ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCES, SCHOOL OF MEDICINE, DEPARTMENT OF ANATOMY

In Vitro **Antifungal Effect of Crude Extracts and Solvent Fractions of** ***Vernonia amygdalina*** **Delile and** ***Croton macrostachyus*** **Hochst. ex Del. against Fungi Isolated from Formalin Fixed Cadavers**

BY: Yossef Teshome Zikarg (BSc.)

July 2018

Addis Ababa, Ethiopia

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Thesis submitted to Department of Anatomy, School of Medicine, College of Health Sciences, Addis Ababa University for the partial fulfillment of the requirements for the Degree of Masters of Science (MSc.) in Human Anatomy.

By: Yossef Teshome Zikarg (BSc.)

July 2018

Addis Ababa, Ethiopia

IDENTIFICATION

Name of investigator: Yossef Teshome Zikarg (BSc.)

Advisors:

Principal Advisor: Abay Mulu (Ass. Prof. in Anatomy), Department of Anatomy, School of Medicine, College of Health Sciences, AAU

Co advisors:

1. Tadesse Kebede (PhD.), Department of Microbiology, Immunology & Parasitology, School of Medicine, CHS, AAU
2. Prof. Kaleab Asres, Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, CHS, AAU
3. Mr. Abera Abdeta, Clinical Bacteriology and Mycology Directorate, EPHI
4. Mr. Birhanu Gizaw, Bacteriology and Mycology case team, EBI
5. Mr. Negero Gemeda, Modern and Traditional Medicine Directorate, EPHI

Full title of project:

In Vitro Antifungal Effect of Crude Extracts and Solvent Fractions of *Vernonia amygdalina* Delile and *Croton macrostachyus* Hochst. ex Del. against Fungi Isolated from Formalin Fixed Cadavers

Address of Investigator:

AAU, College of Health Sciences, School of Medicine, Department of Anatomy

Cell phone: +251-923164791/ 944126949

E-mail: yossef.teshome@gmail.com / yossef.teshome@yahoo.com

P.O. Box: 9086

Addis Ababa University

School of Graduate Studies

Declaration

This is to certify that the thesis prepared by Yossef Teshome, entitled, **"*In Vitro* Antifungal Effect of Crude Extracts and Solvent Fractions of *Vernonia amygdalina* Delile and *Croton macrostachyus* Hochst. ex Del. against Fungi Isolated from Formalin Fixed Cadavers"** and submitted in partial fulfillment of the requirements for degree of Masters of Science in Anatomy complies with the regulation of the University and meets the accepted standards with respect to originality and quality. This thesis has not been presented for degree in any other University, and that all sources of materials used for the thesis have been fully acknowledged.

Candidate: Yossef Teshome Signature: ---------- Date: ----------

Signed by the Examining Committee:

Examiner: ---------- Signature: ---------- Date: ----------

Advisors:

Mr. Abay Mulu	Signature: ----------	Date: ----------
Dr. Taddesse Kebede	Signature: ----------	Date: ----------
Prof. Kaleab Asres	Signature: ----------	Date: ----------
Mr. Abera Abdeta	Signature: ----------	Date: ----------
Mr. Negero Gemeda	Signature: ----------	Date: ----------
Mr. Birhanu Gizaw	Signature: ----------	Date: ----------

Chair of Department

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List of Abbreviations and Acronyms

AAU	Addis Ababa University
ANOVA	Analysis of variance
CIPIH	Commission on Intellectual Property Innovation and Public Health
DMSO	Dimethyl Sulfoxide
EBI	Ethiopian Biodiversity Institute
EPHI	Ethiopian Public Health Institute
IARC	International Agency for Research on Cancer
MIC	Minimum Inhibitory Concentration
OSHA	Occupational Safety and Health Administration
PEL	Permissible Exposure Limit
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
SPSS	Statistical Package for Social Sciences
STEL	Short Term Exposure Limit
TM	Traditional Medicine
TWA	Time-Weighted Average

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ABSTRACT

Cadavers are an excellent tool for medical students to learn the correct anatomic structures, landmarks and relationships. A successful embalming procedure is necessary for better preservation of cadaver for anatomical study. Embalming fluids contain fixatives and disinfectants like formalin and phenol. These chemicals are proven to have diverse toxic effects for embalmers, researchers, students and instructors. Beyond their health and environmental hazard, embalming chemicals are not adequately effective in inhibiting the growth of fungi, bacteria and maggots. The most prevalent problem that anatomists face during handling of cadavers is the growth of fungi on the cadavers. In Ethiopia, no studies have been carried out to find solutions for these problems.

The objective of this study was to isolate fungi grown on formalin-fixed cadavers and test their susceptibilities to the aqueous and 80% methanol extracts as well as solvent fractions of *Vernonia amygdalina* Del. and *Croton macrostachyus* hochst. ex Del. with the aim of finding natural products that improve the preservative capacity of the currently used embalming solutions.

The studied plants were selected based on their ethnomedicinal profiles and reported antifungal effects. Suspected colonies were collected by the swab technique from the surface of formalin-fixed cadavers obtained from the School of Medicine, College of Health Sciences, Addis Ababa University, St. Paul's Millennium Medical College and Africa Medical College and cultured in Sabouraud Dextrose Agar (SDA). Morphological identification confirmed that the fungi belong to the genera *Acremonium, Aspergillus, Geomyces, Madurella, Microsporum, Neosartorya, Penicillium, Scopulariopsis, Talaromyces* and *Trichophyton* with *Penicillium* genus identified with the highest frequency of occurrence followed by *Aspergillus*. Furthermore, some of the fungi were identified up to species level by biolog and used for plant extract activity evaluation. The identified species were *Aspergillus avenaceus, Aspergillus carneus, Aspergillus restrictus Geomyces pulvereus, Neosartorya fischeri*, *Penicillium pinophilum*, *Penicillium restrictum*, *Penicillium roqueforti*, *Scopulariopsis brumptii* and *Talaromyces bacillisporus*. The concentrations of aqueous and 80% methanol extracts used for the agar well diffusion method were 25, 50, 100 and 200 mg/mL, whilst 12.5, 25, 50 and 100 mg/mL concentrations were employed for the hexane, chloroform, acetone and methanol fractions. For the determination of

minimum inhibitory concentrations (MICs) in the agar dilution assay, concentrations of 1, 2, 4 and 8 mg/mL were utilized for all the experiments involving the total extracts and solvent fractions. The results of the study revealed that the 80% methanol extract of *V. amygdalina* showed activity towards *A. avenaceus*, *N. fischeri* and *S. brumptii*, whilst all the tested fungi were resistant to the aqueous extract of the same plant. On the contrary, the aqueous extract of *C. macrostchyus* was active against *A. restrictus*, *P. restrictum*, *S. brumptii* and *T. bacillisporus*, whereas its 80% methanol extract was effective against *P. pinophilum* only. Solvent fractions of both plants were not active against the tested fungi in agar well diffusion method.

In conclusion, the findings of the present study have established the susceptibilities of some of the isolated fungal species to the extracts of *V. amygdalina* and *C. macrostachyus*, demonstrating the usefulness of the studied medicinal plants at least for the modification of the present embalming solution by mixing it with the plant extracts. The study further provides scientific evidence for the use of the leaves of *V. amygdalina* for preservation of corpse by people in southern Ethiopia.

Key words and phrases*; Cadaver, Embalming, Antifungal, Medicinal plants, Inhibition zone*

1 Introduction

1.1 Background of the study

The study of anatomy has been the cornerstone of medical sciences from the beginning. It is not possible to train a health care professional from a strictly theoretical point of view; therefore dissecting of corpse tissues plays a key role in the teaching-learning process. This practice, considered by many to be irreplaceable, allows students to develop skills by handling tissues while giving it a better perception of the features of the structures of the body (Fonseca-Matheus, 2013).

Naturally, physical and chemical changes lead to alterations in the external and internal structures of corpses (Khouri, 2012). The rapid deterioration of the tissues that occurs after death, and the risk of disease transmission from body material, has promoted the development of preservation techniques. Embalming halt these reactions and give us needed time to study human corpses, and could be considered as a suitable tool, especially as an anatomy teaching tool, in medical schools (Bajracharya and Magar, 2006). The ultimate purpose of embalming is to preserve the body material for variable periods of time and to inhibit or destroy microorganisms that may be present in the specimen to be preserved (Fonseca-Matheus, 2013).

The process of human cadaveric preservation uses diverse embalming fluids introduced in cadavers by means of various technical approaches (Bajracharya and Magar, 2006). Embalming is the technique to preserve the dead bodies with its normal anatomy and lifelike appearance for a long period, thus it is the inescapable necessity of the medical sciences.

