PHYTOCHEMICAL, ANTIMICROBIAL AND CYTOTOXICITY EVALUATION OF RHIZOME EXTRACTS OF *HYDNORA ABYSSINICA* FROM *ACACIA NIGRENCES* HOST.

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NGHINAUNYE TEOPOLINA

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Main supervisor: Dr Muvari Connie Tjiurutue (Chemistry and Biochemistry Department, University of Namibia)

Co-supervisors: Dr Iwanette Du Preez (Multidisciplinary Research Centre, University of Namibia)

Associate Prof Erica Maass (Academic Affairs, University of Namibia)

ABSTRACT

Medicinal plants are a rich source of phytochemicals, and are used traditionally for treatment of many ailments, and thus are of great ethnomedicinal importance. This study presents work on phytochemical analysis, *in vitro* antimicrobial activity and cytotoxicity evaluation of medicinal plant *Hydnora abyssinica. H. abyssinica* is a medicinal plant indigenous to Namibia, Kenya, Democratic Republic of Congo (DRC), South Africa and Sudan. Preliminary phytochemical screening was done by colorimetric tests, while total phenol content (TPC), total flavonoid content (TFC) and total tannin content (TTC) were determined using spectrophotometric methods. Antimicrobial activity of the extracts was determined by means of agar diffusion and broth microdilution methods. Cytotoxicity was carried out using the Sulforhodamine B (SRB) assay on a 3T3 embryonic fibroblast mouse cell line. Phytochemical screening revealed the presence of tannins, alkaloids, flavonoids, steroids and saponins. Phenols and tannins were detected in the three extracts of water (H_2O), methanol (MeOH), and methanol: dichloromethane (MeOH-DCM). The MeOH extract exhibited the highest TTC (0. 44 ± 0.01 mg TAE/g) as well as TPC (0.31 ± 0.05 mg GAE/g). In contrast, the MeOH-DCM (1:1) extract exhibited the highest TFC of 0.12 ± 0.01 mg QE/g. Flavonoid quantity was not detectable in the water extract. The MeOH extract demonstrated moderate antimicrobial activity against *Candida albicans* (12.0 ± 1.0 mm), *Listeria monocytogenes* (6.0 ± 3.0 mm), *Enterococcus durans* (6.0 ± 2.8 mm) and weak antimicrobial activity against *Escherichia coli* (4.0 ± 1.0 mm) and *Shigella Sonnei* (4.0 ± 2.6 mm). The MeOH-DCM extract demonstrated moderate antimicrobial activity against *Candida albicans* (10.0 ± 1.0 mm), *Enterococcus durans* (6.0 ± 1.7 mm) and weak antimicrobial activity against *Listeria monocytogenes* (5.0 ± 1.7 mm), *Escherichia coli* (3.0 ± 2.5 mm) and *Shigella Sonnei* (3.0 ± 1.4 mm). The water extract demonstrated moderate antimicrobial activity against *Candida albicans* (6.0 ± 1.0 mm) and weak antimicrobial activity against *Listeria monocytogenes* (4.0 ± 2.3 mm), *Escherichia coli* (4.0 ± 1.5 mm), *Enterococcus durans* (4.0 ± 1.8 mm) and *Shigella Sonnei* (3.0 ± 1.4 mm). Overall, the MeOH (12.0 ± 1.0 mm) and MeOH-DCM (10.0 ± 1.0 mm) extracts were active against the yeast isolate *C. albicans.* The H_2O extract demonstrated weak antimicrobial activity (≤ 6.0 ± 1.0 mm) against *Listeria monocytogenes, Enterococcus durans, Shigella Sonnei* and *Escherichia coli.* The highest

antimicrobial activity of the methanol extract (12.0 ± 1.0 mm and MIC ranging from 3.125 to 6.25 mg/mL) was recorded against *C. albicans*, indicating antifungal activity. Little to no cytotoxicity of the plant is reported against the 3T3 embryonic fibroblast mouse cell line. High tannin and phenolic contents present in the rhizome extracts together with the observed moderate antimicrobial activity supports the folklore use of the plant rhizome as a cure for stomach and throat complaints as well as an astringent in dysentery. However, the findings of this study suggest that the safety of the plant in traditional usage needs to be confirmed by further testing *in vitro, in vivo* and in clinical studies. Further studies could look into the characterization and identification of the bioactive compounds present in the rhizomes of *H. abyssinica.*

KEYWORDS: *Acacia nigrences,* Antimicrobial activity, Cytotoxicity, *Hydnora abyssinica*, Phytochemical analysis.

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AST	Antimicrobial Susceptibility Test
ATCC	American Type Culture Collection
CO_2	Carbon dioxide
DCM	Dichloromethane
DH_20	Distilled water
DMSO	Dimethyl Sulphoxide
DMEM	Dulbecco's Modified Eagle Medium
DRC	Democratic Republic of Congo
FBS	Fetal Bovine Serum
GAE	Gallic Acid Equivalent
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MRC	Multidisciplinary Research Centre
MRC-5	Medical Research Council cell strain-5
OD	Optical Density
PBS	Phosphate-Buffered Saline
QE	Quercetin Equivalent

RPM	Rotation Per Minute
SRB	Sulforhordamine B
TAE	Tannic Acid Equivalent
TCA	Trichloroacetic Acid
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TTC	Total Tannin Content
WHO	World Health Organisation
UREC	University Research Ethics Committee
UV	UtraViolet rays

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DEDICATION

This thesis is dedicated to my loving mother, Olivia Ndinelao Nghinaunye, my late Father, Petrus Hailonga Nghinaunye, my siblings, my cousin Puye Ipawa Ndakolelwa, my uncle Gideon Shimanda, and to the Samuels for their genuine love, unwavering support and prayers throughout my study.

DECLARATIONS

I, TEOPOLINA NGHINAUNYE, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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CHAPTER 1: INTRODUCTION

1.1. Orientation of the study

A medicinal plant is defined as any plant with healing properties. The healing properties are linked to chemical constituents called secondary metabolites which produce a definite physiological action on the human body (1). Phenols, flavonoids, tannins and alkaloids are important medicinal chemical constituents (2). Furthermore, these constituents serve in plant defence mechanisms against predators such as insect, microorganisms and herbivores (3).

Since time immemorial, people have been using traditional medicine. The knowledge of traditional medicine has been handed down from one generation to the next (4). Today, in many parts of the world, such as Africa and Asia, traditional medicine replaces conventional medicines, and in developed nations such as United States, traditional medicine is rapidly gaining appeal (5).The reliance on traditional medicine in Africa has gained popularity since the colonial rule, especially in the rural areas, which is primarily associated with poor or lack of public health service delivery (6). The importance of traditional medicines is attributed to their cultural acceptance and affordability (7).

Traditional medicines are often used in the maintenance of health through disease prevention and treatment (8). Plant species have various active compounds with different biological activities which can be used in the management of diseases (9). Even though such plants are used by communities, scientific studies of many such plants are lacking. Namibia has a large heritage of traditional knowledge of which some is used for health care. *Hydnora abyssinica*, also known as *H. johannis* (Beccari) and *H. solmsiana* (Dinter) (1), is a strange leafless parasitic medicinal herb consisting of only a floral and root body (10). In Namibia, *H. abyssinica* is well-known for its healing properties and

has been used to treat diarrhoea, piles, acne, kidney problems, dysentery, menstrual problems, stomach cramps and haemophilia (1,4). Phytochemicals, antimicrobial activity and cytotoxicity of *H. abyssinica* were previously reported for Kenya, Sudan and South Yemen (1,11,12). However there is no data available for the Namibia species.

The substantial coincidence which exists between scientifically-proved phytochemical properties and traditional medicinal plant uses shows that the traditional remedies are a crucial and effective part of indigenous healthcare system, a call for more detailed phytochemical studies of traditionally-used medicinal plants. Foremost, medicinal plants provide abundant opportunities for local community development and livelihood improvement. However, only few of the plant species (19.5 % of 3159) have been studied and used for medicinal purposes in Namibia (13). Subsequently, considering the abundance of *H. abyssinica* in most parts of Namibia, the present study investigates a Namibian based *H. abyssinica* from *Acacia nigrences* host by screening and quantifying its phytochemical constituents, assessing the plant antimicrobial properties against selected microbial pathogens of interest (*Enterococcus durans, Listeria monocytogenes, Escherichia coli, Shigella Sonnei* and *Candida albicans)* due to their association with dysenteric and intestinal problems (1). The present study also examines the plant cytotoxicity.

1.2. Problem Statement

Although *Hydnora* is among the most commonly known angiosperms in science, little information on the ecology, physiology and phytochemistry is known, and its medicinal value remains virtually poor. This could be due to its hidden nature, an extreme reduction in morphological features including the absence of leaves and often seasonal

appearance (14). Additionally, the phytochemical properties of parasitic plants can be influenced by the host chemistry (15,16) and geography (17).

Biological activities of *H. abyssinica* have not been studied and documented for Namibian *H. abyssinica.* Equally important, there is a pressing need to evaluate the safety of the herbal consumption since some of the bioactive compounds are potentially toxic (18). Moreover, it is essential to continuously search for novel medicines to fight the alarming antimicrobial resistance.

1.3. Objectives of the study

This study aimed at identifying and quantifying the bioactive compounds present in *H. abyssinica* rhizome extracts, evaluating the plant's toxicity in vitro, assessing the antimicrobial activity of the plant and, most importantly, preserving indigenous knowledge by documentation of the bioactivity of the local plant rhizome. The specific objectives of the study were:

a) To investigate major classes of phytochemical constituents present in rhizome crude extracts of *H. abyssinica* employing both qualitative and quantitative methods.
b) To determine antimicrobial activity of the rhizome crude extracts of *H. abyssinica* against *E. durans*, *L. monocytogenes*, *E. coli*, *S. sonnei* and *C. albicans.*
c) To determine cytotoxicity of the rhizome crude extracts of *H. abyssinica* against 3T3 embryonic fibroblast mouse cell line.

1.4. Research hypotheses

(1) The crude extracts of *H. abyssinica* contain major classes of phytochemicals, (2) exhibit antimicrobial activity against *E. durans, S. sonnei, L. monocytogenes, E. coli* and *C. albicans* and (3) are safe for consumption as they are reported to be effective against microbial diseases with little or no toxicity.

1.5. Significance of the study

The study aided in broadening knowledge on the chemical composition of *H. abyssinica* from Namibia and shed some light on the chemical compounds that may be responsible for therapeutic properties of the plant extracts. Furthermore, this study was the first to document the *in vitro* safety of the Namibian based *H. abyssinica* extracts but more studies are needed to confirm the findings. The findings from this study increased our scientific knowledge on bioactivity of *H. abyssinica* parasitizing *A. nigrences*. Studying the Namibian based *H. abyssinica* also contributed in the on-going search for new antimicrobial novel leads since there is a global problem of antimicrobial resistance.

CHAPTER 2: LITERATURE REVIEW

2.1. Plant natural products

Natural products are an important source of drugs (19); they are defined as chemical compounds found in nature which usually have a pharmacological or biological activity that can be of therapeutic benefit in treating diseases (20). Considering their eminent profile of chemical diversity and novel mechanism of action, natural products still play a pivotal role in many drug development and research programs. In 1805, morphine from Opium poppy was discovered as the first pharmacologically active compound (21). Subsequently, a number of active compounds were isolated from natural products. Plants display different compounds from one another with different biological activities (22).

More than 80 % of drug molecules are natural products or natural product derivatives (23). The diversity of plants result in a variety of secondary metabolites with diverse chemical structures which play a vital role in drug discovery and development process (24). Following the fortuitous discovery of penicillin from the filamentous fungus *Penicillum* by Fleming in 1929 and the observation of its broad therapeutic effects in 1940s, drug discovery from nature was encouraged (12). Between 1970 and 1980s, divine techniques such as combinatorial chemistry shifted the focus of drug discovery from nature to *in vitro* laboratory works (25). Many well-known drugs (artemisinin, chloroquine and other antimalarial drugs) listed in modern pharmacopoeia were discovered based on their traditional uses (21). Traditional medical practitioners use different plant parts such as seeds, leaves and roots to cure diseases.

Chemical compounds found in a plant may have varied therapeutic roles such as antioxidant, antimicrobial, neuromodulators, cytotoxicity (26). During the 1990s, the use of plant extracts started gaining popularity (27), indicating the importance of plants as

natural sources of drug products. Examples of medicines that highly benefited from natural products are anticancer, antimigraine and antihypertensive medicines (21).

2.2. Plant description and geographical area

H. abyssinica is the strangest subterranean root parasitic plant (due to its extreme reduction in morphological features, including absence of leaves) previously known as *H. johannis* (Beccari) and *H. solmsiana* (Dinter) (12). The plant is predominantly found in the dry to semi-arid parts of Namibia, across most of Angola and DRC, Ethiopia, Sudan, South Africa and Arabian Peninsula (1). Although *H. abyssinica* is the most widely distributed species in Africa (28), its distribution is very scattered (29). It is entirely dependent on its host for minerals, organic materials and water since it lacks chlorophyll. *H. abyssinica* is rarely collected due to its underground nature of the rhizome and seasonal emergence of the flowers (30). *H. abyssinica,* which previously was classified in the family *Hydnoraceae* and currently placed in *Aristochiaceae* family (31) comprises of two genera; *Hydnora* and *Prosopanche* (14). *Prosopanche* with two species is found in Central and South America while on the other hand, *Hydnora* with five species is mainly found in Africa with few records from Arabian peninsula(14,32). *H. esculanta* is restricted to Madagascar, *H. africana* and *H. abyssinica* are fairly common in Namibia (33).

Hydnora species mainly grow on the roots of *Euphorbia* and *Acacia* species (34). Specifically, *H. abyssinica*, *H. esculanta and H. sinandevu* which are the most widespread species of *Hydnora*, parasitize host plants from *Burseraceae* and *Fabaceae* families while *H. africana*, *H. triceps and H.viseri* parasitize host plants from the genus *Euphorbia.* The plant body of *H. abyssinica* is a root-like rhizome with severely reduced

vegetative features, a dark brown periderm and fleshly pink/red interior (12). Figure 1 below shows *H. abyssinica* rhizomes and its host plant *A. nigrences* during sampling at Eros-Windhoek, Namibia.

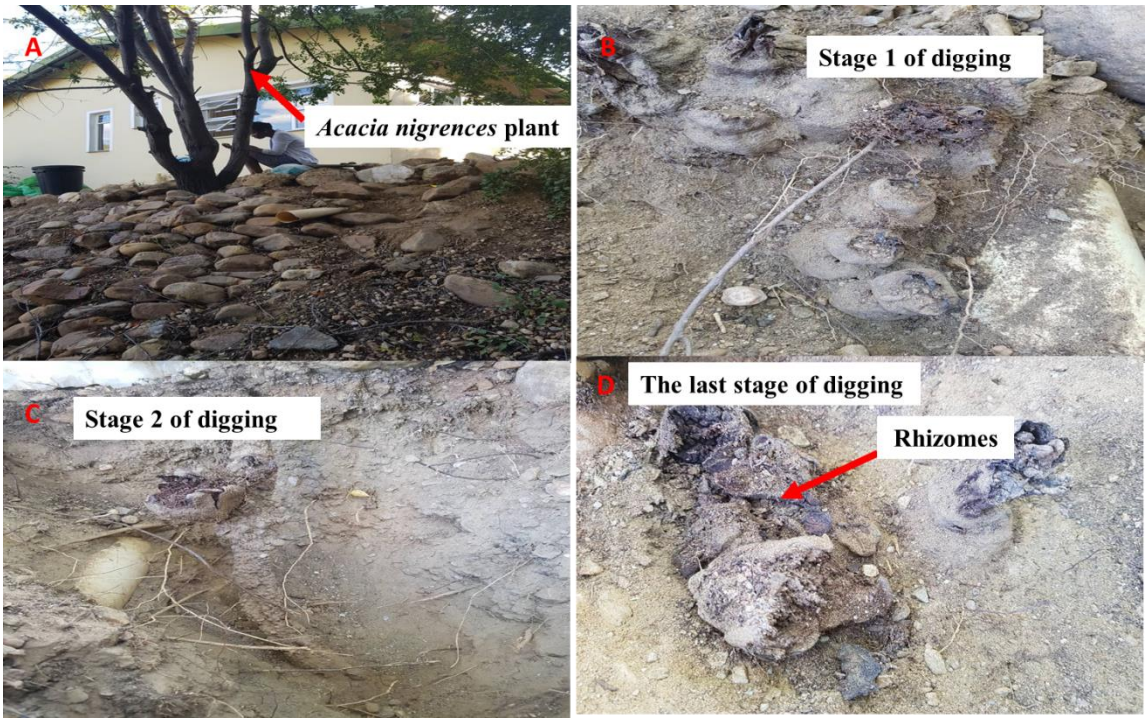

Figure 1: *H. abyssinica's* rhizomes from *A.nigrences* host plant

2.3. Host selection of parasitic plants and the influence of the host plants on parasitic plant chemistry

Parasitic plants make their living by mining out resources such as carbon from another plant called a host plant (35). Interestingly, parasitic plants select their hosts based on the host plant traits and characteristics. Host plants with best traits such as chemical resistance and tolerance to infections to other parasites and antagonists are the most important factors for selection by parasitic plants (35). Many approaches are used by parasitic plants to locate the best hosts. For example, Striga root parasites produce a massive number of small seeds which germinate only after a signal by host-derived

chemical (36). The tiny Orobanche seeds, germinate after a specific root host stimulus (37).

The intimate relationship between the host plant and parasitic plant may influence the chemistry of both the parasitic plant and host plant. For example, one study showed that parasitic plants acquire some or all of their nutrients from their hosts (38). In another study, various secondary metabolites such as defence secondary metabolites are mostly acquired by the parasitic plants from the host (16,39).

The chemistry of the genus *Hydnora* is microscopic (1). Specifically, information about host-parasite relationship of *Hydnora* species is very scanty due to no apparent transpiration of the parasite (29). In one study, six volatile compounds were isolated from *H. abyssinica,* parasitizing *Acacia* species (40), while 31 volatile compounds were reported to be isolated from *H. africana*, parasitizing *Euphorbia species* (40). The difference in the number of isolates obtained might be attributed to the different *Hydnora* species studied, the host plant species that are parasitized or to the geographical origin. To the best of our knowledge, most of the studies on *H. abyssinica* do not report which host species are parasitized but it is evident that other studies on other parasitic plants report that host chemistry can influence its parasite (16,41) . This presents an area of novelty for further scientific investigation.

2.4. Traditional uses of *H. abyssinica*

Scientific records on medicinal uses of *H. abyssinica* are available for different countries but not for Namibia. In Sudan, for example, the rhizome is normally boiled and the infusion is drunk as a cure for diarrhoea, cholera, swelling tonsillitis and as an astringent in dysentery (34). In Eastern Ethiopia, the plant is used for the treatment of

haemorrhage, diarrhoea, wound and mouth infections (40). On the other hand, Kenyan traditional medical practitioners use the root decoction as a cure for throat complaints, stomach ache and removal of placenta during child birth when it does not come out on time (10). The plant is also used for medicinal purposes in Namibia (personal communication, Prof Erika Maass), but scientific records are lacking.

2.5. Previous studies done on *H. abyssinica*

Most studies focused on botany and systematics of the family *Aristochiaceae* and only few focused on the biological activities. A phytochemical study done in Kenya showed that the plant is rich in tannins and phenols (12). Catechin, tyrosol, benzoic acid and tetra-decanoic acid were isolated through Bioassay-guided phytochemical investigation of *H. abyssinica* (12). The biological activity of *H. abyssinica* studied previously in South Yemen was directed on the antimicrobial and antischistosomic properties (40). Antimicrobial activity of *H. abyssinica;* with aqueous, methanolic and chloroform extracts showed ability to inhibit growth of some human pathogenic bacteria and fungi (*Staphylococcus aureus*, *Staphylococcus epididermis*, *E. coli*, *Bacillus pumilus*, *Saccharomyces cerevisiae* and *Candida albicans*), with aqueous extracts being the most inhibitory and chloroform extracts being the least effective (12).

In another study done in Sudan (42), the aqueous extracts showed prominent bioactivity against the fungal isolate of the pathogen *Trichophyton rubrum*. The plant's rhizome toxicity was also investigated against selected human mouth epidermoid carcinoma cell line (KB) and non-cancer human fetal lung cell line (MRC-5) (1). Little cytotoxicity was reported against the cell line used. In a similar study, *H. abyssinica* ethanolic extracts at

the given concentrations of 50, 100, 200 and 400 mg/kg/day were reported to have toxic effect on Wistar rats (18).

2.6. Phytochemicals from plants

Phytochemicals are defined as chemical compounds produced by plants (43). They are divided into two categories namely primary and secondary metabolites. Primary metabolites are defined as metabolites required for the growth and maintenance of cellular functions and are common to all organisms (44). Amino acids, lipids and nucleic acids are examples of primary metabolites. On the other hand, secondary metabolites are defined as the end products of primary metabolism produced by the plant in response to physical and chemical stress (44).

Unlike primary metabolites, secondary metabolites are not directly involved in the plant growth and development. In addition, many of the secondary metabolites are toxic or deterrent to different species such as humans (45), thus classified as chemical defences that repel, poison and kill other species or predators (46). These phytochemicals protect plant cells from environmental hazards such as pollution, stress, drought, ultraviolet (UV) exposure and pathogenic attacks and are responsible for the medicinal properties of a plant. Phenolic, nitrogen-containing substances and terpenes are major secondary metabolites (47). Phytochemicals originate in different parts of the plant (48). However, this thesis only focused on the phytochemicals that originate in the rhizome part of the plant.

2.6.1 Phenolic compounds

Phenolic compounds are ubiquitous chemically heterogeneous compounds in the plant kingdom with a hydroxyl functional group on an aromatic ring (49). Some phenolic compounds are only soluble in organic solvents, some are water soluble while others are insoluble polymers (49,50). The essential groups of phenolic compounds include phenols, tannins, simple phenol propanoids, benzoic acid derivatives, anthocyanin, isoflavones, lignin and flavonoids (50) with phenols being the smallest class (51). Solanum plants such as potatoes (*Solanum tuberosum*) are known to be rich in phenolic compounds (43).

2.6.2 Flavonoids

Flavonoids are a group of plant–derived heterocyclic organic compound which are widely studied compared to other phenolics due to their profound multiple bioactivities (47). They are derived from flavans (43). Flavonoids are divided into 14 different subgroups according to the chemical nature and positions of substituents on the three rings. They have been reported to possess many useful properties including antioxidant, antimicrobial, anti-inflammatory, antitumor and cytotoxicity activity (52). Flavonoids are for defence and floral pigmentation (50). Some flavonoids inhibit the growth of microbes that causes plant diseases (51).

2.6.3. Tannins

Tannins or tannoids are water- soluble polyphenolics of molecular weight between 500 and 3000 Da which are unevenly distributed in the plant kingdom (40,50,51). However, the proportion of these compounds differs in some plants. The biological action of plants

containing tannins has been well documented since millennia. A study has shown that, tannins precipitate proteins such as gelatine and alkaloids, forming their own aqueous solutions (55). The medicinal activities of tannins include anticancer, antioxidant and antimicrobials, as astringents, anti-inflammatory, and as antiseptics (56,57). Black berries, tea, red wine and apple are some few sources of tannins (50).

2.6.4. Alkaloids

Alkaloids are a large class of naturally occurring heterogeneous compounds containing basic nitrogen atom and an amino acid as a precursor in most alkaloids (51), with pronounced physiological actions on human and pharmacological activities. Thus, many of the common drugs are alkaloid based. They are found in approximately 20 % species of vascular plants (50). Alkaloids are not only produced by plants as their secondary metabolites, but they are also produced by other organisms such as bacteria, fungi and animals (51).

Plants such as opium poppy (*Papaver somniferum*) was the first plant from which the medically useful alkaloid morphine was derived in 1805 (50). Structural diversity of alkaloids is also one of their unique characteristics (51). Amines in alkaloids give them the basic form, thus differentiating them from other secondary metabolites. Tyrosine, which is an amino acid, is one of the precursors for alkaloids biosynthesis (51). The common alkaloid ring structures are pyridines, pyrroles, indoles, isoquinolines, piperidines and pyrrolidines. Nicotine and atropines are two examples of alkaloids (49).

2.6.5. Saponins

Saponins are terpene derivatives defined as high-molecular weight glycosides with detergent or surfactant-like properties (50). Triterpene and steroids are the two types of saponins which hydrolyse to form triterpenoidal and steroidal compounds (where the sugar is removed). Because of their detergent-like properties; lipophobic (triterpene or steroids) and lipophilic (a side chain of water-soluble carbohydrates), saponins form very stable foams (58). Because of their surfactant-like properties, saponins are used in the preparation of emulsions for photographic films, ore separation and in cosmetic products such as hair shampoo (58). According to literature, saponins are bitter, toxic and cause irritation to the mucous membrane, thus making them good chemical defences (43,50).

2.5.6. Glycosides

Glycosides are any of the naturally occurring organic compounds formed as a result of condensation products of sugar and yield a sugar and a glycon when hydrolysed (59).

2.6.7. Steroids

Steroids are synthetic or natural hormones that belong to the terpenoid class with a broad spectrum of therapeutic applications (43). Lupeol, betulinic acid and Cardiac glycoside are examples of steroids (43,59). Cardiac glycoside, one of the plant steroids are used as cardiac drugs and arrow poisons (43).

2.6.8 Terpenoids

Terpenoids, sometimes called isoprenoids are unsaturated hydrocarbons and, are the most widespread naturally occurring organic compounds of which over 3000 types of

plant terpenoids with various functions such as hormones, antibiotics, insect attractants and many more have been discussed (49). Mevalonic acid and isoprene units are the precursors of terpenoids (59).

The sources of terpenoids are resins and essential oils (43). Terpenoids are used as anti-inflammatory, anti-cancer, anti-malarial, antimicrobial and as inhibitors of cholesterol synthesis. Nitrogen-containing substances are used as anaesthetic agents (60), while phenolics exhibit antioxidant and anti-inflammatory properties allowing them to be used as potential chemopreventives (61).

2.7. Quantitative determination of phytochemicals

The discovery of medicinally important plants is based on their phytochemicals such as phenols, flavonoids, terpenes, alkaloids, tannins, glycosides, steroids and saponins which can be extracted from their leaves, roots, flowers, barks and countless other parts of the plants (62). These chemicals have extreme diverse pharmacological activities such as antimicrobial action, cytotoxicity, antioxidant, anti-inflammatory and many others depending on the compound (51). In order to extract, purify and identify phytochemicals, a quantitative assay needs to be carried out.

2.7.1 Total Phenolic Content (TPC)

Phenolics are compounds with phenolic groups-not hydroxyl. Depending on the number of phenolic groups, it can be mono-phenol, di-phenols tri-phenols or polyphenols. The Folin-ciocalteu (FC) reagent assay is one of the assays used to estimate the amount of phenolics in a certain sample. Folin-ciocalteu reagent is reduced by phenolic acid to

form a blue complex between molybdenum and tungsten present in FC which can be measured through spectrophotometry (63).

