions of wild oat seeds ($p < 0.001$). Differences in the dimension of conidia were not statistically significant ($p > 0.05$). Phylogenetic analysis based on the β-tubulin Intron 3 region revealed separate clustering of our fungal isolates with strong bootstrap value of 94 from a clade that contains *Claviceps purpurea* (G1), *Claviceps spartinae* (G3) and *Claviceps humidiphila* (G2). From the combined morphological and molecular data, it is confirmed that the *Claviceps* isolates in the current study belong to *Claviceps purpurea*, some difference in the morphological features and phylogenetic analysis might place it into a new variety.

Key words: Ergot fungus, β-tubulin, wild oats, *Claviceps purpurea,* sclerotia

3.1 Introduction

Ergot is a parasitic fungus that belongs to the genus *Claviceps* (Nicholson, 2007). The genus *Claviceps* parasitizes more than 600 monocot plants including economically important crops such as rye, barely, oat, rice, wheat and pearl millet (Bove, 1970) and wild oat (*Avena abyssinica*) (Teshome Demeke *et al.*, 1979). The wild oat (*Avena abyssinica*), the host for the Ethiopian ergot fungus, is an endemic weed in Ethiopia and Yemen (Ladizinsky, 1973). The name 'ergot' is derived from a French word 'Argot' which means Cock's spur which represents the dark brown, horn-shaped fungal structure that projects from the ripening ears of infected crops replacing the grains (Van Dogen and De Groot, 1995; Nicholson, 2007). Ergot is the overwintering sclerotia of the fungus *Claviceps* formed at the end of the infection process by sexual spores (ascospores) or asexual spores (conidia) (Alderman, 2003).

When these overwintering structure (sclerotia) fall on agricultural fields during the time of harvesting they give rise to sexually reproducing structures that produce wind-borne ascospores (Mantle and Shaw, 1976). The ascospores are forcefully ejected from the mature asci and the ascospores that land on the stigma of susceptible host plant geminate and produce fungal hyphae that grow into the ovary. After the infection is stabilized in the ovary of the susceptible host large numbers of asexual spores or conidia are produced and released into the sugary syrup derived from the plant known as honey dew. The asexual spores (conidia) are then disseminated to uninfected plant florets by flower visiting insects leading to secondary infection. Finally, infection resulted from ascospores or conidia lead finally to replacement of the seed of the infected plants by hardened mass of mycelia or sclerotia of the ergot fungus (Alderman, 2003).

Classification of ergot fungus was initially based on morphological features such as size and color of sclerotia, the color of stromata (stipe and capitula), the presence or absence of loose hyphae on the stroma, the size and shape of Perithecia, aci and ascospores(Langdon, 942). Morphological feature of the ergot fungi are generally variable. For example, the length of sclerotia of *Claviceps purpurea* ranges from 2 to 50 mm and the color of stromata, shape and size of conidia found to differ (Sprague, 1950; Loveless, 1971; Tanda, 1979). Variation in the length of conidia of isolates collected from grasses of wet/shady habitats compared to conidia of isolates collected from land grasses was observed with the former generally producing longer conidia than the later(Loveless, 1971). Differences in the dimension of spores from laboratory cultures and natural hosts were also

reported (Loveless, 1971). Overlaps of conidial dimension from the upper range between G1 and G2 groups were reported (Pazoutova *et al*., 2015).

Due to this overlaps, the conidia size cannot be used as a single discriminating marker among *Claviceps* species (Pazoutova *et al*., 2015). Owing to variability in the morphological features, molecular characterization has been used for further classification of the *Claviceps* species. Pazoutova *et al*. (2000), used ITS region of ribosomal DNA for analysis of the genus Claviceps. Comprehensive characterization of *Claviceps purpurea* based on host or habitat preference, phenotypic traits such as conidial morphology, alkaloid types and properties of sclerotia, and molecular characterization using RAPD and EcoRI restriction site polymorphism in the 5.8S rDNA was done by Pazoutova *et al*. (2000).

Based on these combined methods, Pazoutova *et al.* (2000), identified three groups G1, G2 and G3. G1 are groups of *Claviceps purpurea* adapted to grasses from open fields and meadows, G2 are adapted to grasses from shady and wet habitats and G3 are adapted to spartina salt marshes. Based on the alkaloid profile, the sclerotia of G1 contained various ergotamines and ergotoxins, G2 produced ergosine and ergocristine with small amounts of ergocryptine and G3 produced ergocristine and ergocryptine.

3.2 Objective of the study

3.2.1 General objective

- To characterize and identify the ergot fungus (*Claviceps purpurea*) isolated from wild oat (*Avena abyssinica*) using morphological and molecular techniques

3.2.2 Specific objectives

- To characterize and identify the ergot fungus based on morphological and molecular features using the β- tubulin interin3 region
- To grow and asses the features associated with the sexual stages of the ergot fungi

3.3 Materials and Methods

3.3.1 Description of the study area

The study was conducted in three major areas (Fig. 3.1) where outbreak of gangrenous ergotism was previously reported, namely the Digelu area with seven Kebeles (Digelu Kidame, Digelu Bora, Kubsa Bora, Kogo Ashebeka, Jemo, Digelu Araby and Digelu – 01), Tijo area with five Kebeles (Mankula Negele, Shaldo Jigessa, Tite Wajii, Bura Jale and Tijo-01) and Kechema area with nine Kebeles (Kechema Murkicha, Bucho Selassie, Fite Ketara, Lole Abosera, Lole Ketara, Aymura Boledena, Tulu Kite, Burkitu Alkessa and Ashebeka Welkite) , of Sagure district, Arsi zone, Oromiya Regional State, Ethiopia, during the month of October 2011, 2012, 2013, 2014 and 2015. Kebeles are the smallest administrative regions in Ethiopia, and analysis was not done for ergot sclerotia collected during the years 2012 and 2013 as the numbers of sclerotia collected were very small.

The areas were selected purposively owing to former outbreak of gangrenous ergotism that occurred during the year 2001 due to consumption of food contaminated with ergot sclerotia from wild oats. The geographical coordinates of the sampling sites were $7^0$45’17’’N latitude, $39^0$8’16’’ E longitude and $7^0$45’16’’ N latitude, $39^0$8’16’’ E longitude for Bucho Selassie; $7^0$46’29’’ N latitude , $39^0$5’5’’ E longitude for Kechema Murkicha; $7^0$46’35’’ N latitude, 39^0 15’28’’ E longitudes for Digelu Bora, and $7^0$41’19’’ N latitude, $39^0$14’44’’E longitude for Mankula Negele. The Altitude of the study sites were 2510m ± 3m and 2520m ± 3m a.s.l for Bucho Selassie, 2415m ± 3m a.s.l for Kechema Murkicha, 2716m ± 2m a.s.l for Digelu Bora and 2580m ± 3m a.s.l for Mankula Negelle respectively.

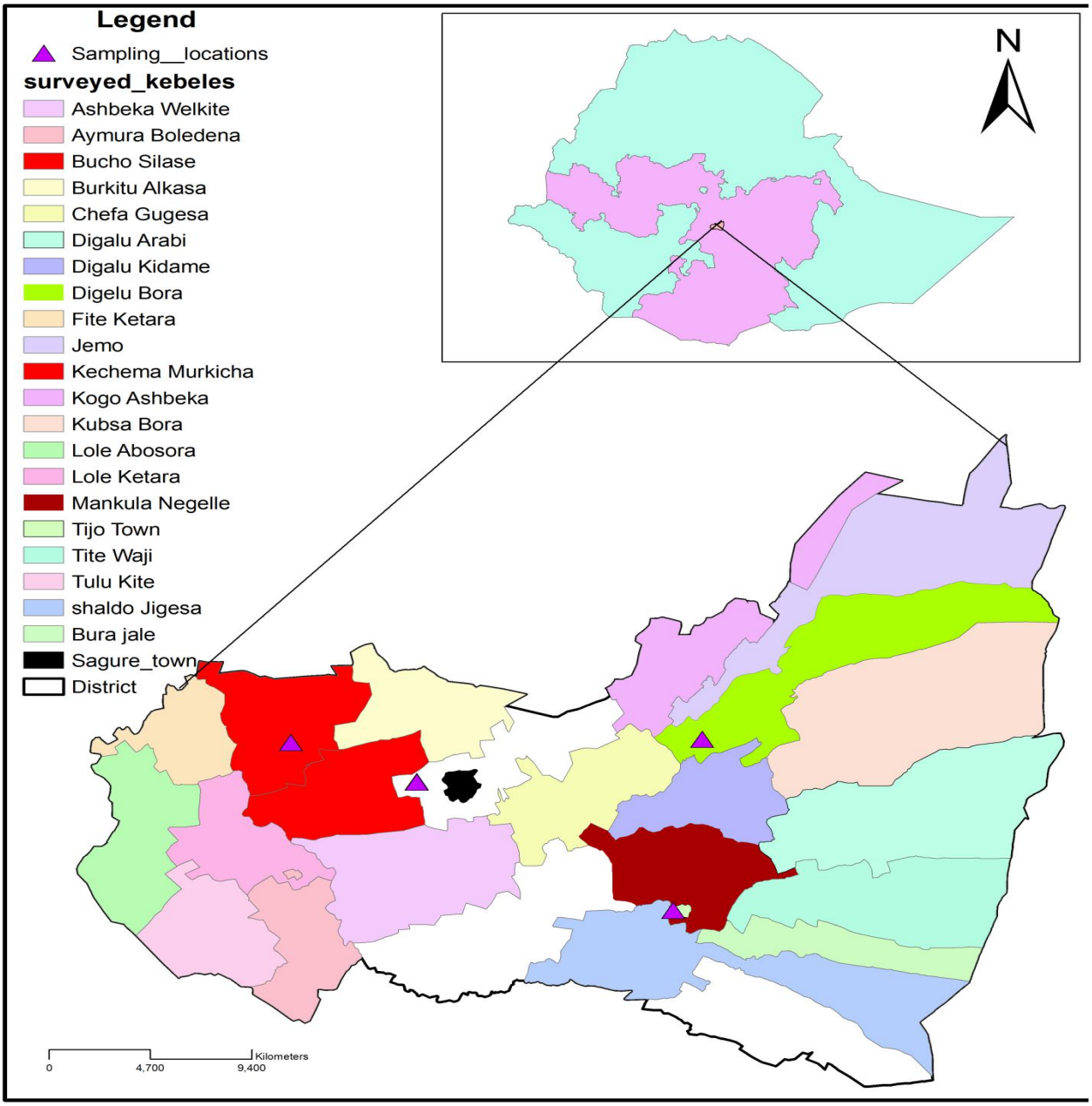

Fig 3.1 Map of the study area

3.3.2. Sample collection and analysis

The sclerotia of the ergot fungus (*Claviceps purpurea*) were collected during the month of October 2011, 2012, 2014 and 2015 from infected heads of the wild oat plant (*Avena abyssinica*) grown as weed in Barley and Wheat fields. The month of October was selected for collection of ergot sclerotia because the prevalence of ergot fungus (*Claviceps purpurea*) was reported to be high during this month.

Ergotized wild oat plants were cut and transported into mycology laboratory in pre-sterilized glass containers. Equal numbers of the collected sclerotia were stored at room temperature and in the refrigerator at – 4 ^{O}C until use. The day of collection and localities were written on the container during collection and geological coordinates of the sample collection sites were registered

3.3.3 Measurement of sclerotia and wild oat seed dimension (Diameter and length)

The length of sclerotia from different collection sites and the wild oat seeds were measured using ruler and the values were recorded in millimeters (mm)

3.3.4 Saprophytic cultivation of the ergot fungus on nutrient media

For saprophytic cultivation of the ergot fungus PDA and Mantle Agar were used. The composition of Mantle Agar per liter is (Sucrose, 150 g; L-asparagine 15 g; KH_2PO4, 25mg, $MgSO_4.7H_2O$, 25 mg; $FeSO_4.7H_2O$, 33mg; $ZnSO_4$, 27mg). Sclerotia were surface sterilized with 1.3% sodium hypochlorite and rinsed three times for 5 minutes in sterile distilled water. The sclerotia were blot dried and cut into two using sterile surgical blade and each of the two pieces were placed on pre-sterilized PDA and Mantle Agar supplemented with Chloramphenicol. Isolates were maintained on PDA agar slant as well as Mantle agar slants at 4 ^{O}C and sub cultured every three to six months with slight modification from (Pazoutova *et al*., 2000)

3.3.5 Cultivation of the sclerotia of the ergot fungus for production of the sexual stages

Growth of the sexual structures of the ergot fungus was studied using sclerotia collected from Bucho Selassie 2015. The sexual stage growth was done in 2016 on washed and autoclave sterilized sand in big Petri dishes (95 mm in diameter). Sand used for this experiment was thoroughly washed several times until the soil component is removed and the cleaning water appeared clear. Then equal amount of sand was spread over big Petri dishes and the Petri dishes with sand were sterilized by autoclaving at 121 ^{O}C for 15 minutes. The sclerotia were sonicated for 10 minutes in three changes of sterile distilled water (SDW), Surface disinfected for three minutes in 1.75 % sodium hypochlorite, rinsed five times with sterile distilled water and blot dried aseptically. Three surface sterilized sclerotia were placed per Petri dish containing sterilized sand and the Petri dishes were sealed with Parafilm (van der Linde and Wehner, 2007).

Duplicate Petri dishes with the sclerotia were placed under the following treatments

1. 7 days at 5^{O}C
2. 7 days at 5^{O}C followed by 7 days at 18 ^{O}C
3. 21 days at 5^{O}C
4. Room temperature (no temperature treatment)

Then all the Petri dishes were incubated at 25 ^{O}C and moistened whenever necessary using sterilized distilled water (van der Linde and Wehner, 2007).

3.3.6 Measurement of length of Stroma and width of capitula

Measurement of these sexual structures of the ergot fungus (*Claviceps purpurea*) was done using ruler and measurements were recorded in millimeter (mm).

3.3.7 Measurement of Perithecial dimension

To measure the length and width of the Perithecia, a total of six capitula from three sclerotia(2 per sclerotia) were sectioned longitudinally using sterile razor blade, and measurements of the

dimensions of the Perithecia was done using calibrated ocular micrometer (eyepiece micrometer) (van der Linde and Wehner, 2007).

3.3.8 Collection and measurement of ascospores

Sclerotia bearing mature stromata were attached by **petroleum jelly** to the underside of a glass Petri dish lid so that capitula lay inverted about 7mm above the surface of a clean glass slide which had been placed on moist filter paper in the bottom of the dish. Discharged ascospores became deposited on the slide in an area about 5mm in diameter. After several hours the deposition clearly visible as an opaque area, was removed with a sharp scalpel edge (Corbett *et al*., 1974). Measurement of the ascospores collected was done using ocular micrometer according to (van der Linde and Wehner, 2007).

3.3.9 Molecular characterization of ergot fungi.

DNA extraction

DNA extraction was conducted according to the CTAB method of Stewart and Via (1993) with modification for fungi. Ten ergot fungal isolates maintained on Mantle Agar slant were sub cultured onto duplicate sterile plastic plates containing pre-sterilized PDA. The inoculated plates were tightly closed with parafilm and incubated at 25 ^{O}C for one week. After a week, a small portion of the cultures grown on one of the duplicate PDA plates was transferred separately into pre-sterilized malt extract broth prepared in 2ml well of a 24 plastic well plate using inoculating loop and incubated at 25 ^{O}C for one week. The fungal mycelia from malt extract broth were transferred into eppendorf tubes and the tubes were centrifuged at 13000 rpm for 3 minutes. The liquid portion was discarded and the eppendorf tubes containing the mycelia were kept in a deep freeze at -80 ^{O}C for 30 minutes. After 30 minutes incubation in deep freeze the eppendorf tubes were placed in freeze dryer overnight.

For the extraction of DNA the freeze dried mycelia was pulverized in the Eppendorf tubes using small sterile plastic pestle pre-cooled in liquid nitrogen. Proteinase K (0.3 mg/sample) was weighed and CTAB buffer (0.5ml/sample) with a composition of (Cetyl-Trimethyl Ammonium Bromide:1%; NaCl , 0.7M; Tris-HCl, 0.1M; Bet-Mercaptoethanol, 0.1%, pH 7.5) was added little by little into the

tube containing proteinase K in a fume hood. From the mixture of Proteinase K and CTAB 0.5 ml was added to the pulverized mycelia, thoroughly vortexed and flicked. Then the eppendorf tubes were placed on a floater and incubated in water bath at 65 ^{O}C for 30 minutes. After incubation the eppendorf tubes were placed on ice for 15 min. To the cooled eppendorf tubes containing the DNA 0.3 ml of Chloroform: isoamyl alcohol (24:1) was added and mixing was done by rotating the tubes with hand until the mixture becomes milky then the tubes were centrifuged at 13000 rpm for 15 minutes. Then 0.3ml of the upper aqueous layer was transferred into new tube and equal volume (0.3ml) of cold (-20 ^{O}C) isopropanol was added. The mixture was gently mixed and centrifuged at 13000rpm for 15 min to allow the formation of DNA pellet. The supernatant formed after centrifugation was discarded and the pellet was washed with 0.15ml of 70% ethanol and centrifuged at 13000rpm for 2 min to remove the isopropanol. After centrifugation the supernatant (ethanol) was removed and the tubes were kept open in a fume hood to air dry the pellet. After drying, the content was re-suspended by 0.05 ml of SIGMA water and kept in water bathe for 5 minutes. Then 2 µl of RNase was added and the content was stored at -20 ^{O}C.

The concentration of DNA for all the isolates was measured using Quantus Flourometer, Promega, USA following the user's guide in the manual and DNA for all isolates were diluted to 10 ng/microlitre using nuclease free water. The working solutions for the primers were prepared using molecular grade water SIGMA according to instructions on the vials containing the primers. The PCR master mix preparation was done according to usage information in the manual of GO Taq G2 DNA polymerase, Promega, USA. Accordingly, 5 µl of 5X green Go Tag reaction buffer, PCR ; nucleotide mix, 1.2 µl; upstream primer, 1 µl; downstream primer, 1 µl; Go Tag G2 DNA polymerase, 0.12 µl; $MgCl_2$, 1 µl , nuclease free water was added to bring the volume to about 23 µl and mixing was done on ice. Finally, to every master mix a volume of 2 µl of template DNA was added in the respective column for the isolates. In the first well of the first row master mix was prepared; hence no master mix was added in the entire row. Addition of the entire master mix was done starting from the second row till the 11th well for the negative control. At this point the master mix contains everything except primer and the template DNA. Once the master mix was distributed in all the wells primer for *Claviceps sorghi* was added to all the isolates in the second row. Primer for *Claviceps africana* was added in the third row, *C. paspali* in the 4th row, *Claviceps purpurea* in the 5th row and *Claviceps fusiformis* in the 6th row. After adding the reverse primer to all of the

wells 2 micro liters of the DNA for each isolate was added down the column. Finally, the plate was sealed with plastic cover and the PCR procedure was followed according to the description in the manual of Progmea, USA. Polymerase chain reaction condition include 30 cycles of 94^{O}C for 15 seconds, 60^{O}C for 15 seconds, 72 ^{O}C for 15 seconds and final extension at 72 ^{O}C for 5 minutes.

The PCR products were subjected to jell electrophoresis on 1.5% agarose jell at 120V for 45 minutes with a 10kb DNA MassRuler DNA Ladder, Mix, ready-to-use 50 – 200 applications, (Thermo Fisher Scientific). At the end of 45 minutes the gel was submerged in ethidium bromide for 30 minutes and jell picture was captured using Gel Doc XR^{+} system integrated with Image Lab^{TM} (BioRad Molecular Image Gel DocXR, USA).

Table 3.1: Primers used to amplify the β-tubulin intron 3 region of ergot fungal isolates (Tooley *et al.,* 2001)

Name of primer	Sequence of the primers
BTPUR	TCGCACAGTTTAGCATGCC
BTFUS	TTTTGCATGCATTCCTTGCC
BTAFR	TATGCTTGCACTCCCTTCGC
BTSOR	CATCCATCTGCCCAACGATT
BTPAS	TTGCCGGATGCCTGTTGGGG
BT5	GCTCTAGACTGCTTTCTGGCAGACC

DNA sequencing

Before PCR amplification the extracted DNA of the ergot fungi were divided in to two, one set of the DNA was used for PCR amplification and the other set was stored for Sequencing. After amplification the PCR product was purified using the E.N.Z.A Cycle Pure Kit (Omega Bio-tek), and the purified PCR products were sequenced in both directions by Macrogen Europe Laboratory Services (The Netherlands) using Primers (BTPUR TCGCACAGTTTAGCATGCC) and (BT5 GCTCTAGACTGCTTTCTGGCAGACC).

Phylogenetic analysis

The sequences obtained were BLASTED to GenBank data base (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the taxonomic report was consulted in order to come up with which organisms to select for construction of phylogenetic tree. The taxonomic report with the highest search hits included species of *Claviceps* such as *Claviceps aff. Purpurea*, *Claviceps purpurea* isolates or strains, *Claviceps spartinae* and *Claviceps humidiphila*. Sequences of β-tubulin intron 3 region of the first two isolates from each of the above four groups and those of *Claviceps sorghicola* and *Claviceps africana* were used to construct the phylogenetic tree.

For rooting of the evolutionary tree of the β-tubulin intron three region *Epichloe typhinia* (ATCC 200736 with accession number of X52616 in GenBank) was used (Tooley *et al.,* 2001). Multiple alignment of the selected β-tubulin gene was done using Clustal X software and the aligned sequences were saved in FASTA format. The construction of phylogenetic tree of the β-tubulin gene was performed using MEGA 7 software under default settings.

3.3.10 Data analysis

Comparison of the dimensions of sclerotia, wild oat seeds and conidia were done using KyPlot software version 5.0. For comparisons of the mean width and length of sclerotia, wild oat seeds and conidia, Kruskal-Wallis statistic was used. Multiple pair wise comparison was done using Steel-Dwass test. SPSS version 24 was used for descriptive analysis of data.

3.4 Results

The sclerotia of the ergot fungus collected from the study sites were dark in color, and were generally larger in size and width than the corresponding dimensions of the wild oat seeds (Fig. 3.2). Variations were observed in the width and length of the sclerotia collected during the years 2011, 2014 and 2015. The mean width (mm) of sclerotia varied from 4.1mm ± 1mm to 5.1mm ± 1.1mm for sclerotia collected from Bucho Selassie in 2014 and Kechema Murkicha in 2011 respectively (Table 3.2). Statistically significant difference was observed in the mean diameter of sclerotia ($n = 30$, $X^2 = 14.39$; $p = 0.006$). The mean width of sclerotia collected from Kechema Murkicha in 2011 significantly varied from the mean width of sclerotia collected from Bucho Selassie 2014 ($p = 0.01$), Bucho Selassie 2015 ($p = 0.02$) and Shaldo Jigessa 2015 ($p = 0.02$)

The lowest mean length (11.7mm ± 2.3 mm) of sclerotia was recorded for sclerotia collected from Bucho Selassie 2015 and the largest mean length (18.6mm ± 2.3 mm) was recorded for sclerotia collected from Kechema Murkicha 2011 (Table 3.2). Statistically significant difference in the length of sclerotia ($n = 30$, $X^2 = 84.43$, $p < 0.001$) was observed with length of sclerotia collected from Kechema Murkicha varying significantly from length of sclerotia collected from Bucho Selassie 2014 ($p < 0.001$), Bucho Selassie 2015 ($p < 0.001$) , Shaldo Jigessa 2015 ($p < 0.001$) and Digelu Bora 2015 ($p < 0.001$).

Table 3.2 Dimension in millimeter (mm) of sclerotia collected from five study sites

No	Place of collection of sclerotia	Year of collection	Mean length (mm) ± SD, n = 30	Mean width(mm) ± SD, n = 30	Color and shape of sclerotia
1	Kechema Murkicha	2011	18.6 ± 2.3	5.1±1.1	Dark, curved or straight, round or tapered tips
2	Bucho Selassie	2014	12.1 ± 2.8	4.1±1	>>
3	Bucho Selassie	2015	11.7 ± 2.3	4.2±1	>>
4	Shaldo Jigessa	2015	12.2 ± 2.7	4.2±1	>>
5	Digelu Bora	2015	11.7 ± 2.2	4.5±1	>>

Fig 3.2 Picture of ergot sclerotia (left) and wild oat seeds (right)

Cultivations of the ergot fungus isolated from heads of wild oat plants were done on PDA. The isolates formed white, leathery rigged colony on the front side and brownish colony in the back side (Fig 3.3) and spherical conidia (Fig 3.4)

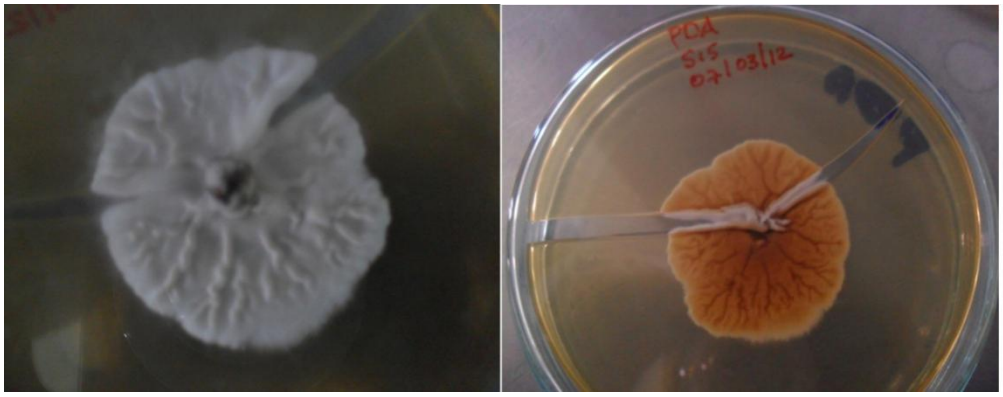

Fig. 3.3 Pictures of the front (left) and back (right) side of ergot fungus on PDA

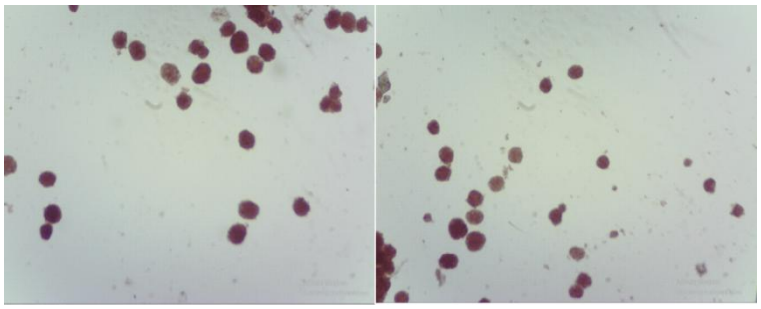

Fig 3.4 Picture of conidia of ergot fungus isolated from wild oat

In this study mean length and width of sclerotia were compared with mean length and width of wild oat seeds. Statistically significant difference differences in mean length ($n = 30$, $X^2 = 64.31$, $p < 0.001$) and width ($n = 30$, $X^2 = 84.43$, $p < 0.001$) between sclerotia and wild oat seeds was observed. The result of the measurements of the length and width in micrometer (µm) of conidia (n = 30) from 30 isolates of ergot fungus collected from Shaldo Jigessa 2015, Digelu Bora 2015, Kechema Murkicha 2011 and Bucho Selassie 2014 was done, and the mean ± standard deviation

values of conidia length and width are listed (Table 3.3). Conidia length (m ± sd) values ranged from 6.3µm ± 0.5 µm for isolate AAU9 from Shaldo Jigessa 2015 to 6.7 µm ± 0.8 µm for isolate AAU 22 from Digelu Bora 2015. Conidia width on the other hand ranged from 3.1 µm ± 0.3 µm for isolates AAU5 from Shaldo Jigessa 2015, AAU 11 and AAU 16 from Kechema Murkicha 2011 and AAU 24 from Digelu Bora 2015 to 3.3 µm ± 0.5 µm for isolate AAU2 from Shaldo Jigessa 2015 (Table 3.3). The minimum length of conidia for all isolates collected from the four study sites was 6 µm and the maximum length of conidia (9 µm) was recorded only for isolate AAU 22 from Digelu Bora

2015. Statistically significant differences were not observed among mean width ($n = 30$, $X^2 = 13.64$, = 0.99), and mean length ($n = 30$, $X^2 = 25.07$, $P = 0.6743$) of conidia of ergot fungi collected from

the four study areas.