The desired properties required for successful embalming of cadavers for gross anatomy teaching include: (1) good long term structural preservation of organs and tissues with minimal shrinkage or distortion; (2) prevention of over hardening, while maintaining flexibility and suppleness of internal organs; (3) prevention of desiccation; (4) prevention of fungal or bacterial growth and spread within a specific cadaver and to other cadavers in the dissection room; (5) reduction of potential biohazards (spread of infection to dissection personnel and students); (6) reduction of environmental chemical hazards and (7) retention of color of tissues and organs while

minimizing oxidation effects that result in browning (Coleman and Kogan, 1998; Suniti, *et al*., 2016)

Typically the embalming fluid is composed of fixative or preservative, disinfectant, modifying agent buffer, anticoagulant, surfactant, humectants, dyes, a perfuming agent, diluent (Bajracharya & Magar, 2006). The most frequently used fixatives and disinfectants are ethanol, formalin, and phenol. It is well known that chemicals used in embalming play an important role in keeping the cadaver free from decomposition and ensuring maximum preservation (Dixit, 2008). Regular embalmed cadavers prepared using conventional methods, exposes medical students, embalmers, and faculty members having contact with cadaveric materials to formaldehyde and phenol fumes that are proven to have diverse toxic effects (Nithya and Nithya, 2014). Beyond its health and environmental hazard, those embalming chemicals are not fully effective in inhibiting the growth of fungi, bacteria and maggots (Natekar and Desouza, 2012).

The balance between the concentrations of formalin and phenol that cause irritation to students and staff, and fungal growth is often difficult to achieve. In other words, raising formalin and phenol concentration are highly irritant while lowering it will open the door for fungi to grow. In Ethiopia, to our knowledge no studies have been carried out to overcome these problems hence the aim of the present study is to test the antifungal activities of *Vernonina amygdalina* and *Croton macrostachyus* extracts against fungi grown on formalin fixed cadavers for the future modification or fashioning of safer and effective new embalming solution to maintain good preservation of cadavers.

1.2 Traditional medicinal plants

According to the World Health Organization (WHO), traditional medicine is defined as the sum total of the knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness.

Medicinal plants are reservoirs of potentially useful chemical compounds that serve as the basis for many standard drugs used in modern medicine (Debella, 2002). Medicinal plants have a promising future, because there are about half million plants around the world, and in most, their medical activities have not yet investigated, and their medical activities could be decisive in the treatment of present or future studies (Singh, 2015).

1.2.1 *Vernonia amygdalina* Del.

Vernonia amygdalina Del. (Figure 1) belong to the flowering plant family Asteraceae. It is a valuable medicinal plant widely spread in East and West Africa (Farombi, 2011). *V. amygdalina* is a shrub of 2-5 meter tall with petiolate leaf of about 6 mm diameter and elliptic shape (Mabekoje *et al*., 2013).

The leaves exhibit a characteristic odor and bitter taste, explaining its common English name of 'bitter leaf' but the bitterness can be abated by boiling or by soaking in several changes of clean water (Farombi, 2011; Ngatu *et al*., 2014). In Ethiopia it is known by its vernacular name "Grawa" in Amharic language. *V. amygdalina* can be commonly found along drainage lines and in natural forests or at home and commercial plantations (Alem and Woldemariam, 2009). It is a common homestead farming vegetable and fodder tree in Nigeria (Ndaeyo, 2007) and has been used as an ingredient to prepare Nigerian (Ogbono soup) or Cameroon (Ndole) dish after removal of its bitter taste (Abosi and Raseroka, 2003; Onabanjo and Oguntona, 2003).

Figure 1: Photograph of *Vernonia amygdalina* taken from Tikur Anbessa Specialized Hospital compound, Addis Ababa, Ethiopia in July 2017.

1.2.1.1 Medicinal properties of *V. amygdalina*

V. amygdalina has been shown to possess diverse traditional therapeutic effects such as anti-malarial, antimicrobial (antibacterial, antifungal, etc.), antidiabetic, anticancer effects. Remedies made from bitter leaf are used in treating 25 common ailments in sub-Saharan African; these include common problems such as fever, and different kinds of intestine complaints, as well as parasite-induced diseases like malaria and schistosomiasis (Echem *et al*., 2013).

This plant is also used for treatment of measles, cough, tuberculosis and for induction of fertility in barren women (Yeap *et al*., 2010). The leaf decoction of *V. amygdalina* is used as a remedy for malaria, gastrointestinal disorders and hepatitis, whereas both root and leaf extracts are used to treat fever, hiccups, kidney disease, diabetes and helminthiasis in western and central African countries (Ngatu *et al*., 2014). The leaves of the plant are also used in the treatment of skin infections such as ringworm, rashes and eczema (Echem *et al*., 2013). Many herbalists and naturopathic doctors have recommended the aqueous extracts for their patients as treatment for emesis, nausea, diabetes, loss of appetite-induced abrosia, dysentery and other gastrointestinal tract problems.

The plant is also reported to have a good nutritional value with its protein and mineral contents (Farombi, 2011). It is a very important protective food and useful for the maintenance of health and prevention and treatment of various diseases (Echem *et al*., 2013). For nutritional purposes, the leaves are cut into small pieces and washed repeatedly until the bitter taste is removed (Farombi, 2011), then boiled and cooked with fish or meat depending on local ethnic eating habits (Ngatu *et al*., 2014).

Due to its bitterness, it can also be used as a bittering agent, a hop substitute and for the control of microbial contamination in beer brewing without affecting the quality of malt. In Ethiopia, it is used to make honey wine called Tej (Eleyinmi *et al*., 2004) and treat skin infections such as ringworm, rashes, eczema and athletic foot (tinea pedis) as traditional medicine by indigenous people.

1.2.2 *Croton macrostachyus* Hochst. ex Del.

Croton macrostachyus Hochst. ex Del. belongs to the family Euphorbiaceae and it is commonly known as broad-leaved *Croton* (English), and Bisana in the Amharic. *C. macrostachyus* (Figure 2) is a deciduous tree which grows up to 3-25 m high, although more commonly it is 6-12 m. The tree is crown rounded and open with large spreading branches commonly found in secondary forests, around lakes, in moist or dry evergreen upland forests and woodlands. *C. macrostachyus* is widely distributed throughout tropical Africa and it is native to Eritrea, Ethiopia, Kenya, Tanzania, Uganda, and Nigeria (Giday *et al*., 2009).

Figure 2: Photograph of *Croton macrostachyus* taken from Ethiopian institute of Architecture, Building Construction and City Development compound, Addis Ababa, Ethiopia in October 2017

1.2.2.1 Medicinal properties of *C. macrostachyus*

Ethnobotanical and pharmacological studies revealed that various parts of *C. macrostachyus* possess a wide range of activities (Giday *et al*., 2007; Giday *et al*., 2009; Mesfin *et al*., 2009; Lulekal *et al*., 2014). These include antidiabetic, purgative and anti-inflammatory, antibacterial and antifungal (Mesfin *et al*., 2009; Wijesundara, 2016; Obey *et al*., 2016) and antimalarial (Mesfin *et al*., 2009) activities. The fruit extract showed promising antimalarial activity (Mohammed *et al*., 2014; Bantie *et al*., 2014). Moreover, the methanol leaf extract exhibited larvicidal activity against late third instar larvae of *Anopiles arabiensis*, a predominant malaria vector in Ethiopia (Karunamoorthi and Ilango, 2010). Stem bark decoction is used for bathing babies against skin infections. Leaf decoction is employed against abdominal discomfort, sores, dermatophilosis, ring worms, mange, scabie, wound and minor bleeding (Lulekal *et al*., 2014). *C. macrostachyus* also has therapeutic effect against fungal infections of skin (tinea versicolor) ('Quaqucha') by rubbing and covering with leaves at the affected area (Amuamuta *et al*., 2015).

In view of the above, the study aimed to evaluate the *in vitro* antifungal effect of extracts of *V. amygdalina* and *C. macrostachyus a*gainst fungi isolated from formalin fixed cadavers.

1.2.2.2 Phytochemistry of *C. macrostachyus*

Multiple classes of phytochemicals have been isolated from *C. macrostachyus*. These include phenolics and triterpenes of the lupine and hopane groups (Tala *et al*., 2013). Phytochemical screening of the hydroalcoholic crude extract of the leaves of *C. macrostachyus* also revealed the presence of alkaloids, saponins, phenolic compounds, cardiac glycosides, tannins, terpenoids, and flavonoids (Bantie *et al*., 2014).