2.7.2. Total Flavonoid Content (TFC)

Flavonoids are polyphenols that form part of phenolic compounds which are assessed by spectrophotometric assay based on aluminium chloride reagent using quercetin as a standard reagent (61). Aluminium chloride reagent is the typical reagent used in this assay, which forms acid stable complexes with the keto carbon of either flavones or flavonols.

2.7.3. Total Tannin Content (TTC)

Tannins are water-soluble phenolic compounds mostly found in the bark of the trees. Folin Denis reagent assay is one of the assays used to estimate the amount of tannins in plant extracts. Folin Denis-reagent is reduced to a blue complex of tungsten and molybdenum of oxides by tannins in alkaline solution (64).

2.8. Natural products as antimicrobials

Microbial infections are still a major threat worldwide (65). Moreover, the rapid increase in multidrug resistant bacteria has urged the search for novel antimicrobials to fight bacterial resistance and assessing the effectiveness of natural plant products against chemical antimicrobial agents (65,66). The effectiveness and success of fighting bacterial resistance depend partly on a better understanding of chemically synthesized drugs that originate from natural sources. Roughly 25 % of all the reported natural products show biological activities, out of those, 10 % are microbial isolates of which 45 % are actinomycetes, 38 % fungi and 17 % bacteria (67).

Antimicrobial drugs are defined as drugs that interfere with the growth of a wide spectrum of microbes such as bacteria, mould, fungi and viruses by killing or inhibiting the growth of such microbes. They are classified as pharmacotherapeutic agents among other medicinal classes (68). The antimicrobial drug can be bacteriostatic or bactericidal. Bacteriostatic means the drug only stops the growth of the microbes and bactericidal means the drug kills the microbes (69). In 1910, the first antimicrobial agent, salvarsan for syphilis remedies was synthesized by Ehrlich. Subsequently, more antimicrobial agents such as sulphonamides and streptomycin were reported in the market in the next decade. These discoveries propelled the golden age of antimicrobial chemotherapies (70).

2.8.1 Microorganisms investigated in the study

Most microorganisms were selected based on their prevalence, availability and serious health effects they have on people. More details are provided below for each specific microorganism.

2.8.1.1. *Candida albicans*

Candida albicans is the most common causative agent of highly rated fungal associated diseases with more than 90 % cases reported (71). *Candida albicans,* an opportunistic human fungal pathogen was first isolated from a sputum of a tuberculosis patient (72,73). It is generally known as normal beneficial flora which exists on the human skin, ears, eyes etc. However, an overgrowth of the microbe triggers common fungal infection called candidiasis (72).

Candida species are found in impaired salivary gland, diabetic people, immunosuppressive conditions, people living with leukaemia and nutritional deficiencies (72). Symptoms of candidiasis are rarely seen but there are a few of the symptoms discovered which include swallowing difficulties and painful sensation (74). In literature (12,40), yeast isolates including *C. albicans* are reported to be the most susceptible microorganisms to *H. abyssinica*'s extracts among others, indicating that *H. abyssinica* has antifungal properties.

2.8.1.2. *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, fast growing, facultative, anaerobic coccobacilli bacteria widely distributed in the environment such as water, sewage and soil. *Listeria monocytogene* bacterium causes listeriosis infection (75). Listeriosis, a serious food borne disease results in serious illnesses such as severe gastrointestinal upset (75). The bacterium is most common in new-borns, immune compromised people and pregnant women (76,77).

In comparison with other outbreaks, WHO ranked South Africa's previous outbreak with the highest impact in the world following the outbreak in the United States of America (USA) (78). Since *L. monocytogenes* is one of the bacterial species associated with diarrheic syndromes (1), it was also assayed with *H. abyssinica* rhizome extracts. Moderate antibacterial activities of *H. abyssinica* were reported against *L. monocytogenes* (1).

2.8.1.3. *Escherichia coli*

Escherichia coli is a gram - negative bacterium which is naturally found in the human gut. It is known to be the most common causative agent of severe diarrhoea (79). *Escherichia coli*, which basically adapts well to environmental stresses such as air quality, is a good indicator for faecal contamination in water (80). The World Health Organisation reported that an average estimate of 88 % of diarrhoeal diseases are said to have been caused by poor sanitation and hygiene, and water contamination (79).

Since *H. abyssinica* is traditionally used as a cure for gastrointestinal ailments and due to the rise in antibiotic resistance (81), *E. coli* was among the bacteria of interest used in the study. Previous antibacterial studies of *H. abyssinica* (1,12,40,42) indicate that the plant showed no activity against *E. coli.*

2.8.1.4. *Enterococcus durans*

Enterococcus durans is a motile gram-positive coccus associated with intestine infections (82). Enterococcus infections are difficult to treat with antibiotics due to their acquired resistance to antibiotics, thus are a threat to human health (83). It is reported that diarrhoea associated with *E. durans* is actually connected to the mechanical interference with digestion and absorption at the brush border of alimentary canals but not with enterotoxin production or mucosal injury (82). *Enterococcus durans* is reported to be the cause of enteritis in piglets (82). Enterococcus species were part of the bacterial strains in antimicrobial studies of *H. abyssinica* reported, however, *E. durans* is reported for the first time in this study.

2.8.1.5. *Shigella sonnei*

Shigella sonnei is a gram-negative, facultative anaerobic, rod shaped, non-spore forming bacterium which is the main causative agent of human large intestine infection called bacillary dysentery (84). Bacillary dysentery commonly known as Shigellosis is a universal health concern across the world characterized by vomiting, watery and bloody diarrhoea, fever, abdominal pain and intestinal cramps (85). The bacterium *S. sonnei* is implicated with the most mortality and morbidity linked to bacillary dysentery, thus labelled the most life threatening diarrhoeal bacteria (86).

Part of the world, particularly in Asia, an estimate of 125 million infections and 14000 deaths due to shigellosis are recorded annually (86). *Shigella sonnei* is mostly transmitted through eating contaminated food and water and from person to person (87). In addition, to the best of our knowledge, no antimicrobial studies testing *H. abyssinica* against *S.sonnei* have been reported. Due to the seriousness of the bacteria on the enteritis infection, *S. sonnei* was one of the bacteria of interest of the current study.

CHAPTER 3: MATERIALS AND METHODS

3.1. Research design

This study investigated the presence/absence of major groups of phytochemicals, antimicrobial activity and cytotoxicity of methanol, water and methanol-dichloromethane extracts of rhizomes of *H. abyssinica* from *A. nigrences* host. The Figure below shows the flow diagram of the research design and methods used.

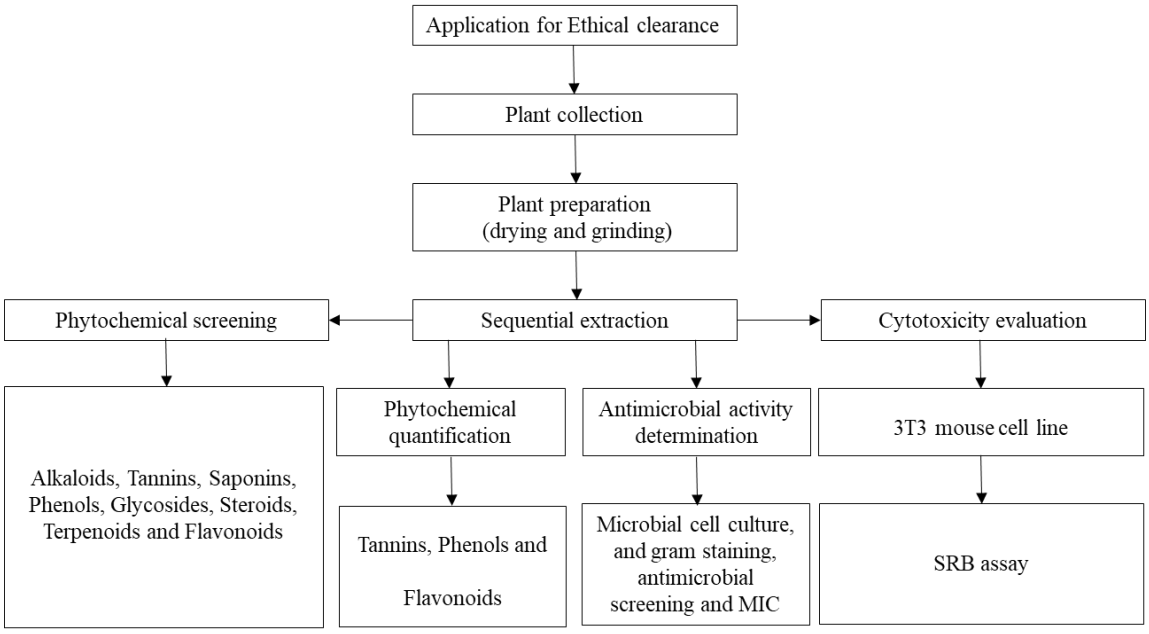

Figure 2: Flow diagram of research design used in the current study

3.2. Research ethical Approval

To be granted the Research ethical approval document, the proposal was submitted to the University Research Ethics Committee (UREC) of the University of Namibia postgraduate committee for evaluation since some of the lab assays such as cytotoxicity and antimicrobial assays conducted during this study needed ethical approval.

3.3. Plant specimen Collection and preparation

The plant rhizomes of *H. abyssinica* were collected from Windhoek, Khomas Region, Namibia on the 16th of May 2018. No collection permit was obtained as the plant sample was collected from a residential area with the permission of the owner. The fresh rhizomes were washed, cut into small pieces and oven dried at 37 ℃ for a week. The dried rhizomes were grounded into powder using a blender and stored at room temperature in a dry place until further analysis (12).

3.4. Preparation of crude extracts

Multiple methods described previously (2,4,65) to prepare extracts were used with slight modifications. In short, the powdered plant specimen was subjected to sequential extraction. Exactly 20 g of the powder was steeped in 200 mL of n-hexane using a 200 mL volumetric flask. The flask was left on the orbital shaker set at 100 rpm and at room temperature for 6 hours. Thereafter, the macerate was filtered by means of Buchner filtration system using Whatman filter paper no 1. The filtrate was discarded as n-hexane was used as a cleaning agent. The plant residues were further steeped in 200 mL of methanol solvent and subjected to shaking for 24 hours to allow efficient extraction.

After extraction, the macerate was further filtered. The filtrate was concentrated using rotary evaporator set at 40 ℃ and transferred to pre-weighed vials to permit drying at room temperature. After drying, the mass of the dried extracts were determined and the crude extracts were stored at room temperature until usage. The process was repeated for the methanol-dichloromethane (1:1v/v) mixture solvent. The water extract did not dry at room temperature; therefore it was subjected to freeze drying for 24 hours. The

percentage extract yield was then calculated using the formula (% yield = (mass of the dried extract/mass used for extraction) × 100).

3.5. Phytochemical analysis

3.5.1 Qualitative Phytochemical screening

Qualitative chemical (colorimetric) tests were carried out on the crude extracts to identify selected secondary constituents present in the plant extracts. Characteristic colour change of the extracts or filtrates or the development of foam signified the type of secondary constituent present (65). The presence/absence of a compound was mainly based on the colour/precipitate formed, however, the colour intensity was used to measure the magnitude of the compound present.

Terpenoids, steroids, alkaloids, phenols, saponins, glycosides, flavonoids and tannins were detected using standard methods (4,88) as shown in Table 1 below.

Table 1: Detection for phytochemical constituents of *H. abyssinica* rhizome methanol, methanol-dichloromethane and water extracts

Phytochemical test	**Detection**
Test for Alkaloids Mayor's test	10 mg of extracts was dissolved in 2 mL of 1 % hydrochloric acid (HCL) in a test tube and heated gently. Mayer's reagent was added thereafter. Yellowish precipitate supported the preliminary evidence for the presence of alkaloids.
Test for Tannins	5 % ferric chloride (0.5 mL) was added to 5 mg of extract. A blue black colour indicated a positive test.
Test for Saponins Foam test	0.5 mg of extracts was dissolved in 2 mL distilled water and the mixture was shaken vigorously. The formation of stable foam indicated the presence of saponins.
Test for Phenols	5 mg of extracts was dissolved in 0.5 mL of 20 % sulphuric acid and then a few drops of aqueous sodium hydroxide solution were added. A blue colour indicated the positive test for phenols.
Test for Steroids	0.5 mg of extracts was mixed with 2 mL chloroform and concentrated sulphuric acid was added sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids.
Test for Terpenoids	10 mg of extracts was dissolved in 2 mL chloroform and evaporated to dryness. 2 mL of concentrated sulphuric acid was added and heated for 2 minutes. A greyish colour indicated the presence of terpenoids.
Test for Flavonoids	10 mg of extracts was mixed with 2 fragments of magnesium ribbons and 2 drops of concentrated hydrochloric acid were added drop wise. The appearance of a scarlet colour after few minutes indicated the presence of flavonoids.
Test for Glycosides	0.5 mg of extracts was dissolved in 1 mL of distilled water. 1M sodium hydroxide was then added. A yellow colour which appeared after few minutes indicated the presence of glycosides.

3.5.2. Quantitative phytochemical analysis

The quantified phytochemical (TPC, TFC and TTC) were experimentally calculated using standard calibration curves achieved from different concentrations of standards (gallic, quercetin and tannic acid) respectively.

3.5.2.1. Analysis of Total Phenol Content (TPC)

Total phenolic content (TPC) of different extracts was determined using Folin-ciocalteu method with some modifications (5,89). Briefly, four millilitres of potassium acetate and 5 mL of 10 % folin- ciocalteu solution were added to 1 mL of plant extracts (prepared by dissolving 1 mg of extracts in 1 mL of sterile distilled water) and to the standard solutions of gallic acid (250, 125, 62.5, 31.25 and 15.63 µg/mL) prepared in sterile distilled water. The resulting mixture was incubated for 30 minutes at room temperature after which the absorbance was read at 765 nm using a micro plate reader. A blank was prepared using sterile distilled water. TPC was determined from extrapolation of a calibration curve. The TPC was expressed as milligram (mg) of Gallic acid (GAE)/ g of the extracts. The experiment was carried out in triplicates.

3.5.2.2. Analysis of Total Flavonoid Content (TFC)

Aluminium chloride method was used with some modifications to determine Total Flavonoid Content (1,61). Briefly, one millilitre of plant extracts that was prepared by dissolving 1 mg of extracts in 1mL of sterile distilled water and the respective standard solutions of quercetin (1000, 500, 250, 125 and 62.5 µg/ml) prepared in sterile distilled water, were mixed with 3 mL methanol, 0.2 mL of 10 % aluminium chloride ($AlCl_3$), 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water.

The mixture was then incubated at room temperature for 30 minutes. The absorbance was measured at 420 nm using a micro plate reader. The blank was prepared using sterile distilled water. Quercetin standard curve of absorbance vs. concentration was constructed using Microsoft Excel. The total flavonoid content was expressed as

milligrams (mg) of quercetin equivalents (QE)/ g of the extracts. The experiment was done in triplicates.

3.5.2.3. Analysis of Total Tannin Content (TTC)

Total tannin content was determined using the Folin-Denis method with slight modifications (90). One millilitre plant extracts prepared by dissolving 1 mg of extracts in 1 mL of sterile distilled water and standard solutions of tannic acid (250, 125, 62.5, 31.25 and 15.63 µg/mL) prepared in distilled water were made up to 7.5 mL with distilled water. Zero point five millilitres of Folin-Denis reagent and 1 mL of 7 % sodium carbonate solution were added simultaneously. Volumes were made up to 10 mL with sterile distilled water. After 30 minutes of incubation at room temperature, absorbance was measured at 700 nm using a spectromax M2 micro plate reader. Tannic acid standard curve of absorbance vs. concentration was constructed using Microsoft Excel. Total tannin content was expressed as milligrams of TAE/g of the extracts. The experiment was done in triplicates.

3.6. Antimicrobial Activity

3.6.1. Cell culture and Gram staining

The microbial strains (*E. durans* ATCC 6056, *L. monocytogenes* ATCC 13932, *E. coli* ATCC 700928, *S. Sonnei* ATCC 25931 and *C. albicans* ATCC 32032) were obtained from the Department of Chemistry and Biochemistry and Department of Biological Science, University of Namibia (UNAM). They were first cultured in both nutrient broth and Mueller Hinton agar to obtain pure cultures. Purity of the isolates was checked

throughout the study through the examination of colony morphology using the light microscope and gram staining procedures (1).

3.6.2. **Antimicrobial susceptibility test**

Antimicrobial screening was performed using both agar disc and well (also known as agar cup plate) diffusion methods (4,47) to determine whether the extracts were active against the test microorganisms at a given extract concentration. Antimicrobial activity of extracts was evaluated against *S. sonnei, E. durans, C. albicans, L. monocytogenes* and *E. coli* (65). Assays were conducted using aseptic techniques.

Firstly, the culture suspensions were adjusted to 0.5 McFarland turbidity using a spectrophotometer (Spectramax M2) to compare activity between the different microorganisms from the study (91,92). A test organism was uniformly inoculated on the solidified Mueller Hinton agar (93) with a spreader.

Thereafter, sterile paper discs of 6 mm in diameter were soaked in plant extracts solutions of different concentrations (50, 25, 12.5, 6.25 and 3.125 mg/mL) and then placed aseptically on the inoculated plate. The plates were allowed to dry for 1 hour at 4 ℃and incubated for 24 hours at 37℃. For the well diffusion method, holes of 6 mm in diameter were made in the seeded agar using a sterile cork-borer. Fifty microlitre aliquots of plant extracts at concentrations of (50, 25, 12.5, 6.25 and 3.125 mg/mL) were placed into the wells using a multichannel pipette. The plates were incubated at 4 ℃and taken to the fridge for 2 hours to allow the extracts to diffuse in agar. After 2 hours of incubation, plates were transferred to the incubator set at 37 ℃for 24 hours.

The antimicrobial activity was based on clear zone formed around the paper disc. Complete inhibition was indicated by a clear zone, while partial inhibition was indicated

by a semi-clear zone. Nystatin (50 mg/mL) and Ampicillin (50 mg/mL) which were in a powder form, were used as positive controls while sterile distilled water was used as negative control. The experiments were performed in triplicates. Zone of inhibition data were all normalised by subtracting 6 mm of the disc paper.

3.6.3. Minimum Inhibitory Concentration (MIC)

Foremost, the MIC of the microorganisms whose growth was suppressed (*C. albicans, E. durans and L. monocytogenes*) was determined using two previously established microdilution methods with minor modifications(1,4,65). In the first method, serial dilutions of the extracts were performed in centrifuge tubes with sterile nutrient broth (50, 25, 12.5, 6.25 and 3.125 mg/mL). Fifty microlitre aliquots of the overnight inoculum adjusted to 0.5 McFarland turbidity standard (93) were transferred into the tubes. Two extra tubes were used as controls.

The tube containing nutrient broth with inoculum was used as a positive control while the tube containing nutrient broth with extracts was used as a negative control. The suspensions were incubated for 24 hours at 37℃. In the second method, nutrient broth (50 µL/wells) was distributed into the wells of a microplate. Fifty microlitre aliquots of extracts were added to the first row of wells and a two-fold dilution was performed into other wells to make up 3.125-50 mg/mL concentrations of each extracts. Fifty microliter of the same overnight inoculum used in the first method was introduced per well and another 100 µL of nutrient broth was introduced into each of the inoculated wells to prepare a total volume of 200 µL/well. The growth inhibition was assessed by observing the absorbance, i.e. the absence of turbidity in the wells. Absorbance was measured at

630 nm before incubation, after 6 hours post incubation as well as after 24 hours post incubation at 37℃.

3.7. Cytotoxicity assay

The cytotoxicity assay was carried out following standard procedures (1,94,95). Briefly, the 3T3 embryonic fibroblast mouse cell line (ATCC-CL-173) was first cultured in Dulbecco's Modified Eagle Medium (DMEM). Cells were then exposed to treatment (*H. abyssinica* rhizome extracts) and further exposed to Sulforhodamine B (SRB).

3.7.1 Cell culture

The mouse (3T3) cell line were obtained from the Biomedical Research laboratory, Department of Biological Sciences, University of Namibia and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 1 mM Sodium pyruvate (Invitrogen 11995-065), 1500 mg/mL Sodium bicarbonate, 10 % (v/v) fetal bovine serum (FBS) (heat inactivated) and 1 % Penicillin-Streptomycin and kept under a humidified 5 % CO_2 atmosphere at 37 ℃ (1).

Cell cultures were maintained and sub-cultured based on the confluent of the cells determined using the light inverted microscope for further propagation of cell line. Fresh culture medium was added to cells every 3 days or as required by the growth rate of the cells to maintain the cell growth. When the cells reached approximately 80 % confluent, the cells were sub cultured or passaged. The cells were then split using a split ratio determined by the confluence of the cells.

The growth medium was carefully aspirated from the cells and the cells were washed with 10 mL of PBS. The PBS was aspirated and the cells were trypsinized by adding 2

mL of trypsin-PBS solution (1:10 v/v) to detach them from the bottom of the flask. The flask was placed in a CO_2 humidified incubator set at 37℃ for 5 minutes for further detachment of the cells. Subsequently, 8 mL of fresh medium was added to the flask with cells to inactivate trypsin and appropriate volume of medium was added to the new flask. The flask was placed in a CO_2 humidified incubator set at 37℃.

3.7.2 Sulforhodamine B (SRB) assay

3.7.2.1 Cell preparation/ seeding of cells

The media was removed from cell monolayers and cells were washed with 10 mL of 1×10 PBS. Three millilitre of (0.25 % w/v) trypsin was added evenly and the flask was incubated for 5 minutes in a CO_2 humidified incubator set at 37 ℃ (95). After incubation, 2 mL of media was added to the flask and the cell suspension was mixed thoroughly with the pipette. The cells concentration was then determined by counting the cells in the haemocytometer chamber under the microscope using a 1:1 mixture of cell suspension and 0.4% trypan blue solution which stains the live cells.

After counting, the cells were seeded on a 96-well microtitre plate at a density of 10^5 cells/well. The cells were treated with sterile extracts/compounds dissolved in DMSO after 24 hours of incubation.

3.7.2.2 Treatment solution preparation

The extracts were removed from the freezer (-20℃) for analysis. Upon removal, the extracts were allowed to thaw at room temperature. Stock solutions of the extracts and those of controls were prepared. Ten milligrams of each extract was dissolved in 1 mL (100 µL of DMSO + 900 µL media) which is equivalent to 0.1 % DMSO. For the

controls, 10 % v/v DMSO was prepared by adding 100 µL of DMSO to 900 µL media and 10 % v/v Triton-x, a detergent known to kill cells (96) was prepared by adding 100 µL of Triton-x to 900 µL media.

The stock solutions were sterile filtered by using a 0.45 µm filter. Working solutions were then prepared from the stock solutions. One milligram of the extracts was prepared by pipetting 100 µL from the stocks into a new Eppendorf tube containing 900 µL of media. One percent v/v DMSO was prepared by pipetting 100 µL from the stock solution into a new Eppendorf tube containing 900 µL of media.

3.7.2.3 Treatment Exposure

Upon completion of the treatment preparation, 100 µL of growth media was added to all the wells in a 96-well plate except the wells with controls. Ninety microliter of media was added to the wells with controls. An extra 80 µL of media was added to the wells with the highest concentration. Twenty microliter (20 µL) from the working solutions of the treatments was added to the wells containing 180 µL of media.

The treatments were then serially diluted (100, 50, 25, 12.5, 6.25, 3.125 µL) by pipetting 100 µL from each well upon mixing. In the last wells (wells with the lowest concentrations), 100 µL of media was discarded off. For the controls, 10 µL of the controls were added to the wells with 90 µL of media making DMSO and Triton-x concentration in the wells to be 0.1 % and 1 % respectively. The wells with no treatment were labelled as negative control and the wells with no treatment and cells were labelled blanks.

The plate containing the cells was then removed from the incubator and medium was removed by inverting the plate and blotted on a tissue paper. The treatment solutions were gently transferred into the plate with cells by using a multichannel pipette. The plate was then incubated for 72 hours in a humidified CO_2 incubator at 37℃.

3.7.2.4 Cell viability assay using Sulforhodamine B (SRB) dye

After 72 hours of exposure, cells were fixed at the bottom of the plate and stained with cold Trichloroacetic acid (TCA) and SRB respectively. Twenty-five microliter of cold 50 % (w/v) TCA was added to each well directly to the media supernatant and incubated for 2 hours at 4℃. Upon incubation, the TCA was washed off four times by submerging the plate in a tub with slow-running tap of 1 % acetic acid. The plate was blotted on a tissue paper and further dried at room temperature for 24 hours. After drying, 100 µL of (SRB) was pipetted into each well and incubated for 30 minutes in a humidified CO_2 incubator at 37℃.

After incubation, the SRB dye was washed off four times with 1% acetic acid and allowed to dry for 24 hours at room temperature. One hundred microliter of 10 mM Tris Base at pH 10.5 was added upon drying to extract protein bound sulforhodamine dye. The plate was placed on an orbital shaker set at room temperature, 90 rpm for 10 minutes. After shaking, the absorbance was measured at 540 nm in a microplate reader and the results were expressed as percentage cell growth using the formula: [(OD540 treated/ OD540 control) ×100]. The experiment was carried out in quadruplicates and repeated twice.

3.8. Statistical analysis

Statistical analysis of phytochemical, antimicrobial activity and cytotoxicity evaluation was performed using statistical program for social sciences (SPSS). In phytochemical analysis, SPSS software was used to determine whether there were significant differences (at 95 % confidence level) for the phytochemical compounds quantified by each solvent. In antimicrobial activity, SPSS software was used to determine whether there were significant differences in the mean diameter of inhibition zones at various concentration and in the susceptibility of microbial strains by the extracts (methanol, methanol: dichloromethane and water) whereas, for cytotoxicity, SPSS was used to determine whether there were significant differences in the percentage cell viability of the extracts.

Foremost, the Shapiro-Wilk normality test was performed for phytochemical analysis, antimicrobial activity and cytotoxicity evaluation data to determine if data were normally distributed. Consequently, non-parametric tests (Kruskal Wallis) were run for the different phytochemicals, antimicrobial activity and cytotoxicity since all the data were not normally distributed. Lastly, post-hoc tests were used for multiple comparisons to find out where the differences lie among the group that tested significantly different. Significance difference was established at $p < 0.05$. Phytochemical and antimicrobial analyses were performed in triplicates whereas cytotoxicity assay was done in quadruplicates. All the experiments were repeated twice and the results were expressed as mean ± standard deviation.

CHAPTER 4: RESULTS

4.1 Plant extraction yield

Table 2 below shows the percentage extracted yield for each crude extract (methanol-dichloromethane, methanol and water). MeOH: DCM extracts had the highest yield (18%) compared to H_2O and MeOH extracts with yields of 3 % and 6% respectively.

Table 2: *H. abyssinica* rhizome crude extracts percentage extraction yields

Crude extracts	**% extraction yield (w/w)**
MeOH-DCM	18 %
MeOH	6 %
H_2O	3 %

4.2. Phytochemical screening

Qualitative screening revealed the presence of alkaloids, tannins, phenols and flavonoids in the intermediate polar extracts (MeOH: DCM) while the polar extracts (MeOH and H_2O), contained alkaloids, tannins, saponins, phenols, steroids and flavonoids (Table 3). This findings are comparable with other similar studies (10,12,40) . Tannins and phenols were found to be present in all the three crude extracts (MeOH: DCM, MeOH and H_2O), with tannins highly present (+++) in all the three crude extracts.