Table 3.3 Colony morphology and conidial dimension

Isolates	Place of collection	Year of collection	Colony morphology	Conidia length (µm)	Conidia width (µm)	Conidia shape
AAU1	SJ	2015	White leathery appearance	6.5 ± 0.5	3.1 ± 0.4	Spherical
AAU2	SJ	2015	White leathery appearance	6.5 ± 0.5	3.3 ± 0.5	Spherical
AAU3	SJ	2015	White leathery appearance	6.6 ± 0.5	3.1 ± 0.4	Spherical
AAU4	SJ	2015	White leathery appearance	6.7 ± 0.5	3.2 ± 0.4	Spherical
AAU5	SJ	2015	White leathery appearance	6.5 ± 0.5	3.1 ± 0.3	Spherical
AAU6	SJ	2015	White leathery appearance	6.5 ± 0.5	3.2 ± 0.4	Spherical
AAU7	SJ	2015	White leathery appearance	6.6 ± 0.5	3.1 ± 0.4	Spherical
AAU8	SJ	2015	White leathery appearance	6.5 ± 0.5	3.1 ± 0.4	Spherical
AAU9	SJ	2015	White leathery appearance	6.3 ± 0.5	3.2 ± 0.4	Spherical
AAU10	SJ	2011	White leathery appearance	6.6 ± 0.5	3.2 ± 0.4	Spherical
AAU11	KM	2011	White leathery appearance	6.6 ± 0.5	3.1 ± 0.3	Spherical
AAU12	KM	2011	White leathery appearance	6.6 ± 0.5	3.2 ± 0.4	Spherical
AAU13	KM	2011	White leathery appearance	6.6 ± 0.5	3.2 ± 0.4	Spherical
AAU14	KM	2011	White leathery appearance	6.6 ± 0.5	3.2 ± 0.4	Spherical
AAU15	KM	2011	White leathery appearance	6.5 ± 0.5	3.2 ± 0.4	Spherical
AAU16	KM	2011	White leathery appearance	6.4 ± 0.5	3.2 ± 0.4	Spherical
AAU17	BS	2014	White leathery appearance	6.5 ± 0.5	3.1 ± 0.3	Spherical
AAU18	BS	2014	White leathery appearance	6.5 ± 0.5	3.2 ± 0.4	Spherical
AAU19	BS	2014	White leathery appearance	6.6 ± 0.5	3.1 ± 0.4	Spherical
AAU20	BS	2014	White leathery appearance	6.4 ± 0.5	3.1 ± 0.4	Spherical
AAU21	BS	2014	White leathery appearance	6.6 ± 0.5	3.2 ± 0.4	Spherical
AAU22	DB	2015	White leathery appearance	6.7 ± 0.8	3.3 ± 0.6	Spherical
AAU23	DB	2015	White leathery appearance	6.7 ± 0.5	3.2 ± 0.4	Spherical
AAU24	DB	2015	White leathery appearance	6.6 ± 0.5	3.1 ± 0.3	Spherical
AAU25	DB	2015	White leathery appearance	6.5 ± 0.5	3.2 ± 0.4	Spherical
AAU26	DB	2015	White leathery appearance	6.6 ± 0.5	3.1 ± 0.4	Spherical
AAU27	DB	2015	White leathery appearance	6.5 ± 0.5	3.1 ± 0.4	Spherical
AAU28	DB	2015	White leathery appearance	6.6 ± 0.5	3.2 ± 0.4	Spherical
AAU29	DB	2015	White leathery appearance	6.5 ± 0.5	3.1 ± 0.4	Spherical
AAU30	DB	2015	White leathery appearance	6.6 ± 0.5	3.1 ± 0.4	Spherical

AAU, Addis Ababa University; BS, Bucho Selassie; DB, Digelu Bora; KM, Kechema Murkicha;SJ, Shaldo Jigessa

For growth of the sexual structures of the ergot fungus from wild oats (*Avenal abyssinica*), three randomly selected sclerotia were placed on pre-sterilized sand in big Petri dishes in duplicate at different sets of temperatures. The sexual structures grown from the sclerotia of the ergot fungus with brown stromata and pale brown colored stipe is indicated in (Fig. 3.5)

Fig 3.5 Pictures of sexual structure of ergot fungus grown from sclerotia.

Growth (100%) occurred on Petri dishes initially incubated for 21days at 5 ^{O}C followed by final incubation at 25 ^{O}C. It took about 2 months for the appearance of the first sexual structure. However, no growth was observed on the other temperature treatments. The maximum length of sclerotia used for this experiment was 18 mm and the minimum was 13 mm. The maximum and minimum numbers of stromata produced per sclerotia were 7 and 4 respectively. The mean length of stromata ranged from 18.5 mm to 19mm, and the mean diameter of the capitula ranged from 1.8 mm to 2mm. The mean length and width of Perithecia (n = 30) was 158.8 µm ± 3.7 µm and 89.2 µm ± 1.7 µm respectively and the shape of Perithecia were cylindrical to flask shaped. Ascospores were filiform shaped with mean length of 77.1 µm ± 4.3 µm and mean width of 3.3 µm ± 0.5 µm (Table 3.4).

Table 3.4: Characteristics of the sexual stages of the ergot fungi

Incubation for 21 days at 5^{O}C	Sclerotia Length (width) (mm)	Stromata number per sclerotia	Mean (n) Stromata length (mm)	Mean (n) Capitula Diameter (mm)	Mean Length (width) (n = 30) of Perithecia (µm)	Mean Length (width) (n = 30) Ascospores (µm)	Asco - spores Shape	Capitula Shape	Capitula Color	Stipe Color
Petri dish 1										
S1	18 (5)	7	19 (7)	1.8 (7)	ND	ND	ND	G / SG	Brown	Light brown
S2	14(4)	5	18.8(5)	1.9(5)	ND	ND	ND	G /SG	Brown	Light brown
S3	15(4)	6	18.5(6)	1.9(6)	157.2 ± 5.1 (89.8 ± 2)	76.4 ± 4.2 (3.3 ± 0.5)	Filiform	G / SG	Brown	Light brown
Petri dish 2										
S1	15(5)	6	19(6)	1.9(6)	159.1 ± 3.0 (89.3 ± 1.8)	77.7 ± 4.3 (3.3 ± 0.5)	Filiform	G / SG	Brown	Light brown
S 2	14(3)	4	18.8(4)	2(4)	ND	ND	ND	G / SG	Brown	Light brown
S3	13(4)	6	18.8(6)	2(6)	160 ± 2.9 (88.5 ± 1.4)	ND	ND	G / SG	Brown	Light brown

G /SG, globose to subglobose; n, total number studied; ND, not determined; S, sclerotia

Gel electrophoresis was conducted for 8 isolates as two isolates (AAU3 and AAU7) were contaminated. Out of the 8 isolates amplification was observed for five isolates (AAU1, AAU5, AAU8, AAU9 and AAU10) (Fig. 3.6).

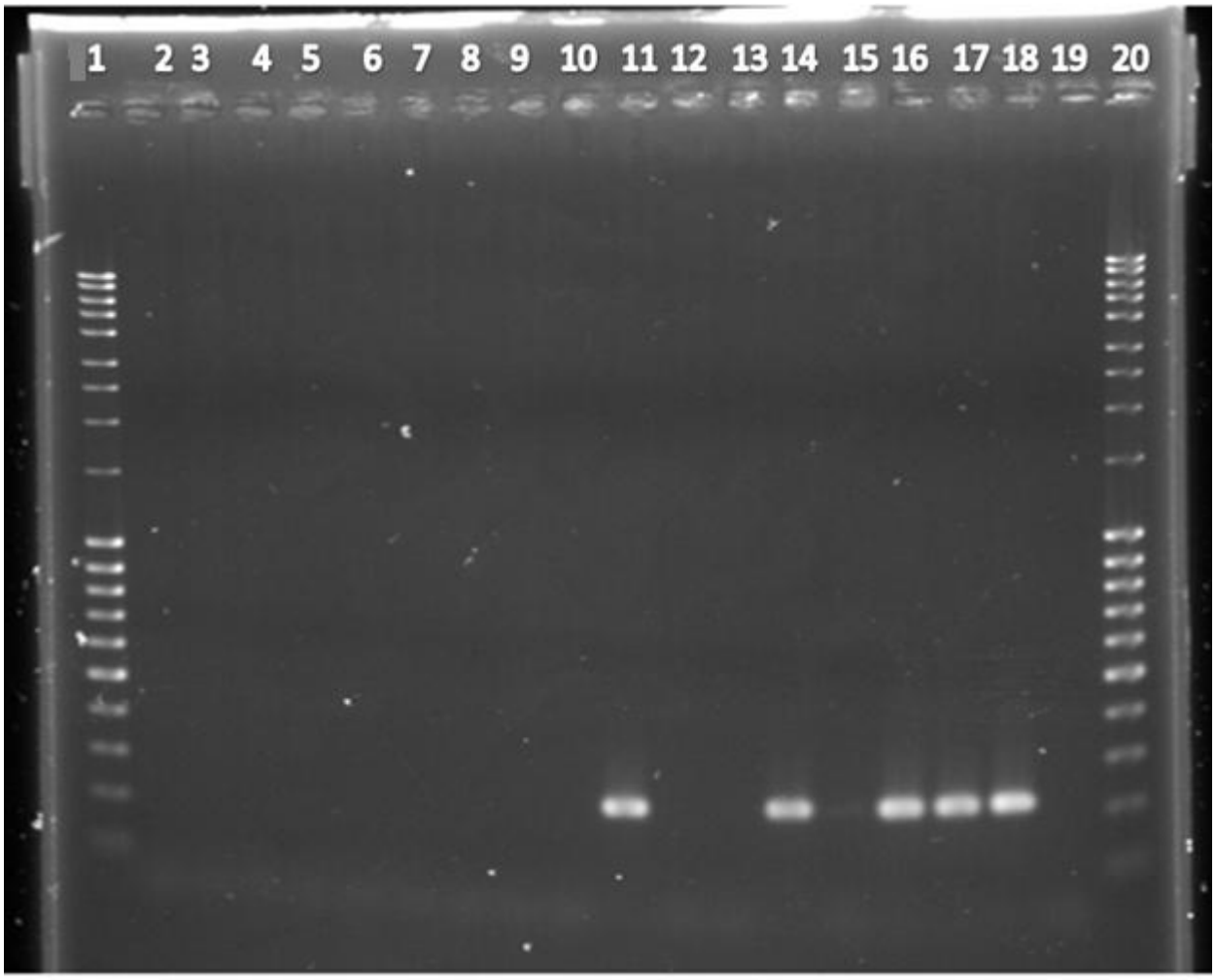

Fig. 3.6 Result of PCR amplification of the β-tubulin intron 3 region using universal primer BT5 and forward primer BTPUR. Lane 11 (AAU1), lane 12 (AAU2), lane 13 (AAU4), lane 14(AAU5), lane 15(AAU6), lane 16(AAU8), lane 17(AAU9), lane 18(AAU10), lane 19 (negative control) and lanes (1 and 20) were molecular weight ladders.

For molecular characterization, the β-tubulin intron 3 region was sequenced for 4 isolates of ergot fungi (AAU1, AAU5, AAU8, AAU9) isolated from wild oats (*Avena abyssinica*). Sequences were edited using bioedit software and saved in FASTA format. The sequences obtained were similar for all the four isolates

(>1.AAU1
CTCCAGACGGCATGCAGCGATGGATTGCATATTGGTAGAGATTTGCTTTATCAATTTAC
ACACCTCATTGAAGTAGACGCTCATGCGCTCGAGCTGTTGCTCCGAGGTACCATTGTAC
ACACCACTGCTGTCGAGGCCGTGCTCGCCAGAGATGGTCTGCCA

>5.AAU5
CTCCAGACGGCATGCAGCGATGGATTGCATATTGGTAGAGATTTGCTTTATCAATTTAC
ACACCTCATTGAAGTAGACGCTCATGCGCTCGAGCTGTTGCTCCGAGGTACCATTGTAC
ACACCACTGCTGTCGAGGCCGTGCTCGCCAGAGATGGTCTGCCA

>6.AAU8

CTCCAGACGGCATGCAGCGATGGATTGCATATTGGTAGAGATTTGCTTTATCAATTTAC
ACACCTCATTGAAGTAGACGCTCATGCGCTCGAGCTGTTGCTCCGAGGTACCATTGTAC
ACACCACTGCTGTCGAGGCCGTGCTCGCCAGAGATGGTCTGCCA

>7.AAU9

CTCCAGACGGCATGCAGCGATGGATTGCATATTGGTAGAGATTTGCTTTATCAATTTAC
ACACCTCATTGAAGTAGACGCTCATGCGCTCGAGCTGTTGCTCCGAGGTACCATTGTAC
ACACCACTGCTGTCGAGGCCGTGCTCGCCAGAGATGGTCTGCCA

The sequences were BLASTED into National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database. The result of the BLAST search revealed 99% identity of our isolates with 12 *Claviceps purpurea* isolates (strains) and 1 *Claviceps cyperi* strain with 100% query coverage (Table 3.5). The habitat specialization of majority of the *Claviceps* species obtained from database belonged to the G1 group; almost all *Claviceps* species except *Claviceps cyperi* were collected either from America or part of Europe.

Table 3.5: The result of NCBI BLAST search of the β-tubulin intron 3 region

Name of the fungus	Habitat	Place of collection	Host	ID	Acc. No.
Claviceps purpurea strain 20.1	_	USA	*Secale cereale*	99%	KP689578.1
Claviceps purpurea isolate CCC763	G1	Slovakia	*Avenella flexuosa*	99%	JX083453.1
Claviceps purpurea isolate CCC734	G1	Norway	*Bromus inermis*	99%	JX083451.1
Claviceps purpurea CCC685	G1	Kazakhstan	*Alopecurus pratensis*	99%	Jx083450.1
Claviceps purpurea CCC597	G1	Greece	*Leymus sp.*	99%	JX083449.1
Claviceps purpurea CCC590	G1	Czech Republic	*Glyceria fluitans*	99%	Jx083448.1
Claviceps purpurea CCC583	G1	Czech Republic	*Lolium sp.*	99%	Jx083447.1
Claviceps purpurea isolate W3	G1	UK	*Agropyron repens*	99%	Jx083444.1
Claviceps purpurea isolate 767	G1	France	*Leymus arenarius*	99%	FJ711486.1
Claviceps purpurea isolate 207	G1	USA	*Triticum aestivum*	99%	FJ711485.1
Claviceps cyperi strain 02/CC	_	South Africa	*Cyperus esculentus*	99%	AY497775.1
Claviceps purpurea strain clp-2	_	USA	*Sclerotia source*	99%	AF263568.1
Claviceps purpurea	_	USA	*Sclerotia source*	99%	AF062646.1

G1, habitat specialization to dry environments; ID, percentage identity, ACC. No, accession number.

Result of maximum parsimony, neighbor joining and maximum likelihood analysis were consistent with one another and showed a clear separation of *Claviceps* species in the same pattern to groups. The most parsimonious tree (length = 1000steps) had a consistency index of 0.8455 and retention index of 0.8273(Fig. 3.7). All *Claviceps africana* isolates from other regions grouped consistently with *Claviceps africana*, the sorghum ergots which are *Claviceps africana* and *Claviceps sorghicola* were grouped together on a branch well supported by bootstrap value of 75 separately from other *Claviceps* species used for phylogenetic analysis.

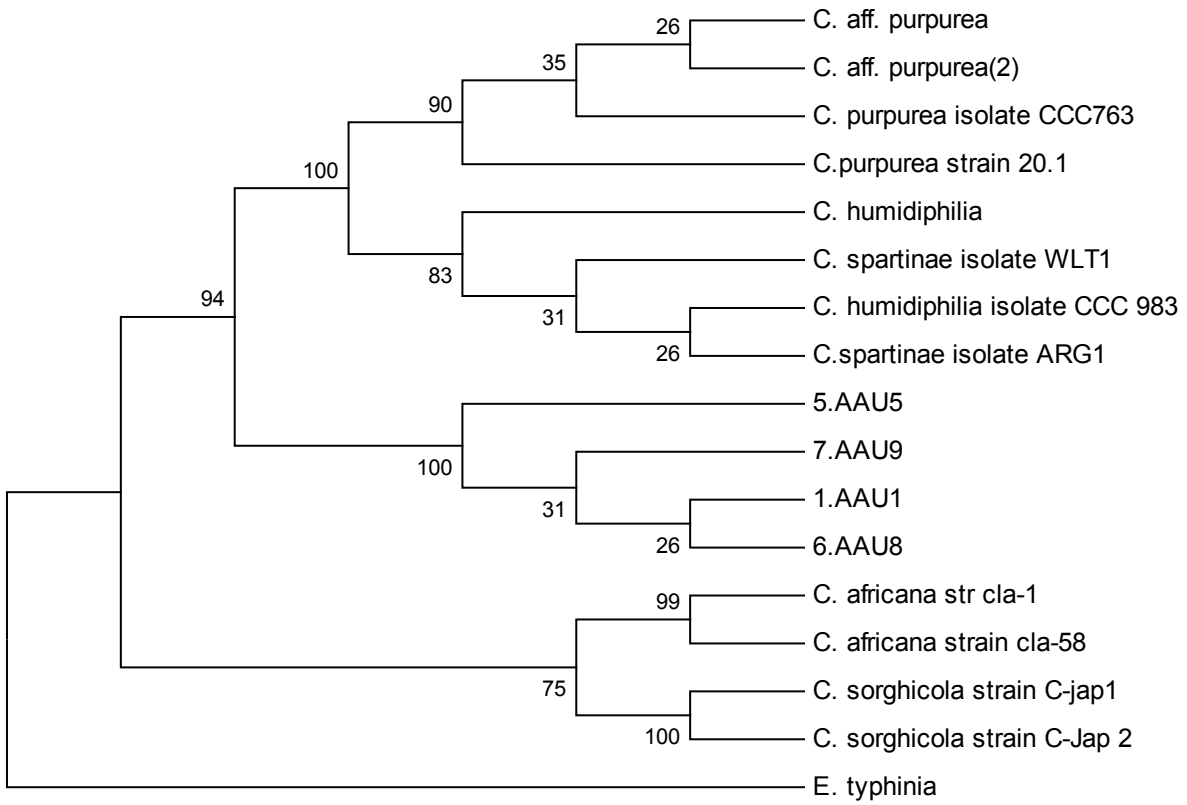

Fig. 3.7 Maximum Parsimony analysis showing relationships of *Claviceps* species based on β-tubulin intron 3 region. Bootstrap values (1000 replications) are shown on the branches. The tree was rooted using out-group species of *Epichloe typhinia.*

3.5 Discussion

In this study morphological and molecular characterization of the ergot fungus (*Claviceps purpurea*) from wild oat (*Avena abyssinica*) was done. The host (*Avena abyssinica*) of the Ethiopian *Claviceps purpurea* is an endemic monocot plant to Ethiopia and Yemen (Ladizinsky, 1975). The taxonomic criteria used to differentiate *Claviceps* species are the color, size and shape of sclerotia, color of stipes and capitula, the presence and absence of loose hyphae on the stroma, the size and shape of Perithecia, asci, ascospores ad conidia (Langdon, 1942). However, Loveless (1964), considered asci as unreliable criteria for classification of ergot due to differences in the length of asci depending on the stage of maturity. According to loveless (1964), species can be identified based on size and shape of conidia as these features of conidia are less dependent on environmental factors compared to asci and ascospores.

The sclerotia collected from the study sites were dark in color which is also in agreement with description by Langdon (1954). However, variation in the descriptions of the color of sclerotia of *Claviceps purpurea* by different authors was also observed. Sprague (1950) and Tanda (1979) described ergot sclerotia as purple black and pale purple to blackish brown respectively. The shape of the sclerotia in the current study varied from straight to curved with round or tapered apex having tissue remnants from the sphacelial stage at the tip. Some of these features are also in agreement with description by Langdon (1954), who described the shape of sclerotia of *Claviceps purpurea* as elongated ovoid to cylindrical with remnants of sphacelial tissues at the tip. Alderman (2003), also described the shape of the sclerotia of the *Claviceps purpurea var purpurea* as cylindrical with round or tapered apex.

Variation in the dimension (length and width) of sclerotia collected from the study sites was also observed with mean length of sclerotia ranging from 11.7 ± 2.3 mm for sclerotia collected from Bucho Selassie in 2015 to 18.6 ± 2.3mm for those collected from Kechema Murkicha in 2011 respectively. Variation in the length of the sclerotia of *Claviceps purpurea* on different hosts was also reported (Pazoutova, 2002). According to this author, the length of sclerotia of *Claviceps purpurea* on Poa annua is only 1 – 2 mm, compared to its length of about 50mm on *Secale cereal.* The author also attributed the differences in sizes of the sclerotia to space available in floral cavity for elongation of the sclerotia. The difference in this result and the result of other researchers might

be due to differences in the host and the ecology of these isolates. Variations in the dimension of the sclerotia among our isolates might be attributed to differences in the environmental factors.

This study also compared the length of conidia of isolates of ergot fungus collected from the seed head of wild oat plants (*Avena abyssinica*) from different sampling sites. The length ($p = 0.6743$) and width ($p = 0.99$) of the conidia did not differ significantly ($p > 0.05$) among ergot isolates from different sampling sites. The length of conidia was between (6.3 ± 0.5 µm) to (6.7 ± 0.8µm) and the width of conidia was between (3.1 ± 0.3 µm) to (3.3 ± 0.5 µm). Pazoutova *et al.* (2015), reported conidial size of (7.1 – 11.6x3.3 – 5.3 µm) for G2 and (3.8 – 9.8x2.2 – 4.5 µm) for G1 group of *Claviceps* species.

A report by Alderman (2003), showed a relatively smaller size of conidia of *Claviceps purpurea var purpurea* with length of 4 – 6 x 2 – 3 µm. According to Loveless (1971), conidia of isolates from wet/shady habitats were longer (6.5 – 8.5 µm) and those found on land grasses (5 – 6 µm). Though isolates in the current study were collected from wild oats grown in open fields, the dimension of the conidia is slightly longer and broader than the dimensions of conidia reported by Loveless (1971) and Alderman (2003), but the dimension of conidia of our isolates lie in the range reported by (Pazoutova *et al*., 2015). Differences between the dimensions of the conidia might be due to differences in the source of conidia. The source of conidia in our study was the culture of the ergot fungus grown under laboratory conditions on (PDA); Loveless (1971), used conidia from honey dew stage of the *Claviceps purpurea*.

Evaluation of the sexual stage of the ergot fungus in the current study was also done, and 100% germination of the sexual stage was observed only for sclerotia incubated at 5 ^{O}C for 21 days before final incubation at 25 ^{O}C. It took about 2 months for the first appearance of the sexual stage out of the sclerotia. A study by van der Linde and Wehner (2007) also showed the highest percentage (51.3) germination of the sclerotia kept at 5 ^{O}C for 21 days prior to incubation at 24 ^{O}C. According to these authors germination of the sclerotia commenced within 4 – 8 weeks of incubation at 24 ^{O}C. In another study by Cooke and Mitchell (1966), sclerotia from *Claviceps purpurea* naturally frosted for six months from October to April were brought to laboratory and allowed to germinate. Germination did not occur until 14 days after the beginning of the first incubation and 100% germination was observed in 23 days. According to Mitchell and Cooke (1968), all sclerotia

irrespective of its origin required chilling period (below 15 ^{O}C) before they germinate and no germination occurred after treatment at – 5 ^{O}C.

The difference in the germination profile of ergot sclerotia from the current study compared to those reported by van der Linde and Wehner (2007) might be due to the difference in the species of the ergot fungi and methodology followed. Even though, this study used methodology of van der Linde and Wehner (2007), there might be variation in moistening of the sand on which the fungus was growing as specific rate was not described. The difference in the percentage growth rate of sclerotia from this study compared to Cooke and Mitchell (1966) and Mitchell and Cooke (1968), might be due to the methodology followed where these authors chilled the sclerotia for longer period compared to the sclerotia in the current study.