1.3 Statement of the problems

A successful and safer embalming fluid is necessary for the long lasting preservation of cadaver for anatomical study. However, most of the standard techniques contain many toxic substances, e.g. formaldehyde and liquefied phenol (Bajracharya and Magar, 2006). Carbolic acid (Phenol, C_6H_5OH) is highly toxic and corrosive. It can be absorbed through the skin as defined by the Occupational Safety and Health Administration (OSHA). Researchers have proved that repeated and prolonged exposure to formaldehyde has been associated with lung and nasal passage cancers and congenital defects. It is also highly irritating to the upper respiratory tract and eyes (Kundu *et al*., 2015; OSHA). The International Agency for Research on Cancer (IARC) (2004) has determined that formaldehyde is carcinogenic to humans and classifying it as a Group 1 carcinogen.

Occupational Safety and Health Administration (OSHA) has currently set the 8-hour time-weighted average (TWA) permissible exposure limit (PEL) for formaldehyde at 0.75 ppm and the short-term exposure limit (STEL) to 0.2 ppm. An 8-hour TWA is the average exposure concentration over the course of an 8-hour workday. A short term exposure limit is the concentration to which workers can be exposed continuously for a short period of time without suffering from irritation, chronic or irreversible damage, or narcosis. However, in anatomical dissecting room the average 8-hour time-weighted average (TWA) for students ranged from 0.81 – 0.92 ppm, all of which are above the OHSA permissible exposure limit (PEL) of 0.75ppm (Ohmichi *et al*., 2006). Another research (Chia *et al*., 1992) also showed that at 0.74 ppm the average 8-hour time-weighted exposure limit had an adverse health effect, which is below the recommendation of OSHA PEL (0.75ppm).

The other problems that face anatomists are the growth of fungi on cadavers (Kupta *et al*., 2013). The embalming chemicals are not effective in inhibiting the growth of fungi, bacteria and maggots (Natekar and Desouza, 2012). This may pose infection risks to people who handle them during embalming procedures or dissections. Elsebai *et al*.(2002) identified five genera of fungi that grew on formalin-fixed cadavers, namely: *Aspergillius, Penicillium, Trichophyton, Epidermophyton* and *Cryptococcus* which could be hazardous and lead to a dangerous risk of exposure for instructors and students. However, different authors suggest using high

concentration of formalin and phenol could hinder the growth of those fungi grown on formalin fixed cadavers, but it causes adverse health effect on instructors and students.

To prevent further fungal growth there is a need for a better fixative with antifungal properties that is safe to use. This study primarily designed to isolate fungi that grow on formalin fixed cadavers and for future fashioning or modifying the present toxic embalming fluids by a better preservative and safer fluids. Medicinal plants are promising for such uses as they are generally considered to be safe and also have long history of use by humans without major adverse effect (Vermani and Garg, 2002).

1.4 Significance of the study

Different fungi were isolated from formalin fixed cadavers. For instance, Elsebai *et al*. (2014) have identified five genera of fungi that grew on formalin-fixed cadavers, namely: *Aspergillius*, *Penicillium*, *Trichophyton*, *Epidermophyton* and *Cryptococcus*. The growth of different fungi on formalin-fixed cadavers is one of the problems that face anatomists and embalmers in the gross anatomy laboratories (Gupta *et al*., 2013). However, fungus can grow on different environments; their distribution and type are majorly varies by the effectiveness of the embalming and environment factors like temperature and humidity (Elsebai *et al*., 2002; Sri-indrasutdhi *et al*., 2015). In accordance with this, in the present study different fungi that could expose the embalmer and dissectors to infection have been isolated from formalin fixed cadavers. Thus, the data could be helpful to take preventive safety measures for embalmers, instructors and students.

Extracts of *V. amygdalina* have been used in various folk medicines as remedies against helminthic, protozoan and bacterial infections with scientific support for these claims (Farombi, 2011). The extracts are also used as a bittering agent (substitute for hops) and to control microbial contamination in beer brewing without affecting the quality of malt. In Ethiopia, the leaves have also been used to make honey wine called Tej and as hops in preparing tela (local alcoholic beverage) (Eleyinmi *et al*., 2004). Furthermore, the plant is used by indigenous people as remedy against skin fungal infection and as a preservative agent for corpse particularly by Kembata, Hadiya Sidama and Wolayta people in southern Ethiopia. However, there is no scientific evidence to support such claims. Similarly, *C. macrostachyus* has been reported possess antifungal activity. Therefore, the present study was undertaken to test the activity, if any, of extracts prepared from *V. amygdalina* and *C. macrostachyus* against some fungal species isolated from formalin-fixed cadavers.

The current investigations were considered significant as the data generated could be used as a baseline to fashion new embalming solution and/or modifying the standard embalming solution by mixing it with the plant extracts. The extract may act synergistically with the standard solutions thus reducing their concentrations to a level that is not hazardous to health.

2 Literature Review

2.1 Reported fungi that grow on formalin-fixed cadavers

Reed *et al*. (1993) isolated *Aspergillus*, *Blastomyces*, *Coccidioides* and *Histoplasma* from formalin-fixed and paraffin-embedded material used as antisera which were originally developed for use in immunodiffusion assays. Moreover, Elsebai *et al*. (2002) found *Aspergillus, Penicillium, Trichophyton, Epidermophyton* and *Cryptococcus* on formalin-fixed cadavers at Iraq. In other research done in Thailand *Penicillium* sp., *Chrysonilia sitophila* and *Trichoderma* sp. (Lakchayapakorn *et al*., 2008) and airborne fungi, namely; *Penicillium oxalicum* and *Cladosporium colocasiae* (Sri-indrasutdhi *et al*., 2015) were found on formalin-fixed human cadavers. These results indicated that some airborne fungi have the ability to grow on formalin fixed cadavers and cause contamination. Osman *et al*. (2014) also isolated five different species that belong to the genera, *Microsporum*, *Aspergillus*, *Cryptococcus, Trichophyton* and *Candida* from formalin fixed human cadavers, and advised instructors and students should to use face masks and rubber gloves for protection. But in none of the above articles, mechanisms were forwarded to prevent the growth of fungi on formalin-fixed cadavers.

2.2 Other microorganisms in formalin-fixed cadavers

Tabaac *et al.* (2013) were isolated sixteen genera of bacteria including pathogenic bacteria, such as *Staphylococcus aureus* and *Gemella spp.* from the surface of formalin fixed cadavers. The perineal region likely had the most growth and most of the organisms found are classified as normal flora of the perineum, oronasal region, and skin. Therefore, this indicates that the source of contamination of cadavers was presence of microorganisms in pre-mortem and cross contamination may also occur during processing. Osman *et al*. (2014) stated that the sources of microbes' contamination were the environmental contamination, instructor, student or cadaver itself and another research (Sri-indrasutdhi *et al*., 2015) showed dissection room humidity, concentration of airborne fungal spores and period of time to fungal inoculation.

Infectious pathogens in the cadavers that present particular risks include *Mycobacterium tuberculosis*, hepatitis B and C viruses, HIV, and prions that cause transmissible spongiform encephalopathies (Demiryurek *et al*., 2002). Embalming of bodies infected with HIV is not

recommended and effectiveness of embalming fluids against HIV in cadavers is unknown. Cadavers infected with HIV are after infected with other organisms such as mycobacterium which may be more infectious than HIV infection itself (Mahajan *et al*. 2011).

Every cadaver should be regarded as an infectious material. It has been recommended that every corpse must have a detailed file, indicating the reason of death and containing previous hospital records and the cases known to be infectious with *Mycobacterium tuberculosis*, hepatitis B and C, HIV and prions should be avoided. But it is impossible to compile such file in our setting since the source of cadavers is unclaimed.

2.3 Phytochemical content and antifungal effect of *V. amygdalina*

A number of useful secondary metabolites such as vernolide vernodalol and epivernodalol (Erasto, 2006) have been isolated from the leaves of *V. amygdalina*. Several other compounds such as saponins, alkaloids, terpenes, steroids, coumarins, flavonoids (like luteolin, luteolin 7-O-glucosides and luteolin 7-O-glucuronide), phenolic acids, lignans, xanthones, anthraquinones, edotides and sesquiterpenes have also been identified from this plant (Farombai, 2011).

2.3.1 Stigmastane type steroidal glucosides

One of the major steroidal glucosides that have been isolated from *V. amygdalina* are the vernoniosides (Osinubi, 2007). These compounds were isolated from the leaf, stem, pith and root parts of the plant (Huffman, 2001). Among these, vernonioside B1 was found in higher concentrations in leaves and responsible for the removal of parasites in primates who sucked the young pith of *V. amygdalina,* for the control of gastrointestinal illnesses.