Table 3: Phytochemical constituents of *H. abyssinica* rhizome extracts

	Crude extracts		
Phytochemical constituents	**MeOH: DCM**	**MeOH**	**H_2O**
Alkaloids	+++	++	-
Tannins	+++	+++	+++
Saponins	-	++	+
Phenols	++	+++	++
Glycosides	-	-	-
Steroids	-	+++	-
Tepernoids	-	-	-

Flavonoids	+++	+++	-

(-) = absent, (+) = present, (++) = moderately present, (+++) = highly present

Negative sign (-), indicates the absence of the reference colour/precipitate; a positive sign (+), indicates that the precipitate/colour was formed; a double positive sign (++), indicates that the colour was moderately present and a triple positive sign (+++) indicates that the colour was highly present.

4.3. Quantitative phytochemical determination

Phytochemical quantitative determination of *H. abyssinica* rhizome extracts is summarized in Table 4. The total flavonoids content (TFC), total tannins content (TTC) and total phenols content (TPC) were expressed as quercetin, tannic acid and gallic acid equivalents respectively.

Specifically, there were significant differences between TPC and TFC ($P < 0.005$), TPC and TTC ($p< 0.05$) and TFC and TTC ($p< 0.005$) respectively. Significant differences between solvents across the three different categories of compounds ($p< 0.05$) were also observed. Post-hoc test showed that there were significant differences between MeOH and H_2O extracts for the different compounds ($p< 0.05$). Specifically, MeOH extracts contained the highest TTC and TPC of 0.44 ± 0.01 mg TAE/g extracts and 0.31± 0.04 mg GAE/g extracts respectively (Table 4) compared to H_2O extracts. On the other hand, MeOH- DCM extracts exhibited the highest TFC of 0.12 ± 0.01 mg QE/g extracts. Furthermore, H_2O extracts did not contain any detectable amounts of TFC, however, it contained more TTC and TPC (0.21 ± 0.01 mg TAE/g extracts and 0.13 ± 0.01 mg GAE/g extracts respectively) compared to the intermediate polar extracts MeOH-DCM (0.14 ± 0.00 mg TAE/g extracts and 0.10 ± 0.01 mg GAE/g extracts respectively).

Table 4: Summary of total flavonoid, phenolic and tannin content in *H. abyssinica* rhizome extracts.

Crude extracts	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	TTC (mg TAE/g extract)
MeOH-DCM	0.10 ± 0.01	0.12 ± 0.01	0.14 ± 0.00
MeOH	0.31 ± 0.05	0.02 ± 0.01	0.44 ± 0.01
H_2O	0.13 ± 0.01	-	0.21 ± 0.01

TFC, total flavonoid content; TPC, total phenolic content; TTC, total tannin content; -, very small to be quantified, GAE, gallic equivalent; QE, quercetin equivalent; TAE, tannin equivalent.

4.4. Antimicrobial activity

4.4.1 Antimicrobial susceptibility test

Clear zones of inhibition were seen in all the extracts. Variation in the zones of inhibition among the extract solvents and positive control were observed. Most of the clear zones were less in diameter compared to positive controls and bigger in diameter compared to the negative control (Table 5 to Table 10).

4.4.1.1 Methanol (MeOH) extracts

Antimicrobial activities of *H. abyssinica*'s methanol extracts against the selected microbial pathogens are summarized below in Table 5 (agar disc diffusion) as well as Table 6 (agar well diffusion). When using agar diffusion methods for assessing antimicrobial activity, the rule of thumb is to consider a diameter of more than 6 mm for the extract to be active against microorganisms (42) and a diameter of more than 15 mm is considered as a high antimicrobial activity (11). These diameters (6 mm and 15 mm) are inclusive of the disc diameter of 6 mm. MeOH extract was highly active (12 mm) than MeOH: DCM and H_2O extracts. The maximum mean zone of inhibition of 12 mm was recorded in agar well diffusion method for *C. albicans*. Even in the lowest extract

concentration used (3.125 mg/mL), the inhibition zones of *C. albicans* compared to other microbes at the same concentration ranged from 1 mm to 3 mm.

It was clear that the gram negative bacteria (*E.coli* and *S. sonnei*) had the lowest average zone of inhibition recorded ranging from 0 mm to 4 mm while the gram positive bacteria (*E. durans* and *L. monocytogenes*) had the mean zone diameter ranging from 0 mm to 6 mm toward the extracts.

Moreover, results also showed that the antimicrobial activity of MeOH extracts increased at higher concentrations. Specifically, at 50 mg/mL extract concentration, a mean zone of diameter of 12 mm was recorded while at 3.125 mg/mL extract concentration, a mean zone of diameter of 0 mm was recorded. Despite this observation, there was no significant difference in diameter (pvalue > 0.05) observed in the activity of the extracts against the microbes (Table 5, Appendix K). However, significant differences in diameter were observed (pvalue < 0.05) across the concentrations used, even after dropping the outliers (Appendix K). The significant differences were found to be between the least concentration used (3.125 mg/mL) with all the other concentrations (50, 25, 12.5 and 6.25 mg/mL) with p values < 0.05 (refer to figures in Appendix K).

On the other hand for the agar well diffusion method (Table 6), apart from the significance differences observed in the concentrations, the extracts behaved differently in terms of the microbes. A significance difference (p value < 0.05) was recorded in the susceptibility of the microbes towards the extracts. The difference was found to be between *C. albicans* and all the other remaining microbes (*E. durans, E.coli, S. sonnei* and *L .monocytogenes*) (*p* values <0.05) and between (3.125 mg/mL and 25 mg/mL), (3.125 mg/mL and 50 mg/mL), (6.25 mg/mL and 25 mg/mL) with p values < 0.05. It is

worth pointing out that agar disc diffusion method (Table 5) findings are showing that the MeOH extracts were able to suppress the growth of all the microbes somehow even though the suppression was not significantly different (p value > 0.05) from H_2O and MeOH-DCM extracts.

All microbes showed significant differences between the highest mean zone diameter and the positive control and between lowest mean zone diameter and negative control respectively except for *C. albicans*. Nystatin (50 mg/mL), which was the positive control for *C. albicans*, had the lowest mean zone of diameter of 1 mm, which is comparable to the mean zone of diameter exhibited by the lowest extracts concentration (3.125 mg/mL) against *C. albicans*. This finding is in contrast with a recent report using the same standard as the positive control (12). Nystatin exhibited good antifungal properties with mean zone of inhibition recorded as 18.15 mm. A significance difference (p value < 0.05) in the antimicrobial activity of the extracts (MeOH, MeOH-DCM and H_2O) and that of the controls (ampicillin and nystatin) were recorded.

Table 5: Mean zone of inhibition (± standard deviation) of MeOH extract of rhizome of *H. abyssinica* collected from *A. nigrences* host using agar disc diffusion method

	Concentration (mg/mL)							
SMP	**50**	**25**	**12.5**	**6.25**	**3.125**	**Ampicillin (50 mg/mL)**	**Nystatin (50 mg/mL)**	**DdH_2 O**
E.c	4.0 ±1.0	3.0 ± 1.4	3.0 ± 0.6	2.0 ± 1.2	0	13 ± 2.6	NT	0
S.s	4.0 ± 2.6	4.0 ± 2.8	3.0 ± 1.0	3.0 ± 1.8	0	13 ± 3.2	NT	0
E.d	5.0 ± 1.5	3.0 ± 2.1	4.0 ± 1.9	3.0 ± 1.0	0	22 ± 8.4	NT	0
C.a	7.0 ± 3.8	4.0 ± 2.4	4.0 ± 1.6	3.0 ± 1.0	1.0 ± 1.0	NT	1.0 ± 2.0	0
L.m	6.0 ± 3.0	4.0 ± 2.0	3.0 ± 1.4	2.0 ± 1.5	0	23 ± 6.1	NT	0

SMP, selected microbial pathogens; *E. c, E. coli; S. s, S. sonnei; E. d, E. durans; C. a, C. albicans; L. m, L. monocytogenes*; NT, not treated; DdH_2O, Double distilled water; ZOI, Zone of inhibition; ZOI > 0 mm, Plant extracts active; ZOI > 6 mm, Plant extracts moderately active, ZOI > 9 mm, Plant extracts highly active. Diameter of inhibition zone excludes the disc diameter of 6 mm.

Table 6: Mean zone of inhibition (± standard deviation) of MeOH extracts of rhizome of *H. abyssinica* collected from *Acacia nigrences* host using agar well diffusion method

	Concentration (mg/mL)							
SMP	**50**	**25**	**12.5**	**6.25**	**3.125**	**Ampicillin (50 mg/mL)**	**Nystatin (50 mg/mL)**	**DdH_2O**
E.c	0.0 ±0.6	0	0	0	0	34 ± 0.0	NT	0
S.s	0	0	0	0	0	30 ± 4.6	NT	0
E.d	6.0 ± 5.0	4.0 ± 4.0	2.0 ± 2.1	0	0	0	NT	0
C.a	12 ± 1.0	10 ± 2.5	7.0 ± 2.4	5.0 ± 1.5	3.0 ± 2.1	NT	0.0 ± 0.6	0
L.m	5.0 ± 3.4	2.0 ± 1.7	0 ± 0.6	2.0 ± 2.9	0	38 ± 4.9	NT	0

SMP, selected microbial pathogens; *E. c, E. coli; S. s, S. sonnei; E. d, E. durans; C. a, C. albicans; L. m, L. monocytogenes*; NT, not treated; DdH_2O, Double distilled water; ZOI, Zone of inhibition; ZOI > 0 mm, Plant extracts active; ZOI > 6 mm, Plant extracts moderately active, ZOI > 9 mm, Plant extracts highly active. Diameter of inhibition zone excludes the disc diameter of 6 mm.

4.4.1.2: Methanol- dichloromethane (MeOH-DCM) extracts

Results on antimicrobial activity of MeOH-DCM extracts of *H. abyssinica* rhizome are presented in Table 7 (agar disc diffusion) as well as in Table 8 (agar well diffusion) below. Similar to methanol extracts, *C. albicans* had the highest average zone of inhibition of 10 mm. For both agar disc and well diffusion methods, gram positive bacteria (*E. durans and L. monocytogenes*) mean zone of inhibitions ranged between 0 mm to 6 mm while gram negative bacteria (*E. coli* and *S. sonnei*) had the lowest average zone of inhibition ranged between 0 mm to 3 mm. The highest zone of inhibition (10 mm) was recorded in agar well diffusion method.

The minimum mean zone of inhibition recorded for *C. albicans* was 1 mm for both agar disc and well diffusion methods. Although, it appears that the extracts had suppressed the growth of *C. albicans* more than it did for the other microbes (Table 7) for the agar disc diffusion method, this was not statistically different. Similar to the MeOH extracts,

the results show that MeOH-DCM extracts inhibition power was increasing with increasing concentrations (*P* value < 0.05).

Again, it is worth pointing out that agar disc diffusion method (Table 7) suppressed the growth of all the microbes compared to the agar well diffusion method (Table 8). Even though a mean inhibition zone of up to 37 mm was observed for the susceptibility of the microbes against the positive control (ampicillin), it was not statistically different from the extract inhibitions.

Table 7: Mean zone of inhibition (± standard deviation) of MeOH-DCM extracts of rhizome of *H. abyssinica* collected from *A. nigrences* host using agar disc diffusion method

	Concentration (mg/mL)							
SMP	**50**	**25**	**12.5**	**6.25**	**3.125**	**Ampicillin (50 mg/mL)**	**Nystatin (50 mg/mL)**	**DdH_2O**
E.c	3.0 ± 2.5	3.0 ± 2.6	3.0 ± 0.3	2.0 ± 1.5	0	13 ± 1.2	NT	0
S.s	3.0 ± 1.4	2.0 ± 1.3	2.0 ± 1.5	3.0 ± 1.8	0	13 ± 3.2	NT	0
E.d	6.0 ± 2.8	4.0 ± 1.3	4.0 ± 1.8	3.0 ± 1.3	0	23 ± 5.4	NT	0
C.a	7.0 ± 0.6	5.0 ± 2.1	4.0 ± 1.3	3.0 ± 1.8	1.0 ± 0.8	NT	1.0 ± 1.5	0
L.m	5.0 ± 1.7	4.0 ± 2.0	4.0 ± 2.3	2.0 ± 2.0	0	23 ± 5.3	NT	0

SMP, selected microbial pathogens; *E. c, E.coli; S. s, S. sonnei; E. d, E. durans; C. a, C. albicans; L. m, L. monocytogenes*; NT, not treated; DdH_2O, Double distilled water; ZOI, Zone of inhibition; ZOI > 0 mm, Plant extracts active; ZOI > 6 mm, Plant extracts moderately active, ZOI > 9 mm, Plant extracts highly active. Diameter of inhibition zone excludes the disc diameter of 6 mm.

Table 8: Mean zone of inhibition (± standard deviation) of MeOH-DCM extracts of rhizome of *H. abyssinica* collected from *A. nigrences* host using agar well diffusion method

	Concentration (mg/mL)							
SMP	**50**	**25**	**12.5**	**6.25**	**3.125**	**Ampicillin (50 mg/mL)**	**Nystatin (50 mg/mL)**	**DdH_2O**
E.c	0.0 ± 0.6	0	0	0	0	32 ± 3.5	NT	0
S.s	0.0 ± 0.6	0	0	0	0	30 ± 4.6	NT	0
E.d	6.0 ± 3.1	2.0 ± 2.3	0.6 ± 0.6	0	0	0	NT	0
C.a	10 ± 1.0	9.0 ± 2.8	6.0 ± 3.3	4.0 ± 3.0	3.0 ± 2.1	NT	0.0 ± 0.4	0
L.m	4.0 ± 1.5	4.0 ± 1.5	1.0 ± 1.0	0	0	37 ± 2.0	NT	0

SMP, selected microbial pathogens; *E. c, E.coli; S. s, S.sonnei; E. d, E. durans; C. a, C. albicans; L .m, L. monocytogenes*; NT, not treated; DdH_2O, Double distilled water; ZOI, Zone of inhibition; ZOI > 0 mm, Plant extracts active; ZOI > 6 mm, Plant extracts moderately active, ZOI > 9 mm, Plant extracts highly active. Diameter of inhibition zone excludes the disc diameter of 6 mm.

4.4.1.2 Water (H_2O) extracts

Results revealed that the water extract of *H. abyssinica* rhizome was the least active extract towards all the microbes since the highest mean zone of inhibition recorded was not more than 6 mm as shown in Table 9 (agar disc diffusion) as well as Table 10 (agar well diffusion) below. A similar study of *Hydnora* (4), which employed agar well diffusion, water extracts were not active against the microbes. The yeast strain (*C. albicans*) and the gram positive bacteria (*E. durans* and *L. monocytogenes*) mean zone of inhibition were both in the range of 0 mm to 6 mm. As with MeOH and MeOH-DCM extracts, the gram negative bacteria (*E. coli* and *S. sonnei*) had the lowest mean zone of inhibition ranging from 0 mm to 4 mm but this was not statistically significant (*P* value > 0.05), for the agar disc diffusion.

There was no increasing trend in antimicrobial activity of water extracts with increasing concentration as expected. At 25 mg/mL extracts concentration, *L. monocytogenes* mean zone of diameter of 6 mm was recorded, which is more than the mean zone of diameter at 50 mg/mL (4 mm). At 6.25 extracts concentration, *C. albicans*, mean zone of diameter of 6 mm was recorded, which is more than the mean zone of diameter at 12.5 mg/mL (4 mm).

Apart from the variation in concentrations, significant differences (p value < 0.05) in the antimicrobial activity across the concentrations were recorded for both the agar diffusion methods. The difference was found to be between all the concentrations and 50 mg/mL for agar well diffusion method and between all the concentrations and 3.125 mg/mL.

Table 9: Mean zone of inhibition (± standard deviation) of H_2O extracts of rhizome of *H. abyssinica* collected from *A. nigrences* host using agar disc diffusion method

	Concentration (mg/mL)							
SMP	**50**	**25**	**12.5**	**6.25**	**3.125**	**Ampicillin (50 mg/mL)**	**Nystatin (50 mg/mL)**	**DdH_2O**
E.c	4.0 ± 1.5	3.0 ± 1.7	3.0 ± 1.0	3.0 ± 1.8	0	12 ± 2.1	NT	0
S.s	3.0 ± 1.4	3.0 ± 2.6	3.0 ± 0.8	2.0 ± 2.1	0	13 ± 0.2	NT	0
E.d	4.0 ± 1.8	3.0 ± 1.4	3.0 ± 1.8	2.0 ± 2.0	0	22 ± 1.1	NT	0
C.a	5.0 ± 2.5	5.0 ± 1.6	4.0 ± 0.9	7.0 ± 6.5	0.0 ± 0.6	NT	1.0 ± 1.7	0
L.m	4.0 ± 2.3	6.0 ± 4.0	3.0 ± 1.0	1.0 ± 1.0	0	23 ± 3.0	NT	0

SMP, selected microbial pathogens; *E. c, E.coli; S. s, S. sonnei; E. d, E. durans; C. a, C. albicans; L. m, L. monocytogenes*; NT, not treated; DdH_2O, Double distilled water; ; ZOI, Zone of inhibition; ZOI > 0 mm, Plant extracts active; ZOI > 6 mm, Plant extracts moderately active, ZOI > 9 mm, Plant extracts highly active. Diameter of inhibition zone excludes the disc diameter of 6 mm.

Table 10: Mean zone of inhibition (± standard deviation) of H_2O extracts of rhizome of *H. abyssinica* collected from *A. nigrences* host using agar well diffusion method

	Concentration (mg/mL)							
SMP	**50**	**25**	**12.5**	**6.25**	**3.125**	**Ampicillin (50 mg/mL)**	**Nystatin (50 mg/mL)**	**DdH_2O**
E.c	0	0	0	0	0	34 ± 0.0	NT	0
S.s	0	0	0	0	0	30 ± 4.6	NT	0
E.d	0.0 ± 0.6	0	0	0	0.0 ± 0.6	0	NT	0
C.a	6.0 ± 1.0	3.0 ± 2.1	2.0 ± 2.4	1.0 ± 1.2	1.0 ± 1.2	NT	1.0 ± 1.2	0
L.m	3.0 ± 0.0	1.0 ± 0.6	0	0	0	38 ± 3.0	NT	0

SMP, selected microbial pathogens; *E.c, E.coli; S.s, S. sonnei; E.d, E. durans; C.a, C. albicans; L.m, L. monocytogenes*; NT, not treated; DdH_2O, Double distilled water; ; ZOI, Zone of inhibition; ZOI > 0 mm, Plant extracts active; ZOI > 6 mm, Plant extracts moderately active, ZOI > 9 mm, Plant extracts highly active. Diameter of inhibition zone excludes the disc diameter of 6 mm.

4.4.2 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration recorded for *H. abyssinica* rhizome extracts in the present study against all assayed microorganisms ranged from 3.125 to 12.5 mg/mL. No MIC of *E. coli* and *S. sonnei* was determined for all the extracts because all the

extracts were not active against *E. coli* and *S. sonnei*. However, the MIC of methanol extract against *C. albicans* was 3.125 mg/mL. The minimum extract concentrations which inhibit microbial growth (MIC) are summarized in Table 11.

Table 11: Minimum inhibitory concentration of *H. abyssinica* rhizome extracts against susceptible microorganisms

	Crude extracts(mg/mL)		
SMP	**MeOH**	**MeOH-DCM**	**DdH_2O**
E. c	> 12.5	> 12.5	>12.5
S. s	> 12.5	> 12.5	>12.5
E. d	12.5	12.5	>12.5
C. a	3.125	6.25	12.5
L. m	12.5	>12.5	>12.5

SMP, selected microbial pathogens; *E. c, E.coli; S. s, S. sonnei; E.d, E. durans; C. a, C. albicans; L. m, L. monocytogenes*; DdH_2O, Double distilled water

4.5 Cytotoxicity Evaluation

The results of cytotoxic activity of *H. abyssinica* rhizome extracts against 3T3 embryonic fibroblast mouse cell line are summarized in Figure 3 below. The percentage cell viability was calculated using the formula: % cell viability = 100 × (OD540 treated/OD540 control).

The cytotoxic activity of all the extracts (MeOH, MeOH-DCM and H_2O) showed concentration dependent effect. Cell viability ranged from 83.1 % to 88.9 % for 100 µg/mL extracts concentration, while cell viability ranged from the range of 98.3 to 100 % at 3.125 µg/mL extracts concentration. In addition, there were significant differences between the different concentrations (P value < 0.05). The significant differences were found to be between 100 and 3,125 and between 50 and 3,125 µg/mL extract concentrations. Negative control and DMSO percentage cell viabilities were both recorded as 100 %. Positive control percentage viability was 8 %. Significance

differences between the positive control and MeOH extracts and between positive and negative controls were observed (P value < 0.05).

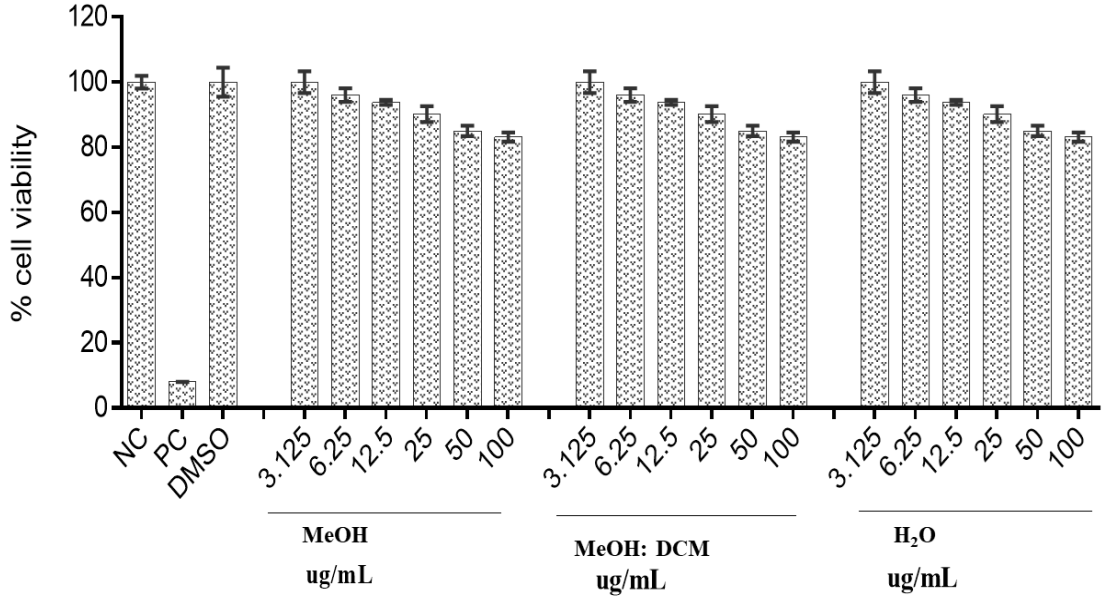

Figure 3: Cytotoxicity activity of *H. abyssinica* rhizome extracts on 3T3 embryonic fibroblast mouse cell line

CHAPTER 5: DISCUSSION

Plants are good sources of phytochemicals and have provided natural remedies to humans since time immemorial (97). Active ingredients from plant materials and their biological activities are intensely dependent on the solvent, treatment and extraction procedures used (4). The type of sample used, whether the sample is dried or fresh, depends on the compounds intended to be isolated. In this study, dried samples were used to prevent the degradation of compounds and thus enhance the preservation of the compounds in the samples.

A study that was carried out to compare the amount of phytochemical compounds extracted between fresh and dried samples revealed that there was no significant difference in the amount of phenolic contents quantified, however the significant difference was observed in the amount of flavonoid content quantified between the dried and fresh sample (98). The dried samples had more flavonoids compared to the fresh samples (98). This implies that dried samples are suitable to use in quantifying phytochemicals than fresh samples since the dried sample may have more phytochemicals.

A sequential extraction technique widely functional in estimating chemical association of trace elements in soils and sediments was employed (99). Sequential extraction also known as serial exhaustive extraction was used to ensure a wide polarity range of compounds could be extracted (100). We observed that the intermediate polarity solvent (MeOH-DCM) had more yield (18%) and the more polar solvent (H_2O) had the least yield (3%). These results are expected as MeOH-DCM is both polar and non-polar and would extract both polar and non-polar compounds compared to H_2O and MeOH extracts, thus a higher yield of MeOH-DCM extracts was not surprising. H_2O is the most

polar solvent and was the least extractor among other solvents (MeOH and MeOH-DCM), this could be due to the fact that most of the biological molecules are poorly soluble in water.

All the phytochemicals screened and revealed in this study are reported to contain health and medicinal benefits (101). In a recent study on *H. abyssinica*, glycosides and saponins were not detected in rhizome extracts using water, chloroform and methanol as solvents for to extract glycosides and saponins (42). In contrast, saponins were present in methanol and water extracts of the current study. In the present study, phenols and tannins were appreciably higher in the rhizome extracts of *H. abyssinica* compared to another similar study (11) which reported phenols, tannins and flavonoids as the main chemical constituents of the root of *H. abyssinica*. These results designate that there are some similarities and differences in chemical profiles of the same species from different geographic locations. This is also evident from one study (38) which indicated that there is a direct relationship in chemical composition between a host and a parasite.

Phenols, flavonoids and tannins were the only compound that were quantified because they have been extensively reported for their antimicrobial and cytotoxicity activities (102,103). Quantitative phytochemical results of the current study (Table 4) clearly indicated that MeOH and H_2O extracts contain significant amount of tannins and phenols while MeOH-DCM extracts contains significant amounts of flavonoids.

As discussed earlier, these results are not surprising as they reflect the extraction properties of the solvents used. These findings would suggest that the extraction value of phenols, flavonoids and tannins were prominently depended on the solvent polarity. The predominance of tannins and phenols in the current study correlates to some studies of

Hydnora species which used H_2O and MeOH solvents as these solvents were also used in the present study (1,104). A recent study of *H. abyssinica* rhizome extracts (H_2O, MeOH and $CHCL_3$) found out that phenols and tannins were the main constituents of *H. abyssinica* rhizome plant part while flavonoid was found to be in minute amount (42).

We found that *H. abyssinica* rhizome had high amount of tannins compared to phenols and flavonoids. Other studies of *H. abyssinica* rhizome have reported similar findings (4,42). Phenols, tannins and flavonoids revealed in the present study are immensely reported for their antimicrobial properties (4,42,105), hence it is likely that the presence of these biological compounds (phenols, flavonoids and tannins) in *H. abyssinica* rhizome extracts contribute to the antimicrobial activity and toxicity of the extracts. The low level of phytochemicals obtained in this study compared to most other studies done on the same plant might have been attributed to the geographical area, age of the rhizome, preparation of the sample and/or different host species.