The number of stromata produced per sclerotia ranged from 4 to 7, with the highest number obtained from the longest sclerotia. However, some sclerotia with smaller size were found to produce more stromata than did the bigger ones. Cooke and Mitchell (1966), on the other hand reported association between size of sclerotia and number of stromata produced per sclerotia with bigger sclerotia generally producing more stromata than did the smaller ones. Production of more stromata by larger sclerotia was reported to be due to the presence of more energy reserve required for germination (Cooke and Mitchell, 1966). Periodic removal of stromata from germinated sclerotia also resulted in a larger number of stromata formed than if ergots are undisturbed (Cooke and Mitchell, 1967).

The mean length of stromata ranged from 18.5 - 19 mm and the length of capitula ranged from 1.8 mm to 2 mm. The stipe length in the current study ranged from 17.5 - 18 mm. In a study by van der Linde and Wehner (2007), a relatively shorter stipe length and width of 10 – 15 mm and 1.5 – 1.8 mm respectively were reported. The color of capitula and stipes for the isolates of *Claviceps* in the current study was brown and pale brown. The capitula color was also reported as pale-fawn (Dickson, 1956), light orange to pale red (Tanda, 1979) and flesh-colored (Sprague, 1950) for *Claviceps purpurea* and greyish-organge to grayish-red for *Claviceps cyperi* (van der Linde and Wehner, 2007). Perithecia in the current study were cylindrical to flask shaped with mean length and width of 158.8 ± 3.7 µm and 89.2 ± 1.7µm respectively. The mean length and width of ascospores on the other hand were 77.1 ± 4.3 µm and 3.3 ± 0.5 µm respectively. Loveless and

Peach (1986), also reported that ascospores of *Claviceps purpurea* were filiform with length of 60 – 120 µm and width of 0.5 – 1 µm. A study by van der Linde and Wehner (2007), found ellipsoidal Perithecia with dimension of 300-360x130-150 µm and filiform ascospores with dimension of 75 – 100x1-1.5 µm.

Differences in the color, length and width of stipe and capitula between our isolates and that of van der Linde and Wehner (2007) might be due to differences in the species of the ergot fungi used in the study. The color of stipe was more related to those reported by Dickson (1956) and Sprague (1950) with slight differences. Perithecia dimension for our isolates were slightly higher than those reported by Loveless and Peach (1986), this might be due to differences in the method of cultivation of the ergot fungi, measurements and stage at which the Perithecia were analyzed. The differences might also be due to differences in the adaptation of the fungi to different geographical areas. Ascospores length on the other hand lied in the range reported by Loveless and Peach (1986) and van der Linde and Wehner (2007) with slight difference in the width of the ascospores.

Phylogenetic analysis of the of the ergot fungi was done using the β-tubulin intron 3 region. The sequence of BLAST search revealed 99% identity of the sequences of the β-tubulin intron 3 region of our isolates with 12 *Claviceps purpurea* isolates (strains) and 1 *Claviceps cyperi* strain. Majority of the *Claviceps purpurea* isolates retrieved from GenBank were groups of *Claviceps purpurea* associated with land grasses which are grouped as G1 (Duncan *et al*., 2002). The maximum parsimony analysis of the taxa based on β-tubulin intron 3, showed clustering of our isolate separately with strong bootstrap value of 94 in a clade that constitutes *Claviceps purpurea* from the G1 group and *Claviceps spartinae* from G3 (on grasses in salt marsh habitats) and *Claviceps humidiphila* (G2) groups (grasses from wet and shady environments (Duncan *et al.,* 2002).

Even when the sequence of β-tubulin intron 3 region of the G2 and G3 were not included in the analysis, isolates in the current study were grouped separately with strong bootstrap value in a clade that contains *Claviceps purpurea*. But, the relatively less related *Claviceps africana* and *Claviceps sorghicola* used for comparison where grouped in a separate clade. This is also supported by study by Tooley *et al*. (2001), who also found grouping of the *Claviceps purpurea* strains in different clade with *Claviceps africana* and *Claviceps sorghicola.* Based on the morphological and molecular features, our isolates belonged to the G1 group of the *Claviceps purpurea* species, but some of the

differences in our isolate from the G1 groups based on both morphological and molecular features, it might be a new strain of the *Claviceps purpurea* species.

3.6 Conclusion and recommendation

In this study, morphological and molecular characterization of ergot fungus isolated from wild oats was done. The morphological features used include both sexual stage features such as the color of stroma, stipe, capitula and their dimensions. Sclerotia dimensions, asexual spores (conidia) dimensions were analyzed and compared with the result of other researches as well as between different localities in the study areas. In addition, phylogenetic analysis of the β-tubulin gene intron 3 regions was also used to assess the relationship of isolates in the current study with *Claviceps* species for which sequences are found from the database. Slight variations between isolates in the current study and *Claviceps purpurea* reported by other researchers was observed in the morphological features studied, and phylogenetic analysis placed isolates in this study in a separate cluster with strong bootstrap support. From the combined analysis, isolates in this study belonged to the G1 groups of *Claviceps purpurea* adapted to open fields. However, it might be a new variety of the widespread *Claviceps purpurea* due to its existence in a relatively dry tropical habitat and also infect a host which is endemic to Ethiopia and Yemen, though the infection has not been reported from Yemen. Further, studies need to be conducted in order to delimit whether it is a new variety or not. This research has provided a baseline data for further study on the ergot fungus.

Chapter 4. Identification of ergot alkaloids from sclerotia of ergot fungus collected from Arsi area, Ethiopia

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Abstract

Ergot alkaloids are toxic secondary metabolites produced by the ergot fungus, mainly by *Claviceps purpurea.* This fungus infects more than 600 monocot plants including economically important cereals such wheat, barley, oats, rye and wild oats. The host for the Ethiopian ergot fungus is the wild oat (*Avena abyssinica*) which is an endemic weed in Ethiopia and Yemen. The current study was initiated with the objective of identifying the ergot alkaloids contained in the sclerotia of ergot fungus collected from areas in Arsi, Ethiopia, where outbreaks of ergotism was reported. Analysis of ergot alkaloids extracted from the sclerotia was done using UPLC-QTOF High Definition Mass Spectrometer. A total of nine ergot alkaloids comprising of eight with their –innine isomers (ergometrine, ergocryptine, ergovaline, ergosine, ergocornine, valine methyl ester, lysergyl valine, lysergyl alanine) and an ergopeptam (ergocryptam) were identified. The presence of the sclerotia in the farmers' field with additional toxic ergot alkaloids signifies the need for intervention towards reduction or elimination of the ergot alkaloids from the food chain.

Key words: Ergot alkaloids, Sclerotia, wild oat, UPLC-QTOF, High definition mass spectrometer

4.1 Introduction

Ergot alkaloids are secondary metabolites produced by various members of the fungal family, *Clavicipitaceae* (Kobel and Snglier, 1986). The genus *Claviceps* parasitizes more than 600 monocot plants including economically important crops such as rye, barely, oat, rice, wheat and pearl millet (Bove, 1970) and wild oat (*Avena abyssinica*) (Teshome Demeke *et al.*, 1979). The Ethiopian host for *Claviceps purpurea* is the wild oat (*Avena abyssinica*) which is endemic to Ethiopia and Yemen (Ladizinsky, 1975).). They are colorless crystals that are readily soluble in various organic solvents, but insoluble or slightly soluble in water (Komarova and Tolkachev, 2001). Most of the ergot alkaloids are composed of a tetracyclic ergoline ring (Fig. 4.1) synthesized from L-tryptophan and an isoprene unit. The ergoline ring is methylated on the N-6 nitrogen atom, substituted on C-8 and possesses a C-8 = C-9 or C-9 = C-10 double bond (Fliger *et al*., 1997). Solubility of ergot alkaloids in polar and non polar solvents depend on the pH of the solution which affects the charge of the N-6 position of the ergoline ring. Ergot alkaloids are generally positively charged in acidic solutions and neutral at higher pH values. Due to this nature of ergot alkaloids, quantitative and qualitative determinations have been performed under alkaline conditions with non – polar organic solvents or with polar solvents under acidic conditions (Krska *et al*., 2008).

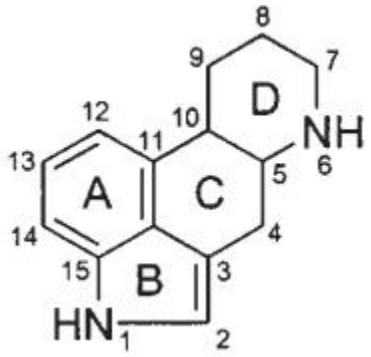
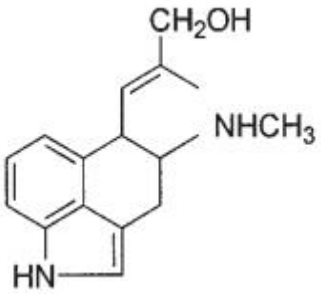


Fig. 4.1 Ergoline ring

Based on the substituent at C-8 position of the ergoline ring, ergot alkaloids are classified into Clavine, simple lysergic acid derivatives, ergopeptines and ergopeptam (Kobel and Sanglier, 1986; Mukherjee and Menge, 2000). Clavine are the simplest ergot alkaloids that contain tri-cyclic (Secoergoline) or tetra-cyclic (ergolines) compound with relation to L-tryptophan, they are substituted at position 8 or 9 with simple constituents such as methyl, hydroxymethyl or have double bond in position 8 = 9 or 9 =10. These groups of ergot alkaloids include Chanoclavines (Fig.

4.2) with an open D-ring between N-6 and C-7 and tetra-cyclic ergoline such as Agroclavine and Elymoclavine (Fig. 4.3) (Flieger *et al*., 1997).



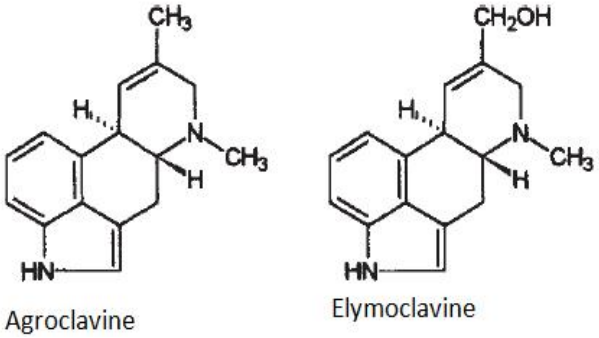


Fig. 4.2 Chanoclavine- I Fig. 4.3 Structure of clavine alkaloids with tetra-cyclic ergoline

Lysergic acid ($\Delta^{9,10}$-6-methyl-ergolene-8-carboxylic acid) is a pharmacologically active compound that has two sterocenters. The ergot alkaloids with C9=C10 double bond undergo epimerization at the C-8 carbon atom (Lehner *et al.,* 2005). The isomer of lysergic acid that has inverted configuration of the carboxyl group at C-8 is known as the *iso*-lysergic acid (Fig. 4.4) (Crews, 2015). Ergonovine (ergometrine) (Fig. 4.5) is one of the representative examples of short chain substituted amide derivative of lysergic acid.

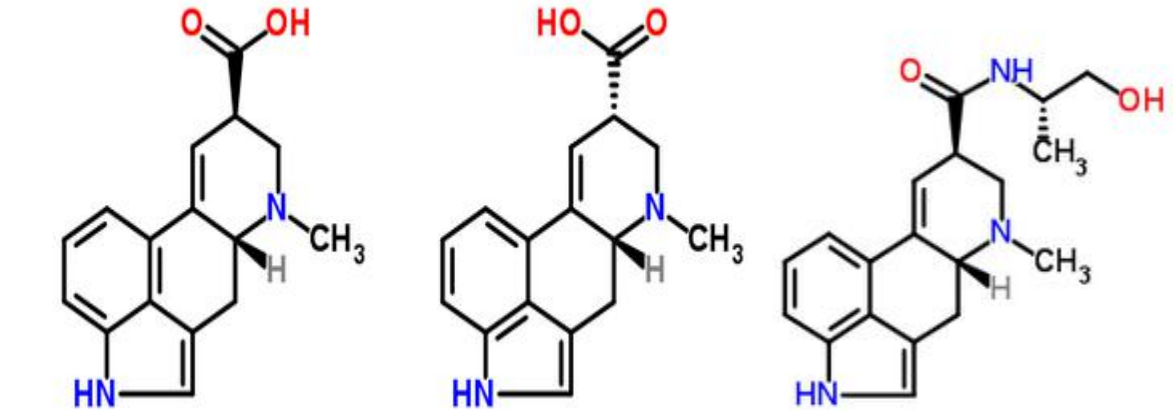


Fig. 4.4 Lysergic acid (left) and *iso*-lysergic acid (right) Fig. 4.5 Ergometrine

Peptide ergot alkaloids (ergopeptines) are tetra-peptide formed from lysergic acid and a tri-peptide cyclic structure, cyclol alkaloids (Fig. 4.6, A). Differences between the types of ergopeptines are

based on substituents at position I and II of the ergopeptines structure (Fig. 4.6, A) as position III is always occupied by proline. Ergopeptam alkaloids (Fig. 4.6, B) are another class of peptide ergot alkaloids that lack the Cyclol Bridge, these alkaloids normally co-occur with their corresponding ergopeptines. Peptide ergot alkaloids (ergopeptines) are generally considered as the end products of ergot alkaloids biosynthesis (Schardl *et al*., 2006).

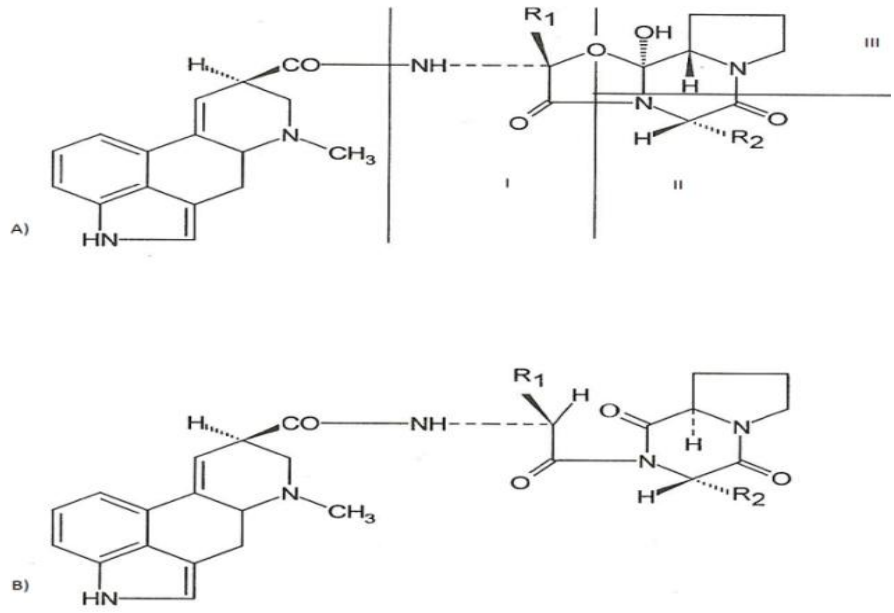


Fig. 4.6 Structure of ergopeptines (A) and ergopeptam (B)

The sclerotia of *Claviceps* species are composed of ergot alkaloids such as ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, ergocorinine and clavine alkaloids such as agroclavine (EFSA, 2005). Kobel and Sanglier (1978), identified differences in the composition of ergot alkaloids in the sclerotia collected from Europe and North America. The common combinations of ergot alkaloids in the sclerotia were ergocornine and ergocryptine (23% of the samples), ergocristine and ergosine (20%) and ergotamine (13%). The ability to produce ergot alkaloids seems to be due to habitat specialization of the *Claviceps purpurea*. The *Claviceps purpurea* grouped under G1 based on habitat preferences are adapted to dry environments such as open fields and meadows. They produce one or more of the seven ergot alkaloids (ergotamine, ergosine, ergocryptine, ergocristine, ergocornine, ergine and ergometrine) in different combinations. The G2 groups which are adapted to shady and wet habitats produce ergosine, ergocristine and small amount of ergocryptine. The G3

groups are specifically adapted to salt marshes, well known to infect Spartina, and produce combinations of ergocristine and ergocryptine (Pazoutova *et al.*, 2000).

Ergot alkaloids are toxic metabolites that were responsible for the mass poisonings in the middle ages in Europe due to consumption of bread made from rye contaminated with sclerotia of the ergot fungus (Barger, 1931). In Ethiopia, gangrenous ergotism was reported by Teshome Demeke *et al*. (1979) in Waro and Gazo-Belay areas of the highlands of Lasta and Wadla Delanta, Northern part of Wollo. According to this report, the epidemic occurred due to consumption of locally grown barley contaminated by ergot sclerotia on wild oats. A total of 93 cases and 47 deaths of ergotism were reported and majority of the cases were between the age of 5 and 34 years. The symptoms of the disease ranged from the general symptoms such as weaknesses, nausea, vomiting and diarrhea to dry gangrene of whole or part of the limbs. The most recent outbreak of gangrenous ergotism in human history occurred in 2001 in Tijo and Digelu areas of the Arsi zone, Ethiopia following the consumption of barley containing ergotized wild oat. During this outbreak 18 cases aged between 5 to 30 years were affected by the disease with three deaths (Kelbessa Urga *et al*., 2002).

These days researchers are analyzing cereals and cereal products intended for human consumption as well as animal feeds for the presence of ergot alkaloids. A study by Diana Di Mavungu *et al*. (2011), analyzed rye and wheat based food samples collected from mills in eight different European countries using HPLC-MS/MS method and reported the contamination of 182 rye and 127 wheat based food samples. In another study Malysheva *et al*. (2014), analyzed 1,065 cereals and cereal products intended for human consumption and animal feed in Europe using liquid chromatography –tandem mass spectrometry. The study revealed contamination of 59% of the food and feed samples analyzed with ergot alkaloids. Scott *et al*. (1992), also detected ergot alkaloids in over 50% of products tested. In a study that analyzed a total of 71 winter wheat samples from Albania using chromatography-tandem mass spectrometry, 48.6% of the samples during the year 2014 and 19.4% during the year 2015 were contaminated with ergot alkaloids ranging in concentration from 17.3 – 975.4 μg kg^{-1} to 10.3 – 390.5 μg kg^{-1} for samples collected during the years 2014 and 2015 respectively (Topi *et al*., 2017). Analysis of 28 rye products such as rye bread, rye crisp bread and products containing mixed cereals from UK showed the presence of ergot alkaloids in different levels in the products tested (Crews *et al.,* 2009)

The aim of the current study is to identify the type of ergot alkaloids contained in the sclerotia of the ergot fungus isolated from wild oat (*Avena abyssinica*) that grew as weed in agricultural fields in the highlands of Arsi, Ethiopia.

4.2. Objectives

4.2.1 General Objective

To identify ergot alkaloids in the sclerotia of the ergot fungus isolated from *Avena abyssinica*

4.2.2 Specific Objectives

To elucidate the structures of ergot alkaloids present in the sclerotia of the ergot fungus isolated from *Avena abyssinica*

4.3 Materials and Methods

4.3.1 Chromatographic conditions

Chromatographic separation was achieved on an ACQUITY UPLC I-class FTN system (Waters, Manchester, UK), using a ZORBAX RRHD Eclipse Plus C18 (1.8 µm, 2.1 x 100 mm). The mobile phase consisted of H_2O:MeOH (99:1, v/v) containing 0.05 % HCOOH and 5mM $HCOONH_4$ [solvent A] and MeOH [solvent B]. A gradient elution program was applied as follows: 0-0.5 min: 5 % B, 0.5-20 min: 5-95 % B, 20-21 min: 95 % B, 21-24 min: 95-5 % B, 24-28 min: 5 % B. The flow rate was 0.3 mL/min. The column temperature was set at 40 °C and the temperature of the autosampler was 10 °C. Five microliters of the sample was injected.

4.3.2 MS conditions

MS analyses were performed using a hybrid quadrupole (Q) orthogonal acceleration time-of-flight (TOF) High Definition Mass Spectrometer, the Synapt G2-Si HDMS (Waters), equipped with an electrospray ionization (ESI) source. Data were acquired as positive ion (ESI^+) polarity runs in resolution mode (>20000 FWHM). The MS parameters were as follows: a capillary voltage of 2.8 kV; a sample cone voltage of 30V; a source temperature of 150 °C; a desolvation gas flow of 800 L/h at a temperature of 550 °C and a cone gas flow of 50 L/h. Nitrogen was used as the desolvation and cone gases. Argon was employed as the collision gas at a pressure of 9.28 x 10^{-3} mbar. The instrument was calibrated using sodium formate clusters. During the MS analysis, a leucine-enkephalin solution (200 pg/µL) was continuously infused into the mass spectrometer at a flow rate of 20 µL/min via the lockspray interface, generating the reference ion ($[M+H]^+$ = 556.2771) used for mass correction. Mass spectra were collected in continuum mode from *m*/*z* 50 to 1200 with a scan time of 0.1 s, an inter-scan delay of 0.01 s and locks pray frequency of 20 s. A data-dependent acquisition (DDA) mode was implemented to obtain the simultaneous acquisition of exact mass data for the precursor and fragment ions. The top five ions were selected for MS/MS from a single MS survey scan. The scan time for MS/MS was 0.2 s. The collision energy in the trap cell was ramped from 10/15 V (low mass, start/end) up to 60/150 V (high mass, start/end).Instrument control and data processing were carried out using Masslynx 4.1 software (Waters).

4.3.3 Alkaloid extraction

Sclerotia were placed in at -20 ^{O}C overnight and powdered with mortar and Pestle and extracted with 6ml of extraction mixture (85% methanol modified with 1ml of NH_4OH per liter) with slight modification to the method by (Pazoutova *et al*., 2000). The ergot alkaloids were extracted from single sclerotium separately overnight on Agitelec overhead shaker (J. Toulemonde and Cie, Paris, France). The extract was then centrifuged and the 5 ml supernatant was evaporated till dryness under stream of nitrogen at 40 ^{O}C. Prior to UPLC-QTOF analysis the residue was reconstituted in 200 µl injection solvent MeOH:ACN:H_2O (20:40:40, v/v/v) (Pazoutova *et al*., 2000).

4.4 Result

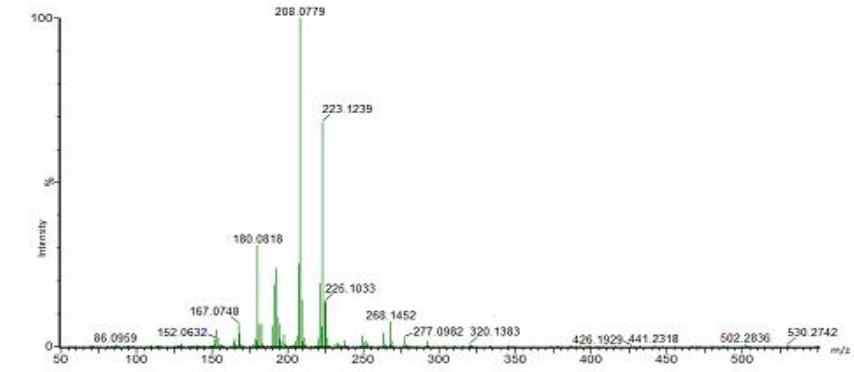
Analysis of ergot alkaloids from the sclerotia of the ergot fungus was done using UPLC-QTOF High Definition Mass Spectrometer. The ergot alkaloids detected from the sclerotia and the retention time of the –ine and their –inine isomers are indicated (Table 4.1)

Table 4.1 Features of ergot alkaloids screened from sclerotia of the fungal isolates

No	Ergot alkaloids	Chemical formula	Mono isotopic mass (Da)	Mono isotopic mass (Da) + proton (M + H^+)	Retention time
1	Ergocornine	$C_{31}H_{39}N_5O_5$	561.295105	562.302105	12.656
2	Ergocorninine	$C_{31}H_{39}N_5O_5$	561.295105	562.302105	15.022
3	Ergocryptam	$C_{32}H_{41}N_5O_4$	560.3231	561.3301	14.180
4	Ergocryptine	$C_{32}H_{41}N_5O_5$	575.310791	576.317791	15.789
5	Ergocryptinine	$C_{32}H_{41}N_5O_5$	575.310791	576.317791	16.789
6	Ergometrine	$C_{19}H_{23}N_3O_2$	325.179016	326.186016	6.14
7	Ergometrinine	$C_{29}H_{35}N_5O_5$	325.179016	326.186016	7.854
8	Ergosine	$C_{30}H_{37}N_5O_5$	547.279480	548.28748	11.094
9	Ergosinine	$C_{30}H_{37}N_5O_5$	547.279480	548.28748	12.218
10	Ergovaline	$C_{29}H_{35}N_5O_5$	533.263794	534.270794	11.052
11	Ergovalinine	$C_{29}H_{35}N_5O_5$	533.263794	534.270794	11.503
12	Lysergyl alanine	$C_{19}H_{21}N_3O_3$	340.1651	341.1721	6.060
13	Lysergyl alanine isomer	$C_{19}H_{21}N_3O_3$	340.1651	341.1721	7.856
14	Lysergyl valine	$C_{21}H_{25}N_3O_3$	368.1964	369.2034	8.341
15	Lysergyl valine isomer	$C_{21}H_{25}N_3O_3$	368.1964	369.2034	10.660
16	Valine methyl ester	$C_{22}H_{27}N_3O_3$	382.2119	383.2189	10.859
17	Valine methyl ester isomer	$C_{22}H_{27}N_3O_3$	382.2119	383.2189	10.942

[M + H^+], Protonated parent molecule; Da, Dalton

The extracted ion chromatogram (EIC) of ergosine standard and ergosine extracted from sclerotia with ($M + H^+$) = 548.29 is indicated in (Fig. 4.7) and (Fig. 4.8) respectively. The major fragments found from the ergosine standard were 208.0779 and 223.1239, and minor fragments were also observed at m/z of 180.0818, 225.1033 and 268.1452. Similarly, the major fragments for ergosine extracted from the sclerotia were observed at m/z of 208.0790 and 223.1259, with the minor fragments of 167.0758, 180.0879, 224.1311 and 268.1482.