V. amygdalina is well known for its bitter taste. Vernoniosides A1, A2, A3 and A4, were found to be part of the constituents that contribute to this characteristic, while vernoniosides B1, B2 and B3, did not show any bitter taste (Osinubi, 2007). Vernoniosides B were found to lack a free hydroxyl end at their C-16, as was present in vernoniosides A. Hydroxylation at C-16 of these steroid glucosides was therefore hypothesized to play an important role in causing bitterness to this plant.

2.3.2 Sesquiterpene lactones

Another major group of bioactive compounds that have been isolated from *V. amygdalina* are the sesquiterpene lactones, consisting of vernodalin, vernolide, vernolepin, vernomenin, vernomygdin, vernolic, vernodalol, hydroxylvernolide, 11,13-dihydrovernodalin, 11,13-dihydrovernorodeline, 4,15-dihydrovernodalin,7,24(28)-stigmastadien-3β-ol and 1,2,3,15,11,13,2',3'- octahydrovernodalin (Farombi, 2011).

2.3.3 Antifungal effect of *Vernonia amygdalina*

Most of the researches on antifungal activity of *V. amygdalina* focused on its water extracts. The water extract of *V. amygdalina* leaves inhibited the growth of *Fusarium moniliforme* on seeds of maize (Zea mays) as well as mycelial and conidial growths of *Colletotrichum gloeosporioides* in rubber tree (Suleiman *et al*., 2008). Cold water extract of stem bark and root bark (but not leaves) was able to inhibit *Colletotrichum capsici* (Synd) isolated from pepper (Nduagu *et al*., 2008). On the other hand, the juice of *V. amygdalina* showed stronger effect than its cold water extract where its juice was more effectively inhibited the seed borne fungi *Fusarium moniliforme*, *Botryodiplodia theobromae*, *Aspergillus niger* and *Aspergillus flavus* both *in vitro* and *in vivo* (Nwachukwu and Umechuruba, 2001).

In crop industry, hot water extract of *V. amygdalina* was able to help to control the infection of *Sclerocium rolfii* and increased the plant height, shelf life, relative water content, chlorophyll content, leaf area index, number of branches, total dry matter, number of pod per plant, weight and also grain yield on cowpea. However, it induced phytotoxic effect where it reduced the recoverable of photosynthethates and transpiration rate of the treated plant. This feature was suggested to maintain high water content in cowpea seedlings through antitranspirants during dry seasons for its survival (Alabi *et al*., 2005).

The methanol extract of *V. amygdalina* was also reported to show strong antifungal activity on *Pseudoperonospora cubensis* and mild activity on *Rhizoctonia solani* (Higashi *et al*., 1991). Interestingly, the ash from *V. amygdalina* also possesses antifungal property where it was shown to inhibit the growth of *Sclerotium rolfsii* Sacc. mycelial growth on wheat and protected seedlings against post-emergence infection through inhibition of fungal growth within the root

and crown zones. High nitrogen level in the ash of *V. amgydalina* which could directly inhibit sclerotial germination and retarding mycelial growth of *Sclerotium rolfsii* contributed to this effect (Enikuomehin, 1998).

3 Objectives of the study

3.1 General objective

- To assess the antifungal effect of aqueous and 80% methanol extracts and solvent fractions of *Vernonia amygdalina* and *Croton macrostachyus* on fungi isolated from formalin fixed cadavers

3.2 Specific objectives

- To isolate fungi from formalin fixed cadavers;
- To determine the minimum inhibitory concentrations of the extracts and solvent fractions on the isolated fungi; and
- To compare the antifungal activities among crude extracts and solvent fractions of both medicinal plants.

4 Methods and Materials

4.1 Study design and period

A laboratory based experimental study was conducted from February 2017 to April 2018 GC.

4.2 Study area

The study was conducted in Addis Ababa University (AAU), College of Health Sciences, St. Paul's Millennium Medical College and Africa Medical College, Addis Ababa, Ethiopia

4.3 Collections and preparation of plant materials

The leaves of *Vernonia amygdalina* were collected in July 2017 from Tikur Anbessa Specialized Hospital (TASH) compound, Addis Ababa University (AAU), Addis Ababa, Ethiopia. The leaves of *Croton macrostachyus* were collected in the month of October 2017 from Ethiopian Institute of Architecture, Building Construction and City Development (EiABC) compound of AAU, Addis Ababa. The plants were identified and authenticated by a taxonomist at the Ethiopian Public Health Institute (EPHI), Addis Ababa. The voucher specimens were deposited for future reference with Collection number YT 001 for *V. amygdalina* and YT 002 for *C. macrostachhyus*. The plant materials were first cleaned by removing extraneous materials and dried under shade and coarsely powdered using mortar and pestle.

4.4 Extractions

4.4.1 Preparation of aqueous extracts

Powdered plant materials (100 g each) were separately macerated in 500 mL of distilled water for 72 h with intermittent agitation. The macerates were then filtered first by using gauze and then Whatman No 1 filter paper (Nduagu *et al*., 2008). The filtrate was freeze dried using Lypholizer (Operan, Korea vacuum limited, Korea).

4.4.2 Preparation of hydroalcoholic extracts

Powdered plant materials (100g each) were separately macerated in 500 mL of 80% methanol for 72 h with intermittent agitation. The same procedure was followed as described above for aqueous extraction, but the organic solvent was first removed in a rotatory evaporator (Buchi Rota-vapor R-200, Switzerland) under reduced pressure before lyophilization was done.

4.4.3 Solvent fractionation

Powdered leaves (50g each) were exhaustively extracted successively in a Soxhlet apparatus using hexane, chloroform, acetone and methanol. Solvents were removed under reduced pressure in a rotatory evaporator.

4.5 Collection and identification of fungi from formalin fixed cadavers

The fungal samples were collected from the surface of non-dissected formalin-fixed human cadavers at the School of Medicine, College of Health Sciences, Addis Ababa University, St. Paul's Millennium Medical College and Africa Medical College, Addis Ababa, Ethiopia. The total number and proportion of cadavers used from each site were based on availability in the study period. Accordingly, five cadavers from School of Medicine, College of Health Sciences, Addis Ababa University, two cadavers from St. Paul's Millennium Medical College and two from Africa Medical College with a total of nine formalin fixed cadavers were used. The suspected fungal colonies were collected from the surface of the cadavers by the swab technique. Sterile moistened cotton plugs were used to pick up mycelia and spores of the suspected fungal colonies and kept in labeled collecting tubes (Sri-indrasutdhi *et al*., 2015) and they were then inoculated into earlier prepared Sabouraud Dextrose Agar (SDA) on the same day of sample collection.

4.5.1 Sterilization and media preparation

The SDA medium was prepared according to the manufacturer's specification on the container. The collected fungal samples were inoculated into SDA by plate spread technique (Sri-indrasutdhi *et al*., 2015). *Candida albican* and free agar media were used as quality control. The samples were incubated at 25 ºC, and the colony growth was checked daily. Different colonies

grown on a plate were transferred (subcultured) to a new medium continuously until axenic cultures were obtained. The descriptions of each colony were recorded, and representative samples were photographed. Finally, each pure culture was transferred and stored in a slant form in a refrigerator at -4 ºC for further identification and antifungal test.

4.5.2 Micro and macro morphological identification of fungal isolates

The genera of all isolated fungal colonies were identified by laboratory personnel in the Bacteriology and Mycology reference laboratory of Ethiopian Public Health Institute (EPHI), Addis Ababa, based on their shape, diameter, margin/border, surface appearance, opacity and color (pigmentation) of the colonies. Furthermore microscopical identifications were based on the presence or absence of septets on hyphae, shape and size of conidia conidiogenous cell and conidiophore. Special characters were demonstrated and compared with the known species in taxonomic keys, and by comparing the micrographic characteristic of the fungi with standard mycology textbook. For the purpose of antifungal tests of the ongoing study, some of the fungi were selected based on their prevalence of occurrence and by considering budget limitation, further identified by biolog micro station up to the species level in the Mycology laboratory of Ethiopian Biodiversity Institute (EBI), Addis Ababa.

4.5.3 Biolog identification of fungal isolates

Biolog identification of the isolated filamentous fungi was done by following the method used by Gizaw *et al*. (2017). Fungi isolates were screened based on primary and secondary colony morphology and transferred to biolog universal growth agar medium. For inoculum preparation, 15 mL suspension of filamentous fungi (FF) were taken and adjusted at 75±2 turbidity by biolog turbidimeter. Using digital pipettor 100 µL of inoculum was transferred into 96 wells of the biolog FF microplate, tagged with different carbon source and incubated at 26 °C for 24-240 h (for filamentous fungi) and read by micro station reader at a single wavelength of 590 nm. Biolog software micro log3 ver. 4.20.05 compared the results obtained with the test strain to the database and provided identification based on distance value of match; separation score produced similarity index value and probability. Acceptable species identification must have similarity index value above or equal to 0.5 or probability above or equal to 75%.