The presence of high tannins in *H. abyssinica* crude extracts is of great significance since tannins have been extensively recorded for their pharmacological properties (4,106). Tannins have astringent and bitterness properties, which form part of their antidiarrheal activity and defence mechanisms against parasites (1). Additionally, tannins exert antimicrobial properties via hydrogen bonds or specific interactions with enzymes in microbial cells as a basis in the treatment of ulcerated organs. Furthermore, tannins also portray anticancer properties (4,107). In Oman and East Africa, tannins are used as tanning agents (40). Due to tannins astringency, it is well known that tannins possess many benefits including using as tanning agents and therapeutic benefits for the

treatment of diarrhoea and dysentery (11). Thus it makes sense why the Oman and East Africa people are seeing the benefits from the use of the plant *H. abyssinica*.

In line with previous studies on *H. abyssinica* (104,108), phenols were also present in this study. The higher level of total phenols compared to other phytochemical constituents analysed in this study could indicate antioxidant properties in the plant extracts (34). Phenols play a major role in inhibiting oxidation, neutralizing free radicals or decomposing peroxides. Due to antioxidant properties of phenols, this would justify the use of *H. abyssinica* to fight acne problems (1,34). Flavonoids are reported to be toxic against foreign cells (109), suppress the progression of cancer, alleviate coronary heart diseases and inhibits menopausal symptoms (4). In addition, flavonoids are also deemed to be responsible for the different colours of the parasitic plant (40). It was reported that *H. abyssinica* is known to treat cancer, menstrual cramps, inflammation and is immunosuppressive (1,7,11).

Antimicrobial activity of plants has been widely reported to be linked to secondary metabolites (4). To fight microbial resistance to antibiotic, a worldwide trending issue, scientists are keen to discover new antibiotics (110). The present study was undertaken to ascertain the *in vitro* antimicrobial effects of MeOH, H_2O and MeOH-DCM extracts of *H. abyssinica* rhizome on selected pathogens.

For the first time, agar disc and well diffusion methods were both employed to establish the antimicrobial activity of *H. abyssinica* rhizome extracts against selected microorganisms. The two methods approach was done for comparison purposes since antimicrobial studies done on *H. abyssinica*'s extracts had only used agar well diffusion method (12,42). Nystatin and ampicillin positive controls were chosen based on their

broad spectrum use as standard antibiotics (111). By using these positive control, it was observed that both organic (MeOH, MeOH-DCM) and aqueous (H_2O) solvents extracted antimicrobial chemical constituents even though *C. albicans* showed some resistance to nystatin (50 mg/mL).

From this study, it was clear that the organic (MeOH and MeOH-DCM) extracts exhibited moderate to high antimicrobial activity on the strains used compared to aqueous (H_2O) extracts. This observation is of more particular interest since the preparation of herbal remedies is done with water. This observation could be as a result of low solubility of biological compounds in water or that the preparation method used was done differently from traditional methods. These findings supports other studies 'findings which indicated that water extracts (aqueous) tend to not extract antibacterial compounds as much as organic compounds do (4).

The MeOH extracts possessed the highest antimicrobial activity when compared to the other extracts (zone of inhibition of 12 mm and MIC of 3125 µg/mL). MeOH had the highest TTC content (0.44 mg TAE/g extract). These findings suggest that tannins could be associated with the antimicrobial activity of *H. abyssinica* rhizome extracts. The results also revealed that MeOH and MeOH-DCM extracts unveiled concentration dependent activities (112). H_2O extracts had some variants in the concentrations contributing to it not unveiling concentration dependent activity. This could be due to improper mixing of solutions which can be improved by doing more efficient mixing methods in future.

Although, there was no significant difference in the susceptibility (unable to grow) of the microbes toward the extracts in the present study, *C. albicans* was more susceptible with

the highest inhibition zone recorded of 12 mm and MIC of 3.125 mg/mL. This is not surprising since another study on *H. abyssinica* reported similar findings on *C. albicans* of which zones diameter of inhibition of 9.41 mm of MeOH extracts and 15.11mm of $CHCl_3$ extracts were observed, using disc diffusion method (12). These results might be attributed to faster bacterial resistance compared to fungi, which lead to drug ineffectiveness (113). Tannins, which were found to be in abundance in the present study could also be the possible instrumental for the suppression of *C. albicans* growth (114). More so, studies have shown the magnificent activities possessed by tannins such as antidiarrheal and anticholera (1).

On the other hand, *E. coli and S. sonnei* were disparagingly less susceptible (mean zone of inhibition in the range of 0 - 4 mm) to the extracts compared to *E. durans* and *L. monocytogenes* (mean zone of inhibition in the range of 0- 6 mm). This is expected since both *S. sonnei* and *E. coli* are gram negative bacteria. Suppression of gram negative bacterial growth by plant extracts is quite infrequent (115) because gram negative bacteria contains a lipopolysaccharide layer which is impermeable to antimicrobial compounds, making the microbe less or not susceptible to the plant extract (4).

In a previous report on antibacterial activity of H_2O, MeOH and $CHCl_3$ extracts of *H. abyssinica* rhizome against *E. coli*, *Staphylococcus aureus*, *Bacillus sub-tilis*, and *Pseudomonas aeruginosa* (42), MeOH extract moderately active against *E. coli* with the mean zone of inhibition of 9 mm. In another report on antimicrobial activity of *H. abyssinica* rhizome's H_2O, MeOH and $CHCl_3$ extracts (12) against *C. albicans* and *S. cerevisiae*, MeOH extract was active against *C. albicans* with mean zone of inhibition of 9.41mm.

In this study, *H. abyssinica* rhizome extracts were less active toward *E. coli* with highest mean zone of 4 mm (12) and highly active toward *C. albicans* with highest mean zone of 12 mm . This variation in *H. abyssinica* rhizome extracts activity may result from different solvents used for the extraction. More so, variation could be due to different geographical regions or host species. On the other hand, gram positive bacteria *L. monocytogenes* and *E. durans* showed susceptibility to all the solvent extracts with the highest mean zone of inhibition of 6 mm and MIC of 12.5 mg/mL. This could be supported by the fact that gram positive bacteria lacks a lipopolysaccharide layer, making their outer membrane more permeable to the plant extracts (1).

Nystatin which was used as a positive control for the fungi (*C. albicans*) in the present study exhibit less to no antifungal activity (highest mean zone of inhibition of 1 mm) less than the microbial growth inhibitions of all the extracts against *C. albicans* (highest mean zone diameter of inhibition of 12 mm). In a more recent study of *H. abyssinica*, nystatin was used as a positive control and had the mean zone diameter of inhibition of 18.15 mm while that of MeOH extracts was at 9.41 mm. It is possible that *C. albicans* is exhibiting microbial resistance to nystatin (50 mg/mL), justifying our findings. However, further studies are needed to confirm these findings. Nystatin (50 mg/mL) has been used as an antibiotic to treat oral candidiasis since it is discovery in 1951 (116). Long term drug exposure would stimulate the production of resistance traits by the microbe (117).

Methanol, methanol-dichloromethane and water extracts showed little to non-toxic effect. The percentage (%) cell viability was in the range of 83.1 to 100 % for all the extracts. Cytotoxic effect of *H. abyssinica's* rhizome extracts showed concentration

dependent effects. This is expected as the highest concentration is likely to contain more active ingredients (112). Dimethyl Sulfoxide (DMSO) did not show any cytotoxic effect (100 % cell growth). This is also not surprising since the chemical is known to be a good diluent with no or little toxicity effect when used at high concentration (118).

We observed that MeOH-DCM extracts had little toxicity when compared to MeOH and H_2O extracts. The little cytotoxic activity of MeOH-DCM extracts could be due to high flavonoids and tannins in the extracts. Flavonoids are connected to toxicity since studies has assessed the potential role of flavonoids in cancer prevention (119). Tannins have high molecular weight. High molecular weight cannot be easily absorbed through the cell membrame, leading to it not reaching the target organ and thus causing toxicity (18). These findings are in line with similar study which reports that MeOH-DCM extracts showed little to no toxicity (11). However, the findings are not in line with another study(18), which revealed moderate toxicity effect of the root extracts of *H. abyssinica* on Wistar rats.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

The study was carried out to determine the phytochemical profile, antimicrobial properties and cytotoxicity of rhizome extracts from *H. abyssinica* parasitizing *A. nigrescens* host. Rhizome extracts of *H. abyssinica* had notable tannins and phenols, moderate to low antimicrobial activity and little to non-toxic effect. It can be suggested that tannins and phenolics are likely the key compounds in *H. abyssinica* that maybe responsible for the management of diseases.

Moderate to high antimicrobial activity demonstrated against *C. albicans*, advocates the use of *H. abyssinica* rhizome extracts as antifungal agents supporting their folklore uses. More so, the study supports the use of agar well diffusion method as it has showed more pronounced inhibition zones compared to agar disc diffusion. Notably, from this study, the use of *H. abyssinica* in the treatment of diseases such as dysentery, diarrhoea, inflammation is justified. From the results, it was evident that H_2O extract was not toxic, which was not surprising since the water extract yield was very low. Since water is the solvent used to prepare infusions in traditional settings, the continual use of water in ethnomedicine is recommended.

Furthermore, a bioassay fractionation, isolation, purification and structure elucidation of active secondary metabolites should be done. An intensive phytochemical analysis is required to closely determine all the bioactive compounds present in *H. abyssinica.* A wide range of solvents should be used to increase the yield of bioactive compounds.

Additionally, more microbial strains should be tested on *H. abyssinica* and further work on the plant's cytotoxicity must be done to establish the safety of this plant in ethnomedicine.

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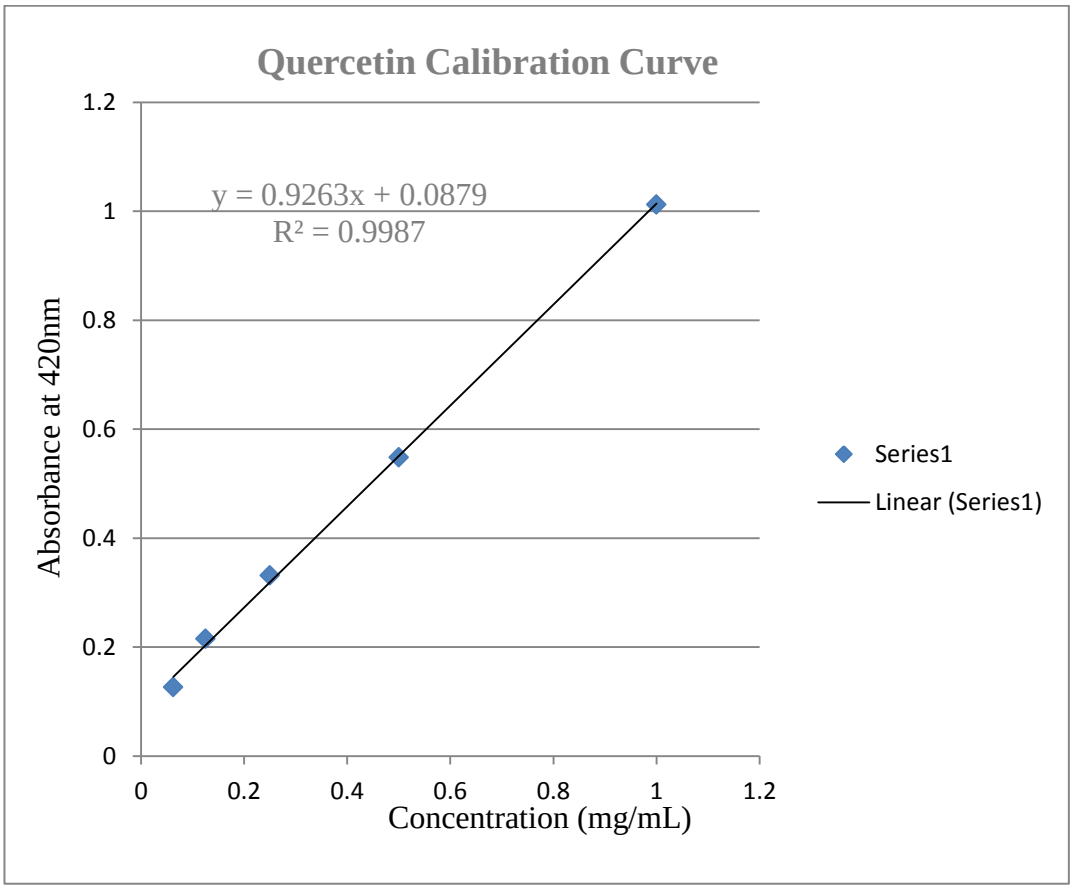
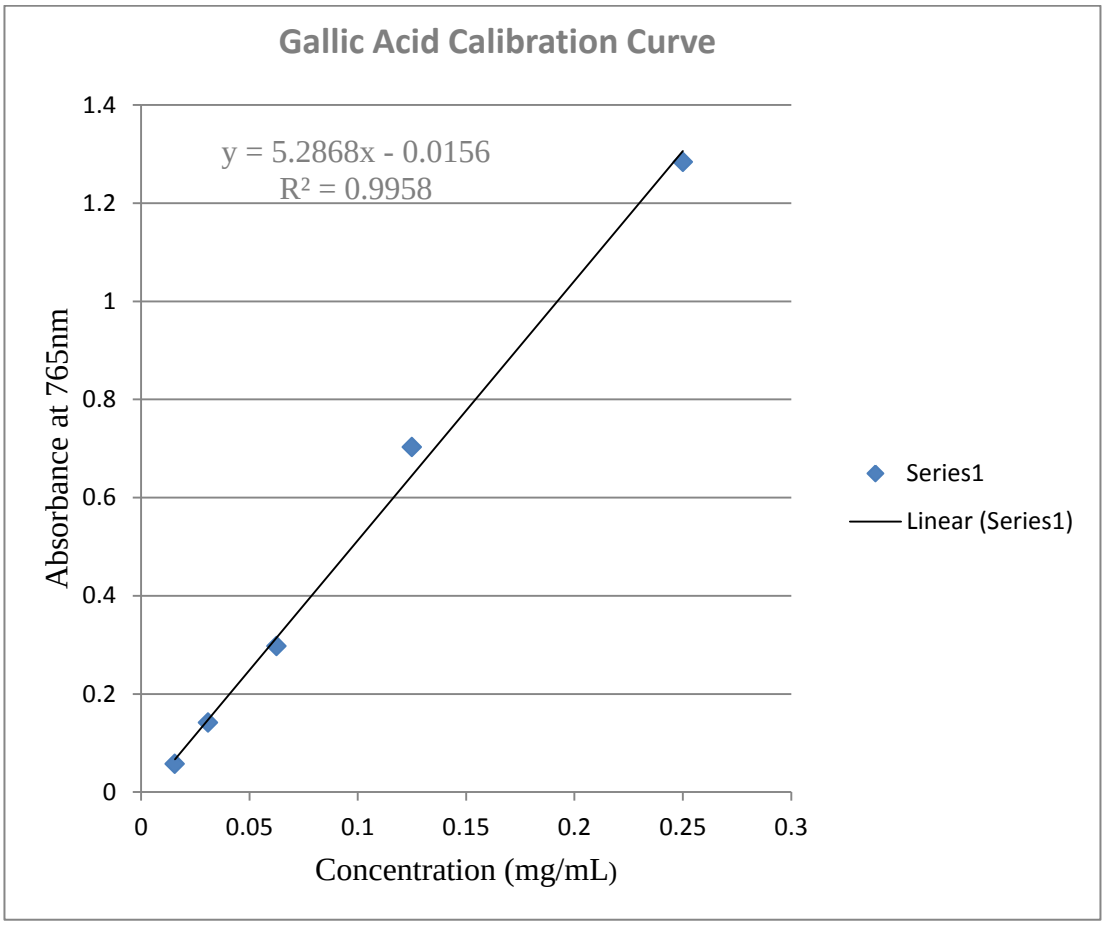
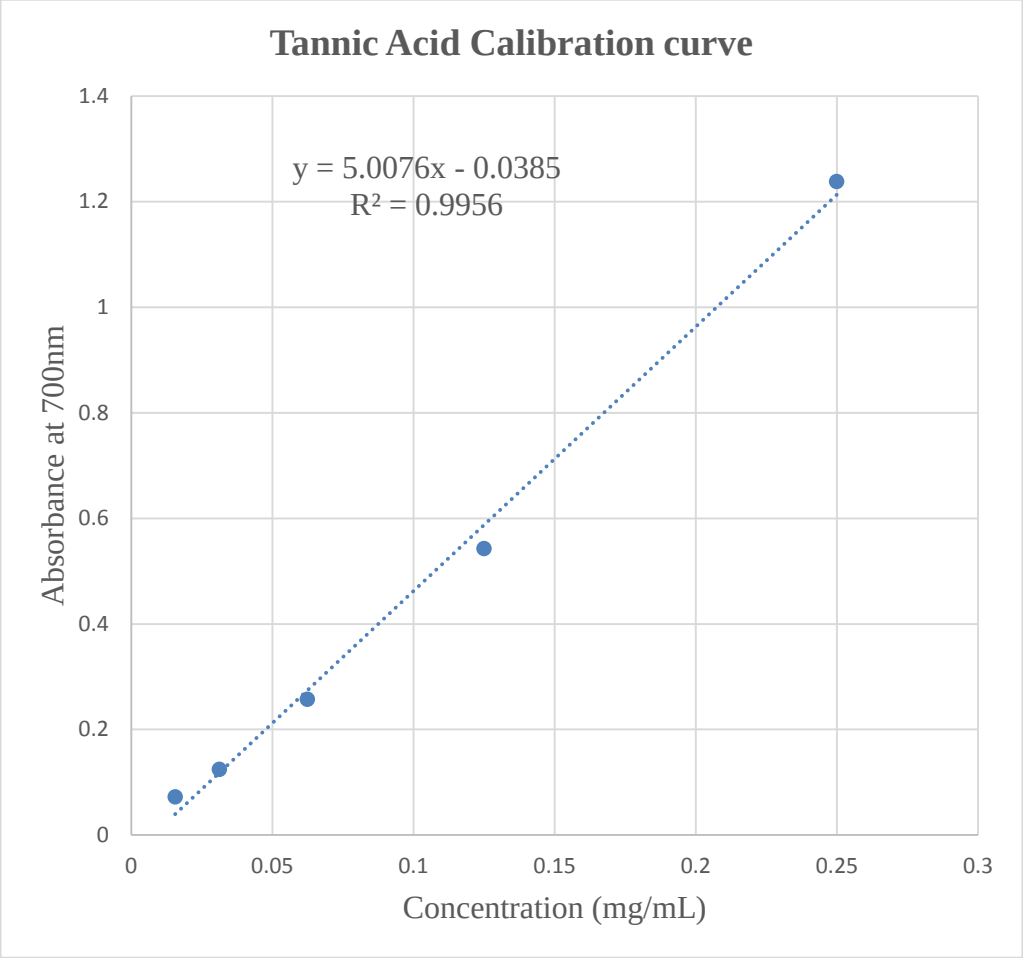
APPENDIX A: Quercetin Calibration Curve



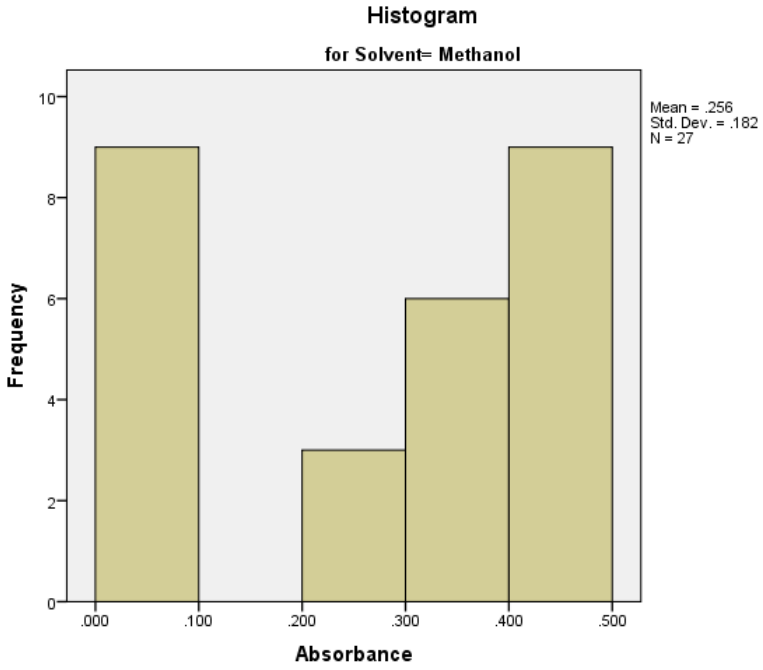
APPENDIX B: Gallic Acid Calibration Curve

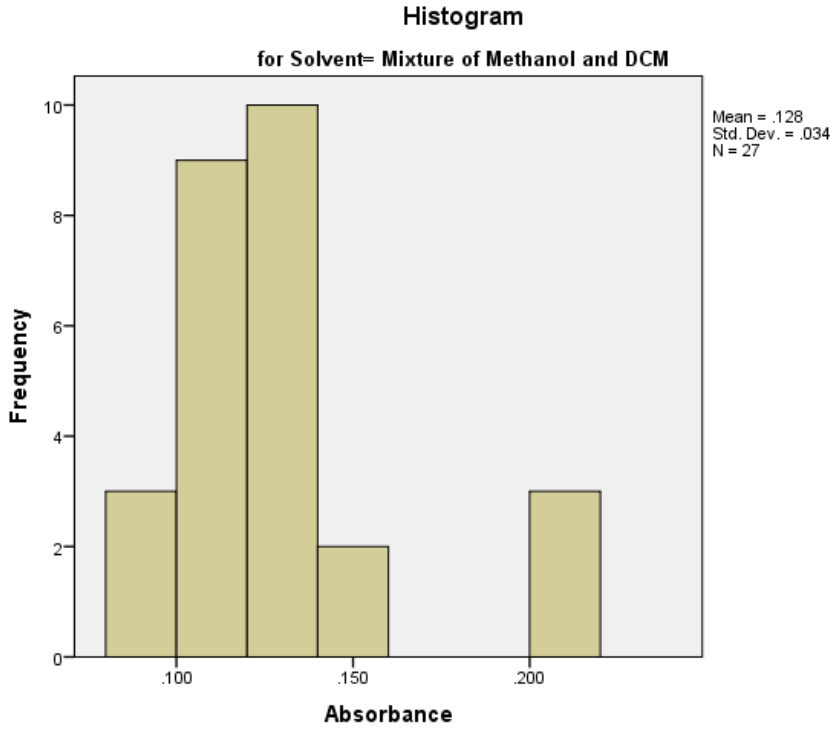


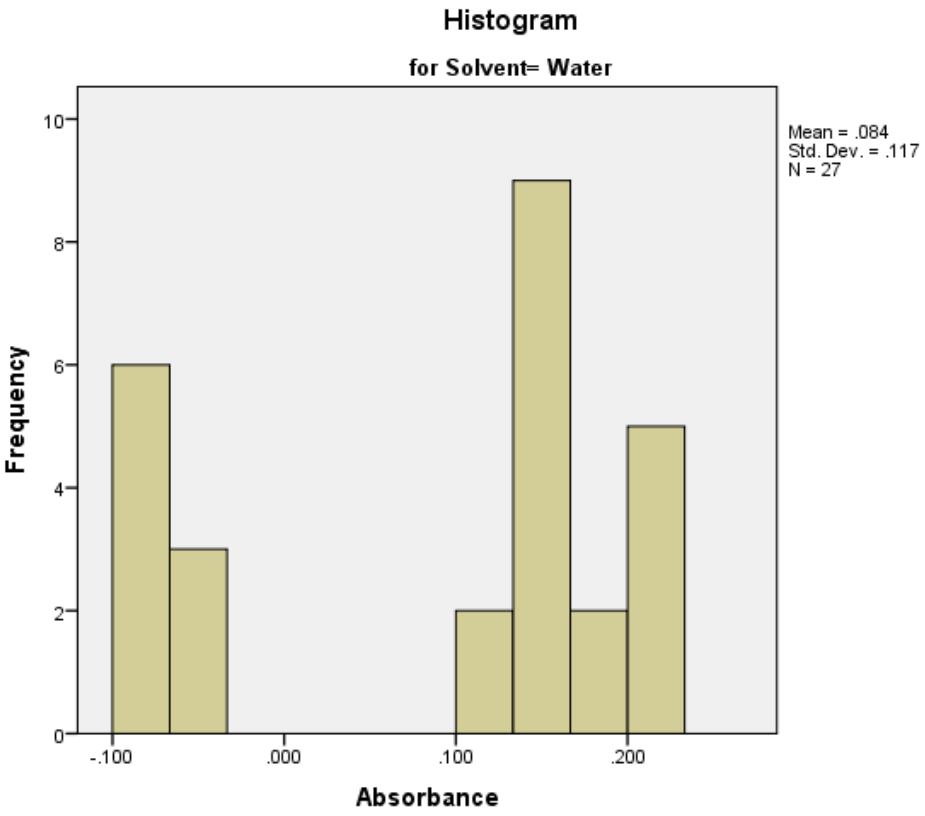
APPENDIX C: Tannic Acid Calibration Curve



APPENDIX D: Histograms showing distribution normality tests for MeOH, MeOH: DCM and H2O






Histogram
for Solvent= Water
Mean = .084
Std. Dev. = .117
N = 27
Frequency
Absorbance
0
2
4
6
8
10
-.100
.000
.100
.200

APPENDIX E: Statistical analyses on absorbance across different solvents

Non-Parametric Tests on absorbance across different solvents

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Absorbance	81	.15627	.145049	-.096	.456
Solvent	81	2.00	.822	1	3

Kruskal-Wallis Test

Ranks

	Solvent	N	Mean Rank
Absorbance	MeOH	27	53.00
	MeOH: DCM	27	35.48
	H_2O	27	34.52
	Total	81	

Test Statistics[a,b]

	Absorbance
Chi-Square	10.563
df	2
Asymp. Sig.	.005

a. Kruskal Wallis Test
b. Grouping Variable: Solvent

Post hoc tests: Multiple comparisons absorbance across different solvents

Mann-Whitney Test: MeOH vs MeOH: DCM

Ranks

	Solvent	N	Mean Rank	Sum of Ranks
Absorbance	MeOH	27	32.00	864.00
	MeOH: DCM	27	23.00	621.00
	Total	54		

Test Statistics[a]

	Absorbance
Mann-Whitney U	243.000
Wilcoxon W	621.000
Z	-2.102
Asymp. Sig. (2-tailed)	.036

a. Grouping Variable: Solvent

MeOH vs H_2O

Ranks

	Solvent	N	Mean Rank	Sum of Ranks
Absorbance	MeOH	27	35.00	945.00
	H_2O	27	20.00	540.00
	Total	54		