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
548.2878	548.2846	3.2	5.8	15.5	C26 H34 N11 O3	476.3	0.787	45.51	26	34	11	3			
	548.2873	0.5	0.9	14.5	C30 H38 N5 O5	476.6	1.004	36.63	30	38	5	5			
	548.2860	1.8	3.3	9.5	C29 H42 N O9	477.5	1.978	13.83	29	42	1	9			
	548.2886	-0.8	-1.5	19.5	C31 H34 N9 O	479.0	3.466	3.12	31	34	9	1			
	548.2833	4.5	8.2	10.5	C25 H38 N7 O7	480.5	4.947	0.71	25	38	7	7			
	548.2905	-2.7	-4.9	6.5	C19 H38 N11 O8	483.1	7.556	0.05	19	38	11	8			
	548.2918	-4.0	-7.3	11.5	C20 H34 N15 O4	483.3	7.765	0.04	20	34	15	4			
	548.2891	-1.3	-2.4	1.5	C18 H42 N7 O12	483.3	7.773	0.04	18	42	7	12			

C

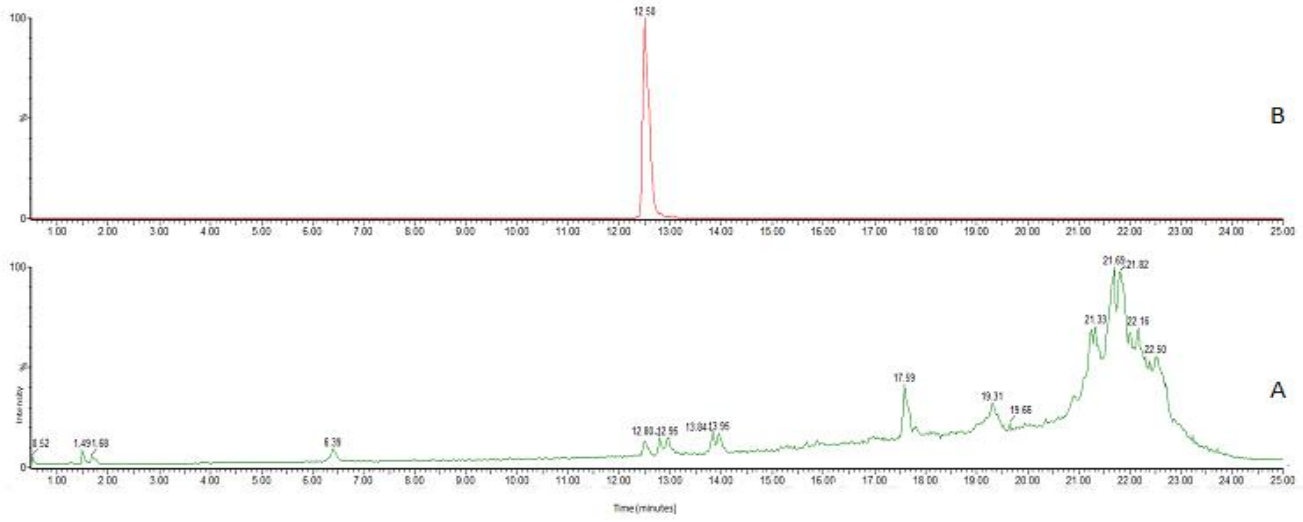
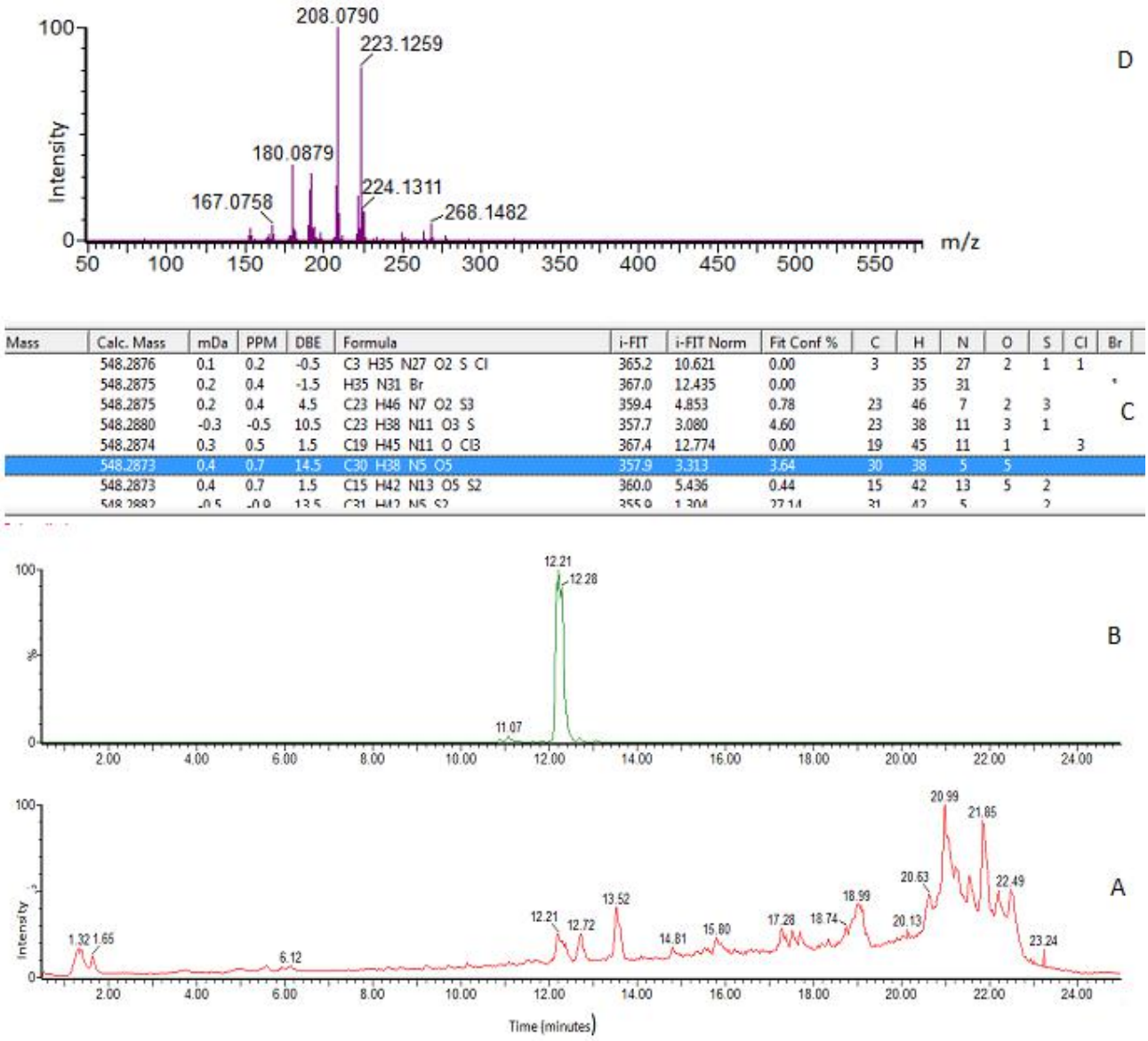


Fig. 4.7 Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of ergosine standard.



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
	548.2876	0.1	0.2	-0.5	C3 H35 N27 O2 S Cl	365.2	10.621	0.00	3	35	27	2	1	1	
	548.2875	0.2	0.4	-1.5	H35 N31 Br	367.0	12.435	0.00		35	31				*
	548.2875	0.2	0.4	4.5	C23 H46 N7 O2 S3	359.4	4.853	0.78	23	46	7	2	3		
	548.2880	-0.3	-0.5	10.5	C23 H38 N11 O3 S	357.7	3.080	4.60	23	38	11	3	1		
	548.2874	0.3	0.5	1.5	C19 H45 N11 O Cl3	367.4	12.774	0.00	19	45	11	1		3	
	548.2873	0.4	0.7	14.5	C30 H38 N5 O5	357.9	3.313	3.64	30	38	5	5			
	548.2873	0.4	0.7	1.5	C15 H42 N13 O5 S2	360.0	5.436	0.44	15	42	13	5	2		

Fig. 4.8 Total ion chromatogram (TIC)(A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of ergosine extracted from sclerotia (D).

The extracted ion chromatogram (EIC) of ergocornine standard and ergocornine extracted from sclerotia with $(M + H^+) = 562.30$ is indicated in (Fig. 4.9) and (Fig. 4.10) respectively. The major fragments found from the ergocornine standard were 208.0773 and 223.1237, and minor fragments were also observed at m/z of 153.0698, 180.0818, 225.1025 and 268.1448. The major fragments for ergocornine extracted from the sclerotia were observed at m/z of 208.0788 and 223.1245, with the minor fragments of 167.0737, 207.0796, 225.1036, 268.1451 and 305.1280

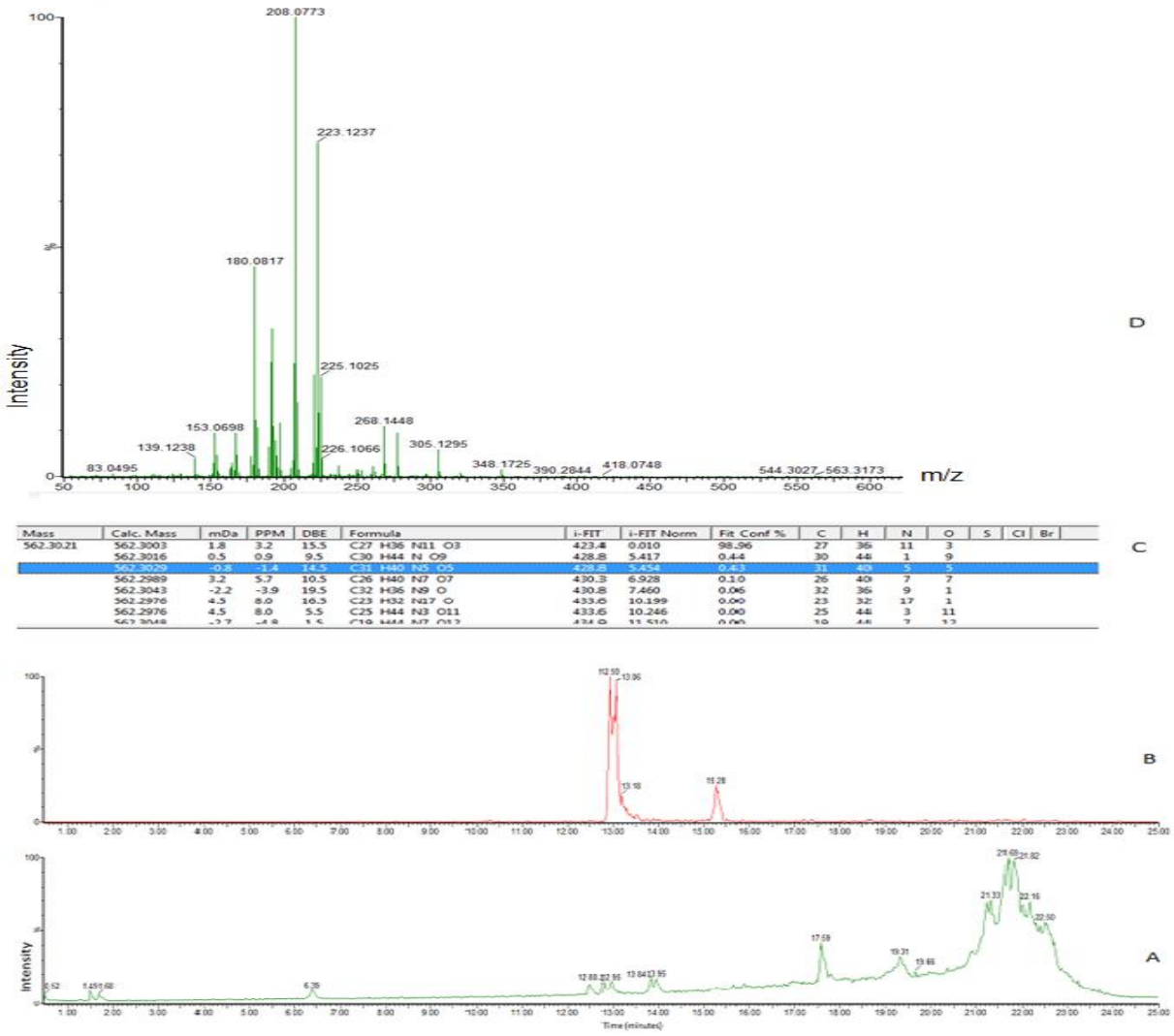


Fig. 4.9 Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of ergocornine standard(D).

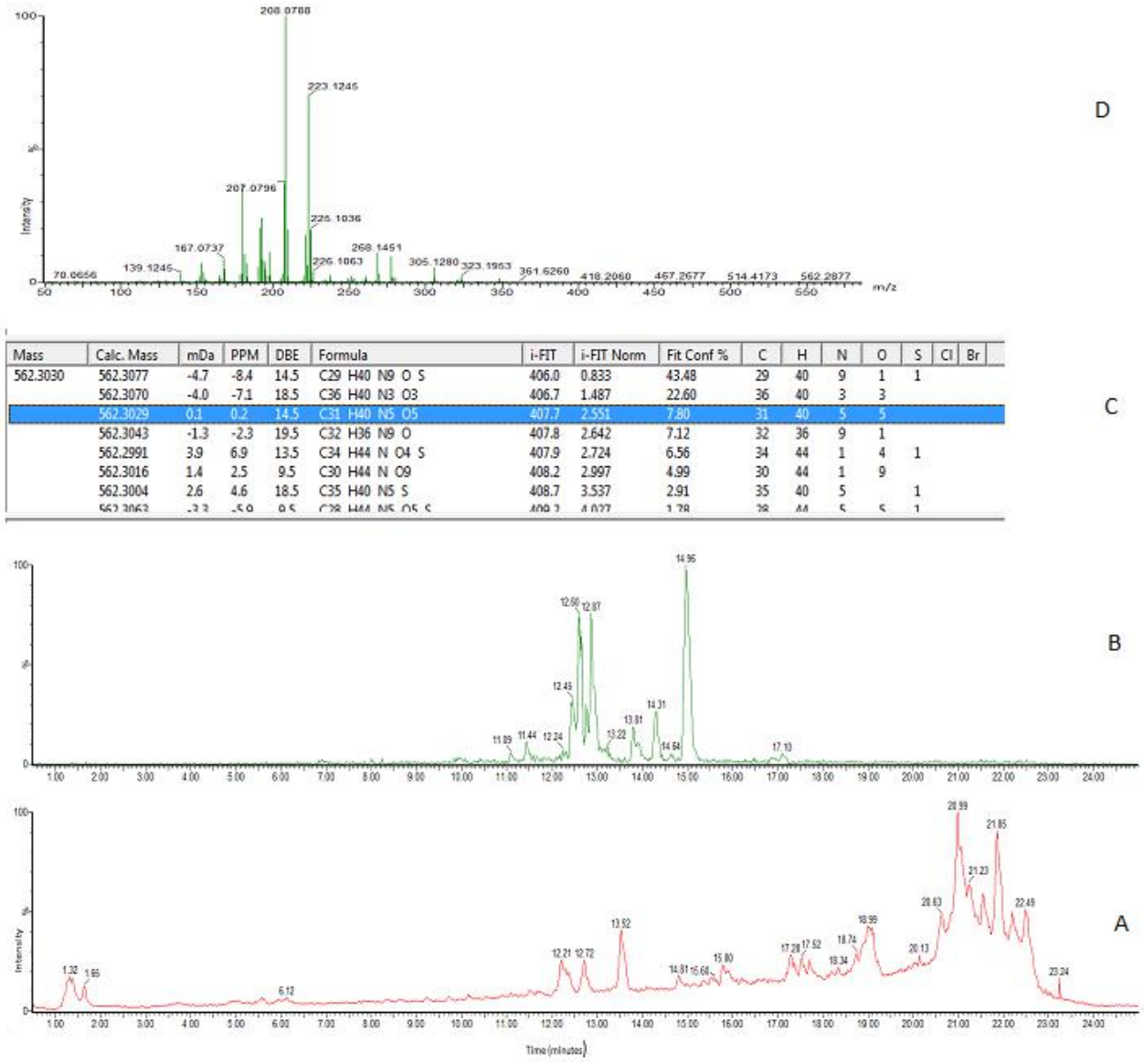
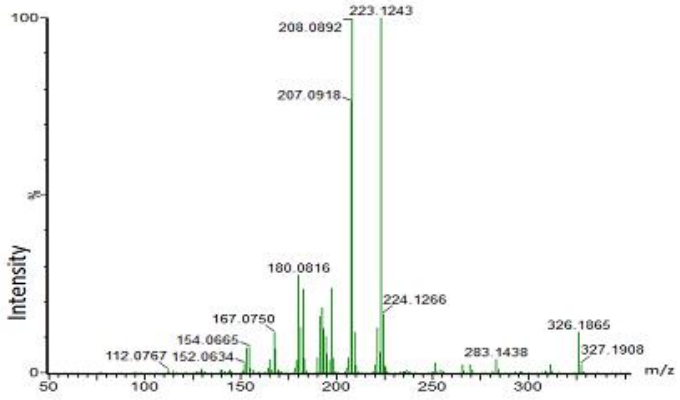


Fig. 4.10 Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of ergocornine extracted from sclerotia (D)

The extracted ion chromatogram (EIC) of ergometrine standard and ergometrine extracted from sclerotia with $(M + H^+) = 326.17$ is indicated in (Fig. 4.11) and (Fig. 4.12) respectively. The major fragments found from the ergometrine standard were 208.0779 and 223.1239, and minor fragments were also observed at m/z of 167.0750, 180.0816, 207.0918 and 224.1266. Similarly, the major fragments for ergometrine extracted from the sclerotia were observed at m/z of 208.0790 and 223.1259, with the minor fragments of 167.0743, 207.0918 and 224.1264.



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
326.1878	326.1869	0.9	2.8	9.5	C19 H24 N3 O2	639.2	0.024	97.67	19	24	3	2			
	326.1842	3.6	11.0	10.5	C15 H20 N9	643.1	3.871	2.08	15	20	9				
	326.1828	5.0	15.3	5.5	C14 H24 N5 O4	645.3	6.121	0.22	14	24	5	4			
	326.1927	-4.9	-15.0	0.5	C12 H28 N3 O7	648.0	8.746	0.02	12	28	3	7			
	326.1909	-3.1	-9.5	13.5	C24 H24 N	648.6	9.389	0.01	24	24	1				
	326.1900	-2.2	-6.7	1.5	C8 H24 N9 O5	649.3	10.090	0.00	8	24	9	5			
	326.1914	-3.6	-11.0	6.5	C9 H20 N13 O	650.8	11.625	0.00	9	20	13	1			
	326.1874	0.4	1.2	2.5	C4 H20 N15 O3	654.0	14.799	0.00	4	20	15	3			

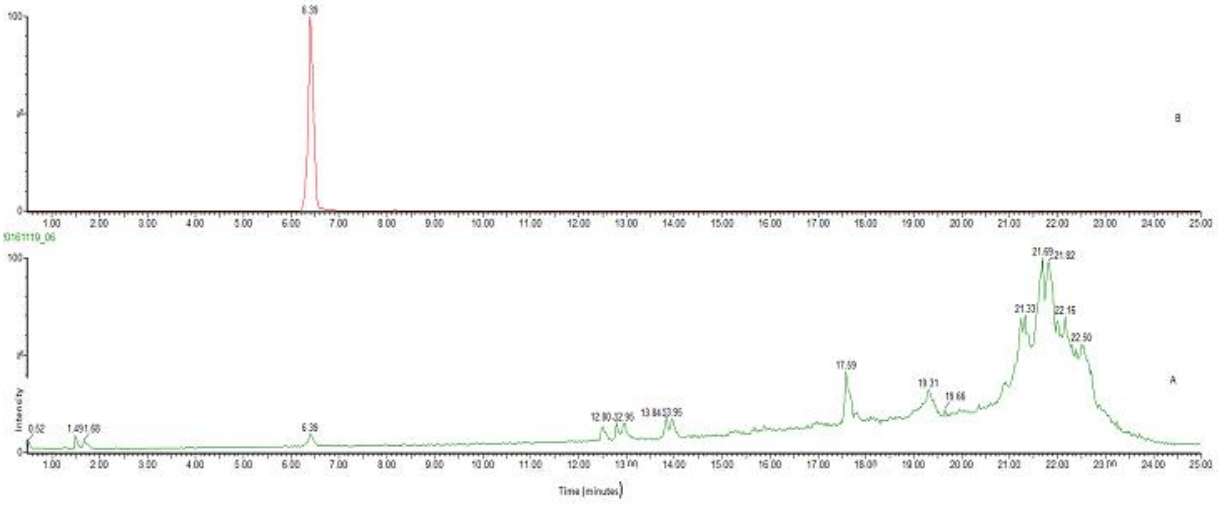


Fig. 4.11 Total ion chromatogram (TIC)(A), extracted ion chromatogram(B), estimated molecular formula (C) and fragmentation pattern of ergometrine standard (D)

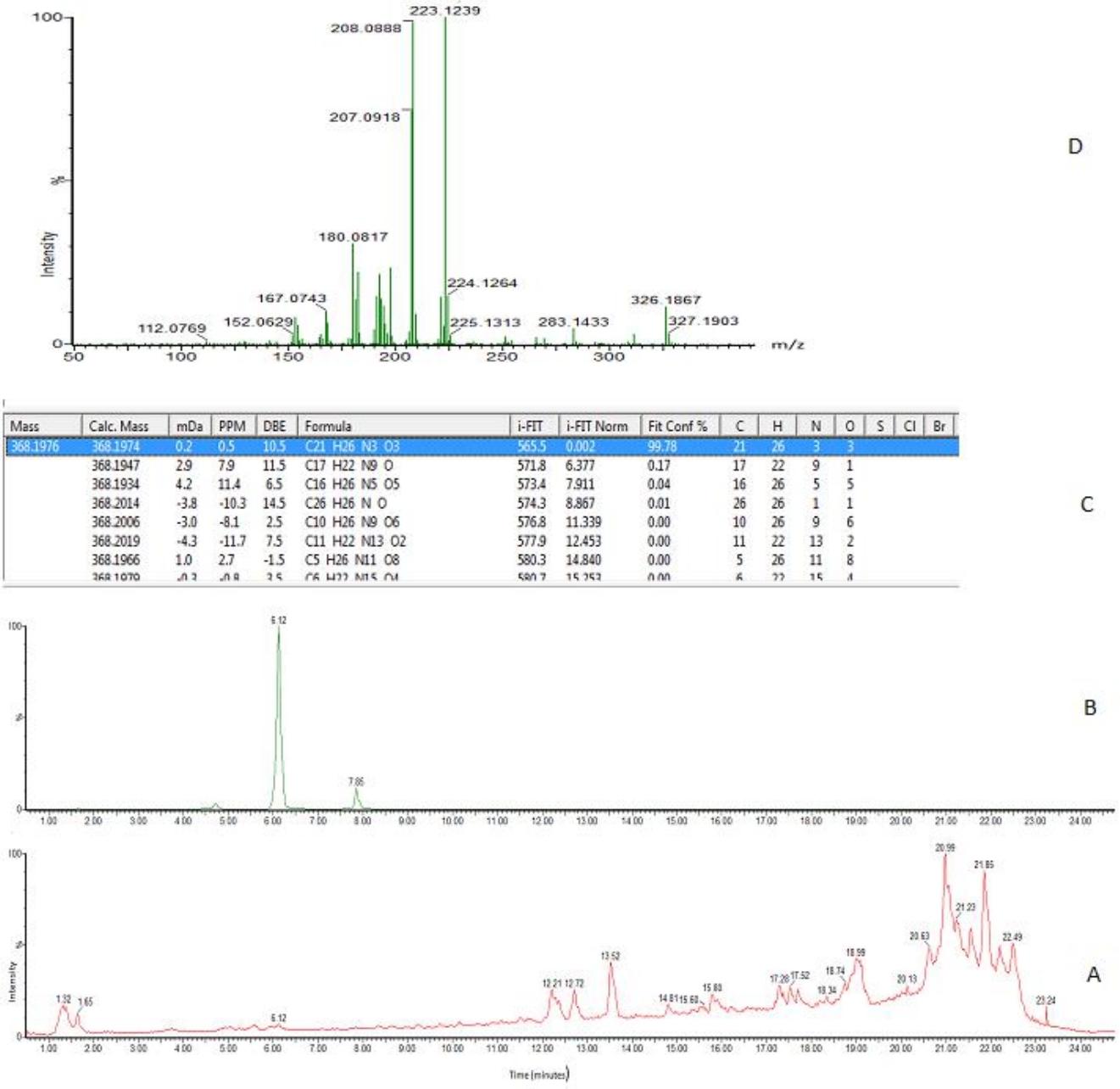


Fig. 4.12 Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of ergometrine extracted from sclerotia (D).

The extracted ion chromatogram (EIC) of ergocryptine standard and ergometrine extracted from sclerotia with $(M + H^+) = 576.32$ is indicated in (Fig. 4.13) and (Fig. 4.14) respectively. The major fragments found from the ergocryptine standard were 208.0784 and 223.1243, and minor fragments were also observed at m/z of 167.0750, 180.0813, 225.1032 and 268.1454. Similarly, the major fragments for ergocryptine extracted from the sclerotia were observed at m/z of 208.0784 and 223.1243, with the minor fragments of 167.0743, 180.0813, 225.1032, 268.1454 and 305.1293.