4.6 Antifungal test

4.6.1 Test organisms

The test organisms used for antifungal test of *V. amygdalina* and *C. macrostychus* are fungi isolated from formalin fixed cadavers and those identified by biolog micro station by this study.

4.6.2 Inoculum preparation and standardization

Inoculum preparation and standardization was performed as recommended by the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2017) (Appendix 1). The fungal test cultures were taken from the stock cultures and refreshed in SDA slants at 25 ºC for 48 h. The mold suspensions were prepared by gently probing the surface of the slant culture with a sterile loop and transferred into a screw cup test tube containing 9 mL of SDB. The suspensions were then mixed by using a vortex mixer (FUSE, Nickel Electro LTD, England). To measure the turbidity spectrophotometrically 3 to 4 mL of homogeneous suspensions were aseptically transferred into cuvette tubes and their optical densities subsequently adjusted in the range of 0.08 - 0.1 at 625nm. According to CLSI guidelines (CLSI, 2008), this turbidity is approximately equivalent to 1 - 5 x 108 colony forming unit (CFU) per mL.

4.6.3 Agar diffusion test

Antifungal activities of the extracts were determined using agar well diffusion method as previously described by Taye *et al*. (2011). The standardized fungal suspensions were streaked by sterilized cotton swab onto preprepared and labeled SDA by rotating each time to ensure even distribution of the inoculum. Following that, five equidistant wells (four for four graded concentration of the extract and the remaining one for the negative control) were punched aseptically using a sterile cork borer. Then, 150 µL of each of the crude extracts or solvent fractions at four different concentrations and negative control were added into the wells using micropipette. The crude extracts were tested at concentrations of 25, 50, 100 and 200 mg/mL, while concentrations of 12.5, 25, 50 and 100 mg/mL were used for the solvent fractions. The solvents used to dissolve the extracts were employed as a negative control. These solvents are distilled water for aqueous and 80% methanol extracts, 4% tween 80 for hexane, chloroform and acetone fractions and 5% DMSO for methanol fraction. The plates were then placed undisturbed

at room temperature for 2 h to allow proper diffusion of the extracts and incubated at 25 ºC for 48 h. At the end of each incubation period, the diameter of inhibition zone including the diameter of the well was measured with a ruler and registered after subtracting the diameter of the well. The experiment was performed in triplicates and the mean of inhibition zones was calculated for each extract.

4.6.4 Determination of minimum inhibitory concentration (MIC)

The lowest concentration of the extract that inhibited the visible growth of a particular fungus was selected as the MIC (Baljeet *et al*., 2015). The agar dilution method was applied to all crude extracts and fractions to determine MIC values and as an alternative method to agar well diffusion method to reveal the antifungal activities of the extracts. A serial dilution of both the crude extracts and solvent fractions were separately prepared by two fold starting from 1 mg/mL up to 8 mg/mL to obtain concentrations of 1, 2, 4 and 8 mg/mL (Appendix 2). Serial dilutions (2 mL) of each of the extracts were aseptically transferred to test tubes containing 18 mL of sterile molten (45 ºC) SDA. This mixture was thoroughly mixed and transferred to sterile and prelabeled Petri dishes. After the media congealed each standardized fungi was inoculated to its corresponding labeled part of the Petri dish (ten fungi per Petri dish) and incubated at 25 ºC for 48 h. At the end of this incubation period the lowest concentration that inhibited the growth of the fungi was taken as the MIC value for the extract. Extract-free agar media were taken as a negative control. All tests were performed in triplicate.

4.7 Statistical analysis

Zone of inhibition were measured in mm. The data were analyzed by using statistical package for social sciences (SPSS) software version 23.0. The statistical differences of the mean inhibition zone of extracts for individual fungus of different concentrations were carried out by employing one way analysis of variance (ANOVA) followed by Tukey Post Hoc Multiple Comparison test and statistical significances of different concentrations of the plant extracts were considered at $p<0.05$. The analyzed data were then presented as the mean ± standard error of the mean (SEM) for each triplicate experiments.

4.8 Ethical considerations

This study was carried out after ethical clearance was obtained from Departmental Research Ethics Review Committee (DRERC) of the Department of Anatomy, School of Medicine, AAU. The samples were collected in accordance with the ethics of cadavers.

5 Results

5.1 Fungi isolated from formalin fixed cadavers

Twenty-seven fungi belonging to ten genera were isolated from formalin-fixed cadavers obtained from the School of Medicine, College of Health Sciences, AAU, St. Paul's Millennium and Africa Medical Colleges (Table 1). Ten of them were identified up to species level by biolog and preserved with vial number in the Gene Bank of EBI, Addis Ababa, Ethiopia (Table 2). In the current study, only these ten fungal species were used for the antifungal activity tests.

Table 1: Genera of fungi isolated from formalin fixed cadavers obtained from the School of Medicine, College of Health Sciences Addis Ababa University (AAU), St. Paul's Millennium and Africa Medical Colleges, Addis Ababa, Ethiopia, September 2017

Genera of isolated fungi	**Frequency**	**Percentage**
Acremonium	1	3.70
Aspergillus	5	18.51
Geomyces	1	3.70
Madurella	2	7.40
Microsporum	1	3.70
Neosartorya	1	3.70
Penicillium	12	44.44
Scopulariopsis	1	3.70
Talaromyces	1	3.70
Trichophyton	2	7.40
Total	27	100

Table 2: Fungal species identified by biolog isolated from formalin fixed cadavers obtained from the School of Medicine, College of Health Sciences, AAU, St. Paul's Millennium and Africa Medical Colleges, Addis Ababa, Ethiopia, January 2018

Fungal species	Vial no.	Genera
Aspergillus avenaceus G.Sm.	15	*Aspergillus*
Aspergillus carneus (v. Tiegham) Blockwitz	9	*Aspergillus*
Aspergillus restrictus G. Sm	18	*Aspergillus*
Geomyces pulvereus Hocking and Pitt	12	*Geomyces*
Neosartorya fischeri (Wehmer) Malloch & Cain	11	*Neosartorya*
Penicillium pinophilum Hedge. BGB	16	*Penicillium*
Penicillium restrictum Gilman & Abbott BGA	14	*Penicillium*
Penicillium roqueforti Thom BGE	10	*Penicillium*
Scopulariopsis brumptii Salvanet-Duval	13	*Scopulariopsis*
Talaromyces bacillisporus (Swift) C.R. Benjamin	17	*Talaromyces*

5.2 Antifungal test results

5.2.1 Agar well diffusion method

The mean diameters of inhibition zone of the 80% methanol extract of *V. amygdalina* against the fungal species tested are shown in Table 3. Among the test fungi, *Scopulariopsis brumptii, Neosartorya fischeri* and *Aspergillus avenaceus* were more susceptible to the 200 mg/mL concentration of the extract with mean inhibition zone of 17.67, 13 and 8.33 mm, respectively. However, the aqueous extract of *V. amygdalina* failed to show any activity against the fungal species tested.

Table 3: Zones of inhibition (mm) of the 80% methanol extract of *Vernonia amygdalina* against fungi isolated from formalin fixed cadavers

Fungal species	**Concentration (mg/ mL)**			
	25	**50**	**100**	**200**
Aspergillus avenaceus	4.5±0.5	6.67±1.67	6.00±0.57	8.33±0.88
Aspergillus carneus	0.00	0.00	0.00	0.00
Aspergillus restrictus	0.00	0.00	0.00	0.00
Geomyces pulvereus	0.00	0.00	0.00	0.00
Neosartorya fischeri	10±5	10.5±5.5	12.5±2.5	13±1.52
Penicillium pinophilum	0.00	0.00	0.00	0.00
Penicillium restrictum	0.00	0.00	0.00	0.00
Penicillium roqueforti	0.00	0.00	0.00	0.00
Scopulariopsis brumptii	5.00±5.00^{d1}	9.33±2.60	11.67±3.33	17.67±1.45^{a1}
Talaromyces bacillisporus	0.00	0.00	0.00	0.00

Values are expressed as Mean ± SEM (n=3), analysis was performed with One-Way ANOVA followed by Tukey test; a: compared to 25 mg/mL; b: compared to 50 mg/mL; c: compared to 100 mg/mL; d: compared to 200 mg/mL, 1 $P< 0.05$, 2 $P < 0.01$. The negative control showed no antifungal activity.