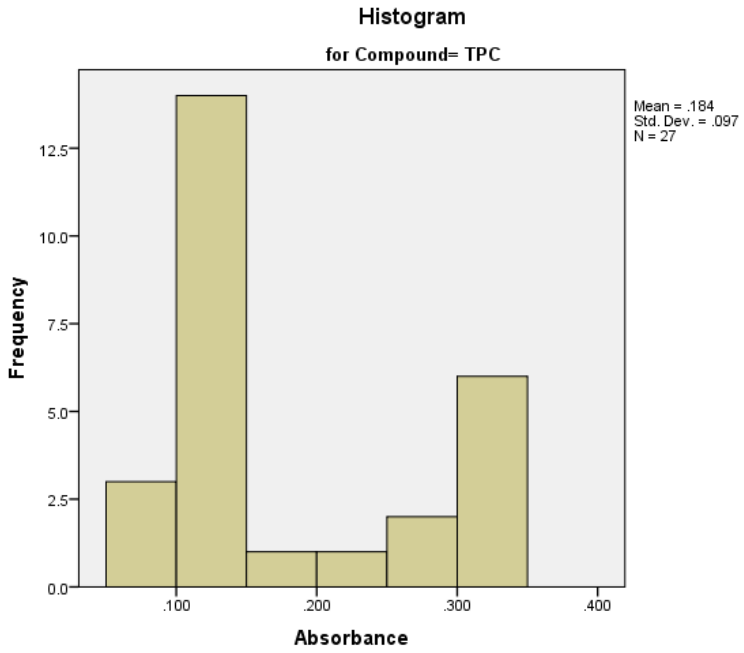
Test Statistics[a]

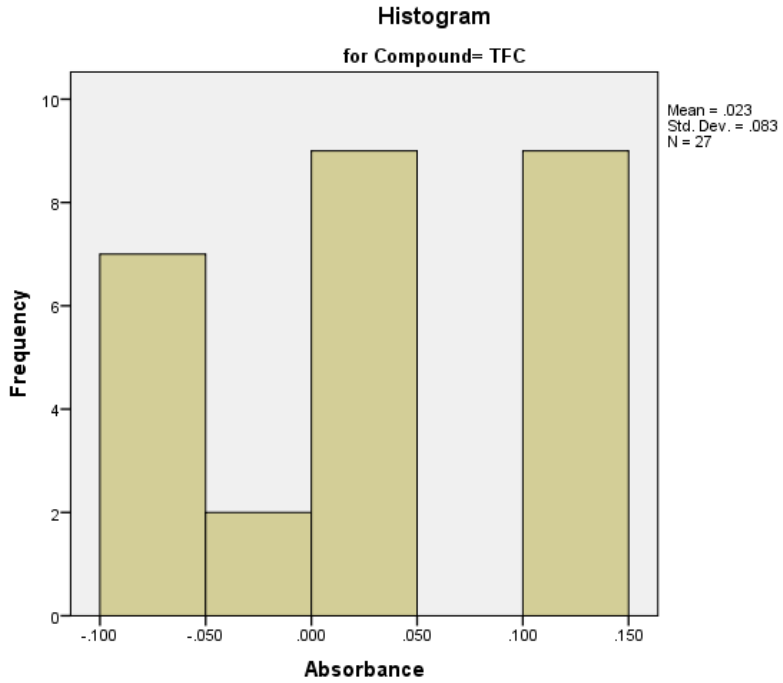
	Absorbance
Mann-Whitney U	162.000
Wilcoxon W	540.000
Z	-3.504

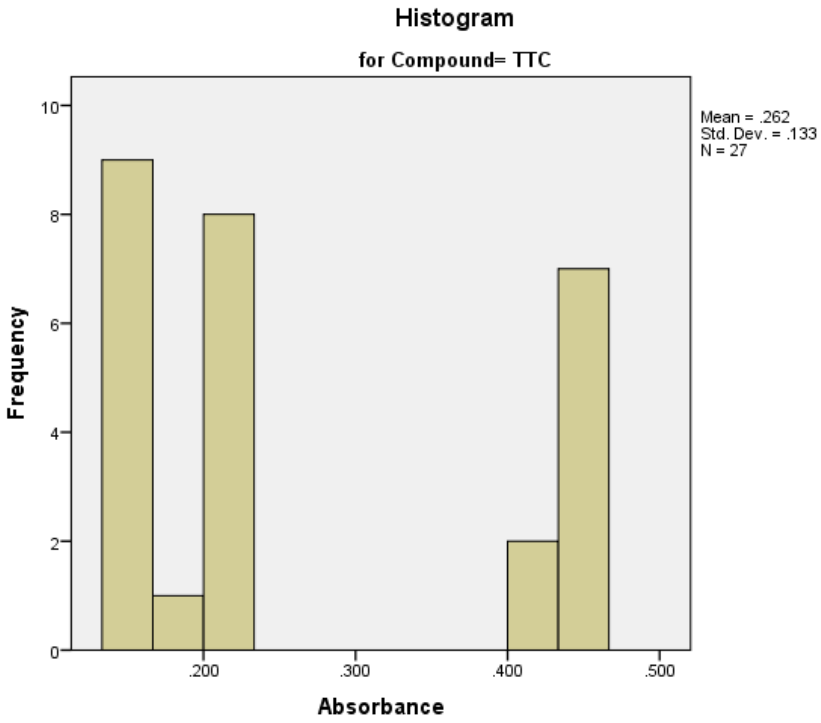
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: Solvent

APPENDIX F: Histograms showing normality tests for TPC, TFC and TTC






Histogram
for Compound= TTC
Mean = .262
Std. Dev. = .133
N = 27
Frequency
Absorbance
0
2
4
6
8
10
.200
.300
.400
.500

APPENDIX G: Statistical analyses on absorbance across different compounds

Non-parametric tests on absorbance across different compounds

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Absorbance	81	.15627	.145049	-.096	.456
Compound	81	2.00	.822	1	3

Kruskal-Wallis Test

Ranks

	Compound	N	Mean Rank
Absorbance	TPC	27	45.54
	TFC	27	17.46
	TTC	27	60.00
	Total	81	

Test Statistics[a,b]

	Absorbance
Chi-Square	45.653
df	2
Asymp. Sig.	.000

a. Kruskal Wallis Test
b. Grouping Variable: Compound

Post-hoc tests: Multiple comparisons on absorbance across different compounds

TPC vs TFC:

Ranks

	Compound	N	Mean Rank	Sum of Ranks
Absorbance	TPC	27	37.54	1013.50
	TFC	27	17.46	471.50
	Total	54		

Test Statistics[a]

	Absorbance
Mann-Whitney U	93.500
Wilcoxon W	471.500
Z	-4.689
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: Compound

TPC vs TTC:

Ranks

	Compound	N	Mean Rank	Sum of Ranks
Absorbance	TPC	27	22.00	594.00
	TTC	27	33.00	891.00
	Total	54		

Test Statistics[a]

	Absorbance
Mann-Whitney U	216.000
Wilcoxon W	594.000
Z	-2.570
Asymp. Sig. (2-tailed)	.010

a. Grouping Variable: Compound

TFC vs TTC:

Ranks

	Compound	N	Mean Rank	Sum of Ranks
Absorbance	TFC	27	14.00	378.00
	TTC	27	41.00	1107.00
	Total	54		

Test Statistics[a]

	Absorbance
Mann-Whitney U	.000
Wilcoxon W	378.000
Z	-6.308
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: Compound

APPENDIX H: Statistical Analyses for Agar disc diffusion method

MeOH extracts

Tests of Normality

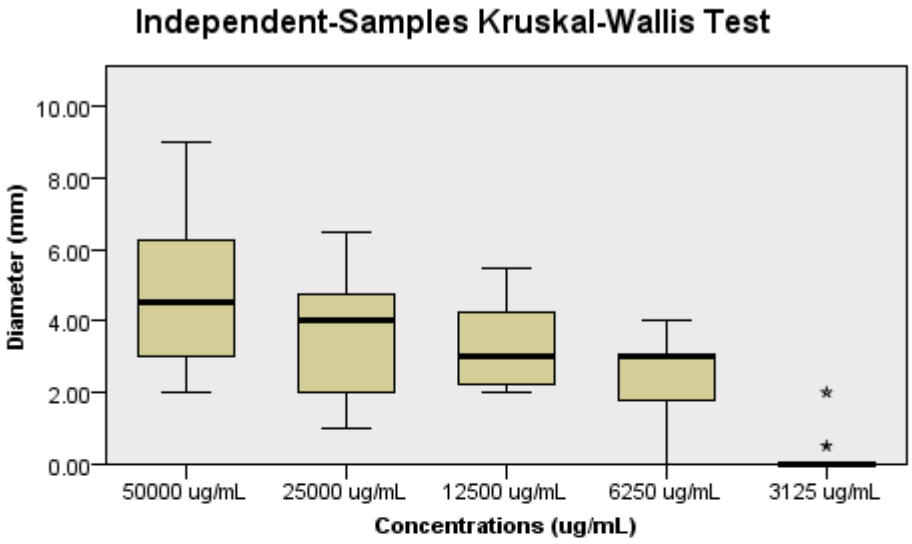
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Diameter (mm)	.138	75	.001	.926	75	.000

a. Lilliefors Significance Correction

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

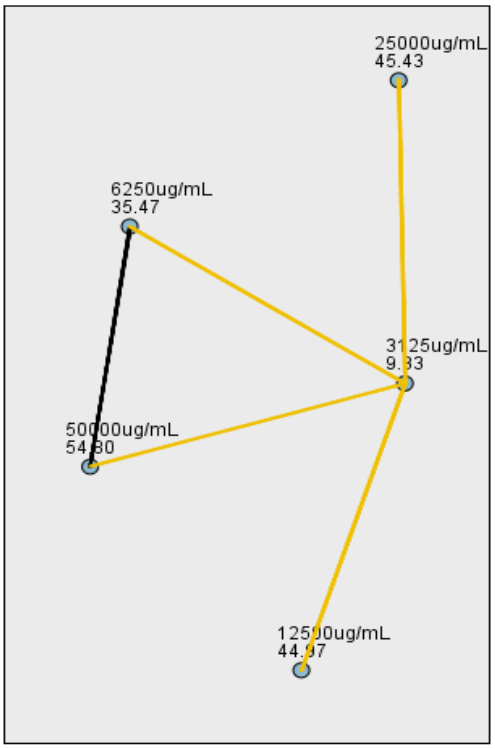
Asymptotic significances are displayed. The significance level is .05.



Total N	75
Test Statistic	39.135
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

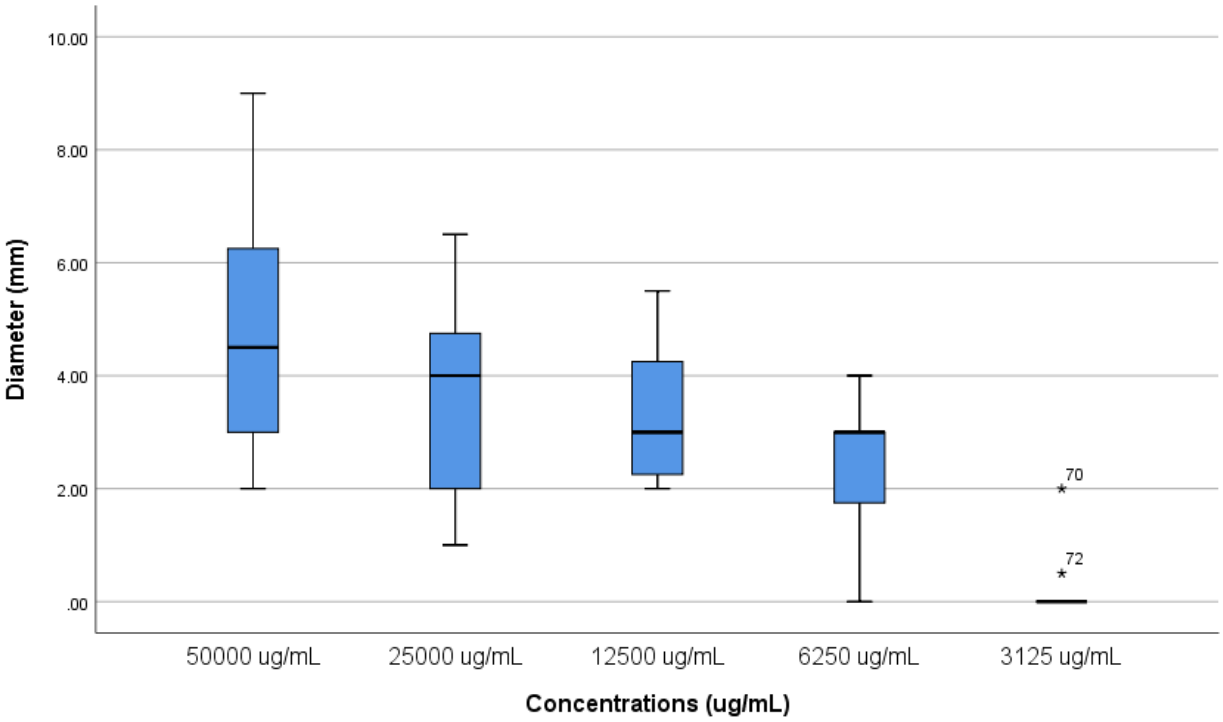
Pairwise Comparisons of Concentrations (ug/mL)



Each node shows the sample average rank of Concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3125ug/mL-6250ug/mL	26.133	7.877	3.318	.001	.009
3125ug/mL-12500ug/mL	35.633	7.877	4.523	.000	.000
3125ug/mL-25000ug/mL	36.100	7.877	4.583	.000	.000
3125ug/mL-50000ug/mL	45.467	7.877	5.772	.000	.000
6250ug/mL-12500ug/mL	9.500	7.877	1.206	.228	1.000
6250ug/mL-25000ug/mL	9.967	7.877	1.265	.206	1.000
6250ug/mL-50000ug/mL	19.333	7.877	2.454	.014	.141
12500ug/mL-25000ug/mL	.467	7.877	.059	.953	1.000
12500ug/mL-50000ug/mL	9.833	7.877	1.248	.212	1.000
25000ug/mL-50000ug/mL	9.367	7.877	1.189	.234	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.



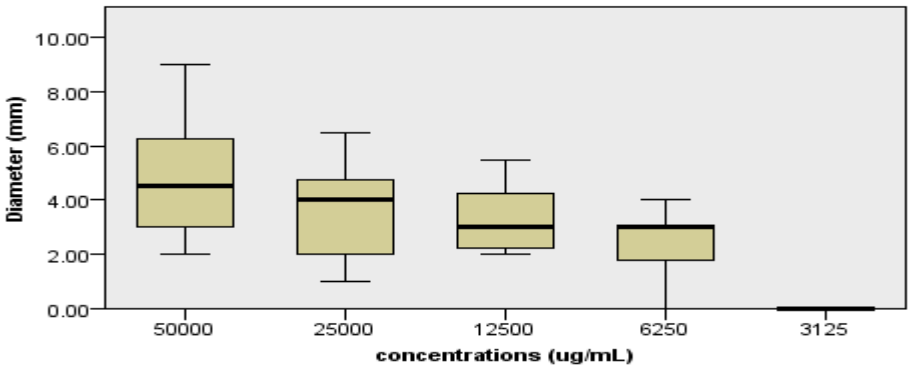
After removing the outliers

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

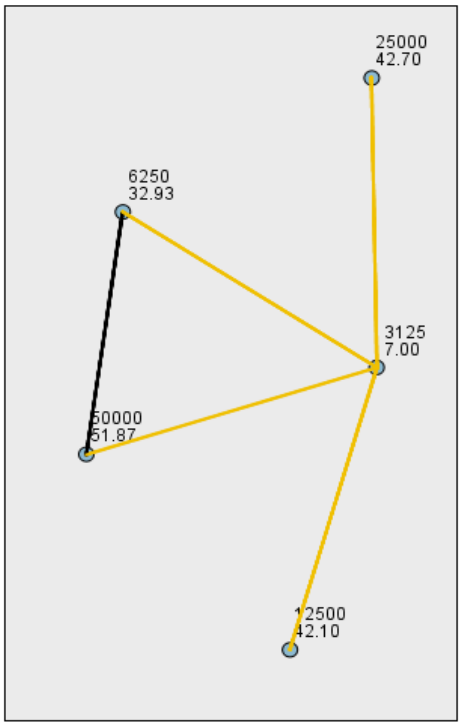
Independent-Samples Kruskal-Wallis Test



Total N	72
Test Statistic	35.469
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

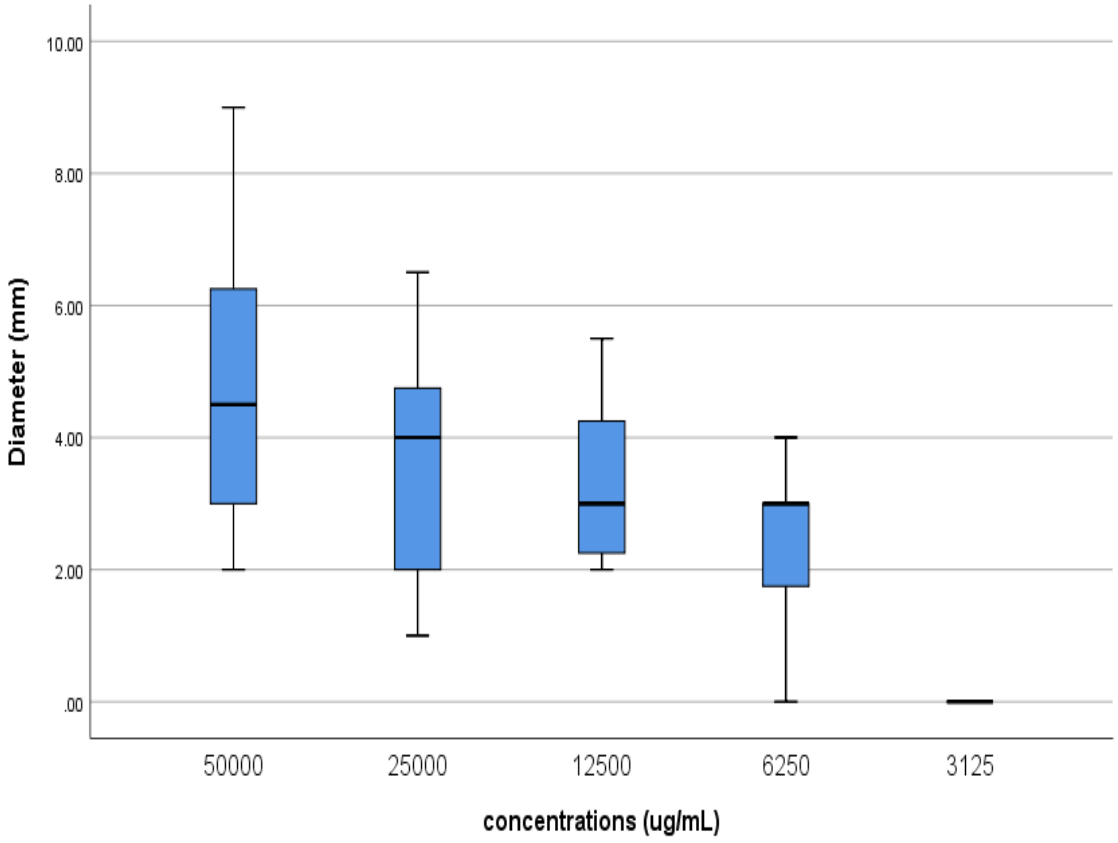
Pairwise Comparisons of concentrations (ug/mL)



Each node shows the sample average rank of concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3125-6250	25.933	8.024	3.232	.001	.012
3125-12500	35.100	8.024	4.375	.000	.000
3125-25000	35.700	8.024	4.449	.000	.000
3125-50000	44.867	8.024	5.592	.000	.000
6250-12500	9.167	7.565	1.212	.226	1.000
6250-25000	9.767	7.565	1.291	.197	1.000
6250-50000	18.933	7.565	2.503	.012	.123
12500-25000	.600	7.565	.079	.937	1.000
12500-50000	9.767	7.565	1.291	.197	1.000
25000-50000	9.167	7.565	1.212	.226	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.

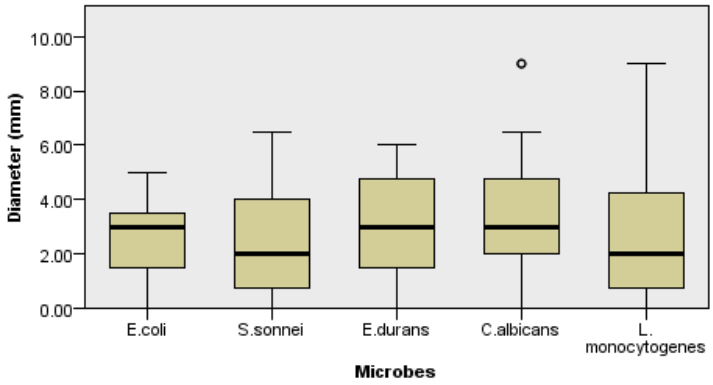


Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Microbes.	Independent-Samples Kruskal-Wallis Test	.839	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Independent-Samples Kruskal-Wallis Test



Total N	75
Test Statistic	1.431
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.839

1. The test statistic is adjusted for ties.
2. Multiple comparisons are not performed because the overall test does not show significant differences across samples.

MeOH: DCM extracts

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Diameter (mm)	.119	75	.011	.938	75	.001

a. Lilliefors Significance Correction

Hypothesis Test Summary

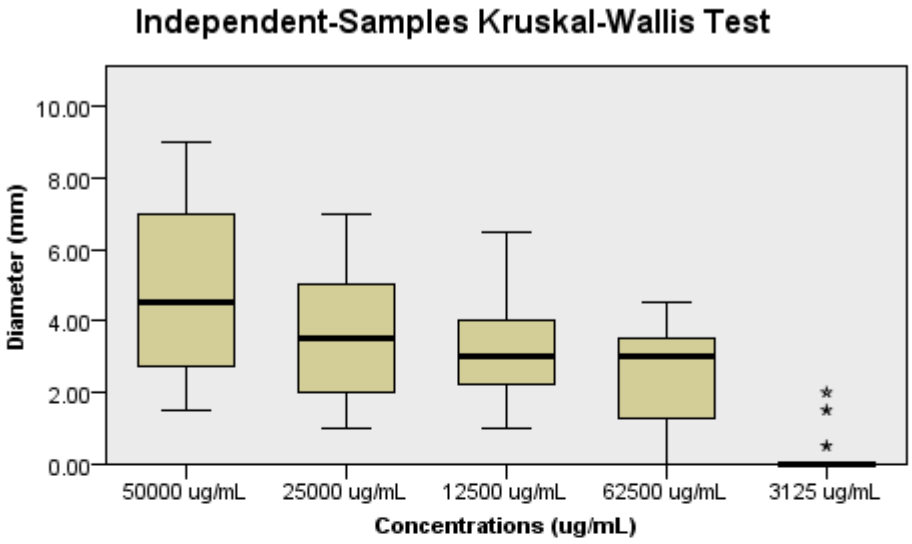
	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Microbes.	Independent-Samples Kruskal-Wallis Test	.113	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

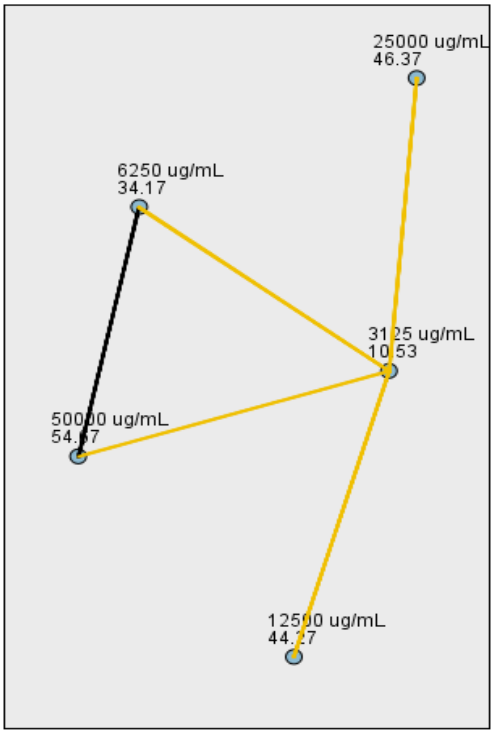
Asymptotic significances are displayed. The significance level is .05.



Total N	75
Test Statistic	37.039
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

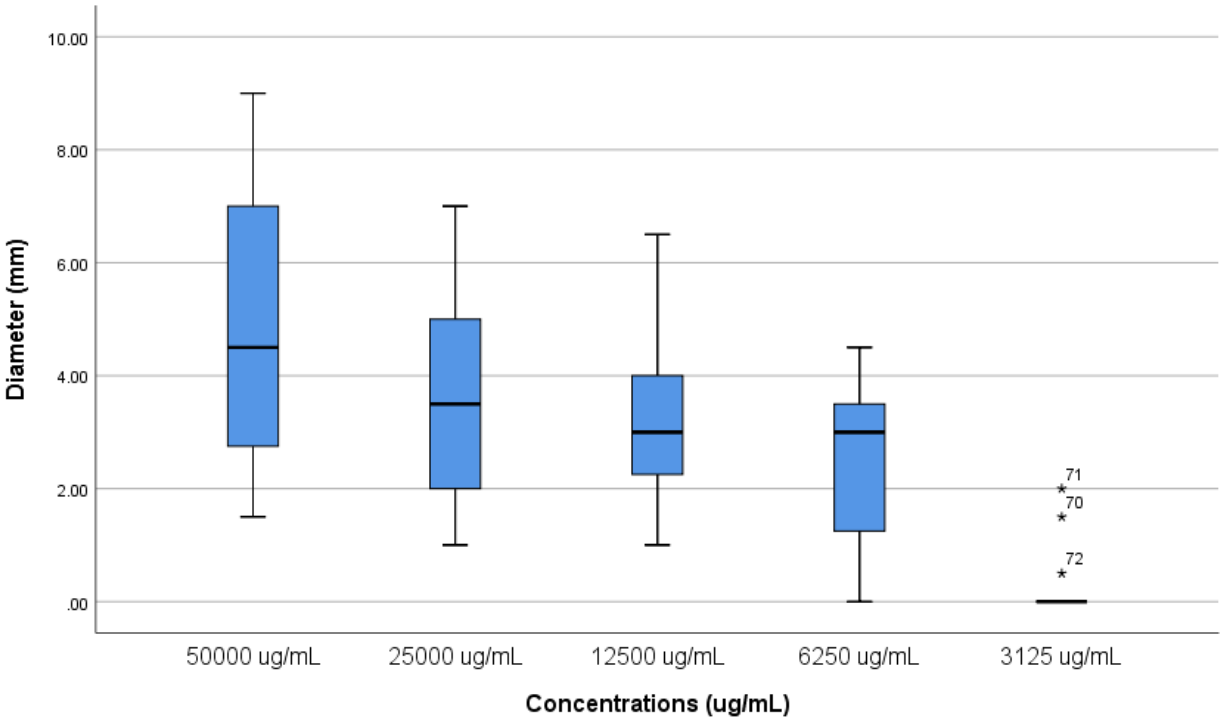
Pairwise Comparisons of Concentrations (ug/mL)



Each node shows the sample average rank of Concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3125 ug/mL-6250 ug/mL	23.633	7.901	2.991	.003	.028
3125 ug/mL-12500 ug/mL	33.733	7.901	4.269	.000	.000
3125 ug/mL-25000 ug/mL	35.833	7.901	4.535	.000	.000
3125 ug/mL-50000 ug/mL	44.133	7.901	5.586	.000	.000
6250 ug/mL-12500 ug/mL	10.100	7.901	1.278	.201	1.000
6250 ug/mL-25000 ug/mL	12.200	7.901	1.544	.123	1.000
6250 ug/mL-50000 ug/mL	20.500	7.901	2.595	.009	.095
12500 ug/mL-25000 ug/mL	2.100	7.901	.266	.790	1.000
12500 ug/mL-50000 ug/mL	10.400	7.901	1.316	.188	1.000
25000 ug/mL-50000 ug/mL	8.300	7.901	1.050	.294	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.