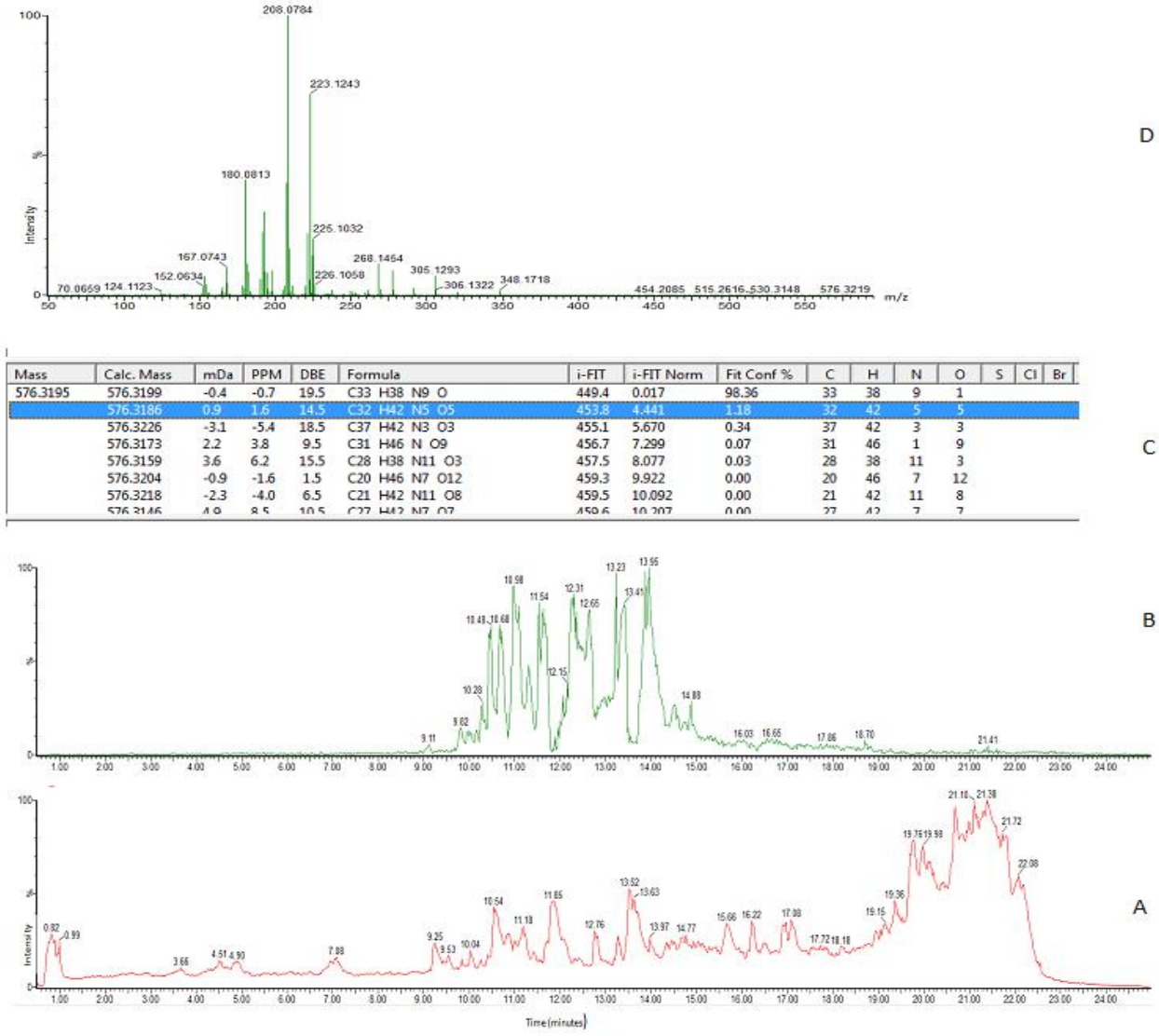
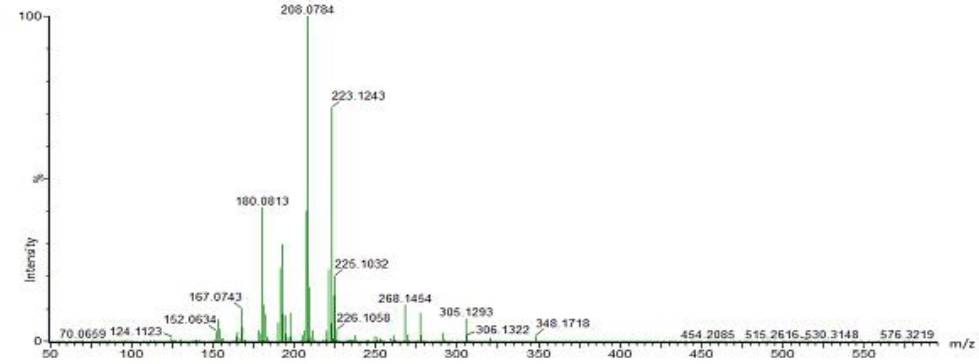


Fig. 4.13 Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of ergocryptine standard (D).



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
576.3195	576.3199	-0.4	-0.7	19.5	C33 H38 N9 O	449.4	0.017	98.36	33	38	9	1			
	576.3186	0.9	1.6	14.5	C32 H42 N5 O5	453.8	4.441	1.18	32	42	5	5			
	576.3226	-3.1	-5.4	18.5	C37 H42 N3 O3	455.1	5.670	0.34	37	42	3	3			
	576.3173	2.2	3.8	9.5	C31 H46 N O9	456.7	7.299	0.07	31	46	1	9			
	576.3159	3.6	6.2	15.5	C28 H38 N11 O3	457.5	8.077	0.03	28	38	11	3			
	576.3204	-0.9	-1.6	1.5	C20 H46 N7 O12	459.3	9.922	0.00	20	46	7	12			
	576.3218	-2.3	-4.0	6.5	C21 H42 N11 O8	459.5	10.092	0.00	21	42	11	8			
	576.3146	4.9	8.5	10.5	C27 H42 N7 O7	459.6	10.207	0.00	27	42	7	7			

C

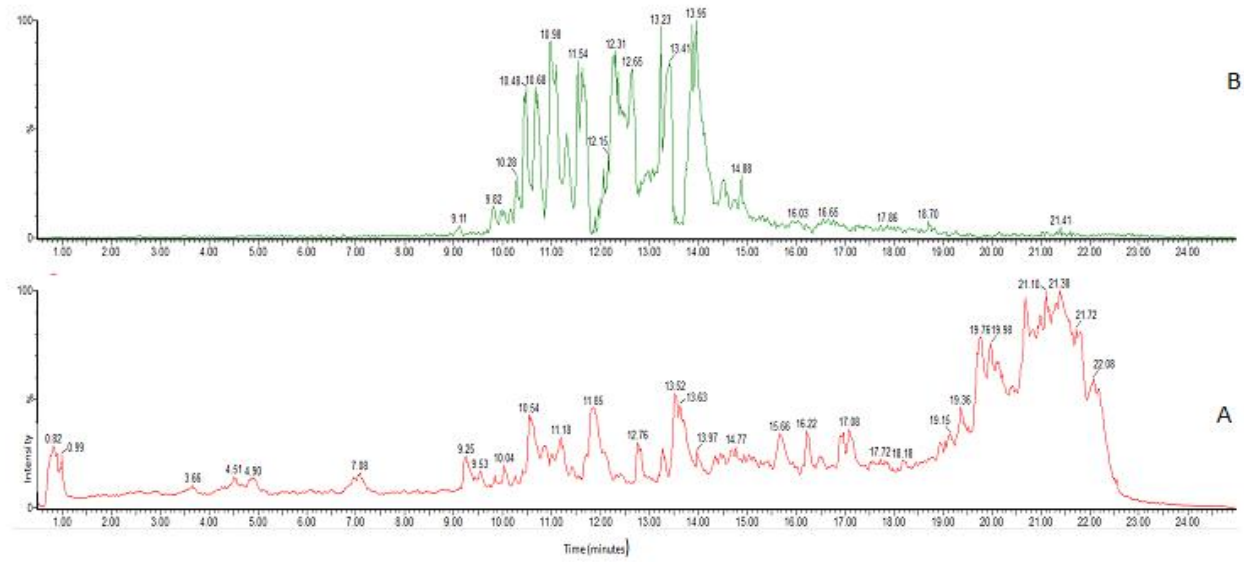


Fig. 4.14 Total ion chromatogram (TIC) (A), extracted ion chromatogram(B), estimated molecular formula (C) and fragmentation pattern of ergocryptine extracted from sclerotia (D).

4.5 Discussion

This study used UPLC-QTOF High Definition Mass Spectrometer for analysis of ergot alkaloids from sclerotia of ergot fungus isolated from wild oat plants, *Avena abyssinica*. Six ergot alkaloids (ergotamine, ergometrine, ergocornine, ergosine, ergocryptine, ergocristine) were used as standard. Identification of ergot alkaloids from the sclerotia in the current study was done by comparing with the fragmentation patterns of the respective standards. For ergot alkaloids for which standards were not found fragmentation patterns with daughter ions typical to ergot alkaloids were used for tentative identification.

In this study the major fragment ions formed from the fragmentation of most of peptide ergot alkaloids and ergoamides resulted in the formation of daughter ions at m/z 223 and 208, with minor fragments at m/z 167, 180, 225, 268 and 305. The formation of the major fragment ions in the fragmentation pattern of ergot alkaloids is also in line with (Paulke *et al*., 2014), for ergometrine where the fragment ions 223 and 208 appeared to be predominant. A study by Arroyo-Manzanares *et al.* (2014), proposed identification of the ergot alkaloids based on characteristics fragments (products ions) that are composed of 180, 208, 223, 251, 320 and 348 which were also observed in our study of ergot alkaloids from sclerotia. The fragmentation patter of the lysergyl alanine also follows similar trend to the pattern reported by Paulke *et al*., 2014).

A study by Lehner *et al.* (2004) also performed fragmentation experiment and found characteristic fragments such as 180, 197, 208, 223, 225 and 251, majority of which are also detected in our study. Fragmentation pattern of ergopeptam followed two paths the first path was transitions from m/z: 350.1854 ($C_{21}H_{24}N_3O_2^+$) → 322.1915 ($C_{20}H_{24}N_3O^+$)→ 251.1180 ($C_{16}H_{15}N_2O^+$)→223.1231 ($C_{15}H_{15}N_2$). The second proposed path was m/z: 350.1854 ($C_{21}H_{24}N_3O_2^+$) → 307.1437 ($C_{19}H_{19}N_2O_2^+$) → m/z:279.1490 ($C_{18}H_{19}N_2O^+$) → m/z:208.0754 ($C_{14}H_{10}NO^+$)(Arroyo-Manzanares et al., 2014). Our study also revealed the presence of these product ions in the ergopeptam (ergocryptam) analyzed.

A total of nine ergot alkaloids namely ergometrine, ergocornine, ergosine, ergocryptine, ergovaline, lysergyl alanine, lysergyl valine, valine methyl ester and ergocryptam were detected. The –inine isomers for the ergot alkaloids listed above other than for ergocryptam were also detected from the sclerotia of ergot fungus isolated from wild oat, Avena abyssinica. Ergotamine and ergocristine were not detected in the current study from the sclerotia samples.

Differences in the alkaloid profile from the sclerotia of Claviceps purpurea adapted to different habitats was reported by Pazoutova *et al.* (2000), with the G1 groups producing the seven ergot alkaloids(ergotamine, ergosine, ergocryptine, ergocristine, ergocornine, ergine and ergometrine) in different combinations.

Blaney *et al*. (2009), analyzed sclerotia samples from rye, barley, oats and wheat using HPLC-UV and HPLC-FLD and found Ergotamine, α-ergocryptine, ergocornine, ergosine and small amount of ergocristine. Ergotamine and ergocristine were also detected from rye ergot sclerotia using HPLC-FLD (Franzmann *et al.,* 2010). Appelt and Ellner (2009), also detected ergocristine, ergotamine, ergosine and ergocornine using HPLC-FLD). A study by Negard *et al*. (2015), used HPLC-PDA-MS analysis for analysis of ergot alkaloids from sclerotia of *Claviceps purpurea* and found higher concentration of ergocristine and ergocryptine in the G1 groups.

In a Matrix-assisted laser desorption ionization (MALDI)-time-of – flight MS based study Sivgnam *et al.* (2016) identified ergocristine, ergocryptine, ergosine and ergocornine. Ergocristine, ergosine and ergotamine were also identified as the major ergot alkaloids from sclerotia of ergot fungus from artificially infected rye verities using HPLC-FLD (Mainka *et al*., 2007). A report by Porter *et al.* (1987) indicated the presence of ergotamine, ergocristine, ergosine as the major ergot alkaloids and ergocryptine, ergocornine, ergostine, ergovaline, ergoptine and ergonine in the sclerotia of Claviceps purpurea analyzed by Tandem mass spectrometry.

A study by Kelbessa Urga *et al.* (2002), analyzed the grain samples using TLC and revealed the presence of ergotamine and ergometrine. Similarly, a study by Teshome Demeke *et al*. (1978), analyzed grain samples using TLC and found ergometrine.

But none of the profile of ergot alkaloids obtained from the sclerotia by other authors exactly fitted to the profile of ergot alkaloids identified in the current study even though the occurrence of some of the ergot alkaloids in the current study overlaps with the results of the study reported by other researchers. This might be due to differences in the geographical locations from which the ergot sclerotia were collected and in some of the cases the differences in the method of analysis might also contribute to the overall profile of the ergot alkaloids in the sclerotia. Regarding differences in the type of ergot alkaloids in the current study and the studies conducted in Ethiopia, the presence of ergometrine is similar but the presence of ergotamine might be due to differences in the sample

source and method of analysis. This study used sclerotia as the source of ergot alkaloids and advanced analytical technique where as the source of ergot alkaloids in the other studies in Ethiopia were grain samples and the analytical techniques used were Thin Layer Chromatography (TLC). The slight differences in the fragmentation patterns might be due to the MS methods used in the analysis.

4.6 Conclusion and recommendation

This study revealed the presence of ergot alkaloids such as ergometrine, ergocryptine, ergovaline, ergosine, ergocornine, valine methyl ester, lysergyl valine, lysergyl alanine, ergopeptam and their corresponding –*innine* isomers. To the knowledge of the researcher, the pattern of ergot alkaloids in the sclerotia are distinctly different compared to those reported by other researchers elsewhere in the world. Additional toxic ergot alkaloids have been identified from the sclerotia of ergot fungus collected from the formerly reported sites of ergotism outbreak in Arsi, Ethiopia. In addition, the presence of additional toxic metabolites such as ergocryptine, ergovaline, ergosine and ergocornine in the sclerotia needs special attention to control the fungus. Finally, the researchers recommend awareness creation towards removal of the host (wild oat) and control of ergot poisoning in the future.

Chapter 5. Study of the awareness of people living in the highlands of Arsi (Tijo, Digelu and Kechema areas), Ethiopia, about the ergot fungus (C. purpurea) and ergotism

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Abstract

A cross-sectional study was conducted in three Kebeles (Mankula Negele, Digelu Kidame, and Kechema Murkicha) of Sagure District near Assela town, Ethiopia, to assess the awareness of the study participants about ergot fungus and ergotism. Of the total of 385 study participants, only 100 (26 %) knew about ergot fungus and ergotism. From the study participants who were aware of ergot fungus and ergotism, majority of them 31(31%) described 'cutting off legs' as the common symptom of the disease and removing wild oats (*Avena abyssinica*) from crop fields as the main method of preventing the disease. Among the study participants who were shown the coloured picture of ergot fungus majority 55 (32.7%) described it as "Black wild oat", saw it in farm 100 (59.5%), on wild oat plant 129 (76.8%) and remove ergot from harvested grains by hand picking 99(59%). In this study among socio-demographic factors, education level was found to be significantly associated with the knowledge of ergot ($p < 0.001$) showing participants from secondary education and above, having better knowledge about the ergot fungus. From A multiple logistic regression model, study site had statistically significant association with knowledge of ergot ($p < 0.05$). In conclusion, as majority of the study participants do not know about ergot fungus and ergotism, there is a need to create awareness about the fungus and the disease in order to prevent possible future outbreak of the disease in the study area.

Key words: Ergot fungus, Ergotism, *Avena Abyssinica*

5.1 Introduction

Ergot is a parasitic fungus that belongs to the genus *Claviceps* (Nicholson, 2007). The genus *Claviceps* parasitizes more than 600 monocot plants including economically important crops such as rye, barely, oat, rice, wheat and pearl millet (Bove, 1970) and wild oat (*Avena abyssinica*) (Teshome Demeke *et al*., 1979). The name 'ergot' is derived from an old French word 'Argot' which means Cock's spur which represents the dark brown, horn-shaped fungal structure that projects from the ripening ears of infected crops replacing the grains(Van Dogen and De Groot, 1995; Nicholson, 2007). Ergot is the overwintering sclerotia of the fungus *Claviceps* formed at the end of the infection process by sexual spores (ascospores) or asexual spores (conidia) (Alderman, 2003).

The sclerotia of the ergot fungus are composed of secondary metabolites known as ergot alkaloids. The main ergot alkaloids produced by *Claviceps* species are ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, ergocornine and agroclavine (EFASA, 2005). Ergot alkaloids are toxic metabolites that were responsible for the mass poisonings in the middle ages in Europe due to consumption of bread made from rye contaminated with sclerotia of the ergot fungus (Barger, 1931). The disease that results from consumption of foods contaminated with the ergot sclerotia containing the toxic ergot alkaloids is Ergotism. There are two forms of the disease, the gangrenous ergotism also known as "St. Anthony's fire" or "Holy Fire" and Convulsive ergotism "St. Vitus' Dance". The gangrenous ergotism is characterized by an intense burning pain resulting from vasoconstrictive effects of the ergot alkaloids and subsequent loss of fingers, hands, feet and even entire limbs (Gabbai, 1951). The convulsive forms were characterized by symptoms such as hallucination, delirium and Epileptic-type seizures (De Costa, 2002; Kokkonen and Jestoi, 2010).

In France and other European countries west of the Rhine River, outbreaks of ergotism were generally of the gangrenous type, where as in Central, Eastern Europe and Scandinavia, outbreaks were of the convulsive type (De Costa, 2002; Edie, 2003). According to Gabbai *et al.* (1951), epidemic of ergot poisoning occurred in France due to consumption of bread prepared from ergotized rye. Both gangrenous and convulsive symptoms began to appear on August 1951, following a period of 6 - 48 hrs after the consumption of the ergot contaminated rye bread. During this epidemic 25 cases were of severe delirious forms and four cases comprising of 3 men and 1 woman died of cardiovascular collapse.

In Ethiopia, gangrenous ergotism was reported by Teshome Demeke *et al*. (1979) in Waro and Gazo-Belay areas of the highlands of Lasta and Wadla Delanta, Northern part of Wollo. According to this report, the epidemic occurred due to consumption of locally grown barley contaminated by ergot sclerotia on wild oats. A total of 93 cases and 47 deaths of ergotism were reported and majority of the cases were between the age of 5 and 34 years. The symptoms of the disease ranged from the general symptoms such as weaknesses, nausea, vomiting and diarrhea to dry gangrene of whole or part of the limbs. The most recent outbreak of gangrenous ergotism in human history occurred in 2001 in Tijo and Digelu areas of the Arsi zone, Ethiopia following the consumption of barley containing ergotized wild oat. During this outbreak 18 cases aged between 5 to 30 years were affected by the disease with three deaths (Kelbessa Urga *et al*., 2002).

The aim of the current study is therefore to assess the level of awareness of the local community living in the most recent ergotism outbreak region of the Arsi, zone, Ethiopia about the ergot fungus and ergotism.

5.2. Objectives

5.2.1 General objective

To assess the level of awareness of the local community living in the study area about ergot fungus and ergotism

5.2.2 Specific objectives

- To assess study participant's knowledge about ergot fungus and ergotism
- To assess practices used by the study participants to eliminate the ergot fungus
- To investigate the socio –demographic factors associated with knowledge of ergot fungus and ergotism

5.3 Methodology

5.3.1 Study area and study period

The survey was conducted in three Kebeles (the smallest administrative units) of the Sagure District of the Arsi region, namely Digelu areas (Digelu Kidame) Tijo areas (Mankula Negele) and Kechema areas (Kechema Murkicha) of the Arsi zone, Ethiopia during the month of May 2017. Tijo (Mankula Negele) is located about 50 Km from Assela, Digelu (Digelu Kidame) is located about (50) Km from Assela and Kechema area (Kechema Murkicha) is located about 38 km from the capital of Assela town.

5.3.2 Research Design

The study employed both quantitative and qualitative research designs. Both close and open ended questions were used as instrument for gathering information from the study participants. A cross-sectional research design was conducted in three different Kebeles of the Sagure district namely Mankula Negele (Tijo area), Digelu Kidame (Digelu area) and Kechema Murkicha (Kechema Murkicha areas), Arsi zone, Ethiopia. Qualitative data was gathered though focus group discussion, key informants interview and snow ball sampling techniques.

5.3.3 Sample size determination

A total of 385 study participants took part in the current study which was designed to assess the level of awareness of the local community about the “ergot fungus” and the disease “ergotism”. The sample size for this study was calculated using the formula indicated below with 95 % CI, 5 % marginal error and 50% estimated level of awareness of the study participants about ergot fungus and ergotism (Cochran, 1963).

$N = Z^2p\ (1 – p)/e2$

Where N = required sample size

Z = confidence level at 95% (1.96 standard value)

p = estimated awareness in the study area (0.5)

e = marginal error at 5% (0.05 standard value) or the level of precision.

Sampling

Quantitative study

The sampling sites were divided into three areas namely Digelu area, Tijo area and Kechema area based on proximity of the Kebeles to each other. There are seven Kebeles near Digelu areas, 5 Kebeles near Tijo areas and 9 Kebeles near Kechema areas. Digelu Kidame, Mankula Negele and Kechema Murkicha from Digelu area, Tijo area and Kechema area respectively were randomly selected by lottery method. After the Kebeles were selected the total population size of the selected Kebeles was obtained from the administrative heads of each Kebele and proportion of the study participants was estimated. Accordingly, 110 study participants from Digelu Kidame (Digelu area), 110 study participants from Mankula Negele (Tijo area) and 165 study participants from Kechema Murkicha (Kechema area) were randomly selected. Questionnaires were delivered to the selected households by data collectors trained for one day and the principal investigator. Each questionnaire had two parts; the first part was about general information of the study participants and their socio-demographic characteristics, the second part has two sub-parts seven questions asking about ergotism without showing the picture of the ergot fungus and the second sub-part four questions showing the colored picture of ergot sclerotia taken side by side with the wild oat seeds.

Qualitative study

For qualitative study purposive sampling was used to recruit participants to the study. To collect information regarding the ergot fungus and ergotism from the community, Focus group discussion, key informants interview and snow ball sampling were used. The focus group discussion was conducted with health professionals from Assela Hospital comprising of (4 medical doctors and two public health officers from department of internal medicine. Key informants interview was conducted with community members who had prior training on the ergot fungus or have first hand information about ergot fungus and ergotism as well as a staff of the department of agriculture located at the Sagure town. Snow ball sampling was used to gather information from the cases of ergotism from Tijo areas to assess their level of awareness about the disease. Though, it was possible to find four of the cases of ergotism in the area only one was willing to participate in the current study.

5.3.4 Limitation of the study

The information for the current study is based on an outbreak which occurred about 15 years back, for which there might be loss of memory of the disease condition as it occurred only once in the area.

5.3.5 Data analysis

All of the completed questionnaires were coded and entered into SPSS version 24. Frequency and percentage were used for descriptive analysis of the data collected and logistic regression was used to examine the association between some important variables and knowledge about the ergot fungus.

5.4 Results

5.4.1 Result of quantitative study

Majority of the study participants in the current study were males (87.5%), married (98.7 %), farmers (96.9%) and lived in the study area for more than 16 years (98.7%). About 46.2 % of the study participants were between the age of 30 and 41. Nearly 51 % and 42.6 % of the study participants respectively were Christians and have 3-5 households (Table 5.1)

Table 5.1. Socio-demographic characteristics of the study participants

Characteristics	Frequency	Percent	Characteristics	Frequency	Percent
Sex			**Number of households**		
Male	337	87.5	1 – 2	27	7
Female	48	12.5	3 – 5	164	42.6
Marital status			6 – 8	144	37.4
Married	380	98.7	9 and more	49	12.7
Single	4	1			
Widowed	1	0.3			
Age			**Residence (years)**		
18 – 23	5	1.3	1 – 5	2	0.5
24 – 29	46	11.9	6 – 10	2	0.5
30 – 35	89	23.1	11 – 15	1	0.3
36 – 41	89	23.1	16 years and more	380	98.7
42 – 47	53	13.8			
48 – 53	46	11.9			
54 – 59	25	6.5			
60 and more	32	8.3			
Education level					
Illiterate	40	10.4			
No formal education but can read and write	50	13			
Primary education	220	57.1			
Secondary education and above	75	19.5			
Occupation					
Farmer	373	96.9			

Merchant	4	1
Self employed	5	1.3
Government employee	1	0.3
Student	2	0.5
Religion		
Christian	196	50.9
Muslim	187	48.6

From the total of 385 study participants recruited from the three Kebeles (Digelu Kidame, Kechema Murkicha and Mankula Negele) only 26% know about ergot and 25.7 % about ergotism. Among the study participants who were aware of ergotism only 30.3% knew a person or family affected by the disease (Table 5.2). Regarding the common symptoms of the disease, the study participants answered that the disease "Cuts off legs" 31(31.3%), "Cuts off arms" 1(1%), is characterized by "Blackening, wounding, drying, severe pain of the legs and lack of sleep" 5 (5.1%), leads to "swelling of limbs" 25 (25.3%), "head ache" 2 (2%) and "nausea, vomiting, diarrhea and stomach ache" 26 (26.3%), 10 (10%) provided unrelated responses or did not respond at all.

From the study participants who responded that they had heard of ergotism, majority of them 72 (72.7%) think that the disease cannot be transmitted from patients to healthy person and 65 (65.7%) think that the disease can be prevented (Table 5.2). Out of the 65 respondents who think the disease is preventable, 35(53.8%) described that the disease can be prevented by removing wild oats from agricultural fields, 15 (23.1%) removing ergot from harvested grains and 12(18.5%) removing wild oats from threshed seeds of wheat and barley just before going to the millhouses and 3 (4.6%) didn't respond.