The mean diameters of inhibition zone of the aqueous and 80% methanol extracts of the *C. macrostachyus* are shown in Tables 4 and 5, respectively. At a concentration of 200 mg/mL, the aqueous extract was most active against *Penicillium restrictum*, *Talaromyces bacillisporus* and *Scopulariopsis brumptii* with mean inhibition zones of 24.33, 22.33 and 15.00 mm, respectively (Table 4). This extract also displayed weak inhibitory activity against *Aspergillus restrictus*. The current study demonstrated that only *Penicillium pinophilum* was susceptible to the 80% methanol extract of *C. macrostachyus* (Table 5).

All solvent fractions obtained from the leaves of *V. amygdalina* and *C. macrostachyus* did not inhibit growth of any of the test fungi when tested by the agar well diffusion method.

Table 4: Zones of inhibition (mm) of the aqueous crude extract of *Croton macrostachyus* against fungi isolated from formalin fixed cadavers

Fungal species	**Concentration (mg/mL)**			
	25	**50**	**100**	**200**
Aspergillus avenaceus	0.00	0.00	0.00	0.00
Aspergillus carneus	0.00	0.00	0.00	0.00
Aspergillus restrictus	12.00±2.81	11.00±0.00	10.67±2.60	9.33±1.67
Geomyces pulvereus	0.00	0.00	0.00	0.00
Neosartorya fischeri	0.00	0.00	0.00	0.00
Penicillium pinophilum	0.00	0.00	0.00	0.00
Penicillium restrictum	12.67±2.88	18.00±2.64	19.00±3.00	24.33±2.88
Penicillium roqueforti	0.00	0.00	0.00	0.00
Scopulariopsis brumptii	5.67 ± 1.20^{c1d1}	7.67 ± 0.88^{c1d2}	14.67 ± 0.88^{a1b1}	15.00 ± 1.73^{a2b2}
Talaromyces bacillisporus	11.33±2.60	17.33±4.37	14.33±2.02	22.33±0.88

Values are expressed as Mean ± SEM (n=3), analysis was performed with One-Way ANOVA followed by Tukey test; a compared to 25 mg/mL; b compared to 50 mg/mL; c compared to 100 mg/mL; d compared to 200 mg/mL, 1 $P< 0.05$, 2 $P < 0.01$. The negative control showed no antifungal activity.

Table 5: Zone of inhibition (mm) of the 80% methanol extract of *Croton macrostachyus* against fungi isolated from formalin fixed cadavers

Fungal species	**Concentration (mg/mL)**			
	25	**50**	**100**	**200**
Aspergillus avenaceus	0.00	0.00	0.00	0.00
Aspergillus carneus	0.00	0.00	0.00	0.00
Aspergillus restrictus	0.00	0.00	0.00	0.00
Geomyces pulvereus	0.00	0.00	0.00	0.00
Neosartorya fischeri	0.00	0.00	0.00	0.00
Penicillium pinophilum	4.00 ± 1.00^{d1}	5.50±0.50	8.00 ± 1.52^{d1}	16.67 ± 1.45^{a1c1}
Penicillium restrictum	0.00	0.00	0.00	0.00
Penicillium roqueforti	0.00	0.00	0.00	0.00
Scopulariopsis brumptii	0.00	0.00	0.00	0.00
Talaromyces bacillisporus	0.00	0.00	0.00	0.00

Values are expressed as Mean ± SEM (n=3), analysis was performed with One-Way ANOVA followed by Tukey test; a compared to 25 mg/mL; b compared to 50 mg/mL; c compared to 100 mg/mL; d compared to 200 mg/mL, 1 $P< 0.05$, 2 $P < 0.01$. The negative control showed no antifungal activity.

5.2.2 Agar dilution method

The MIC values of the crude extracts and solvent fractions of *V. amygdalina* and *C. macrostachyus* were determined by agar dilution method (Appendix1). As shown in Table 6, the maximum MIC value of 80% methanol extract of *V. amygdalina* was 6.67 mg/mL against *Neosartorya fischeri*, while the lowest was 3.33 mg/mL against *Aspergillus avenaceus*. The aqueous extract of *V. amygdalina* failed to show activity against the tested fungi by the agar dilution method. However, some of the solvent fractions of *V. amygdalina* exhibited weak activity against *Penicillium restrictum* and *Talaromyces bacillisporus* in the agar dilution assay.

Table 6: Minimum inhibitory concentrations (mg/mL) of the crude extract and solvent fractions of the leaves of *Vernonia amygdalina* against fungi species isolated from formalin fixed cadavers

Fungal species	**Crude extract**		**Solvent fractions**			
	80% methanol	**Aqueous**	**Hexane**	**Chloroform**	**Acetone**	**Methanol**
Aspergillus avenaceus.	3.33±1.15	-	-	-	-	-
Aspergillus carneus	-	-	-	-	-	-
Aspergillus restrictus	-	-	-	-	-	-
Geomyces pulvereus	-	-	-	-	-	-
Neosartorya fischeri	6.67±2.31	-	-	-	-	-
Penicillium pinophilum	-	-	-	-	-	-
Penicillium restrictum	-	-	3.33±1.15	2.00±0.00	8.00+2.31	-
Penicillium roqueforti	-	-	-	-	-	-
Scopulariopsis brumptii	5.33±2.31	-	-	-	-	-
Talaromyces bacillisporus	-	-	2.00±0.00	-	4.00±0.00	-

Values are the average of triplicate test ±SEM (n=3).

As shown below in Table 7, in the agar dilution method of the 80% methanol extract of *C. macrostachyus* had an effect against *Penicillium pinophilum* only with MIC value was 6.67 mg/mL. For the aqueous extract of *C. macrostachyus,* the maximum MIC was 8 mg/mL against *Aspergillus restrictus* and *Scopulariopsis brumptii* and the minimum MIC was 3.33 mg/mL against *Penicillium restrictum*.

Penicillium restrictum was the most susceptible to the acetone fraction of *C. macrostachyus* with MIC value of 1.00 mg/mL. However, *Aspergillus avenaceus*, *Aspergillus carneus*, *Neosartorya fischeri* and *Penicillium roqueforti* were not susceptible neither to the crude extract nor the solvent fractions of *C. macrostachyus*. Similarily, the crude extracts and solvent fractions prepared from both medicinal plants were inactive against *Aspergillus carneus* and *Penicillium roqueforti*.

Table 7: Minimum inhibitory concentrations (mg/mL) of the crude extract and solvent fractions of the leaves of *Croton macrostachyus* against fungi species isolated from formalin fixed cadavers

Fungal species	**Crude extract**		**Solvent fractions**			
	80% methanol	**Aqueous**	**Hexane**	**Chloroform**	**Acetone**	**Methanol**
Aspergillus avenaceus	-	-	-	-	-	-
Aspergillus carneus	-	-	-	-	-	-
Aspergillus restrictus	-	8.00±0.00	-	-	-	-
Geomyces pulvereus	-	-	-	6.67±2.31	-	-
Neosartorya fischeri	-	-	-	-	-	-
Penicillium pinophilum	6.67±2.31	-	-	-	-	8.00±0.00
Penicillium restrictum	-	3.33±1.54	8.00±00	2.00±0.00	1.00±0.00	-
Penicillium roqueforti	-	-	-	-	-	-
Scopulariopsis brumptii	-	8.00±0.00	4.00±0.00	-	-	-
Talaromyces bacillisporus	-	4.00±0.00	-	-	-	-

Values are the average of triplicate test ±SEM (n=3).

6 Discussion

A successful embalming procedure is necessary for prolonged preservation of cadavers for anatomical study. However, the embalming fluid contains hazardous chemicals like formalin and phenol (Bajracharya and Magar, 2006), and these chemicals have proven adverse health effect on instructors, students and medical staff members who handle the cadaver (Kundu *et al*., 2015; Ohmichi *et al*., 2006, OSHA). The present study was undertaken to test the antifungal activity of *V. amygdalina* and *C. macrostachyus* extracts against fungi isolated from formalin fixed cadavers. The study was conducted with the aim of finding new and safe product(s) that may replace or enhance the preservative effect of the currently used embalming fluids.