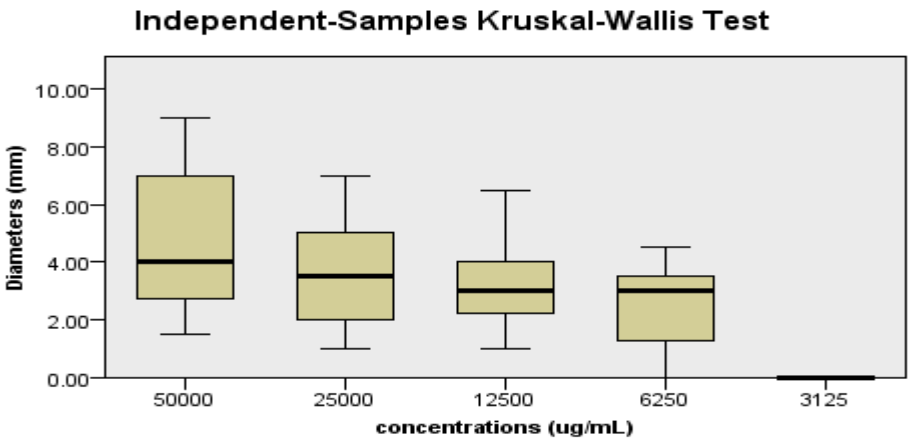


After removing the outliers

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameters (mm) is the same across categories of concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

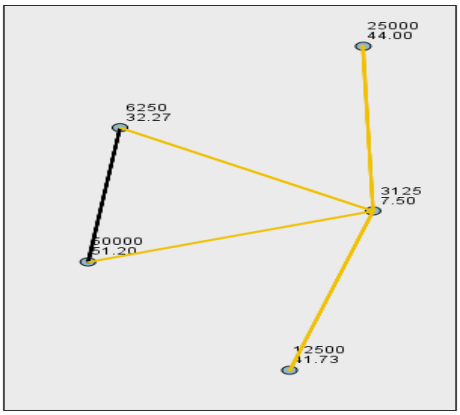
Asymptotic significances are displayed. The significance level is .05.



Total N	72
Test Statistic	34.470
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

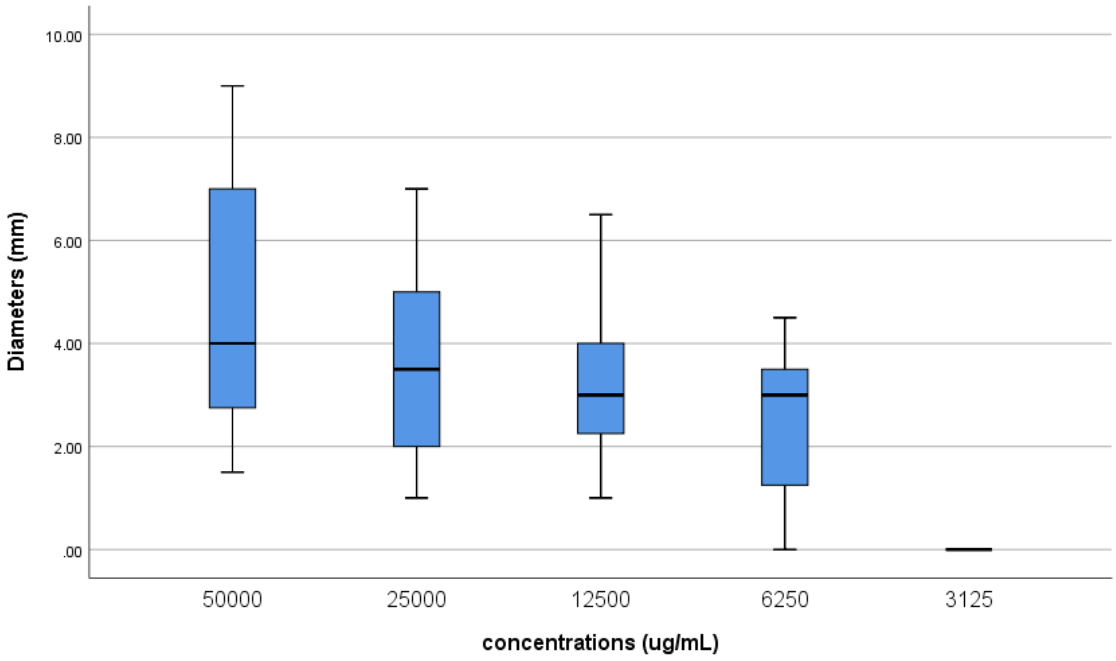
Pairwise Comparisons of concentrations (ug/mL)



Each node shows the sample average rank of concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3125-6250	24.767	8.041	3.080	.002	.021
3125-12500	34.233	8.041	4.258	.000	.000
3125-25000	36.500	8.041	4.539	.000	.000
3125-50000	43.700	8.041	5.435	.000	.000
6250-12500	9.467	7.581	1.249	.212	1.000
6250-25000	11.733	7.581	1.548	.122	1.000
6250-50000	18.933	7.581	2.498	.013	.125
12500-25000	2.267	7.581	.299	.765	1.000
12500-50000	9.467	7.581	1.249	.212	1.000
25000-50000	7.200	7.581	.950	.342	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for



H_2O extracts

Tests of Normality

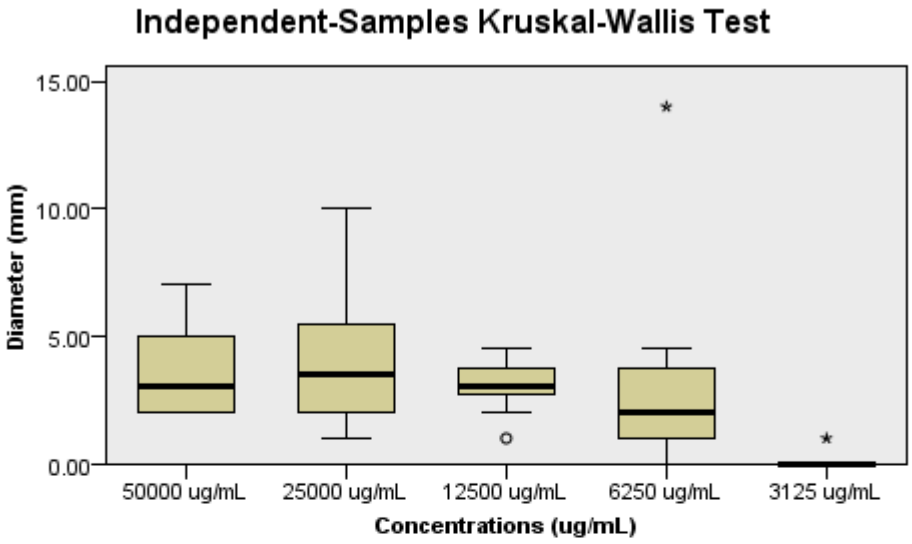
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Diameter (mm)	.140	75	.001	.862	75	.000

a. Lilliefors Significance Correction

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

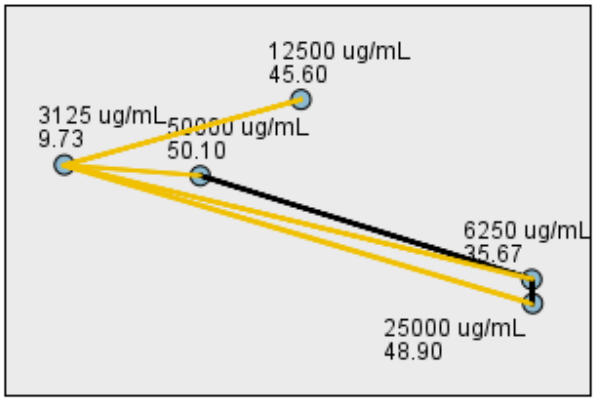
Asymptotic significances are displayed. The significance level is .05.



Total N	75
Test Statistic	36.580
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

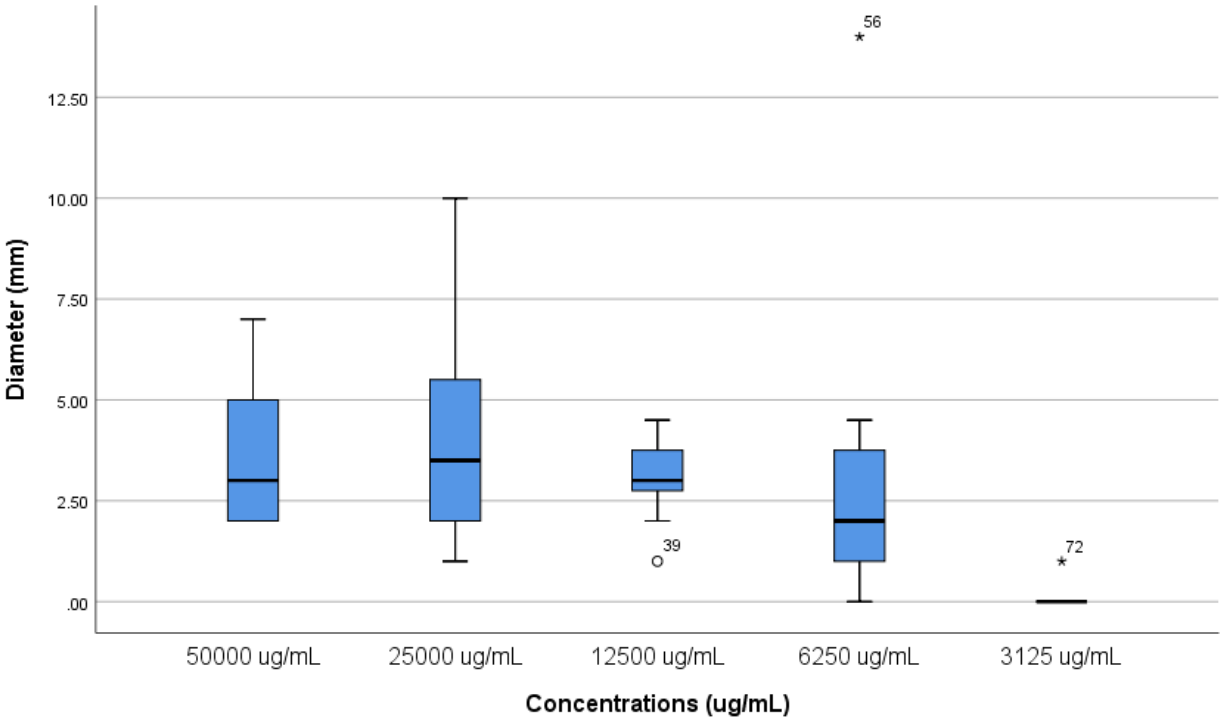
Pairwise Comparisons of Concentrations (ug/mL)



Each node shows the sample average rank of Concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3125 ug/mL-6250 ug/mL	25.933	7.851	3.303	.001	.010
3125 ug/mL-12500 ug/mL	35.867	7.851	4.568	.000	.000
3125 ug/mL-25000 ug/mL	39.167	7.851	4.989	.000	.000
3125 ug/mL-50000 ug/mL	40.367	7.851	5.141	.000	.000
6250 ug/mL-12500 ug/mL	9.933	7.851	1.265	.206	1.000
6250 ug/mL-25000 ug/mL	13.233	7.851	1.686	.092	.919
6250 ug/mL-50000 ug/mL	14.433	7.851	1.838	.066	.660
12500 ug/mL-25000 ug/mL	3.300	7.851	.420	.674	1.000
12500 ug/mL-50000 ug/mL	4.500	7.851	.573	.567	1.000
25000 ug/mL-50000 ug/mL	1.200	7.851	.153	.879	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.

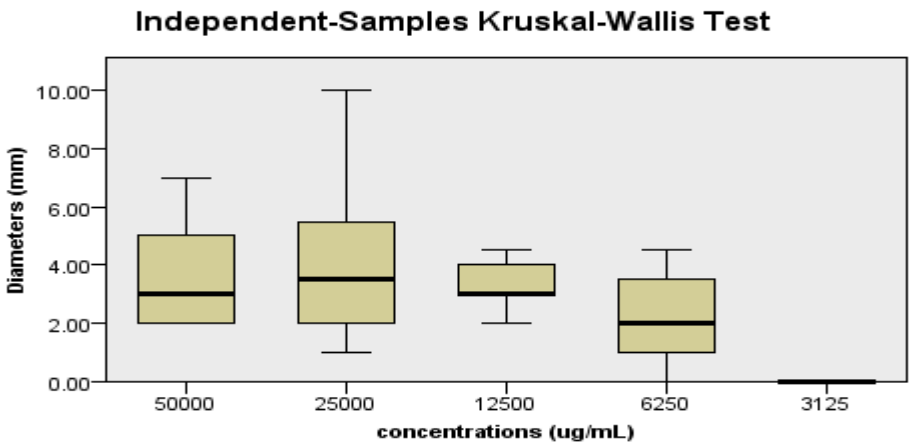


After removing the outliers

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameters (mm) is the same across categories of concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

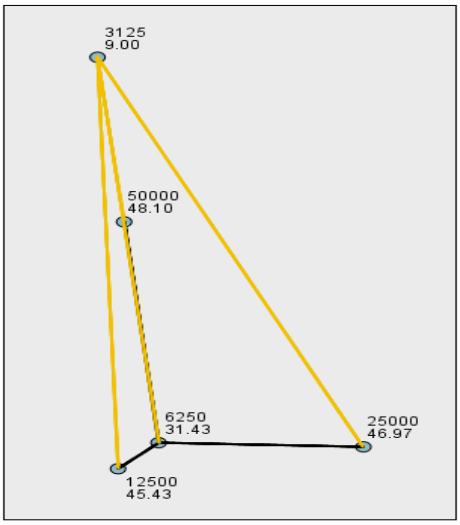
Asymptotic significances are displayed. The significance level is .05.



Total N	72
Test Statistic	37.011
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

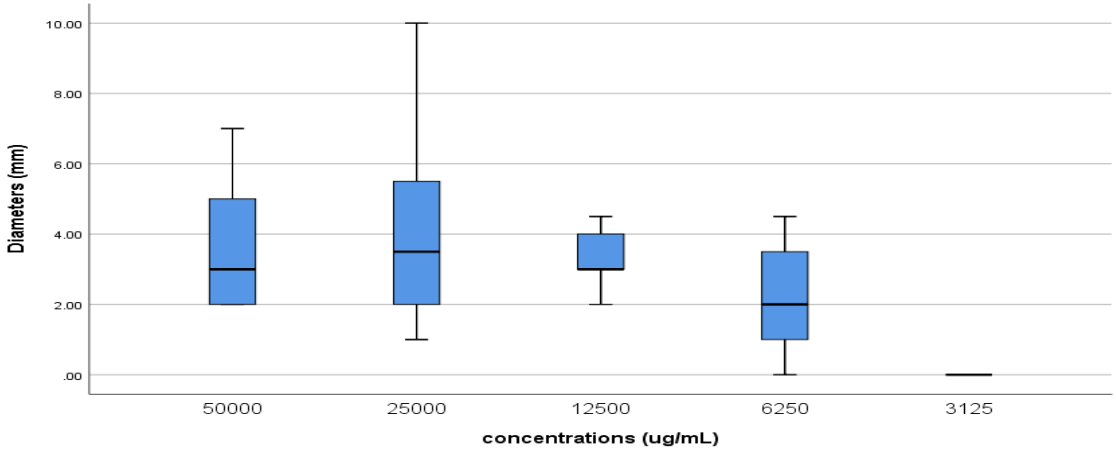
Pairwise Comparisons of concentrations (ug/mL)



Each node shows the sample average rank of concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3125-6250	22.429	7.791	2.879	.004	.040
3125-12500	36.429	7.791	4.676	.000	.000
3125-25000	37.967	7.660	4.957	.000	.000
3125-50000	39.100	7.660	5.104	.000	.000
6250-12500	14.000	7.791	1.797	.072	.723
6250-25000	15.538	7.660	2.028	.043	.425
6250-50000	16.671	7.660	2.176	.030	.295
12500-25000	1.538	7.660	.201	.841	1.000
12500-50000	2.671	7.660	.349	.727	1.000
25000-50000	1.133	7.527	.151	.880	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.



APPENDIX I: Statistical Analyses for Agar well diffusion method

MeOH extracts

Tests for Normality

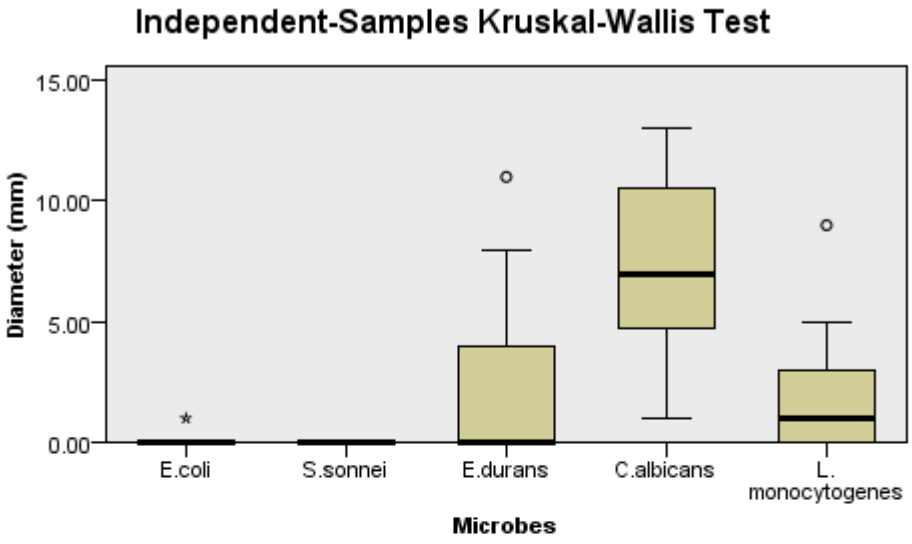
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Diameter (mm)	.319	75	.000	.679	75	.000

a. Lilliefors Significance Correction

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Microbes.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

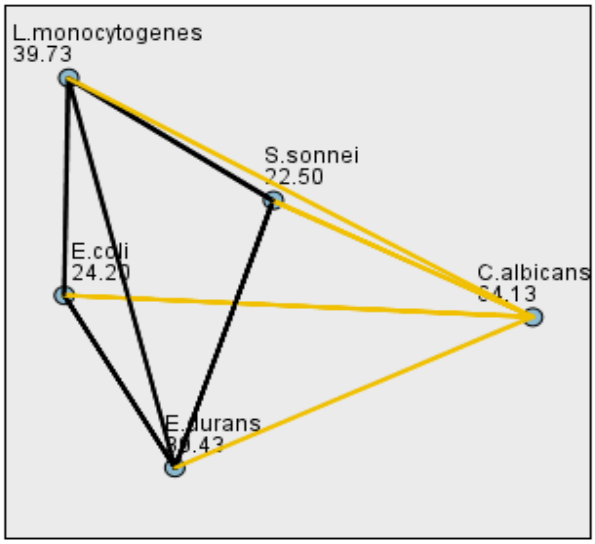
Asymptotic significances are displayed. The significance level is .05.



Total N	75
Test Statistic	44.318
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

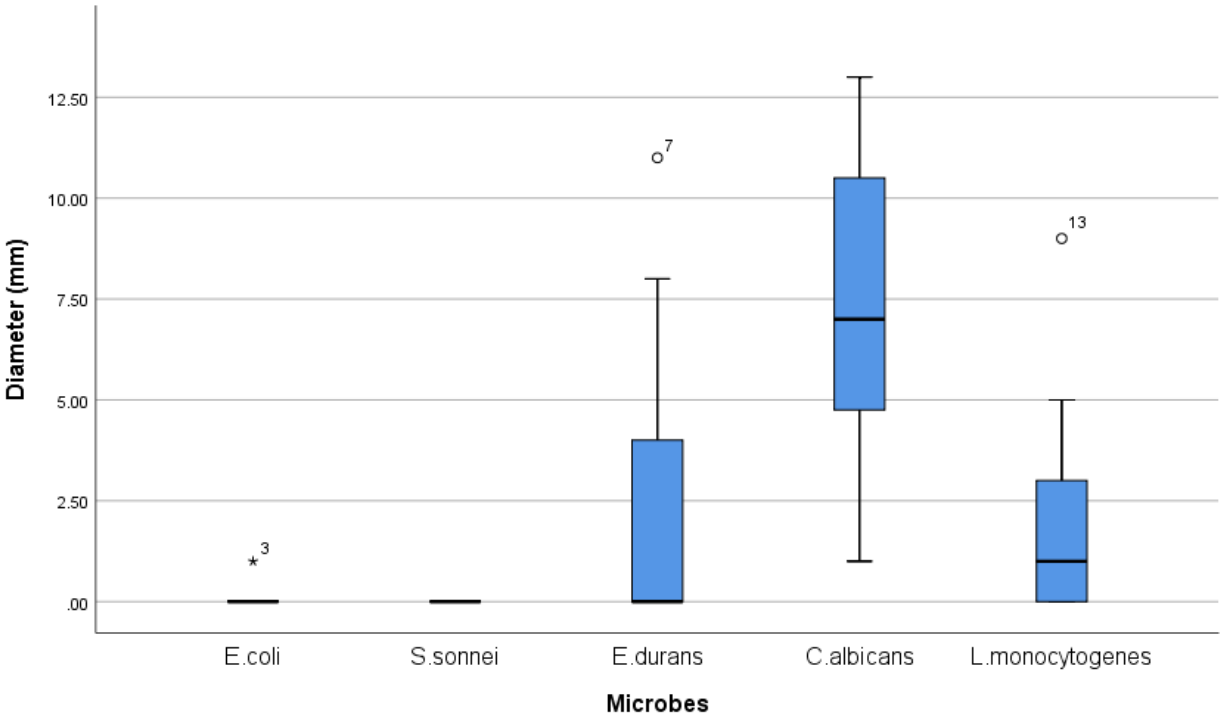
Pairwise Comparisons of Microbes



Each node shows the sample average rank of Microbes.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
S.sonnei-E.coli	1.700	7.105	.239	.811	1.000
S.sonnei-E.durans	-16.933	7.105	-2.383	.017	.172
S.sonnei-L.monocytogenes	-17.233	7.105	-2.425	.015	.153
S.sonnei-C.albicans	-41.633	7.105	-5.860	.000	.000
E.coli-E.durans	-15.233	7.105	-2.144	.032	.320
E.coli-L.monocytogenes	-15.533	7.105	-2.186	.029	.288
E.coli-C.albicans	-39.933	7.105	-5.620	.000	.000
E.durans-L.monocytogenes	-.300	7.105	-.042	.966	1.000
E.durans-C.albicans	-24.700	7.105	-3.476	.001	.005
L.monocytogenes-C.albicans	24.400	7.105	3.434	.001	.006

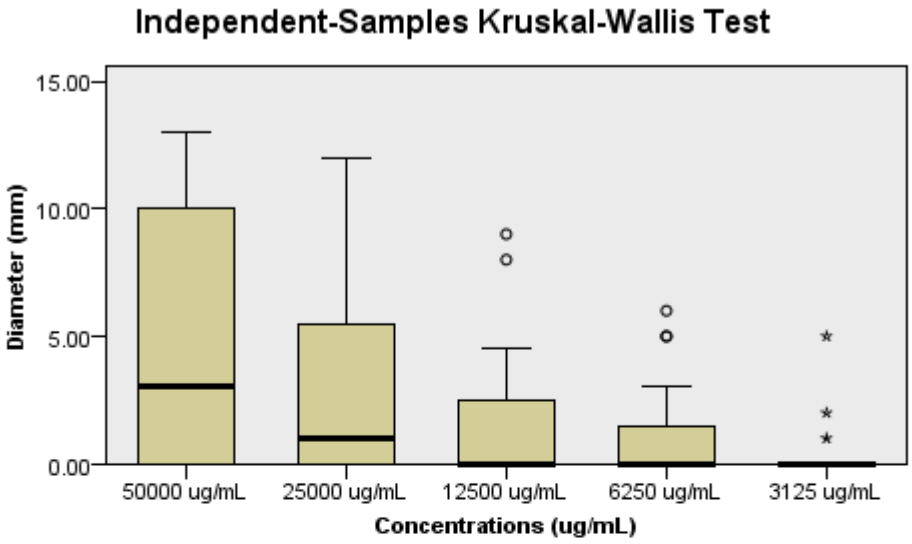
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.