Table 5.2 Knowledge of the study participants about ergot fungus and ergotism

Questions	Response	Frequency	Percent
Do you know ergot?	Yes	100	26
	No	285	74
Have you heard of the disease ergotism?	Yes	99	25.7
	No	286	74.3
Do you know a person or family affected by ergotism?	Yes	30	30.3
	No	69	69.7
Do you think the disease is transmittable?	Yes	2	2
	No	72	72.7
	I don't know	25	25.3
Do you think the disease is preventable?	Yes	65	65.7
	No	30	30.3
	I don't know	4	4

After showing the coloured picture of the ergot fungus, 43.6% of the study participants responded that they recognized the picture of the fungus (Table 5.3). Then these study participants were asked about the local name of the fungi in the picture " ergot picture", 55 (32.7%) answered "Sinara Guracha" in local name which means "Black wild oat", 25(14.9%) "Sinara Dama" or "Honey wild oat", 23(13.7%) "Dhukuba Sinara" or "The disease of wild oats", 22(13.1%) "Dhufiyee" which based on the description fits to "Smut", 39 (23.2%) of the study participants either don't know 4(2.4%) or didn't give response.

From the study participants who recognized the picture of the ergot fungus majority 100(59.5%) of them saw the fungus in farm and 5(5%) did not give any response (Table 5.3). The study participants were also asked on which crop in the farm they saw the ergot fungus. Majority of them 129 (76.8%) answered on wild oats, 4 (2.4%) on wheat, 1 (0.6%) on barley and 34 (20.2%) didn't give response.

Majority of the study participants who recognized the picture of ergot fungus 142 (84.5%), answered farmers remove the ergot fungus from crop fields or harvested grains (Table 5.3), either by hand picking 99 (69.7%), cutting the wild oat and the ergot fungus together using sickles 15(10.6%) and some of the study participants 28 (19.7%) didn't respond or provided unrelated responses. Regarding wild oat removal from crop fields, majority of the study participants 254 (66 %) responded that farmers remove wild oats from their crop fields (Table 5.3), by hand weeding 120 (47.2%), hand weeding and using herbicides 89(35%), using herbicides 24(9.5%), cutting wild oats 7(2.8%), or others14 (5.5%).

Table 5.3. Practices of the study participants regarding removal of ergot and wild oats from harvested grains and crop fields

Questions	Responses	Frequency	Percent
Do you know what this is? (colored picture of ergot shown)	Yes	168	43.6
	No	217	56.4
Where did you see it?	In farm(a)	100	59.5
	In harvested grains (b)	28	16.7
	In both (ab)	35	20.8
	Missing	5	5
Do farmers remove ergot from crop fields or harvested grains?	Yes	142	84.5
	No	25	15
	Missing	1	0.6
Do farmers remove wild oats from their crop fields?	Yes	254	66
	No	128	33.2
	I don't know	1	0.3
	Missing	2	0.5

In both (ab), saw ergot in farm as well as harvested grains

From the demographic-factors used in the current study, statistically significant association ($p < 0.05$) was found between education level and knowledge of ergot where respondents from higher education category had more knowledge about ergot and those with lower education levels have less knowledge about ergot. However, based on the multiple logistic regression model fitted, taking variables associated with the response from univariate analysis, only study sites have statistically significant association ($P < 0.001$) with the study participants' knowledge of ergot. Better knowledge of the study participants about the ergot fungus was recorded from Kechema Murkiacha followed by Mankula Negle and the least knowledge about ergot was recorded from Digelu Kidame.

5.4.2 Result of the qualitative study

5.4.2.1 The result of key informants' interview

Knowledge of the ergot fungus and the disease ergotism varied over the key informants interviewed. However, the response of majority of them regarding the description of the disease symptoms was similar and was as described below.

"I know ergot it is the name of the black wild oats that produces honey on the head of wild oat plants. In our area it is generally called honey wild oat or wild oat with honey. If this type of wild oat is consumed with food it can cause a disease that leads to loss of legs by sucking blood out of the big toe and gradually separating the flesh from the bone. The disease symptoms start with the big toe; first it sucks blood out of the fore toe and dries it up leading to very painful burning sensation and sleeplessness."

"Ergotism is the disease that affected households in our neighborhood in Shaldo Jigessa Kebele of the Tijo and Digelu areas. It occurred around 1995 – 1996 E.C (2002 – 2003 G.C) initially we thought it is caused by bad spirit that targeted some family. Later on people from the health center gave us training and we knew that it was caused by the black wild oats with sticky substances or glue. The affected people had no sleep and their legs starting from their big toe gradually turned into black, the flesh is separated from the bone and the bone was clearly visible protruding out of the flesh of the patients."

Regarding the source of information, transmission and prevention of the disease, majority of the study participants said:

"The disease occurred in our localities of the Tijo areas 'Tite Waji', 'Mankula Negele' and 'Shaldo Jigessa'. It cannot be transmitted from patients to healthy person and it is possible to prevent the disease by removing black wild oats or honey wild oats from harvested grains or crop fields".

Three of the key informants differed in response regarding prevention of the disease from the other key informants even though they had similar responses as the other key informants regarding the fungus, symptoms and transmission of the disease. The responses were as follows "It is possible to prevent the disease by taking great care not to touch the ergot fungus", "The disease cannot be prevented and "It is possible to prevent the disease by eating balanced diet".

Regarding the removal of ergot from their crops majority of the key informants said that "They remove the ergot body from harvested grain and also from the field. But, the wild oat is given to our cattle as feed". They were then asked if it can cause disease to their cattle "No, it does not cause disease to cattle, it is merely the disease of humans".

Some differed in their response to whether they remove the ergot sclerotia "Frankly speaking if somebody in this area tells you that he removes the black wild oat from his harvested crops all the time he is misinforming you. In this area we only remove black wild oat if the food is intended for ourselves or family members. In that case, all impurities including the black wild oat are removed before taking the cereals to the mill houses. But, if we are to sell the crop in market, nobody cares about the black wild oat and other impurities."

The key informants were asked about what favors the growth of the black wild oat or ergot fungus. Majority of them said "We don't know what favors the growth of the black wild oat, but we can find few black wild oats in our farm land almost every year"

However, one informant answered "The black wild oats normally grow in large number in our farm land when the rainfall condition is prolonged. In other words, if there is prolonged rain condition in the environment the black wild oat can grow in large numbers. But, if it rains during late October the number of the black wild oat decreases"

For the question about how to eliminate the wild oat from agricultural fields as a means of prevention of the disease, majority of them said

"We used to weed out the wild oats from the barley as well as the wheat fields by hand. Now a days we use herbicides such as Axial and Pallas to remove weeds. Axial is used to eliminate wild oats and other grasses from barley fields and Pallas is used to eliminate weeds from wheat fields."

Two of the Key informants said "We use hand weeding because the herbicides are not effective for eliminating wild oats from barley as well as wheat fields"

A key informant from the Department of Agriculture was asked about the effectiveness of the herbicides and explained why farmers think it is ineffective.

According to the key informant "The reason why farmers think the herbicide is not effective include the fact that they over dilute the active ingredients in order to cover more areas of agricultural lands with less active ingredients. This act is partly because of shortage and delayed supply of the herbicides by the government agencies. The other reason for the failure of the herbicides in removing the wild oat is associated with timing of application, where early or late application leads to decreased effectiveness of the herbicides. The other reason might be the mode of application of the herbicides; some of the farmers use aerial application which is less effective as the active ingredients can be taken by wind"

5.4.2.2 The result of Focus group discussion

The focus groups were asked whether they know ergot fungus and ergotism. All of them answered "We have never heard of ergot and ergotism". They were then asked if they know what ergot alkaloids are some of them answered "I think ergot alkaloids are pharmaceutically important chemicals such as ergometrine which are used to prevent post partum hemorrhage." The group was asked about the cause of gangrene, if they know any case of gangrene in the area and how they were treated.

"Gangrene can be caused by different bacteria and other toxic substances. When we were in the OPD there were two cases of gangrene in the area and both were women. One of the two women had acute onset of gangrene and the other women was chronic gangrene case. The woman with

chronic gangrene was referred to the Black Lion Hospital. Ergot alkaloids were never associated with the two cases of gangrene during the morning report”

5.4.2.3 Result of the Snowball sampling

This report is based only on the information gathered from one case of ergotism to assess his overall awareness about the disease. He responded to all the questions saying “Ergot the black wild oat. It is a very dangerous disease that results due to consumption of food contaminated by black wild oats. It occurred in Mankula Negele, Tite Waji and Shaldo Jigessa Kebeles. The disease symptoms initially started from my big toe with burning sensation; it sucked blood out of my toe and dried it up. It was very painful and is even hard to explain. I didn’t have sleep the whole night and day. The disease is not transmissible and it is possible to prevent it. The disease can be prevented by using balanced diet and removing wild oats from agricultural fields.”

5.5 Discussion

In the current cross-sectional study about ergotism in the highlands of Arsi, Ethiopia, the awareness of a total of 385 study participants from three Kebeles under Sagure district was assessed. The finding of this study shows that there is limited knowledge of the study participants where only 26% know about ergot fungus and ergotism. Majority of the study participants (97%) were farmers. A study by Ephrem Guchi *et al.* (2015), also found out that 98.7% of farmers, 96.7% traders and 70% consumers were unaware of aflatoxin contamination and its consequence. All (100%) of the farmers participated in the study by Ephrem Guchi *et al*. (2015), never heard about aflatoxin. This comparison is made just due to lack of information about survey of ergotism in Ethiopia, even though it is not appropriate due to differences in the study topic and population.

The difference in the level of awareness between our study and study by Ephrem Guchi *et al.* (2015) could be due to differences in the study populations and the topic of the study. The lack of awareness of majority (74%) of our study participants about the fungus and the disease might be due to loss of memory since the disease outbreak occurred in the area only once about 15 years ago. There is high likely hood of loss or reduction of memory of past events due to aging (Small, 2002).

Among the study participants who were aware of the ergot fungus and ergotism (26%) majority of them described the local name of the fungus as “Sinara Guracha” which means Black wild oat due to the black coloured sclerotia of the ergot fungus (Van Dogen and De groot, 1995). Some also described it as “Sinara Dama” which means honey wild oat; this is also due to the fact that fungal infection of the flower head of the wild oat plants leads to oozing of sugary substances “Honey dew” that contains sucrose, glucose, fructose and the asexual spores of the fungus (Alderman, 2003). Some of the study participants also described the ergot fungus as “Dhukuba Sinara” which means the disease of wild oat. This is also supported by Bove (1970), who described ergot fungus as the disease of more than 600 monocotyledon plants including rye, barely, oat, rice, wheat and millet. There were also misconceptions about the ergot fungus where some of the study participants describe the ergot fungus as “Dhufiyee” which based on the description by the study participants fits to “smut”. This misconception might be due to the blackish appearance of both fungi on the heads of crops.

Among the study participants who were shown the coloured picture of the ergot fungus, majority 33.5% of them said they saw it on wild oats which is also in agreement with Teshome Demeke *et al.* (1979) who reported ergotism in highlands of Wollo, Ethiopia due to consumption of barely contaminated by ergot fungus from wild oats. Kelbess Urga *et al*. (2002), also reported ergotism in the highlands of Arsi that might be due to consumption of food contaminated by ergot sclerotia from wild oats. Besides, the presence of ergot sclerotia on wild oat plats was confirmed from five years (2011 – 2015) survey visits to crop fields in the study area by the principal investigator of the current study.

The symptoms of ergotism were described by the study participants as "cuts off legs", "cuts off arms", "Blackening, wounding, drying, severe pain of the legs and lack of sleep", "Swelling of limbs", "Head ache" and "Nausea, vomiting, Diarrhea and stomach ache". Some of these disease symptoms were also reported by (Kelbessa Urga *et al*., 2001).

According to Rey *et al.* (2003), the most common signs of ergot poisoning include gastro intestinal symptoms such as nausea, vomiting and abdominal pain and neurological symptoms such as head ache, dizziness and decreased level of consciousness. Symptoms such as intense pain resulting from vasoconstriction and subsequent gangrene with loss of fingers, hands, feet and even entire limbs, vomiting and insomnia (Gabbai, 1951) are also in agreement with our study. However, hallucination which was described by the same author does not fit to the description of the disease symptoms in the current study. This might be because, the symptoms of ergotism that occurred in Arsi, highlands were typical symptoms of gangrenous ergotism, and hallucination is one of the symptoms of convulsive ergotism (De Costa, 2002).

Regarding the prevalence of ergot fungus in the study area, majority of the key informants do not know why the prevalence of the fungus is different from year to year. But, one key informant said "Prevalence of ergot in the study area is higher during prolonged period of rainy season. This result is also in agreement with a report by Craig and Hignight (1991), who stated that the prevalence of ergot species is dependent on climatic conditions and especially pronounced during seasons with very heavy rainfall and wet soil conditions. This might be because wet soil conditions facilitate the formation of sexual structures that produces infective sexual spores that can forcefully disseminate to infect more flower heads of the host plants.

Of the 65 study participants who think the disease is preventable, majority of them 35 (9.1%) think the disease can be prevented by removing wild oats from agricultural fields and 15 (3.9%) by removing the black wild oat "ergot" from harvested grains before going to Mill house. This practice is also in agreement with Posner and Hibbs (1997), who reported that effective cleaning techniques at the mil houses enables removal of 82% of the sclerotia from grains. The cleaning procedure however becomes less reliable when the intact sclerotia are broken into fragments or small sclerotia with similar size to the grains are produced due to dry environmental conditions (Labuber *et al*., 2005). Even when the ergot sclerotia are completely removed, ergot alkaloids can still be detected in food and feed commodities (EFASA, 2005).

Among the socio – demographic factors in the current study, education level is significantly associated with Knowledge of ergot ($X^2 = 13.34$, $p = 0.004$). The knowledge about ergot was the highest for the study participants with secondary education and above. This is also in agreement with a study by Ephrem Guchi *et al.* (2015), where respondents from higher education level had better awareness than those from lower education level. People with higher education level was also found to be positively related to some types of risk of food or pesticides in food than those from lower education levels (Dosman *et al*., 2002). This might be because people with higher education levels might be better informed than those from the lower education categories.

Based on the multiple logistic regression model fitted taking variables associated with the response from univariate analysis study site was found to be significantly associated with the knowledge of ergot ($P < 0.001$). This might be because of awareness trainings given in the area by the member of the department of agriculture who is still working in the area of Kechema Murkicha. The second highest knowledge of ergot fungus was observed among the study participants recruited from the Tijo area (Mankula Negele) this is due to the occurrence of the disease in the area.

5.6 Conclusion and Recommendation

In the current study majority of the study participants don't know about ergot and ergotism, despite the occurrence of the disease outbreak in the study area. Even among the study participants who said they know about ergot fungus and the disease ergotism, there are some misconceptions regarding their knowledge of ergot where some study participants associated the ergot fungus with smut.

The practices in the study area regarding prevention of the ergot fungus and ergotism among the study participants who knew about the ergot fungus was fairly good as they think removing the ergot fungus by hand picking, cutting from the field and removing the host of the fungus "the wild oat" are some of the practices that can reduce the prevalence of the ergot fungus in agricultural fields in the study area. The response of one of the key informants, however, shows that the ergot fungus is only removed if the intention is to sell the cereals. If this perception is also shared by other community members this may lead to ergot problems in the consumers who buy and use these ergot contaminated cereals elsewhere in the country.

Detailed disease symptoms were also described by the case of ergotism in the study area, which was also shared by some of the study participants in the area. The two cases of gangrene treated in Assela Hospital in recent years, might be due to consumption of the ergot contaminated foods as both cases are living in remote areas near Assela Town, Ethiopia. Besides, the causes of the gangrene were not attributed to specific causative agents according to the information from the focus group report.

Some of the misconception about the prevention of the disease includes not touching the ergot fungus because it cuts the arms and using balanced diet. This shows that there is lack of awareness of the study participants about how the disease is caused. The reason for the prevalence of the ergot fungus in the study area was also linked to extended period of rain fall, which is also supported by other studies.

This study has provided a baseline report on the awareness of the study participants about ergot fungus and ergotism which could be used for further action. However, it has been conducted only in three Kebeles (Mankula Negele, Kechema Murkicha and Digelu Kidame) of Sagure District, further in depth study in the other Kebeles need to be done in order to assess the knowledge level of the

community living in the area and develop intervention mechanisms in controlling the prevalence of ergot fungus. The major intervention in these cases is making the community aware of the fungus and its effect on human and animals, and devising possible mechanisms of elimination of the fungus from agricultural fields.

Chapter 6. General Conclusion and Recommendations

The wild oat ergots which were responsible for the outbreak of gangrenous ergotism in Arsi area, Ethiopia, have been studied. Morphological features such as dimensions and color of sclerotia, sclerotia shape, conidia shape and size, dimension of the perithecia, shape of perithecia, shape and dimensions of ascospores, color and dimensions of stromata have been studied. Based on these features, slight variations of our isolates of the ergot fungi compared to *Claviceps purpurea* reported elsewhere by other researchers were recorded, though the conidial and other features grouped our isolates into the G1 group of *Claviceps purpurea*.

Molecular characterization based on the β-tubulin Intron 3 region was also conducted; the result of the NCBI BLAST revealed 99% similarity with 100% sequence coverage to *Claviceps purpurea* species, form the G1 group. Phylogenetic analysis based on the sequences of β-tubulin Intron 3 showed clustering of our isolates separately in the Clade that contains *Claviceps purpurea* G1 groups, *Claviceps humidiphila* (G2) and *Claviceps spartinae* (G3).

Ergot alkaloids profiles of our isolates has shown that they belong to the *Claviceps purpurea* G1, but the pattern of ergot alkaloids in the sclerotia of the ergot fungus in this study showed variation from those reported by other researchers. Differences in the type and number of ergot alkaloids detected in the current study were observed compared to the previous reports in Arsi, area. This study revealed the presence of additional toxic ergot alkaloids in the sclerotia of the ergot fungus. Overall, information from the morphological analysis, molecular analysis and the pattern of ergot alkaloids confirmed that our isolates belong to the *Claviceps purpurea* species, however, slight variations in the three parameters (morphological features, molecular features and ergot alkaloid profiles) might further group our isolates into a new variety. Further studies need to be conducted to understand taxonomic position of the *Claviceps purpurea* isolated in the current study from wild oats.

Awareness of randomly selected study participants from Tijo, Digelu and Kechema areas, where there was a report of an outbreak of gangrenous ergotism due to consumption of food contaminated with sclerotia of the ergot fungi was assessed. The study revealed that the awareness of the study participants about ergot fungus and ergotism is very low and majority of the study participants didn't know about ergot and ergotism. Despite the presence of ergot fungus in agricultural fields in

the study area, the awareness about the fungus and the disease it causes is very low. If the problem persists in the environment unrecognized, at some point when conditions are suitable for mass production of the sexual structure of the ergot fungi further infection of the wild oat plants may happen leading to another episode of mass poisoning in the study area or elsewhere. Therefore, awareness creation on the ergot fungus, ergotism and mechanisms of its control need to be devised.

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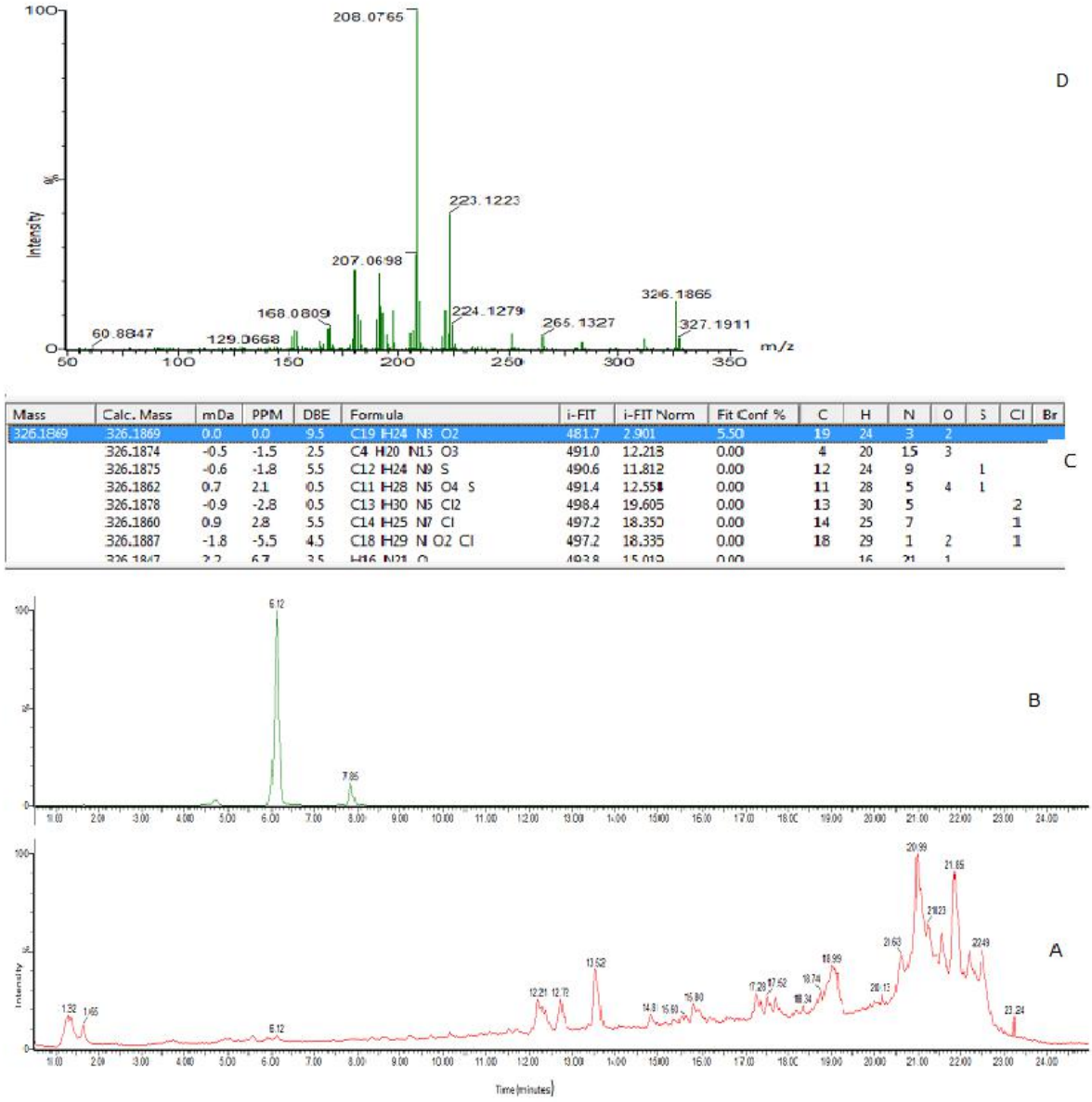
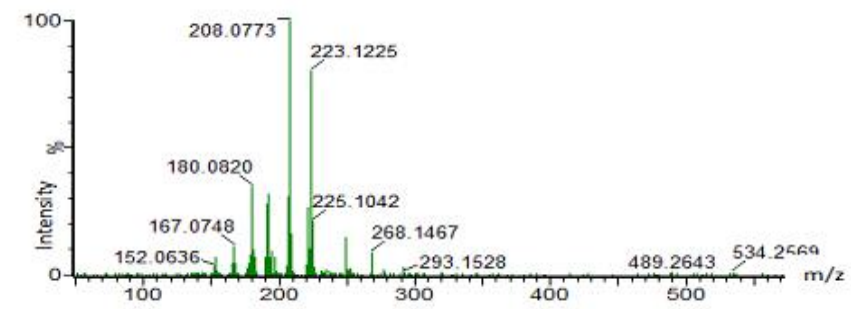
Appendices

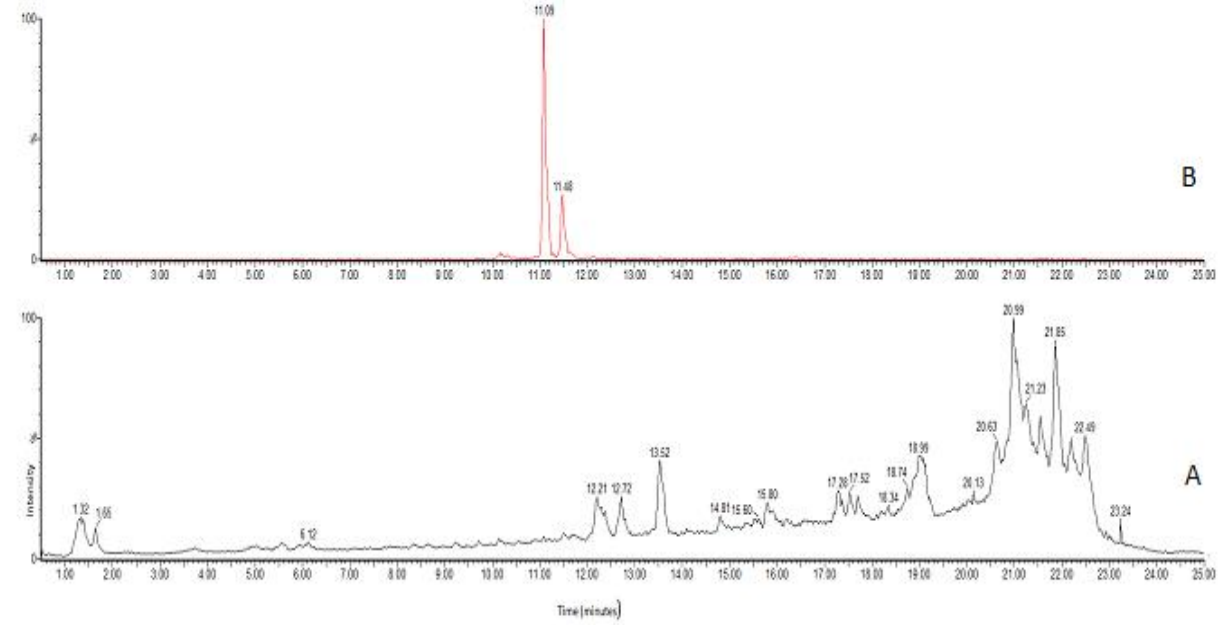


Annex I. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of ergometrinine extracted from sclerotia((D)