As shown in Table 1, fungi that belong to ten genera, namely, *Acremonium*, *Aspergillus*, *Geomyces*, *Madurella*, *Microsporum*, *Neosartorya*, *Penicillium*, *Scopulariopsis*, *Talaromyces* and *Trichophyton* were isolated from formalin fixed human cadavers. The result indicates that the embalming fluids were not effective to prevent the growth of fungi on the embalmed body, which is concurrent with previous research findings (Elsebai *et al*., 2002; Osman *et al*., 2014; Sri-indrasutdhi *et al*., 2015; Sambasivarao and Amruta, 2017). The study further revealed that *Penicillium* (44.44%) was the most prevalent fungus genus followed by *Aspegillus* (18.51%). Earlier reports have also shown the frequent occurrence of fungi that belong to the two genera in human cadavers (Elsebai *et al*., 2002; Sri-indrasutdhi *et al*., 2015).

Previously, Elsebai *et al*. (2002) isolated *Aspergillus*, *Penicillium* and *Trichophyton* spp. from the surface and internal organs of formalin fixed cadavers (Sambasivarao and Amruta, 2017). *Canidida* spp., *Cryptococcus* spp. and *Microsporum* spp. have also been shown to grow on the surface of human cadavers (Osman *et al*. 2014). To the best of our knowledge, this is the first report on the occurrence of *Acremonium*, *Geomyces*, *Madurella*, *Neosartorya*, *Scopulariopsis* and *Talaromyces* spp. on the surface of human cadaver. The fungi isolated in this study were different in type and frequency from those reported previously (Osman *et al*., 2014). The speculations behind these variations are related to the effectiveness of the embalming and environmental factor like temperature and humidity of the dissecting room (Elsebai *et al*., 2002; Sri-indrasutdhi *et al*., 2015).

In the present work, ten fungal samples were selected and further identified by biolog to species level. These were identified as *Aspergillus avenaceus* G.Sm., *Aspergillus carneus* (v. Tiegham) Blockwitz, *Aspergillus restrictus* G. Sm., *Geomyces pulvereus* Hocking and Pitt, *Neosartorya fischeri* (Wehmer) Malloch and Cain, *Penicillium pinophilum* Hedge. BGB, *Penicillium restrictum* Gilman & Abbott BGA, *Penicillium roqueforti* Thom BGE, *Scopulariopsis brumptii* Salvanet-Duval and *Talaromyces bacillisporus* (Swift) C.R. Benjamin. Although *Aspergillus* and *Penicillum* species are known to cause respiratory, skin and urinary infections, mycotic keratitis, external otomycosis and endocarditis, further studies are needed to identify and determine the pathogenicity of the other fungi isolated in this study.

Evaluation of the effect of total extracts and solvent fractions prepared from the leaves of *V. amygdalina* and *C. macrostachyus* against all the isolated fungal species resulted in growth inhibition of some of them. Previous studies have shown that these extracts possess antifungal activity against common fungal pathogenes such as *Aspergillus niger, Aspergillus flavus Candida albicans, Trichophyton mentagrophyts Trichophyton rubrum, Trichophyton soudanense, and Trichophyton violaceum* (Nwachukwu and Umechuruba, 2001; Teugwa *et al*., 2013; Obey *et al*., 2016; Hailu *et al*., 2017). However, this is the first report on the effect of *V. amygdalina* and *C. macrostachyus* extracts against the unique fungal species that were found to grow on the surface of human cadaver.

Among the tested extracts, the 80% methanol extract of *V. amygdalina* was found to inhibit growth of, *A. avenaceus N. fischeri* and *S. brumptii* in both the agar well and agar dilution methods (Tables 3 and 6). Previous study by Higashi *et al*. (1991) demonstrated that only the methanol extract of *V. amygdalina* possesses mild antifungal activity against *Pseudoperonospora cubensis* and *Rhizoctonia solani*. In the present study, all the solvent fractions obtained from *V. amygdalina* failed to inhibit fungal growth when tested by the agar well diffusion method. This could be that the active components present in the polar 80% methanol extract are not be soluble enough in the relatively nonpolar organic solvents used or the active ingredients might have distributed themselves in the different organic solvents that their concentrations were insufficient to show fungal growth inhibition. It should also be noted that synergy may exist among the active components present in the hydroalcoholic extract thus enhancing antifungal activity. Other

reports have also revealed that the total extracts of some plants show good antimicrobial activity whilst their solvent fractions are devoid of any activity (Molla *et al.*, 2016).

In the current study, it was also noted that the hexane, chloroform and acetone fractions of *V. amygdalina* possess activity against *P. restrictum* and *T. bacillisporus* by the agar dilution method but unable to cause growth inhibition by the agar well diffusion assay. There are several reports in the literature which indicate that failure for a compound to cause growth inhibition in the agar disk or well diffusion method does not necessarily mean that the compound is inactive, particularly if it is a nonpolar compound. Generally, nonpolar compounds diffuse slowly into the polar culture medium owing to their hydrophobic nature which prevents them from diffusing uniformly (Mann and Markham, 1998; Moreno *et al*., 2006; Klancnik *et al*., 2010). Even when substances diffuse properly in the agar medium, it is not possible to quantify the amounts that diffused (Balouiri *et al*., 2016).

As shown in Table 4, the aqueous extract of *C. macrostachyus* inhibited the growth of *Aspergillus restrictus*, *Penicillium restrictum*, *Scopulariopsis brumptii* and *Talaromyces bacillisporus*, while the 80% methanol extract was active against *Penicillium pinophilum* only (Table 5). This finding is consistent with the results of Hailu *et al*. (2017) who reported that both the methanol and aqueous extracts of *C. macrostachyus* possess antifungal activity against *Candida albicans* and *Trichophyton mentagrophyts*. During testing of the plant extracts, it was noted that the mean diameter of zones of inhibition increased when the concentration of the test substance increased up to a certain level and then started to decline when the concentration of the plant extract further increased in some of the test fungi. The reduction in growth inhibition with increasing concentrations may be due to the low diffusion of the extract through the agar medium at high concentrations, as a result of forming relatively thicker liquid (Wijesundara *et al*., 2016). However, in most of the test fungi, the activity of the extracts was dose-dependent (Tables 3 and 4). In the present study, the negative controls used (distilled water, 4% tween 80 or 5% DMSO,) were confirmed to have no fungal growth inhibition property.

By and large, the MICs obtained by the agar dilution assay were consistent with the diameter of zones of inhibition observed in the agar well diffusion method. For example, the aqueous crude extract of *C. macrostachyus* which showed highest inhibition zone in agar well diffusion test

inhibited the growth of test fungi with the lowest concentration. However, most solvent fractions showed growth inhibition in the agar dilution assay but failed to exhibit antifungal property in the agar well diffusion method. As stated earlier, this could be due to the relatively nonpolar nature of the bioactive metabolites which finds it difficult for these substances to diffuse through the polar agar medum (Mann and Markham, 1998; Moreno *et al*., 2006; Klancnik *et al*., 2010).

Aspergillus carneus, *Aspergillus restrictus*, *Geomyces pulvereus*, *Penicillium pinophilum* and *Penicillium roqueforti* were not susceptible to all solvent fractions and the total extracts of *V. amygdalina*. Similarly, *Aspergillus avenaceus*, *Aspergillus carneus, Neosartorya fischeri* and *Penicillium roqueforti* were resistant to all extracts of *C. macrostachyus*, while *Penicillium restrictum* was mostly sensitive to both plant extracts. The above results confirm that the extracts contain compounds with genuine antifungal activity as the negative control displayed no activity what so ever.

From the foregoing, it can be seen clearly that most of the test fungi were not susceptible to the plant extracts under study. This is not an unexpected since all the test fungi were isolated from embalmed human cadavers which have already developed high levels of resistance to corrosive chemicals like formalin and phenol, contained in the embalming fluids (Osman *et al*., 2014). It has been well established that clinical microbial specimens are resistant to antimicrobial agents. Several plant extracts have also been shown to display good activity against standard bacterial and fungal strains with little or no activity against clinical isolates of the same species (Molla *et al*., 2016). This could be due to decreased cell permeability to and active efflux of the test substances, the ability of the organisms to modify drug receptor site or synthesize resistant metabolic pathway, among others.

7 Conclusion

The present study confirmed that several fungal species grow on the surface of formalin-fixed human cadavers obtained from College of Health Sciences, Addis Ababa University, St. Paul's Millennium and Africa Medical Colleges in Addis Ababa, Ethiopia. The results further proved that the currently used embalming solutions which contain formalin and phenol are not good enough to prevent growth of fungi on cadavers. Although *Aspergillus, microsporum, Penicillium* and *Trichophyton* species have been previously shown to grow on the surface of formalin-fixed human cadaver, this is the first report on the isolation of *Acremonium, Geomyces, Madurella, Neosartorya, Scopulariopsis* and *Talaromyces* spp. from the same material.