Hypothesis Test Summary

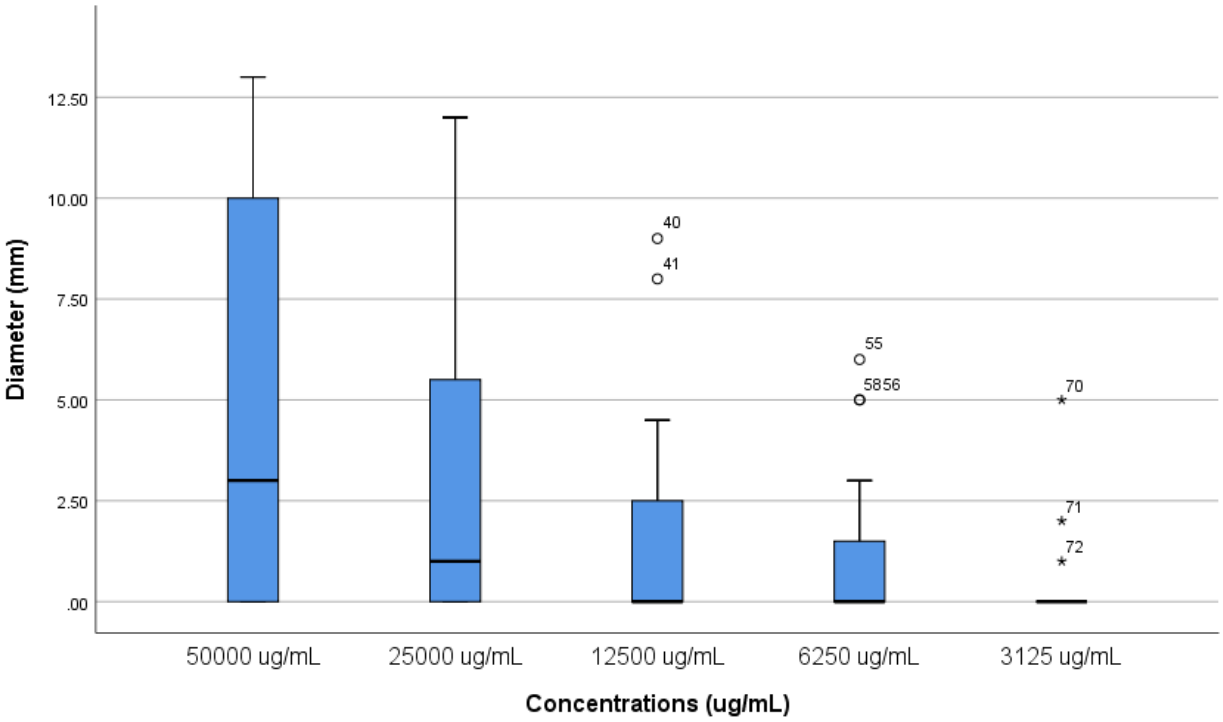
	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.030	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

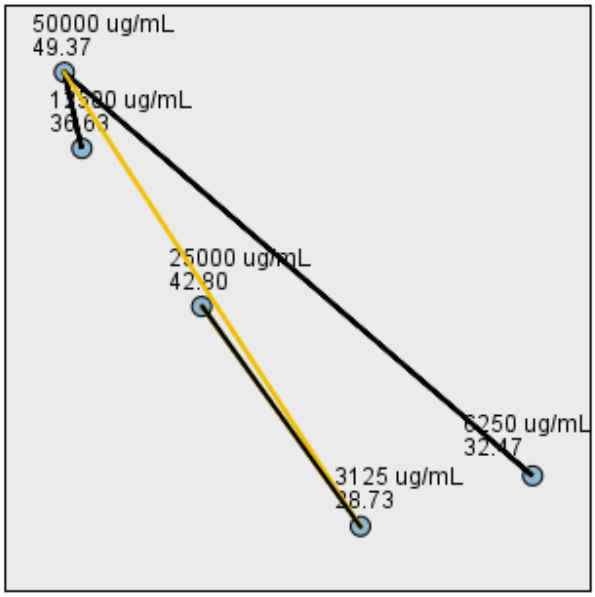


Total N	75
Test Statistic	10.720
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.030

1. The test statistic is adjusted for ties.


Diameter (mm)
12.50
10.00
7.50
5.00
2.50
.00
40
41
55
5856
70
71
72
50000 ug/mL
25000 ug/mL
12500 ug/mL
6250 ug/mL
3125 ug/mL
Concentrations (ug/mL)

Pairwise Comparisons of Concentrations (ug/mL)



Each node shows the sample average rank of Concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3125 ug/mL-6250 ug/mL	3.733	7.105	.525	.599	1.000
3125 ug/mL-12500 ug/mL	7.900	7.105	1.112	.266	1.000
3125 ug/mL-25000 ug/mL	14.067	7.105	1.980	.048	.477
3125 ug/mL-50000 ug/mL	20.633	7.105	2.904	.004	.037
6250 ug/mL-12500 ug/mL	4.167	7.105	.586	.558	1.000
6250 ug/mL-25000 ug/mL	10.333	7.105	1.454	.146	1.000
6250 ug/mL-50000 ug/mL	16.900	7.105	2.379	.017	.174
12500 ug/mL-25000 ug/mL	6.167	7.105	.868	.385	1.000
12500 ug/mL-50000 ug/mL	12.733	7.105	1.792	.073	.731
25000 ug/mL-50000 ug/mL	6.567	7.105	.924	.355	1.000

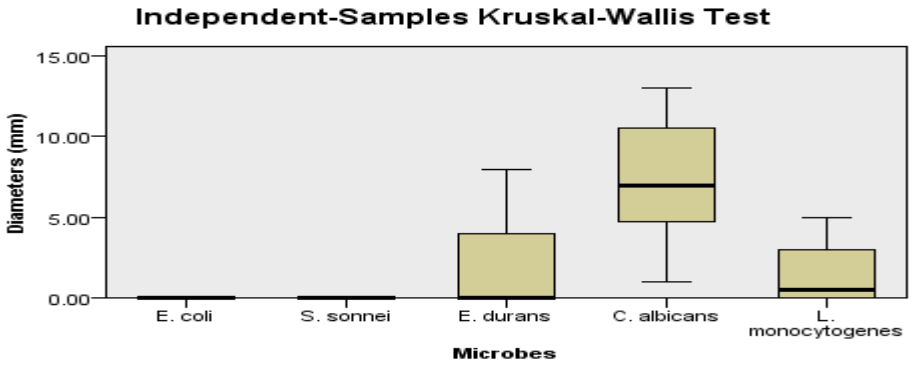
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.

After removing the outliers

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameters (mm) is the same across categories of Microbes.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

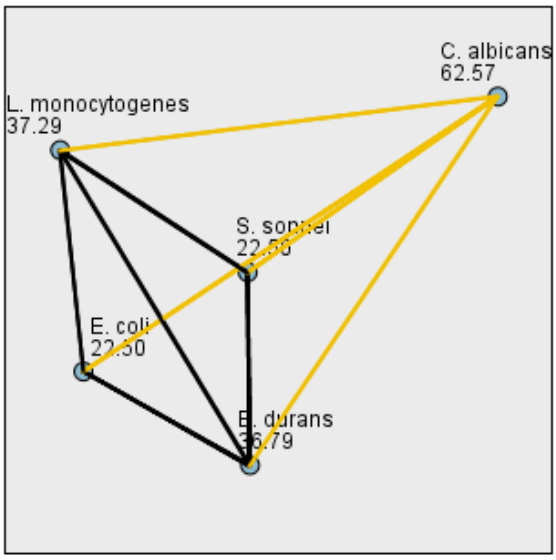
Asymptotic significances are displayed. The significance level is .05.



Total N	72
Test Statistic	47.038
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

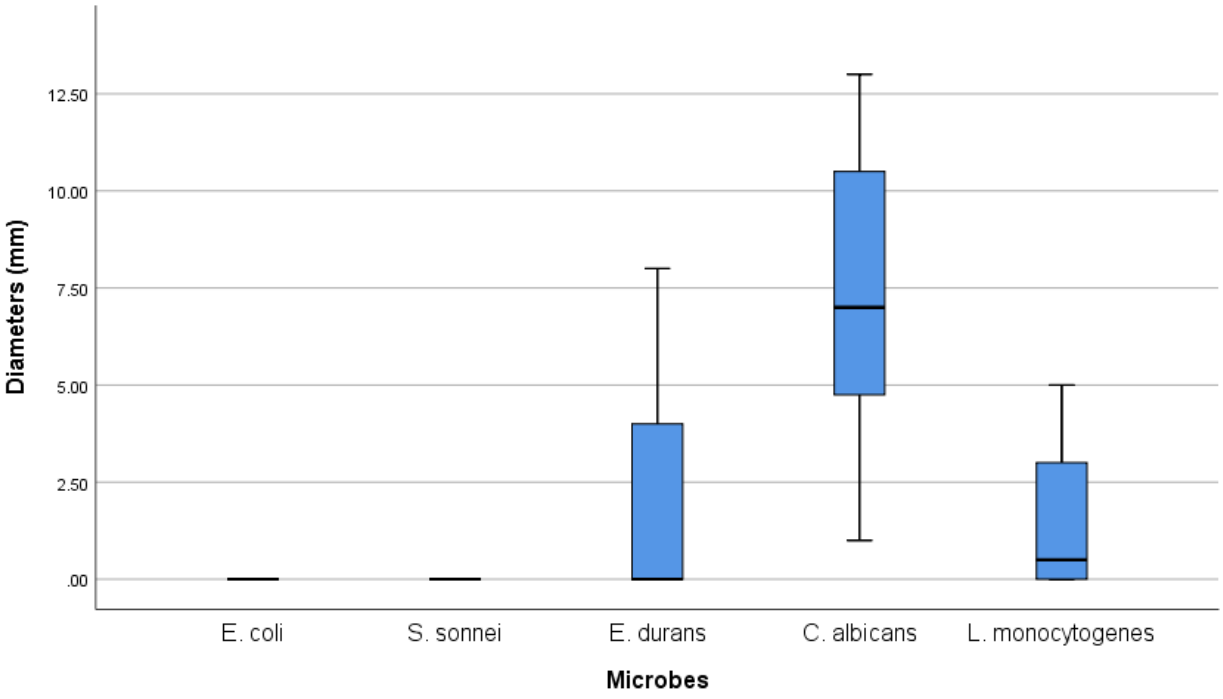
Pairwise Comparisons of Microbes



Each node shows the sample average rank of Microbes.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
E. coli-S. sonnei	.000	6.829	.000	1.000	1.000
E. coli-E. durans	-14.286	6.946	-2.057	.040	.397
E. coli-L. monocytogenes	-14.786	6.946	-2.129	.033	.333
E. coli-C. albicans	-40.067	6.829	-5.867	.000	.000
S. sonnei-E. durans	-14.286	6.829	-2.092	.036	.365
S. sonnei-L. monocytogenes	-14.786	6.829	-2.165	.030	.304
S. sonnei-C. albicans	-40.067	6.710	-5.971	.000	.000
E. durans-L. monocytogenes	-.500	6.946	-.072	.943	1.000
E. durans-C. albicans	-25.781	6.829	-3.775	.000	.002
L. monocytogenes-C. albicans	25.281	6.829	3.702	.000	.002

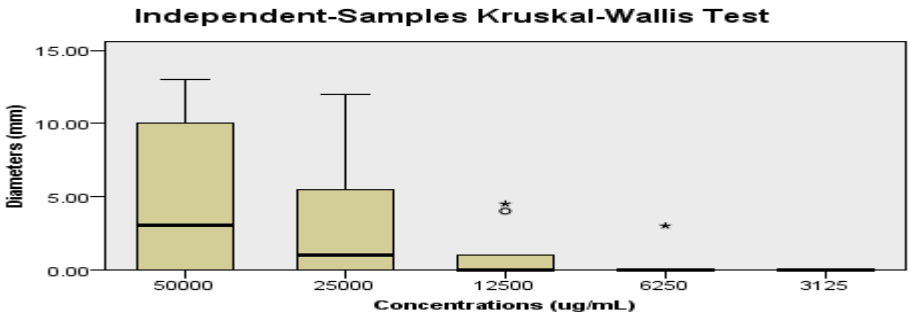
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.



Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameters (mm) is the same across categories of Concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

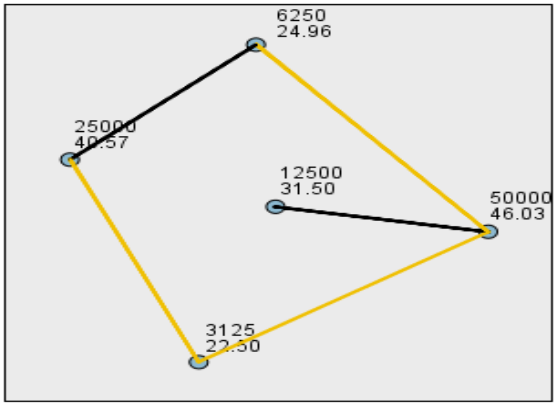
Asymptotic significances are displayed. The significance level is .05.



Total N	67
Test Statistic	20.116
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

Pairwise Comparisons of Concentrations (ug/mL)



Each node shows the sample average rank of Concentrations (ug/mL).

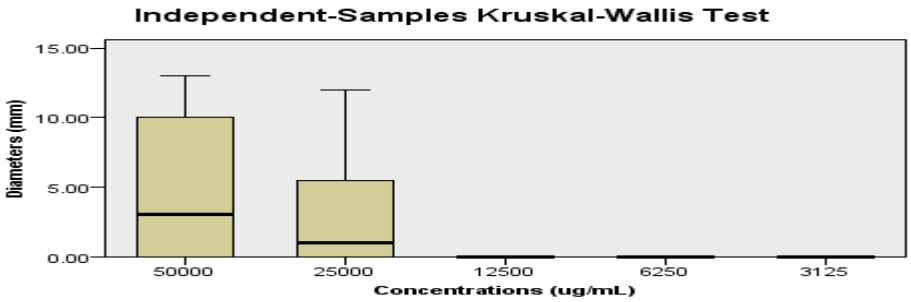
Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3125-6250	2.458	6.731	.365	.715	1.000
3125-12500	9.000	6.600	1.364	.173	1.000
3125-25000	18.067	6.386	2.829	.005	.047
3125-50000	23.533	6.386	3.685	.000	.002
6250-12500	6.542	6.600	.991	.322	1.000
6250-25000	15.608	6.386	2.444	.015	.145
6250-50000	21.075	6.386	3.300	.001	.010
12500-25000	9.067	6.248	1.451	.147	1.000
12500-50000	14.533	6.248	2.326	.020	.200
25000-50000	5.467	6.020	.908	.364	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameters (mm) is the same across categories of Concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

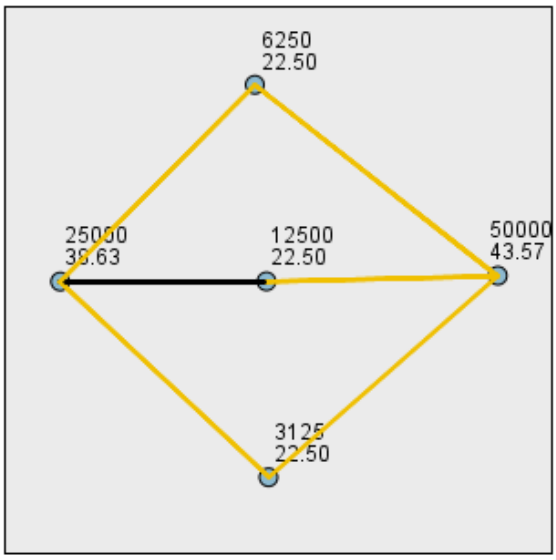
Asymptotic significances are displayed. The significance level is .05.



Total N	62
Test Statistic	26.494
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

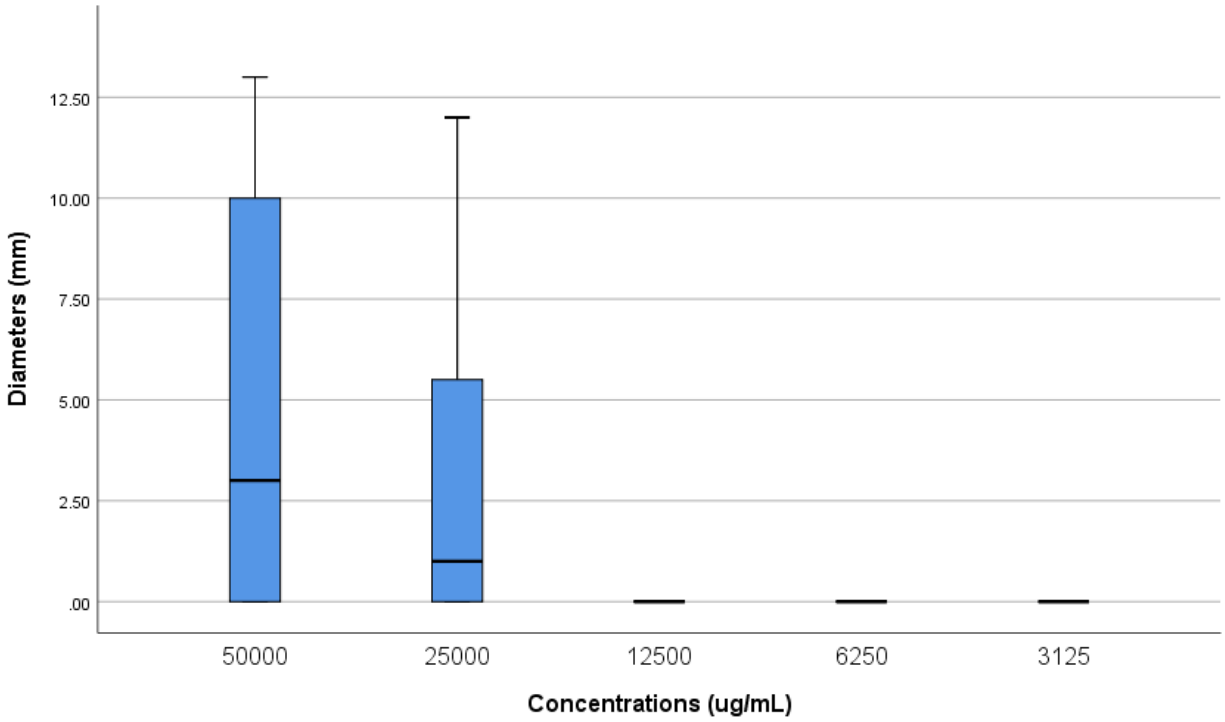
Pairwise Comparisons of Concentrations (ug/mL)



Each node shows the sample average rank of Concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
12500-6250	.000	6.499	.000	1.000	1.000
12500-3125	.000	6.376	.000	1.000	1.000
12500-25000	16.133	6.097	2.646	.008	.081
12500-50000	21.067	6.097	3.455	.001	.005
6250-3125	.000	6.036	.000	1.000	1.000
6250-25000	16.133	5.740	2.811	.005	.049
6250-50000	21.067	5.740	3.670	.000	.002
3125-25000	16.133	5.600	2.881	.004	.040
3125-50000	21.067	5.600	3.762	.000	.002
25000-50000	4.933	5.280	.934	.350	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.



MeOH: DCM extracts

Tests of Normality

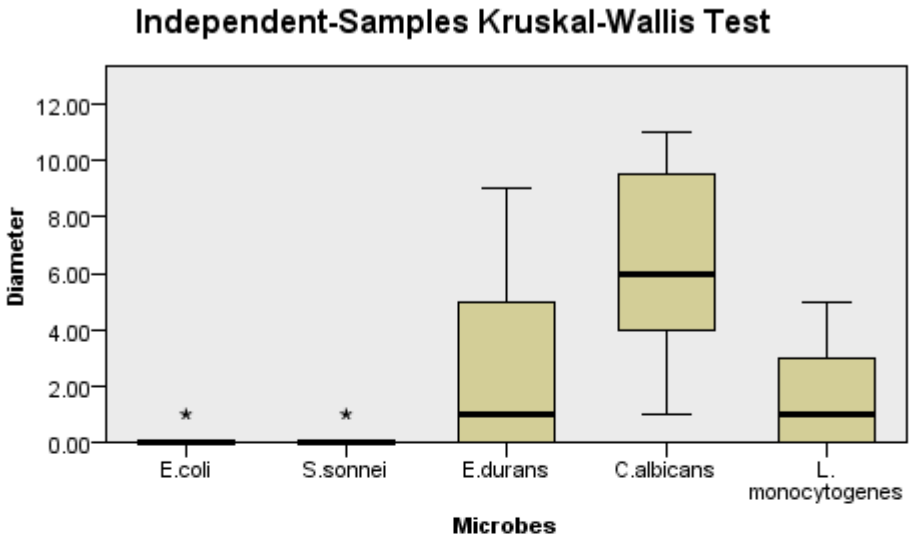
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Diameter	.295	75	.000	.712	75	.000

a. Lilliefors Significance Correction

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter is the same across categories of Microbes.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

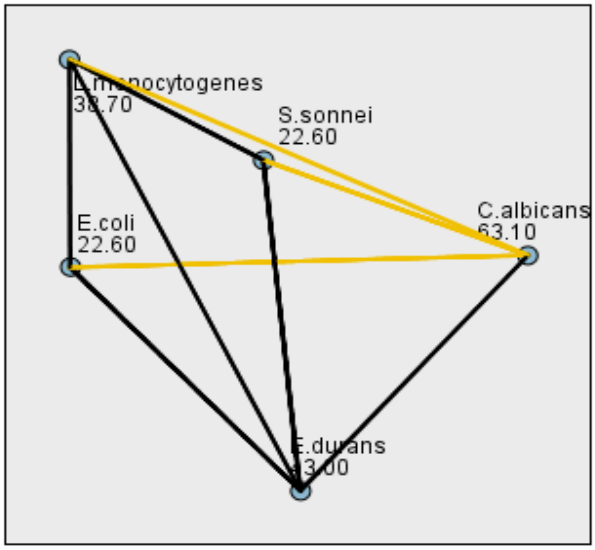
Asymptotic significances are displayed. The significance level is .05.



Total N	75
Test Statistic	42.712
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

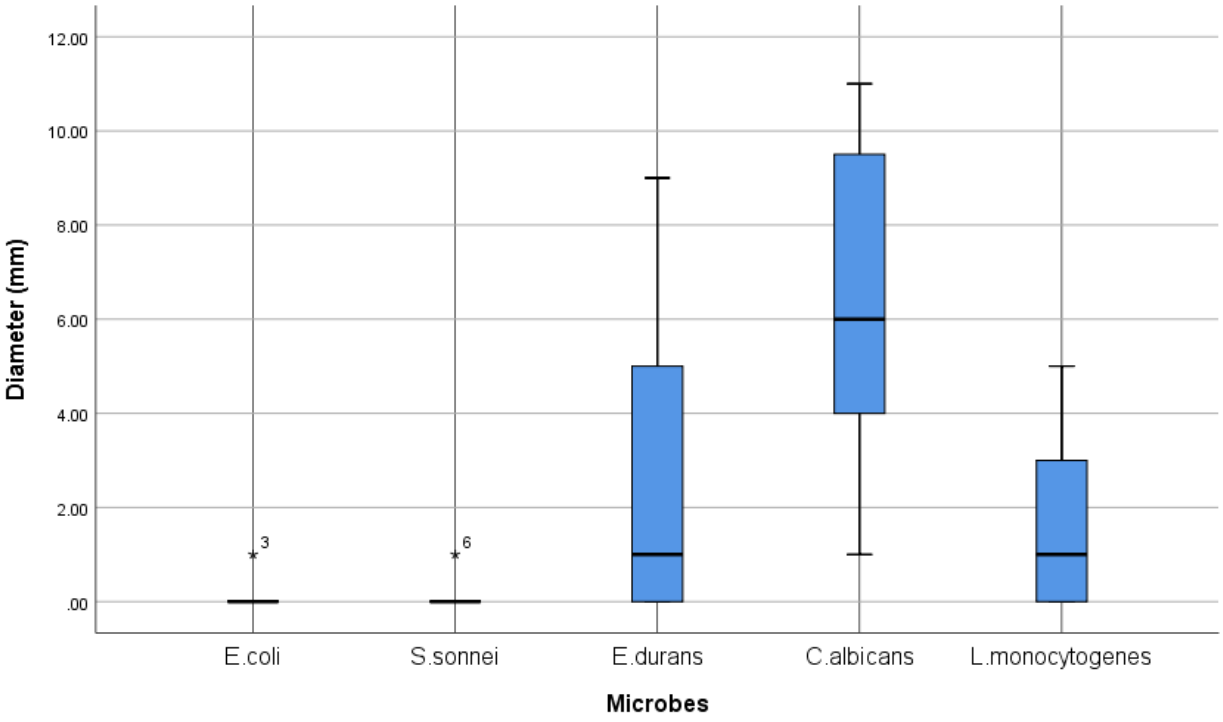
Pairwise Comparisons of Microbes



Each node shows the sample average rank of Microbes.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
E.coli-S.sonnei	.000	7.273	.000	1.000	1.000
E.coli-L.monocytogenes	-16.100	7.273	-2.214	.027	.269
E.coli-E.durans	-20.400	7.273	-2.805	.005	.050
E.coli-C.albicans	-40.500	7.273	-5.568	.000	.000
S.sonnei-L.monocytogenes	-16.100	7.273	-2.214	.027	.269
S.sonnei-E.durans	-20.400	7.273	-2.805	.005	.050
S.sonnei-C.albicans	-40.500	7.273	-5.568	.000	.000
L.monocytogenes-E.durans	4.300	7.273	.591	.554	1.000
L.monocytogenes-C.albicans	24.400	7.273	3.355	.001	.008
E.durans-C.albicans	-20.100	7.273	-2.763	.006	.057

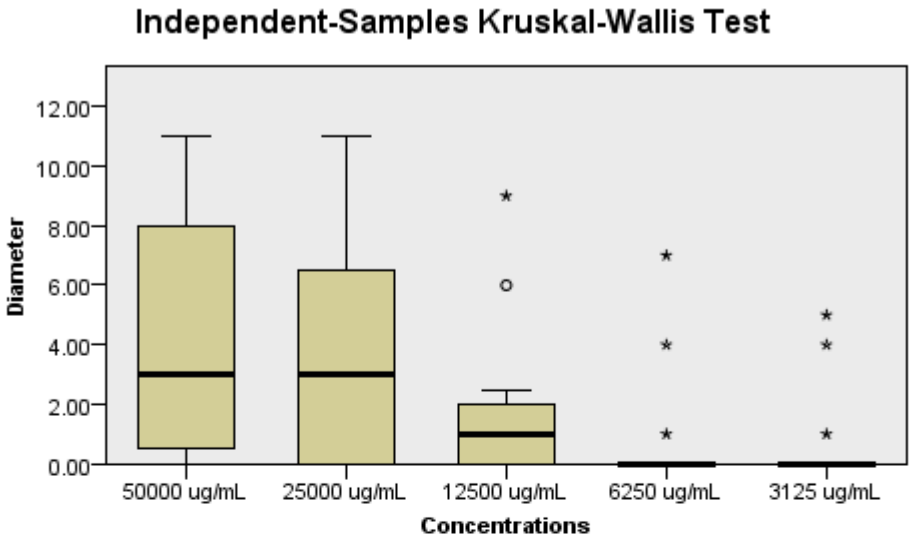
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.



Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter is the same across categories of Concentrations.	Independent-Samples Kruskal-Wallis Test	.003	Reject the null hypothesis.

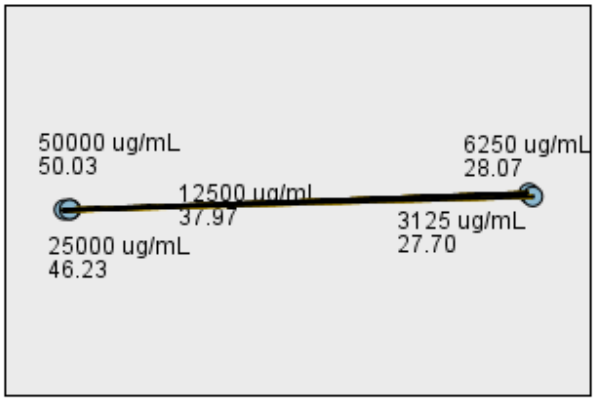
Asymptotic significances are displayed. The significance level is .05.