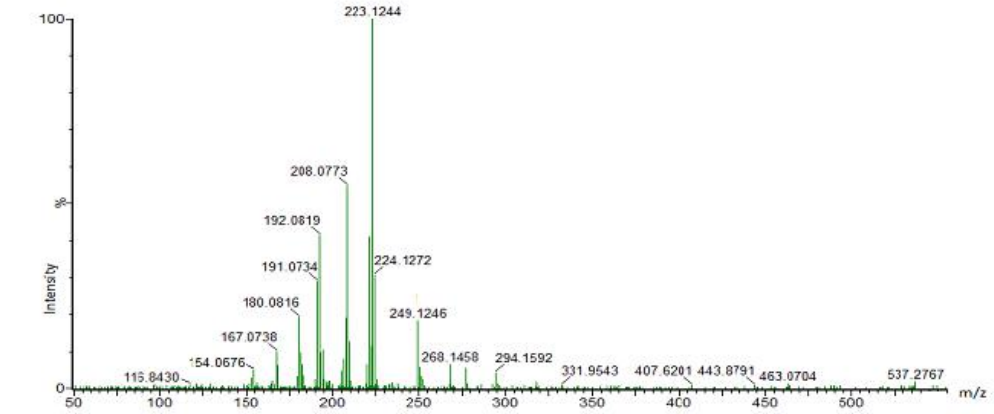


Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
534.2723	534.2716	0.7	1.3	14.5	C29 H36 N5 O5	464.8	0.551	57.63	29	36	5	5			
	534.2730	-0.7	-1.3	19.5	C30 H32 N9 O	465.5	1.291	27.50	30	32	9	1			
	534.2703	2.0	3.7	9.5	C28 H40 N O9	466.3	2.120	12.01	28	40	1	9			
	534.2757	-3.4	-6.4	18.5	C34 H36 N3 O3	468.1	3.898	2.03	34	36	3	3			
	534.2690	3.3	6.2	15.5	C25 H32 N11 O3	469.4	5.187	0.56	25	32	11	3			
	534.2676	4.7	8.8	10.5	C24 H36 N7 O7	470.8	6.588	0.14	24	36	7	7			
	534.2735	-1.2	-2.2	1.5	C17 H40 N7 O12	472.1	7.852	0.04	17	40	7	12			
	534.2748	-2.5	-4.7	6.5	C18 H36 N11 O8	472.2	7.994	0.03	18	36	11	8			

C

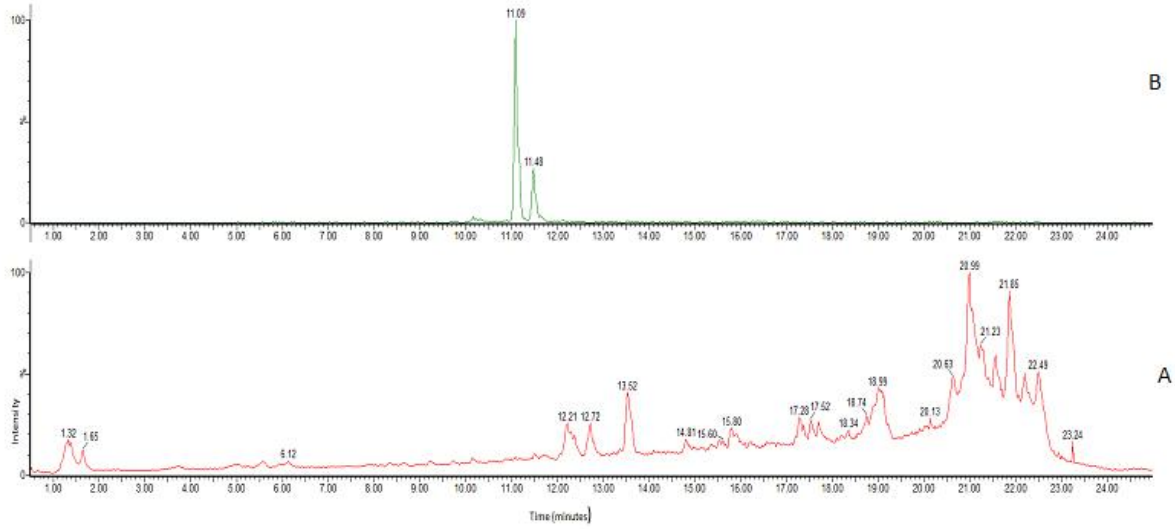


Annex II. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of ergovaline extracted from sclerotia (D)

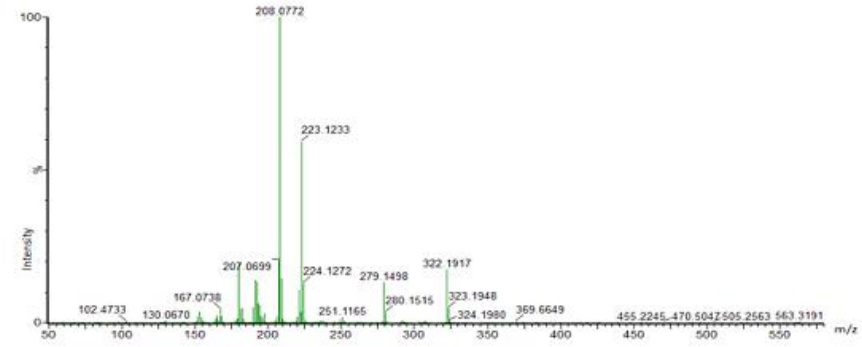


Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
	534.2717	1.4	2.6	1.5	C14 H40 N13 O5 S2	284.9	14.973	0.00	14	40	13	5	2		
	534.2745	-1.4	-2.6	3.5	C26 H48 N O4 S3	286.1	16.103	0.00	26	48	1	4	3		
	534.2745	-1.4	-2.6	3.5	C9 H34 N23 Cl2	290.6	20.582	0.00	9	34	23			2	
	534.2716	1.5	2.8	14.5	C29 H36 N5 O5	270.0	0.032	96.83	29	36	5	5			
	534.2715	1.6	3.0	6.5	C17 H37 N15 O S Cl	289.6	19.617	0.00	17	37	15	1	1	1	
	534.2747	-1.6	-3.0	-1.5	C6 H37 N21 O4 S Cl	289.7	19.682	0.00	6	37	21	4	1	1	
	534.2715	1.6	3.0	-1.5	C6 H36 N19 O8 S	284.6	14.663	0.00	6	36	19	8	1		
	534.2748	-1.7	-3.2	6.5	C18 H36 N11 O8	276.5	6.558	0.14	18	36	11	8			

C

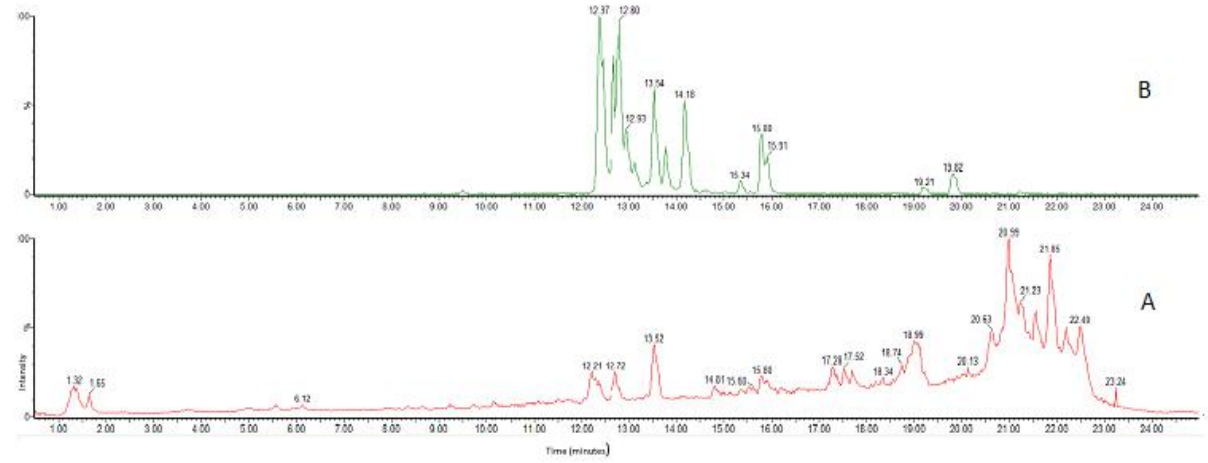


Annex III. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of erogvalinine extracted from sclerotia(D)

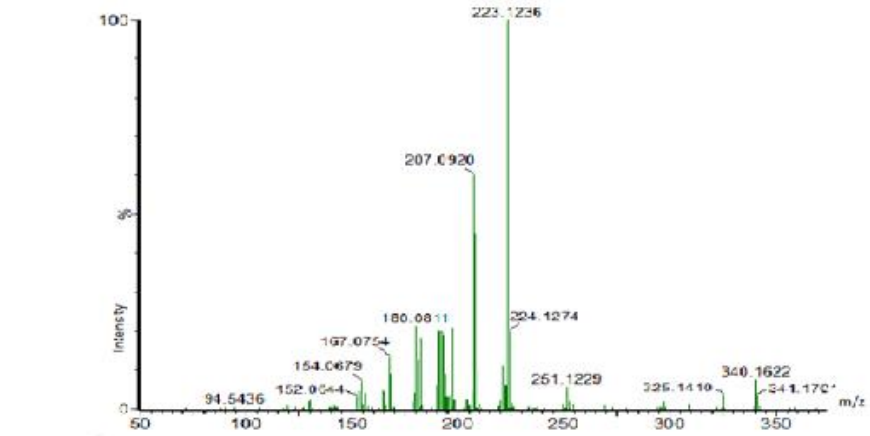


C

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
	560.3257	-0.9	-1.6	4.5	C28 H50 N O8 S	580.5	6.610	0.13	28	50	1	8	1		
	560.3257	-0.9	-1.6	-0.5	C24 H55 N5 O S3 Cl	579.0	5.186	0.56	24	55	5	1	3	1	
	560.3239	0.9	1.6	4.5	C25 H50 N7 O S3	577.8	3.966	1.89	25	50	7	1	3		
	560.3238	1.0	1.8	1.5	C21 H49 N11 Cl3	582.3	8.480	0.02	21	49	11			3	
	560.3237	1.1	2.0	14.5	C32 H42 N5 O4	581.9	8.030	0.03	32	42	5	4			
	560.3237	1.1	2.0	1.5	C17 H46 N13 O4 S2	579.9	6.019	0.24	17	46	13	4	2		
	560.3260	-1.2	-2.1	5.5	C3 H30 N33 O2	582.8	8.930	0.01	3	30	33	2			
	560.3260	-1.2	-2.1	2.5	C16 H43 N15 O5 Cl	578.4	4.587	1.02	16	43	15	5		1	



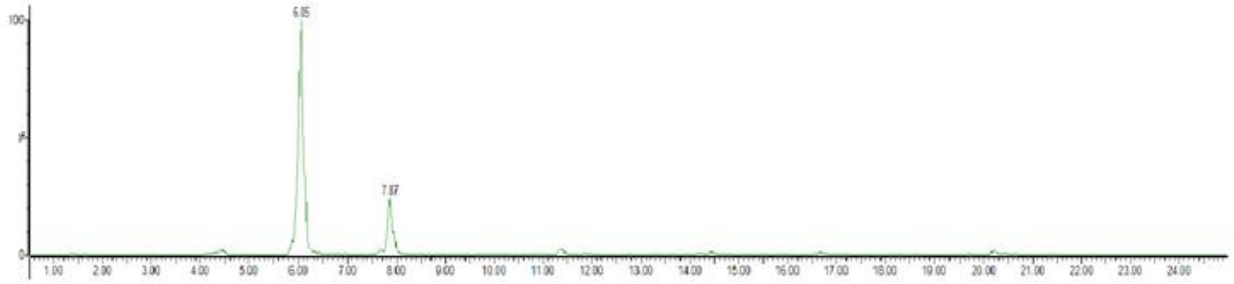
Annex IV. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of erocryptam extracted from sclerotia(D)



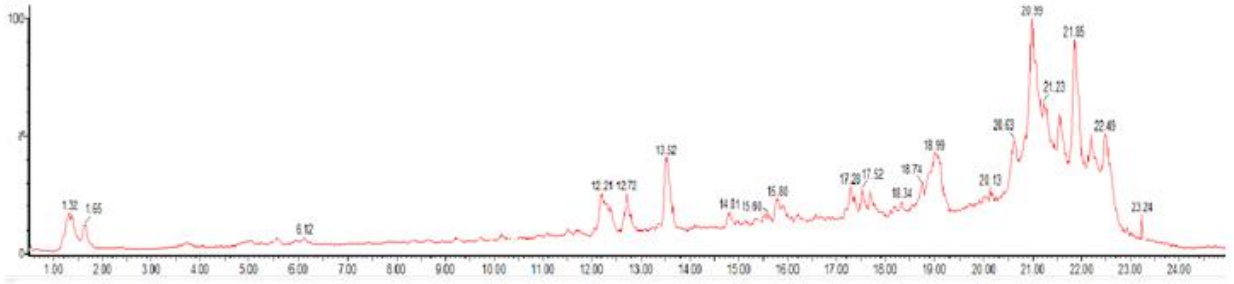
D

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
340.1667	340.1668	-0.1	-0.3	6.5	C12 H22 N9 O S	503.1	3.658	2.58	12	22	9	1	1		
	340.1666	0.1	0.3	3.5	C4 H18 N15 O4	503.0	3.485	3.07	4	18	15	4			
	340.1671	-0.4	-1.2	1.5	C13 H28 N5 O Cl2	504.5	5.022	0.66	13	28	5	1		2	
	340.1663	0.4	1.2	0.5	C12 H30 N5 S3	503.8	4.317	1.33	12	30	5		3		
	340.1661	0.6	1.8	10.5	C19 H22 N3 O3	501.4	1.936	14.42	19	22	3	3			
	340.1674	-0.7	-2.1	-1.5	C15 H35 N S Br	504.3	4.863	0.77	15	35	1		1		1
	340.1679	-1.2	-3.5	5.5	C18 H27 N O3 Cl	503.4	3.909	2.01	18	27	1	3		1	
	340.1655	1.2	3.5	1.5	C11 H26 N5 O5 S	502.9	3.430	3.24	11	26	5	5	1		

C

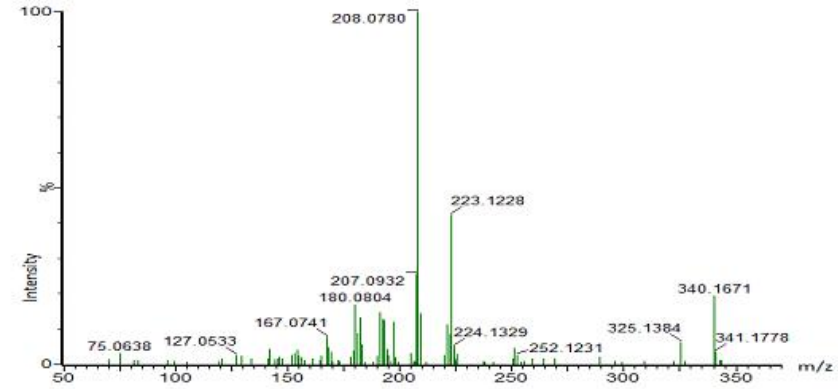


B



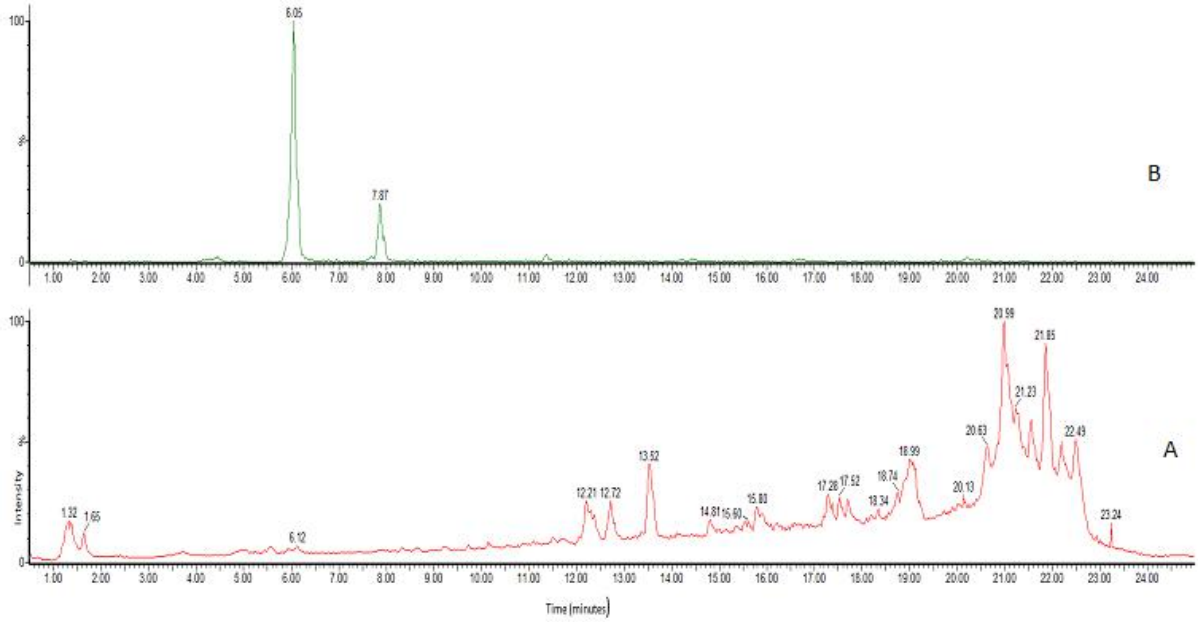
A

Annex V. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of lysergyl alanine extracted from sclerotia(D)

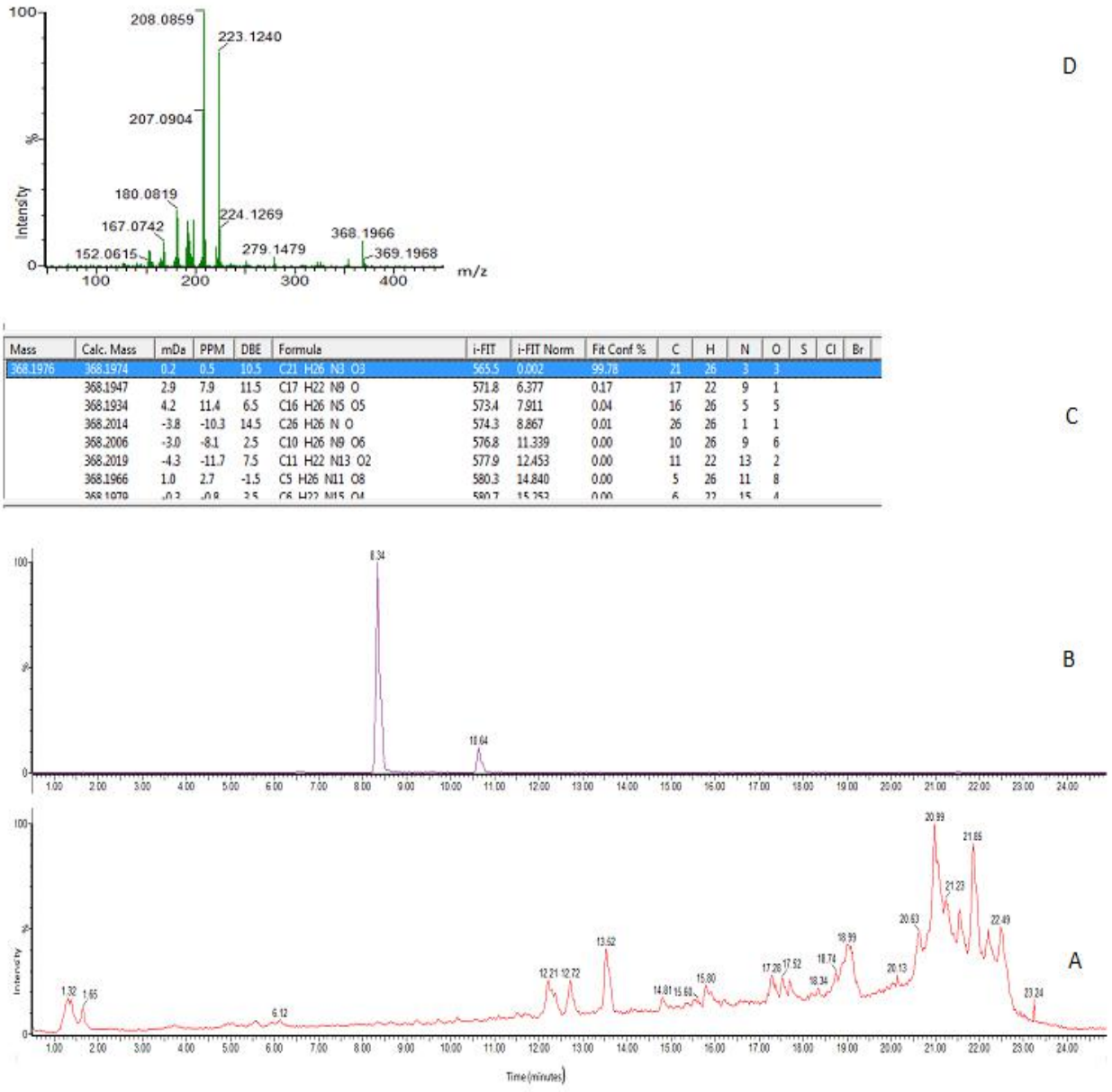


C

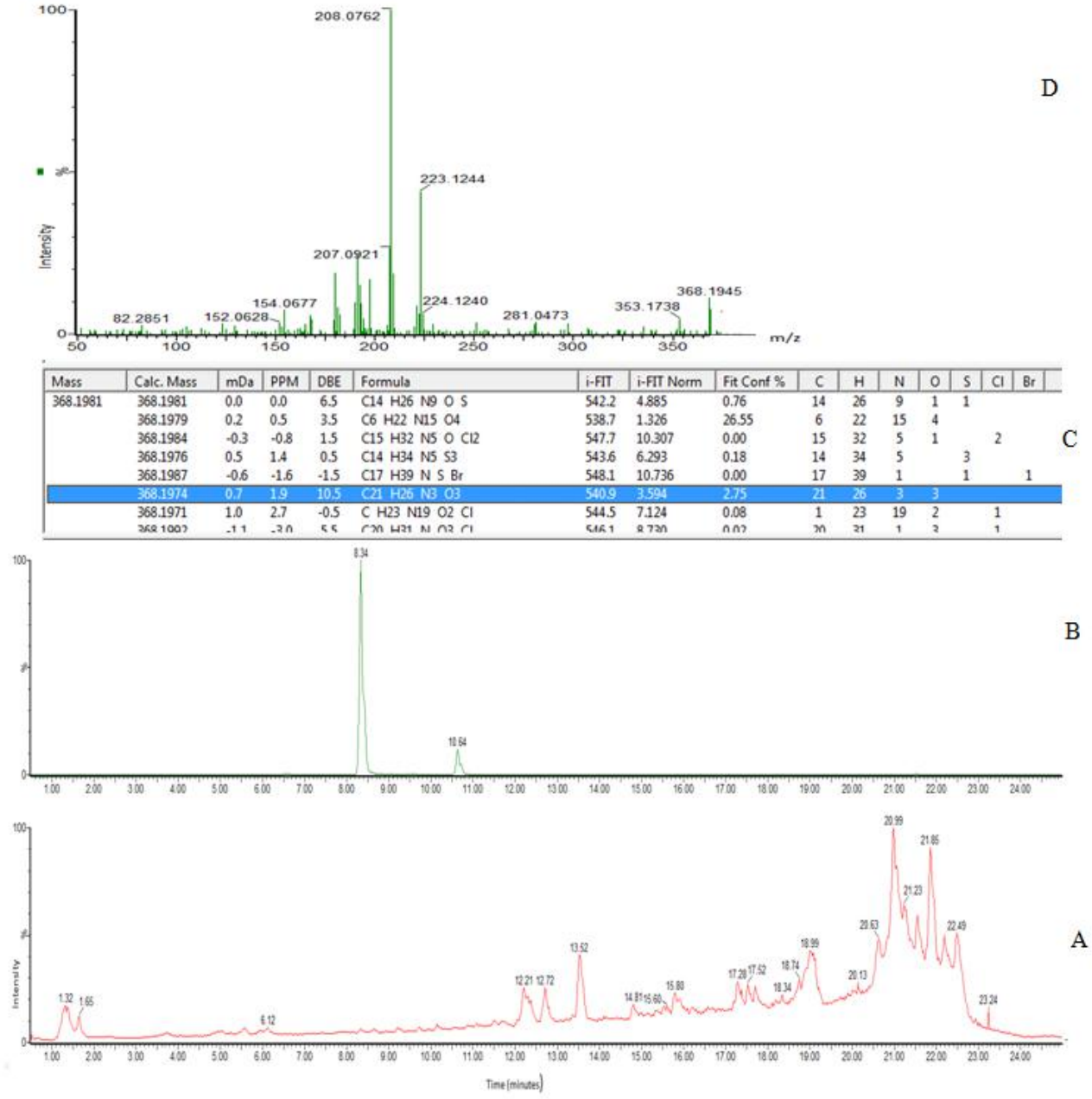
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
340.1661	340.1661	0.0	0.0	10.5	C19 H22 N3 O3	592.9	1.669	18.84	19	22	3	3			
	340.1663	-0.2	-0.6	0.5	C12 H30 N5 S3	596.8	5.588	0.37	12	30	5		3		
	340.1666	-0.5	-1.5	3.5	C4 H18 N15 O4	594.8	3.560	2.85	4	18	15	4			
	340.1655	0.6	1.8	1.5	C11 H26 N5 O5 S	596.8	5.584	0.38	11	26	5	5	1		
	340.1668	-0.7	-2.1	6.5	C12 H22 N9 O S	596.6	5.420	0.44	12	22	9	1	1		
	340.1653	0.8	2.4	6.5	C14 H23 N7 O Cl	598.4	7.198	0.07	14	23	7	1		1	
	340.1653	0.8	2.4	-1.5	C3 H22 N11 O8	594.0	2.830	5.90	3	22	11	8			
	340.1671	-1.0	-2.9	1.5	C13 H28 N5 O Cl2	600.2	9.041	0.01	13	28	5	1		2	