The findings of the present study have established the susceptibilities of some of the isolated fungal species to the extracts of *V. amygdalina* and *C. macrostachyus*. Among the various extracts tested, the aqueous extract of *C. macrostachyus* exhibited promising activity against *Aspergillus restrictus, Penicillium restrictum, Scopulariopsis brumptii* and *Talaromyces bacillisporus*. Concurrently the 80% methanol extract of *V. amygdalina* also showed the same against *Aspergillus avenaceus, Neosartorya fischeri* and *Scopulariopsis brumptii*. Therefore, the current generated data could be used as a baseline at least to modifying the present embalming solution by mixing it with the plant extracts. The study further provides scientific evidence for the use of the leaves of *V. amygdalina* for preservation of corpse by people in southern Ethiopia.

8 Recommendations

Based on the results of the study, the following recommendations are forwarded:

- ✓ To minimize exposure to fungal infection appropriate safety measure should be taken while handling and dissecting the cadavers, otherwise, beyond the hazardous effect of embalming solution, staff and students would be exposed to harmful fungi on a regular basis during routine dissection and handling of the cadavers.
- ✓ The antifungal activities of the plant should also be tested on other fungal species grown on formalin fixed cadavers, which were not addressed by this study.
- ✓ Further cross sectional comparative study should be conducted for the application of the extract of the medicinal plants included in the present study; synergistically with the low level of formalin and phenol for the embalming of the cadavers in life like appearance with minimal health and environmental effect.
- ✓ Further large scale investigation of other microorganisms (not only fungi but including bacteria) from formalin fixed cadavers should be required.

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Appendix

Appendix 1: Inoculate preparation and agar well diffusion assay method

Required equipment

- Laminar air flow hood
- Incubator
- Spectrophotometer
- Autoclave
- Micropipette
- Vertex
- Inoculating Loops
- Cylinder gas
- Bunsen burner
- Refrigerator
- Sterile crock borer
- Analytical balance

Samples

- Crude extract
- Solvent fractions
- Solvents (distilled H_2O, 5%DMSO and 4% Tween 80) for comparison as negative controls

Procedure

1. Prepared 48 h slant of fungi by SDA
2. Inoculate a portion of four to five discrete colonies of the representative of the fungi into 10 mL of SDB.
3. Spectrophotometrically, the optic density was adjusted to 0.08 - 0.1 OD at 625 nanometer wave length. Note this OD is equivalent to $1\text{-}5*10^8$ CFU and the media inoculated within 30 minutes.
4. Pre prepared SDA media were inoculated by swab technique and then made equal distance hole by crock borer

5. Poured 150 micro liter of the test sample, dissolved in the appropriate solvent, into each appropriately pre labeled holes using micropipette. The corresponding solvent used as negative controls.
6. Place the plates at room temperature for 2hr, to allow diffusion of the sample.
7. Incubate the plate face upwards at 25 ^{0}C for 2-5 days and antifungal reading assays were made.

Appendix 2: Agar serial dilution preparation procedure

1. Prepare the extract at 10 times the concentration required in the final test (concentration in millimeter of media)
2. Weight the extract using analytical balance to the amount found by the equation V*C =W
3. Dissolve the weighted sample in the specified volume of solvents (Distilled water, 4% Tween 80 or 5% DMSO) to prepare the stock solution.
4. Visually look at the homogeneity of the solution (solubility)
5. Use Log_2 or twofold dilution for determining MICs and prepare according to the volumetric schedule shown below.
6. Dilution schedule should be selected to include a concentration of 1 µg/mL to permit comparison of results from different laboratories and their easy expression as log_2 statistical manipulation
7. The dilution method shown below in Table is convenient and economical in pipettes because only one need be used for each block of antibiotics dilutions.

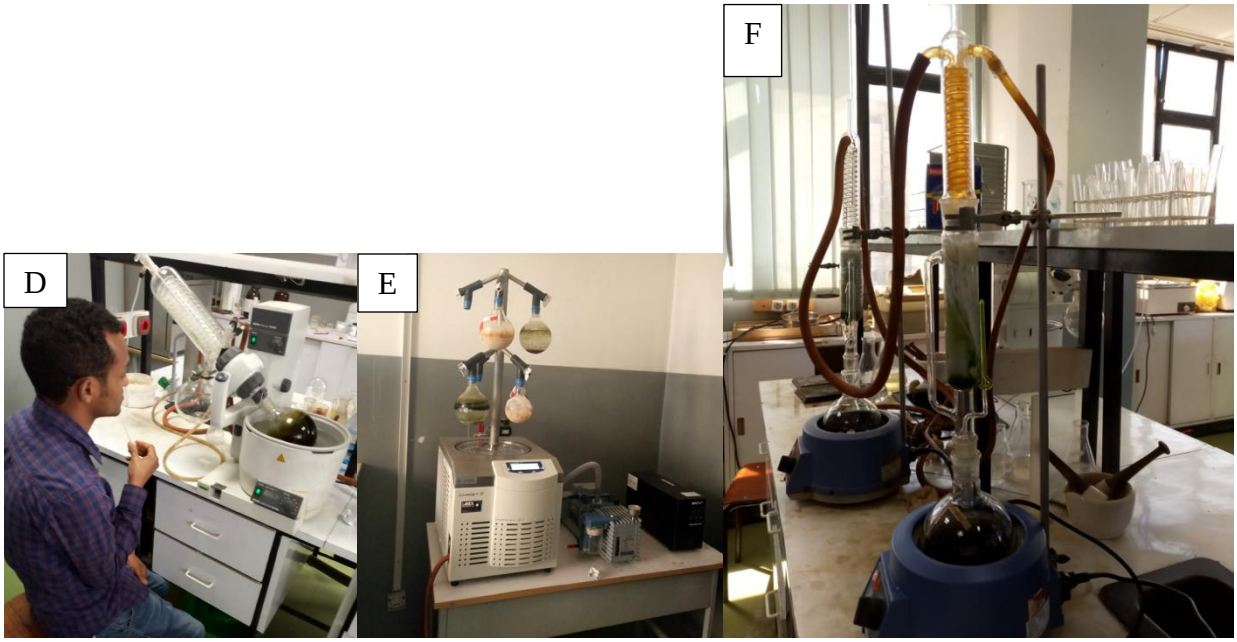
Table1: System for preparing dilution for agar dilution method

Weight (mg) of extract	Volume of solvent in mL	Intermediate concentration (mg/mL)	Final concentration At 1:10 in agar plate (mg/mL)
560	7	80	8
280	7	40	4
140	7	20	2
70	7	10	1

Note: The solvents are distilled water for aqueous and 80% methanol extracts, 4% tween 80 for Hexane, Chloroform and Acetone fractions and 5% DMSO for Methanol fraction.

Appendix 3: Photographs illustrating some aspects of the laboratory activity at Pharmaceutical Chemistry and Pharmacognosy laboratory (School of Pharmacy, AAU)

A. Air drying leaves B. Weighing powdered leaves C. Macerating leaves for crude extraction

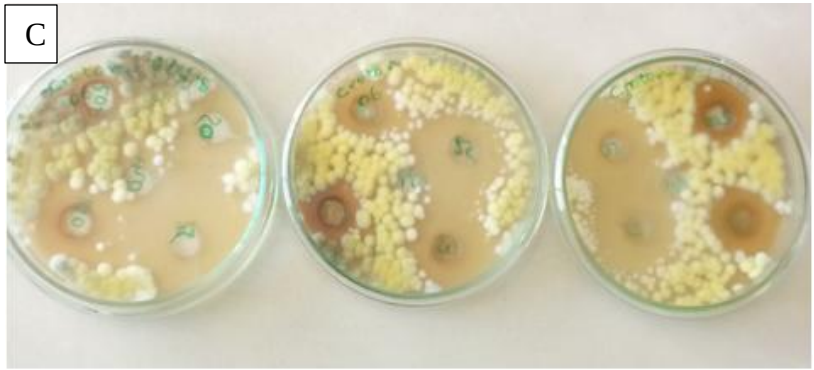


D. Removing methanol (80%) from crude extract by rotary vapor, E. Concentrating crude extracts by lypholizer, F. Photograph illustrating serial solvent fractions by Soxhlet apparatus

Appendix 4: Photographs illustrating some aspects of the laboratory activity of culturing and antifungal test at Ethiopian Public Health Institute (EPHI)

A. Photograph taken during inoculating collected fungal sample and sub culturing

B. Photograph taken during antifungal test by agar well diffusion method



C. Antifungal effect of aqueous crude extract of *Croton macrostachyus* against *Penicillium restrictum* Gilman & Abbott BGA