Annex VI. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of lysergyl alanine isomer extracted from sclerotia(D)

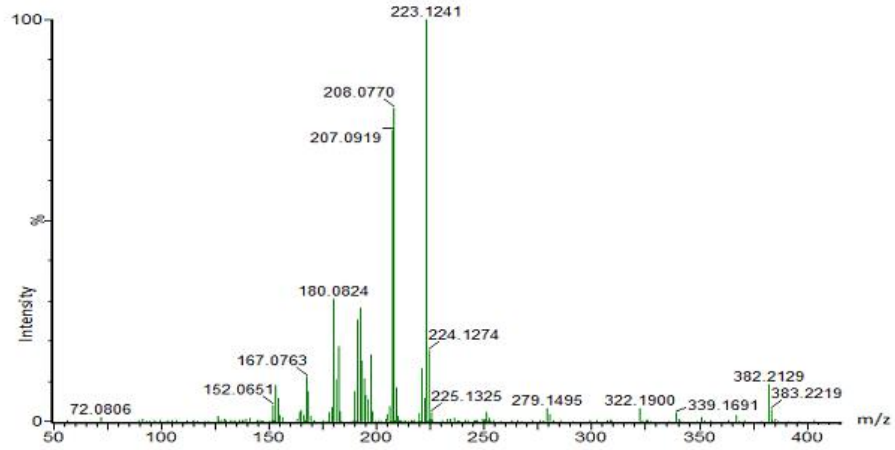


Annex VII. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of lysergyl valine extracted from sclerotia(D)



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
368.1981	368.1981	0.0	0.0	6.5	C14 H26 N9 O S	542.2	4.885	0.76	14	26	9	1	1		
	368.1979	0.2	0.5	3.5	C6 H22 N15 O4	538.7	1.326	26.55	6	22	15	4			
	368.1984	-0.3	-0.8	1.5	C15 H32 N5 O Cl2	547.7	10.307	0.00	15	32	5	1		2	
	368.1976	0.5	1.4	0.5	C14 H34 N5 S3	543.6	6.293	0.18	14	34	5		3		
	368.1987	-0.6	-1.6	-1.5	C17 H39 N S Br	548.1	10.736	0.00	17	39	1		1		1
	368.1974	0.7	1.9	10.5	C21 H26 N3 O3	540.9	3.594	2.75	21	26	3	3			
	368.1971	1.0	2.7	-0.5	C H23 N19 O2 Cl	544.5	7.124	0.08	1	23	19	2		1	

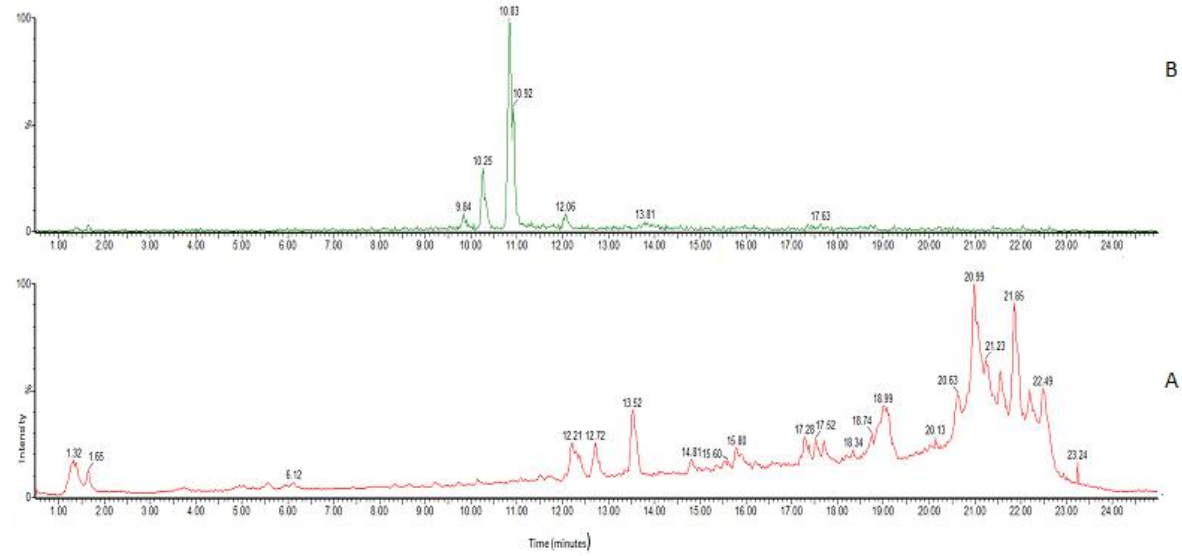
Annex VIII. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of lysergyl valine isomer extracted from sclerotia(D)



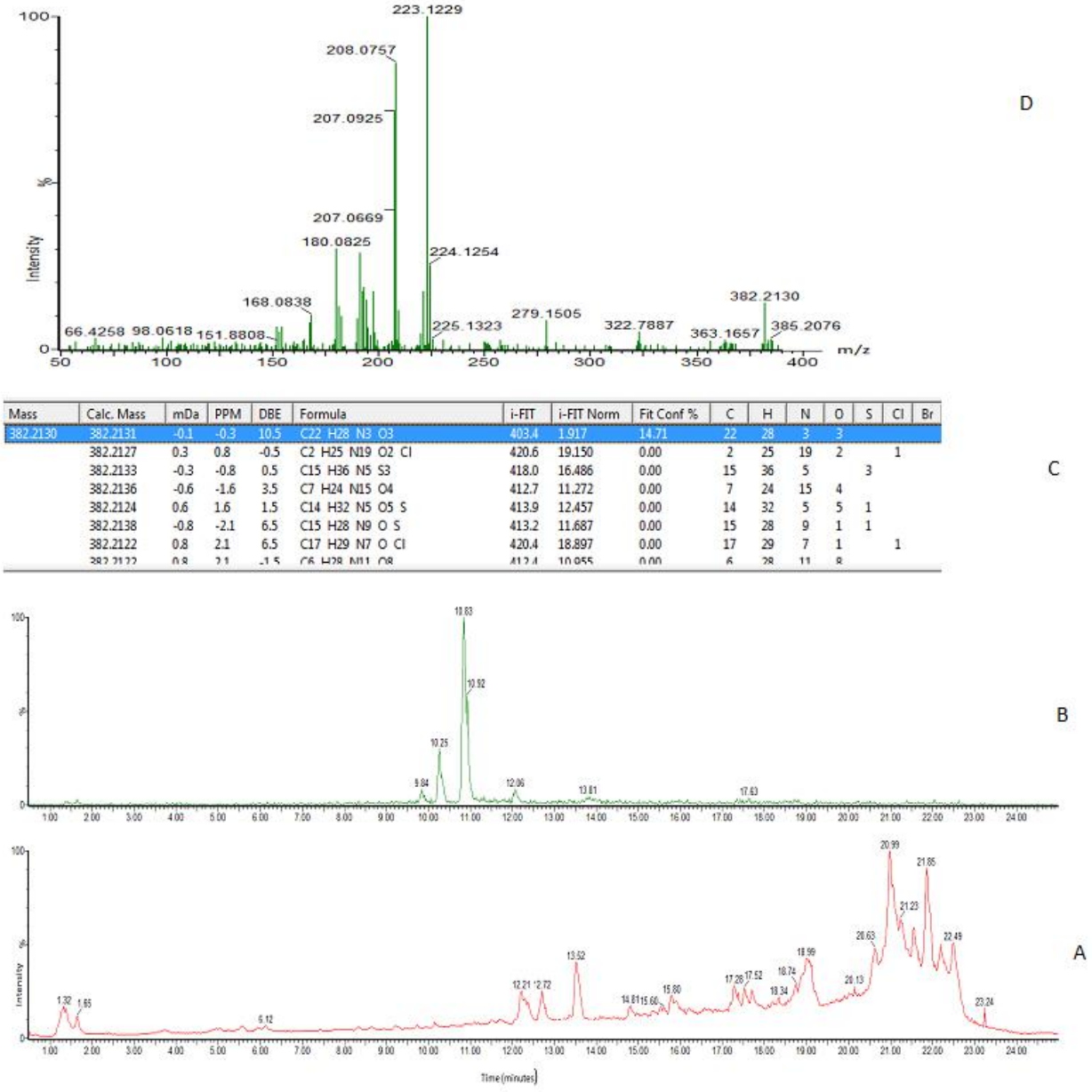
D

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
382.2135	382.2136	-0.1	-0.3	3.5	C7 H24 N15 O4	433.6	13.320	0.00	7	24	15	4			
	382.2133	0.2	0.5	0.5	C15 H36 N5 S3	440.9	20.608	0.00	15	36	5		3		
	382.2138	-0.3	-0.8	6.5	C15 H28 N9 O S	435.2	14.964	0.00	15	28	9	1	1		
	382.2131	0.4	1.0	10.5	C22 H28 N3 O3	420.3	0.008	99.23	22	28	3	3			
	382.2140	-0.5	-1.3	1.5	C16 H34 N5 O Cl2	444.5	24.205	0.00	16	34	5	1		2	
	382.2143	-0.8	-2.1	-1.5	C18 H41 N S Br	444.6	24.351	0.00	18	41	1		1		1
	382.2143	-0.8	-2.1	-0.5	H24 N21 O2 S	439.4	19.122	0.00		24	21	2	1		
	382.2127	0.8	2.1	-0.5	C2 H25 N19 O2 Cl	443.1	22.846	0.00	2	25	19	2		1	

C



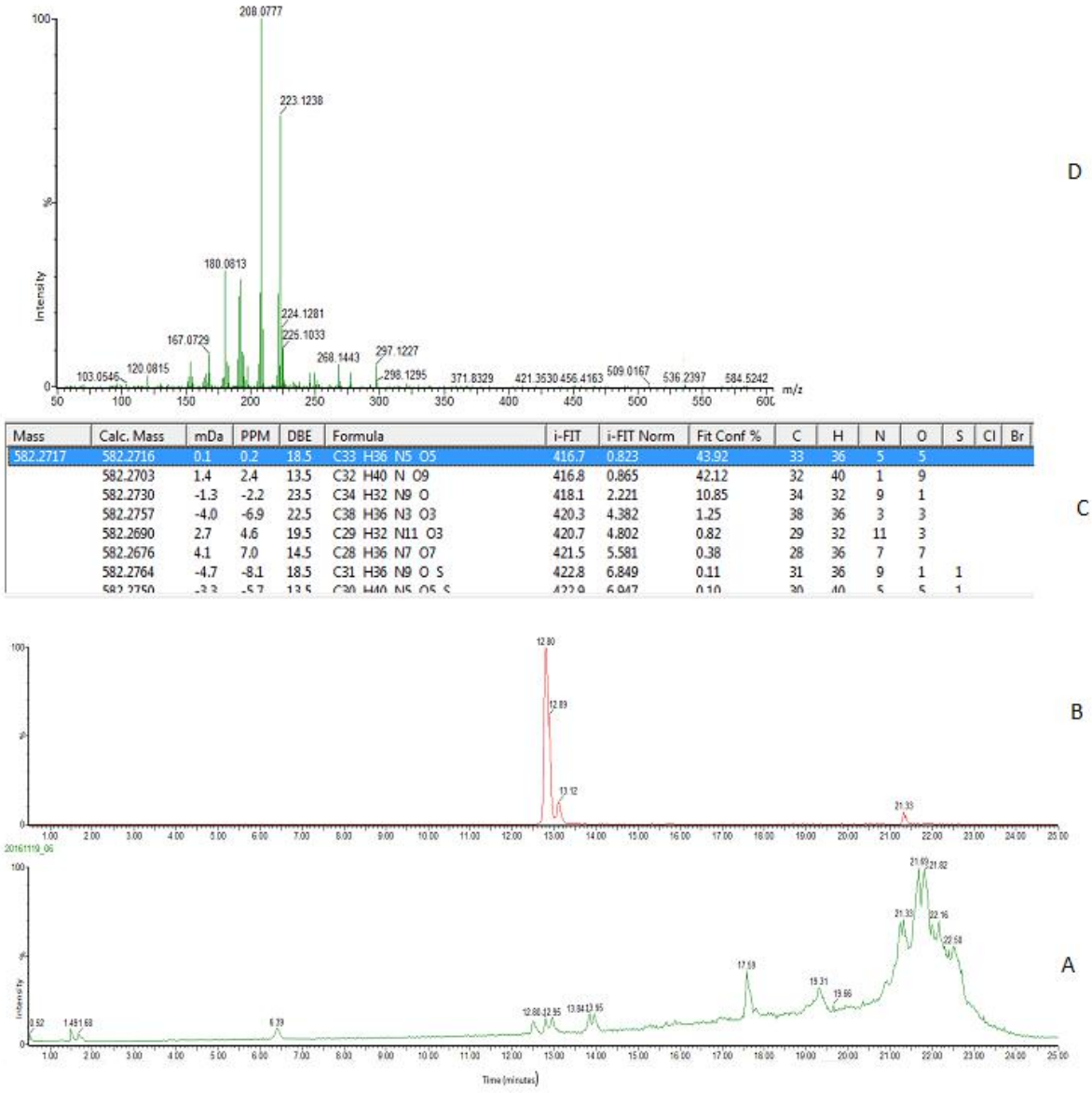
Annex IX. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of valine methyl ester extracted from sclerotia(D)



C

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
382.2130	382.2131	-0.1	-0.3	10.5	C22 H28 N3 O3	403.4	1.917	14.71	22	28	3	3			
	382.2127	0.3	0.8	-0.5	C2 H25 N19 O2 Cl	420.6	19.150	0.00	2	25	19	2		1	
	382.2133	-0.3	-0.8	0.5	C15 H36 N5 S3	418.0	16.486	0.00	15	36	5		3		
	382.2136	-0.6	-1.6	3.5	C7 H24 N15 O4	412.7	11.272	0.00	7	24	15	4			
	382.2124	0.6	1.6	1.5	C14 H32 N5 O5 S	413.9	12.457	0.00	14	32	5	5	1		
	382.2138	-0.8	-2.1	6.5	C15 H28 N9 O S	413.2	11.687	0.00	15	28	9	1	1		
	382.2122	0.8	2.1	6.5	C17 H29 N7 O Cl	420.4	18.897	0.00	17	29	7	1		1	
	382.2122	0.8	2.1	-1.5	C6 H28 N11 O8	412.4	10.955	0.00	6	28	11	8			

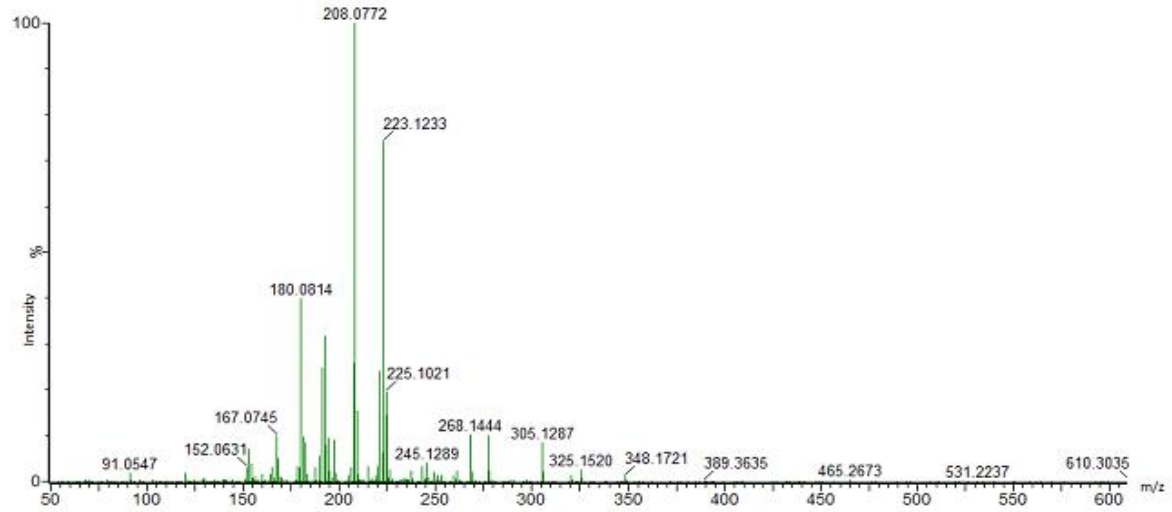
Annex X. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of valine methyl ester isomer extracted from sclerotia(D)



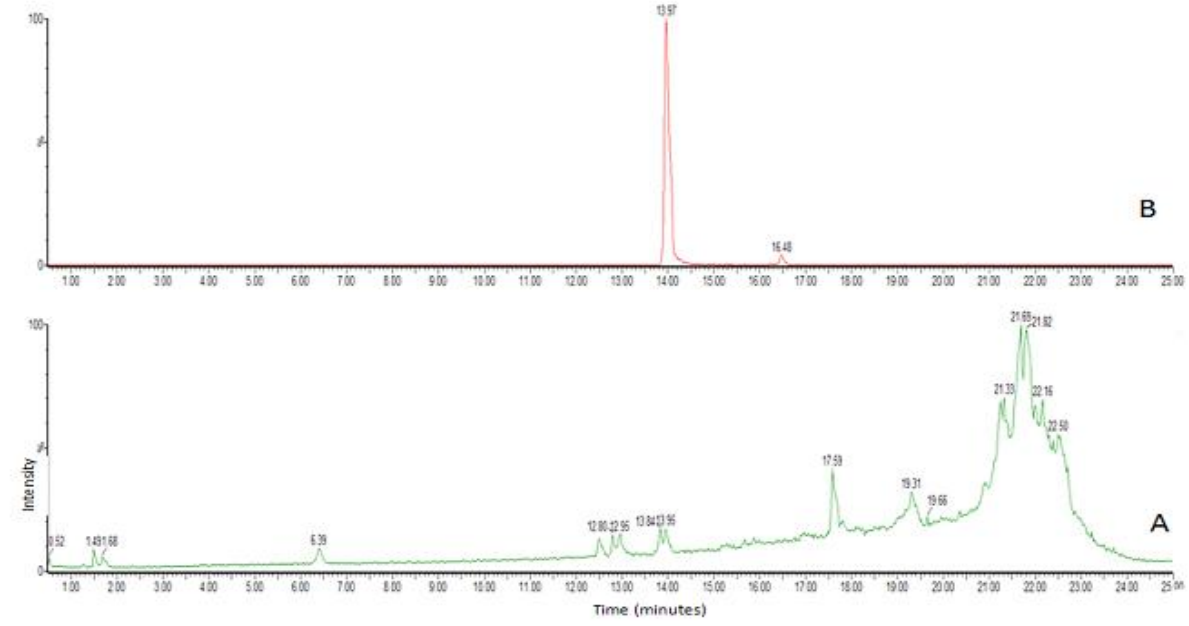
C

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
582.2717	582.2716	0.1	0.2	18.5	C33 H36 N5 O5	416.7	0.823	43.92	33	36	5	5			
	582.2703	1.4	2.4	13.5	C32 H40 N O9	416.8	0.865	42.12	32	40	1	9			
	582.2730	-1.3	-2.2	23.5	C34 H32 N9 O	418.1	2.221	10.85	34	32	9	1			
	582.2757	-4.0	-6.9	22.5	C38 H36 N3 O3	420.3	4.382	1.25	38	36	3	3			
	582.2690	2.7	4.6	19.5	C29 H32 N11 O3	420.7	4.802	0.82	29	32	11	3			
	582.2676	4.1	7.0	14.5	C28 H36 N7 O7	421.5	5.581	0.38	28	36	7	7			
	582.2764	-4.7	-8.1	18.5	C31 H36 N9 O S	422.8	6.849	0.11	31	36	9	1	1		
	582.2750	-3.3	-5.7	13.5	C30 H40 N5 O5 S	422.9	6.947	0.10	30	40	5	5	1		

Annex XI. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of ergotamine standard(D)



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O	S	Cl	Br
610.3023	610.3016	0.7	1.1	13.5	C34 H44 N O9	441.7	0.044	95.65	34	44	1	9			
	610.3029	-0.6	-1.0	18.5	C35 H40 N5 O5	445.5	3.863	2.10	35	40	5	5			
	610.3003	2.0	3.3	19.5	C31 H36 N11 O3	445.5	3.911	2.00	31	36	11	3			
	610.2989	3.4	5.6	14.5	C30 H40 N7 O7	448.2	6.554	0.14	30	40	7	7			
	610.3043	-2.0	-3.3	23.5	C36 H36 N9 O	448.7	7.066	0.09	36	36	9	1			
	610.2976	4.7	7.7	9.5	C29 H44 N3 O11	451.4	9.768	0.01	29	44	3	11			
	610.2976	4.7	7.7	20.5	C27 H32 N17 O	452.2	10.576	0.00	27	32	17	1			



Annex XII. Total ion chromatogram (TIC) (A), extracted ion chromatogram (B), estimated molecular formula (C) and fragmentation pattern of ergocristine standard(D)

Annex XIII: English information sheet, consent form and questionnaires

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Title of the research: "Ergot fungus, ergot alkaloids and ergotism in the highlands of Ethiopia"

Introduction

This research is about the ergot fungus, ergot alkaloids and ergotism in the highlands of Arsi, Ethiopia. We would like to ask your volunteer participation for the research entitled "Ergot fungus, ergot alkaloids and ergotism in the highlands of Arsi".

Purpose of the study

The purpose of this survey is to assess the level of awareness of the study participants about ergot fungus, ergot alkaloids and ergotism in the highlands of Arsi, Ethiopia. Information gathered from this survey will be used for future intervention of the occurrence of the ergot fungus and ergotism.

Participant selection

The study participants will be randomly selected from the three Woredas namely Tijo, Degelu and Kechema. Key informant interviews will be conducted for selected participants from Agricultural Bureau and focal group discussion will be conducted for participants selected from Assela Hospital. All the participants for this study will be 18 years of age or above

Participation

Participation in this study is entirely voluntary and it is your choice to participate in the study or not. Even after giving your consent to participate in this study, you have a full right to withdraw from the study at any time, and your withdrawal from the study will not affect you in any way. If you agree to participate in this study we will provide you with questionnaire to fill or we will interview

you. If you cannot read and write, a local translator trained for this purpose will assist you by reading the questionnaire for you and you will select your choice of interest.

Risks or Discomforts

There is no risk associated with participation in this study

Incentives and payments

There is no incentive or payment for participation in this study, but your participation is important for intervention of the condition in the future.

Confidentiality

The information you provide for this study will be confidential and will not be used for purposes other than this study. Any information about your profile will be used with codes, and your names will not be disclosed in any publication.

Communication

If you have any question regarding this research you can ask the principal investigator indicated hereafter anytime during the research as well as after the completion of the research.

Address of the principal investigator

Name: Asnake Desalegn

Email: Asnake.g2008@gmail.com or Asnake.desalegn@aau.edu.et

Tele: + 251 911 30 17 23

Consent form

I _____, the resident of _____ have read the information sheet, or it has been read for me and understand the information indicated above. I have obtained answers to all the questions I have asked to my satisfaction. I agree to participate in this study; I have been informed that I can withdraw from participation in the study at any time without consequences. I have been given a copy of this form to keep.

Participant's Name:_____ Signature:_____ Date _____
Investigator's Name: _____ Signature: _____Date _____
Witness _____ Signature: _____Date _____

Part I. General information

Introduction

i. Full Name of the study participant _____

ii. Study Participant's Identification code _____

iii. Date of Interview/Questionnaire_____

iv. Place of Interview/Questionnaire _____

Part II. Socio-demographic characteristics

1. Sex Female ☐

Male ☐

2. Age _____

3. Marital Status Single ☐

Married ☐

Divorced ☐

Widowed ☐

4. Education level: can't read and write ☐

Can read and write/no formal education ☐

Primary education ☐

Secondary education ☐

College/University ☐

5. Occupation : Farmer ☐

Merchant ☐

Self employed ☐

Government employee ☐

Student ☐

Others (Specify) _____

6. Religion: Orthodox ☐

Muslim ☐

Protestant ☐

Catholic ☐

Others (Specify) _____

7. Number of household: 1 -2 ☐

3 – 5 ☐

6 – 8 ☐

>9 ☐

8. For how long have you lived here? 1 -5 years ☐

6 – 10 years ☐

11 – 15 years ☐

>16 years ☐

Part III. Knowledge about ergot and ergotism

1. Do you know what ergot is?

Yes ☐

No ☐

If yes please describe it _____

2. Have you heard of the disease ergotism?

Yes ☐

No ☐

If yes please specify _____

3. What is the common symptom of the disease?

_____

_____

_____

_____

4. Have you heard of any body affected by this disease?

Yes ☐

No ☐

If yes please specify the region where it occurred

_____

_____

5. Does the disease transmit from a patient to other person?

Yes ☐

No ☐

I don't know ☐

6. If yes, please specify how it is transmitted

_____

_____

_____

_____

7. Do you think the disease can be prevented?

Yes ☐

No ☐

If yes please specify

_____

_____

_____

Part IV. Questions after colored picture of the ergot fungus was shown to the study participants

NB: If your answer for the first question is No, please do not answer question number 2 and 3.

1. Do you know what is presented in this picture?

Yes ☐

No ☐

If your answer is yes, please write the local name in the space provided?

_____

2. Where did you see it?

In the farm ☐

In harvested grains ☐

In both ☐

If your answer is in the farm, describe on which crop you have seen it?

_____

_____

_____

3. Do farmers in your locality remove the ergot fungus you have seen earlier in the picture?

Yes ☐

No ☐

If yes please explain in what way the farmers remove the ergot fungus

_____

_____

_____

4. Do farmers in your locality remove wild oats from their crop fields?

Yes ☐

No ☐

If your answer is yes, please describe when and how?

_____

_____