Total N	75
Test Statistic	15.778
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.003

1. The test statistic is adjusted for ties.

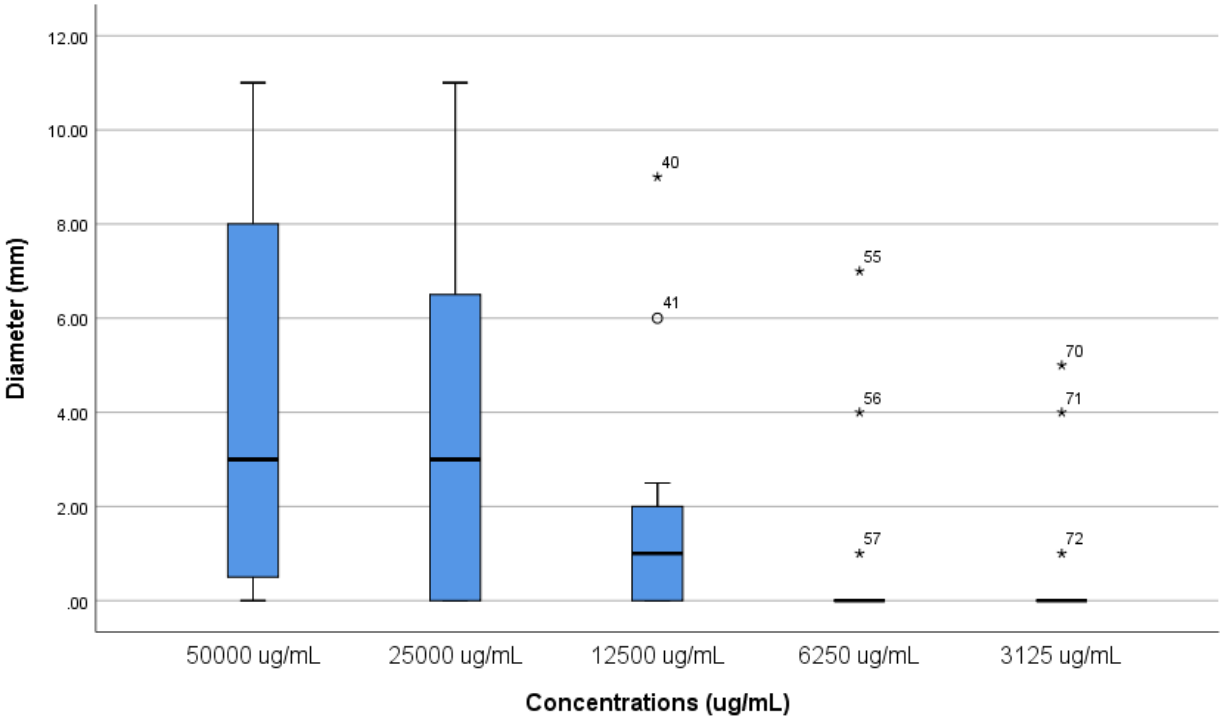
Pairwise Comparisons of Concentrations



Each node shows the sample average rank of Concentrations.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3125 ug/mL-6250 ug/mL	.367	7.273	.050	.960	1.000
3125 ug/mL-12500 ug/mL	10.267	7.273	1.412	.158	1.000
3125 ug/mL-25000 ug/mL	18.533	7.273	2.548	.011	.108
3125 ug/mL-50000 ug/mL	22.333	7.273	3.071	.002	.021
6250 ug/mL-12500 ug/mL	9.900	7.273	1.361	.173	1.000
6250 ug/mL-25000 ug/mL	18.167	7.273	2.498	.013	.125
6250 ug/mL-50000 ug/mL	21.967	7.273	3.020	.003	.025
12500 ug/mL-25000 ug/mL	8.267	7.273	1.137	.256	1.000
12500 ug/mL-50000 ug/mL	12.067	7.273	1.659	.097	.971
25000 ug/mL-50000 ug/mL	3.800	7.273	.522	.601	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.

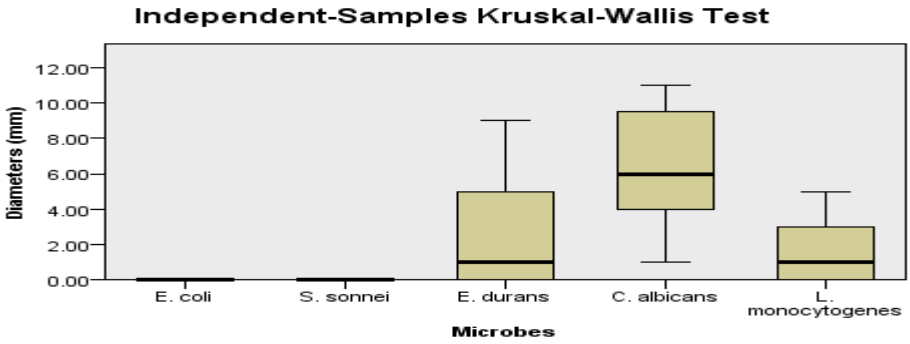


After removing the outliers

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameters (mm) is the same across categories of Microbes.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

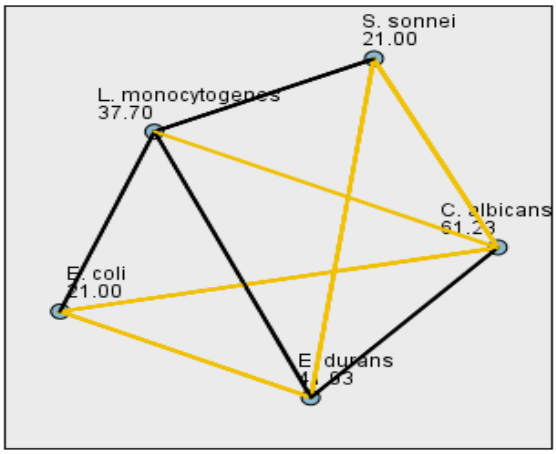
Asymptotic significances are displayed. The significance level is .05.



Total N	73
Test Statistic	44.185
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

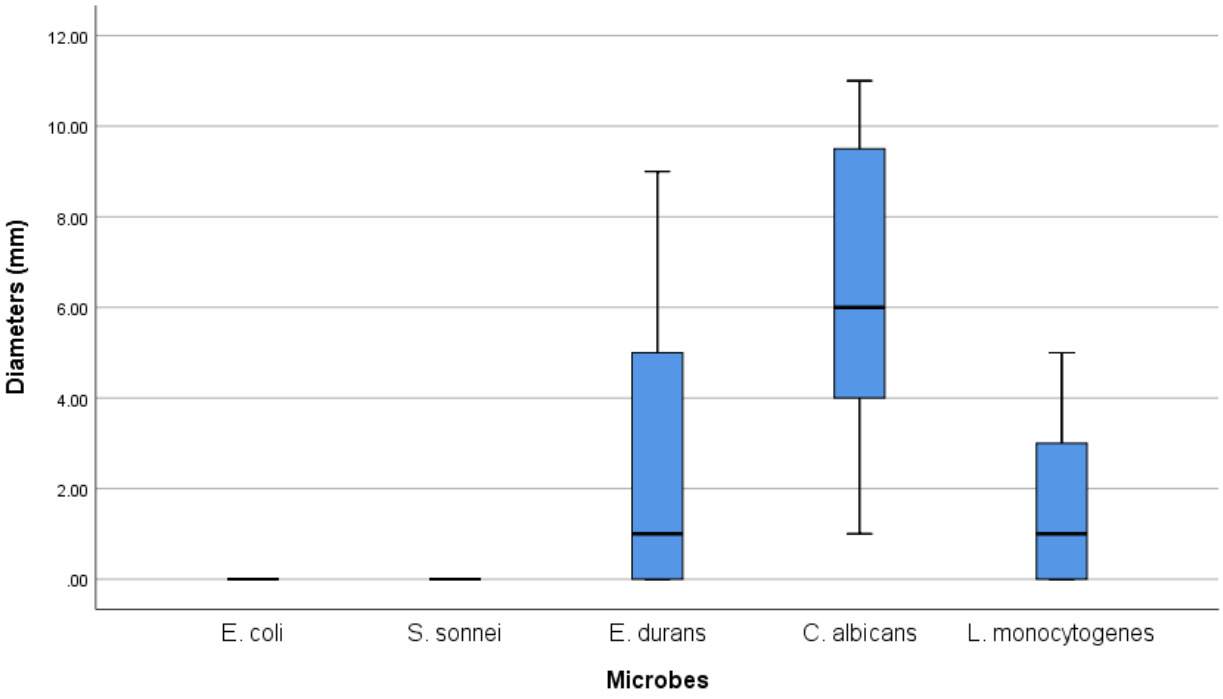
Pairwise Comparisons of Microbes



Each node shows the sample average rank of Microbes.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
E. coli-S. sonnei	.000	7.270	.000	1.000	1.000
E. coli-L. monocytogenes	-16.700	7.148	-2.336	.019	.195
E. coli-E. durans	-20.933	7.148	-2.928	.003	.034
E. coli-C. albicans	-40.233	7.148	-5.628	.000	.000
S. sonnei-L. monocytogenes	-16.700	7.148	-2.336	.019	.195
S. sonnei-E. durans	-20.933	7.148	-2.928	.003	.034
S. sonnei-C. albicans	-40.233	7.148	-5.628	.000	.000
L. monocytogenes-E. durans	4.233	7.024	.603	.547	1.000
L. monocytogenes-C. albicans	23.533	7.024	3.350	.001	.008
E. durans-C. albicans	-19.300	7.024	-2.748	.006	.060

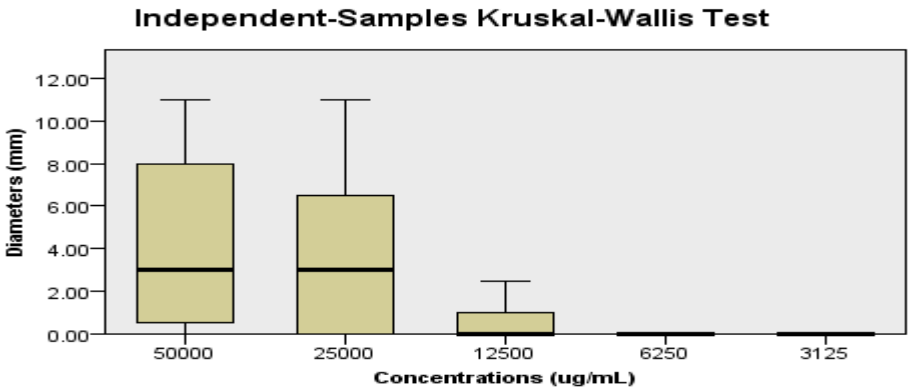
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.



Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameters (mm) is the same across categories of Concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

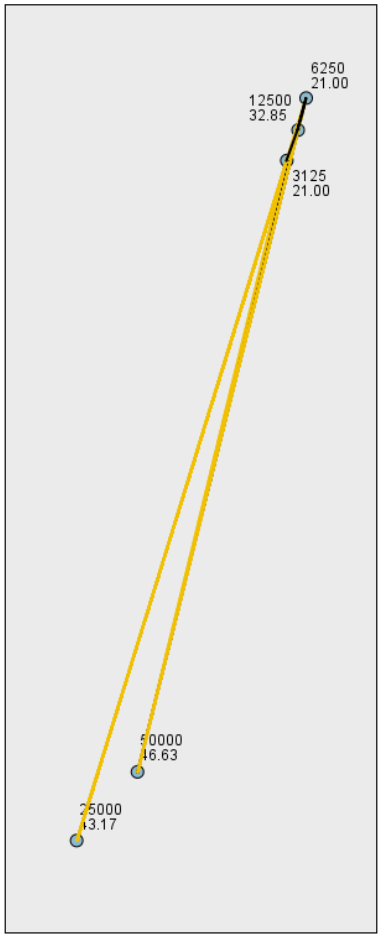
Asymptotic significances are displayed. The significance level is .05.



Total N	67
Test Statistic	26.429
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

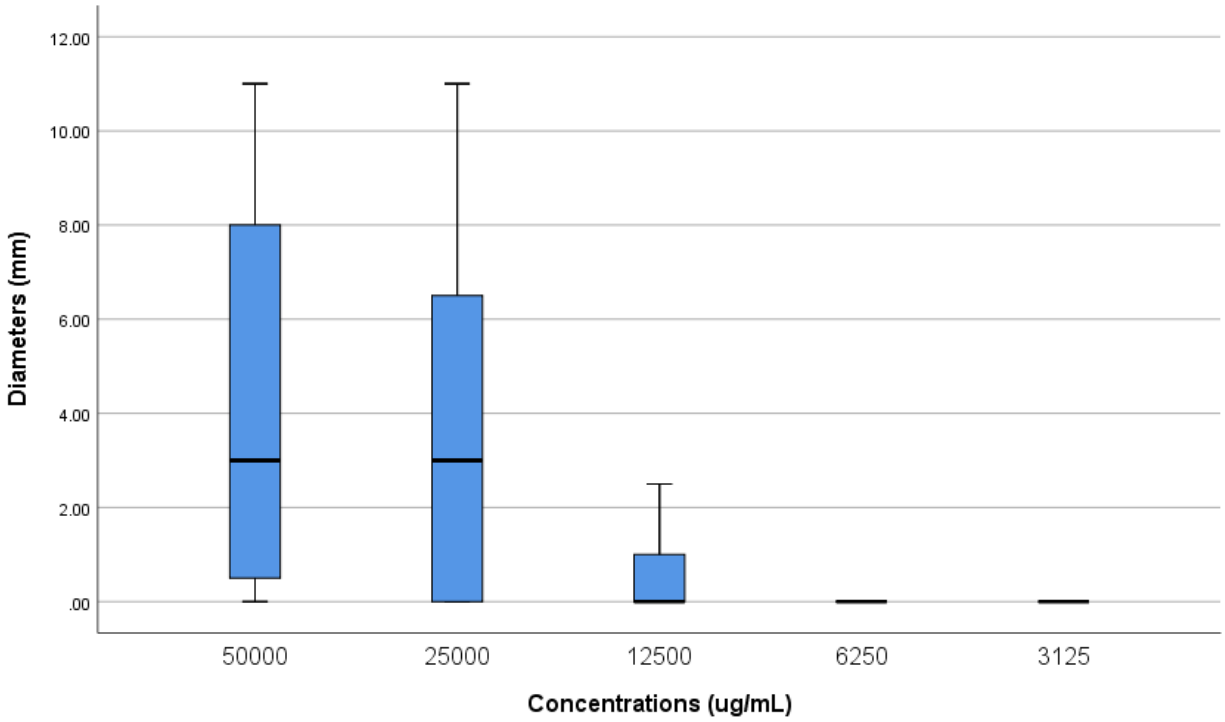
Pairwise Comparisons of Concentrations (ug/mL)



Each node shows the sample average rank of Concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
6250-3125	.000	6.981	.000	1.000	1.000
6250-12500	11.846	6.845	1.731	.084	.835
6250-25000	22.167	6.623	3.347	.001	.008
6250-50000	25.633	6.623	3.871	.000	.001
3125-12500	11.846	6.845	1.731	.084	.835
3125-25000	22.167	6.623	3.347	.001	.008
3125-50000	25.633	6.623	3.871	.000	.001
12500-25000	10.321	6.480	1.593	.111	1.000
12500-50000	13.787	6.480	2.128	.033	.334
25000-50000	3.467	6.244	.555	.579	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.



H_2O extracts

Tests of Normality

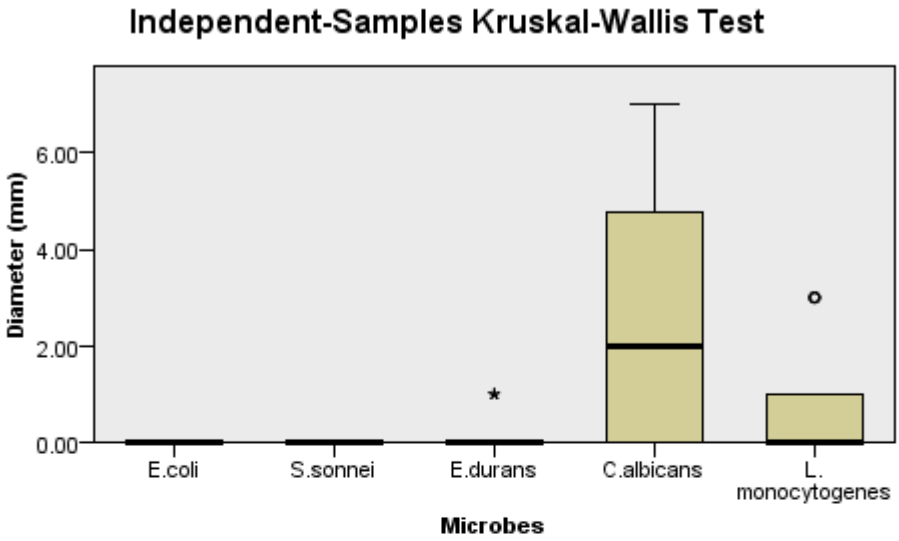
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Diameter (mm)	.441	75	.000	.506	75	.000

a. Lilliefors Significance Correction

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Microbes.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

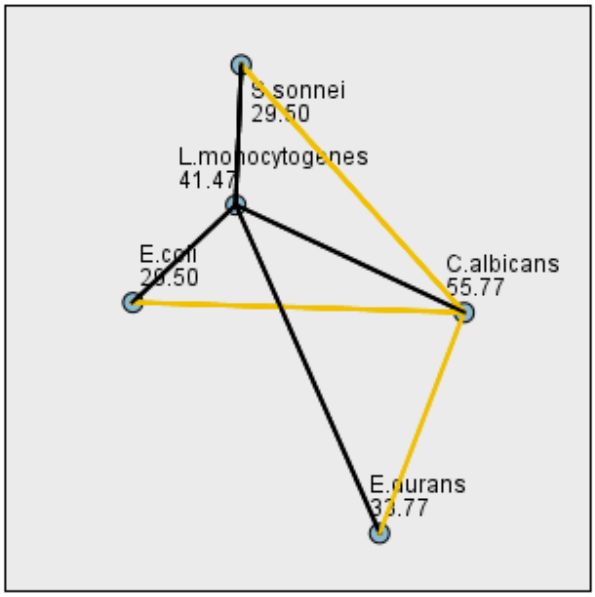
Asymptotic significances are displayed. The significance level is .05.



Total N	75
Test Statistic	28.821
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

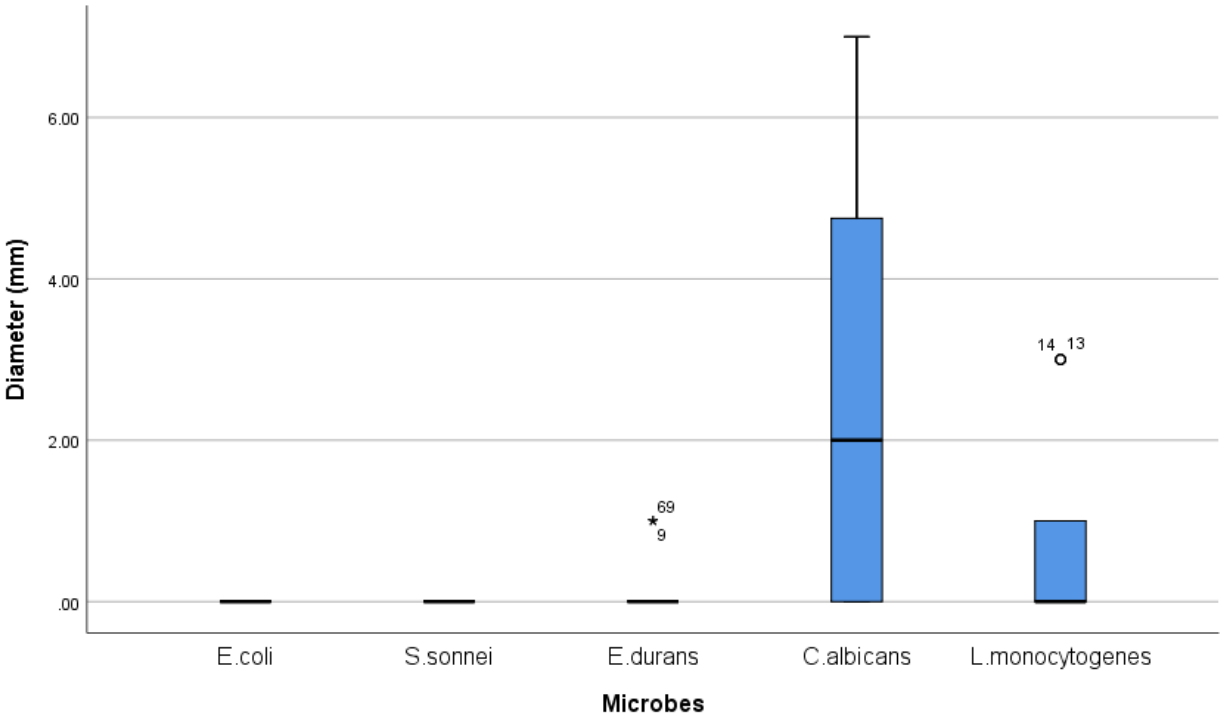
Pairwise Comparisons of Microbes



Each node shows the sample average rank of Microbes.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
E.coli-S.sonnei	.000	5.832	.000	1.000	1.000
E.coli-E.durans	-4.267	5.832	-.732	.464	1.000
E.coli-L.monocytogenes	-11.967	5.832	-2.052	.040	.402
E.coli-C.albicans	-26.267	5.832	-4.504	.000	.000
S.sonnei-E.durans	-4.267	5.832	-.732	.464	1.000
S.sonnei-L.monocytogenes	-11.967	5.832	-2.052	.040	.402
S.sonnei-C.albicans	-26.267	5.832	-4.504	.000	.000
E.durans-L.monocytogenes	-7.700	5.832	-1.320	.187	1.000
E.durans-C.albicans	-22.000	5.832	-3.772	.000	.002
L.monocytogenes-C.albicans	14.300	5.832	2.452	.014	.142

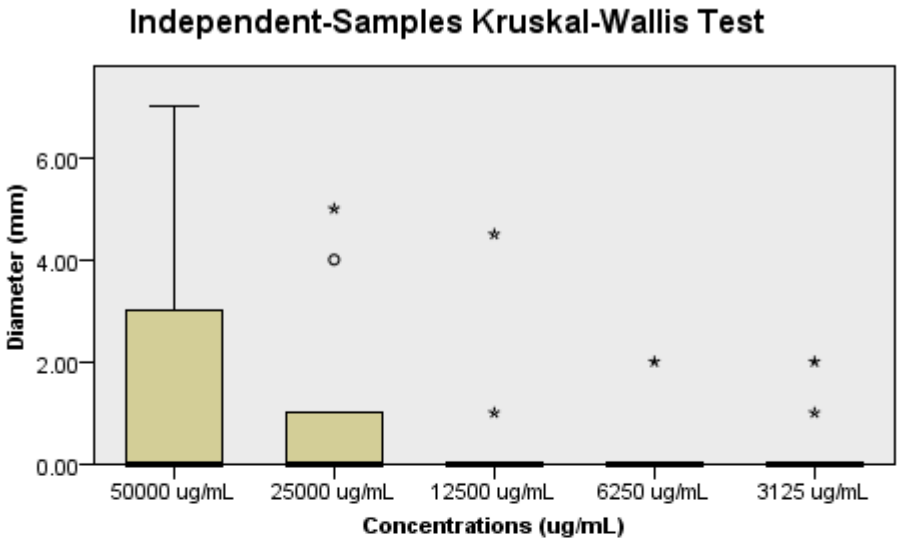
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.



Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.030	Reject the null hypothesis.

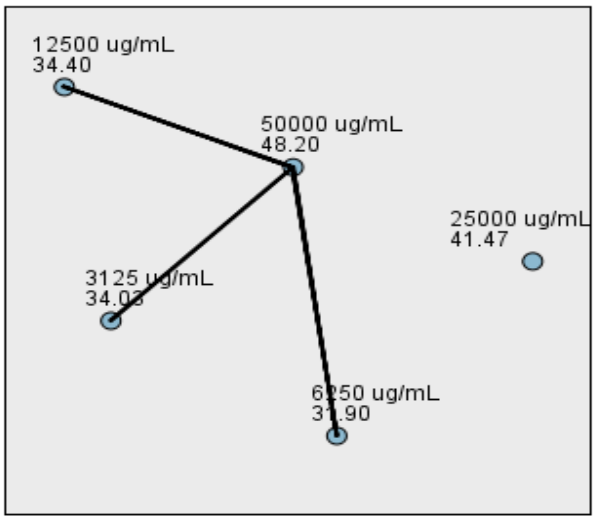
Asymptotic significances are displayed. The significance level is .05.



Total N	75
Test Statistic	10.701
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.030

1. The test statistic is adjusted for ties.

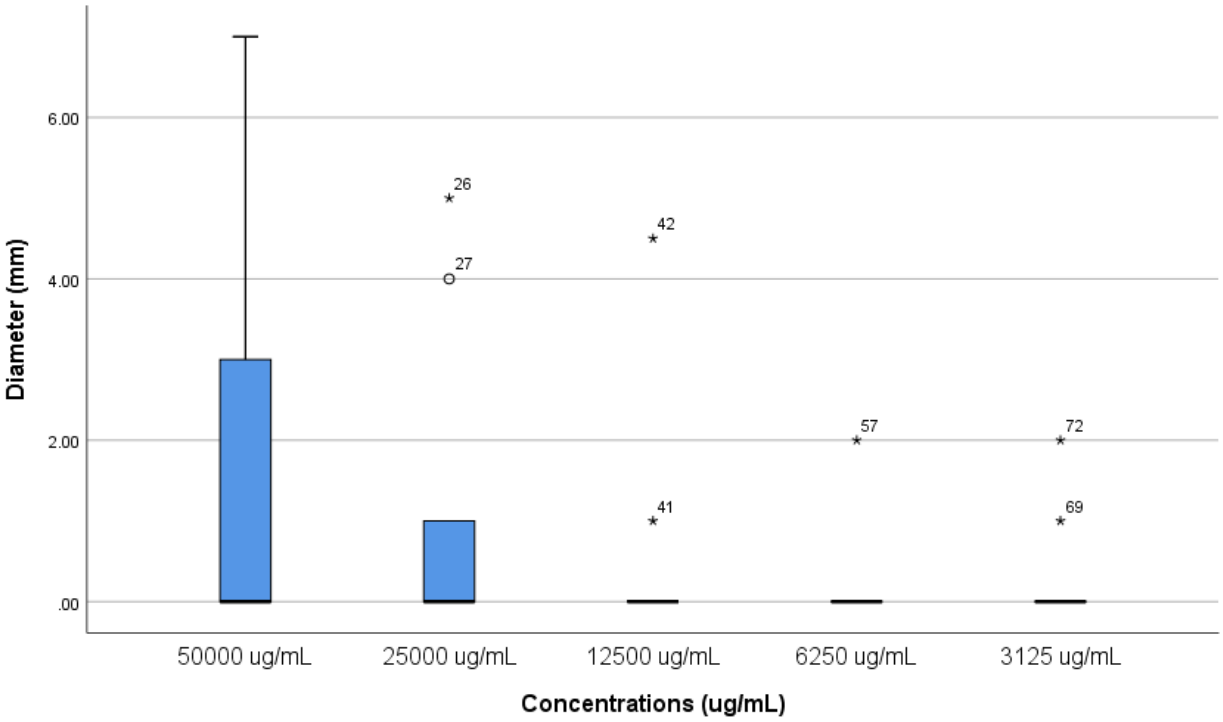
Pairwise Comparisons of Concentrations (ug/mL)



Each node shows the sample average rank of Concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
6250 ug/mL-3125 ug/mL	-2.133	5.832	-.366	.715	1.000
6250 ug/mL-12500 ug/mL	2.500	5.832	.429	.668	1.000
6250 ug/mL-25000 ug/mL	9.567	5.832	1.640	.101	1.000
6250 ug/mL-50000 ug/mL	16.300	5.832	2.795	.005	.052
3125 ug/mL-12500 ug/mL	.367	5.832	.063	.950	1.000
3125 ug/mL-25000 ug/mL	7.433	5.832	1.275	.202	1.000
3125 ug/mL-50000 ug/mL	14.167	5.832	2.429	.015	.151
12500 ug/mL-25000 ug/mL	7.067	5.832	1.212	.226	1.000
12500 ug/mL-50000 ug/mL	13.800	5.832	2.366	.018	.180
25000 ug/mL-50000 ug/mL	6.733	5.832	1.155	.248	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.

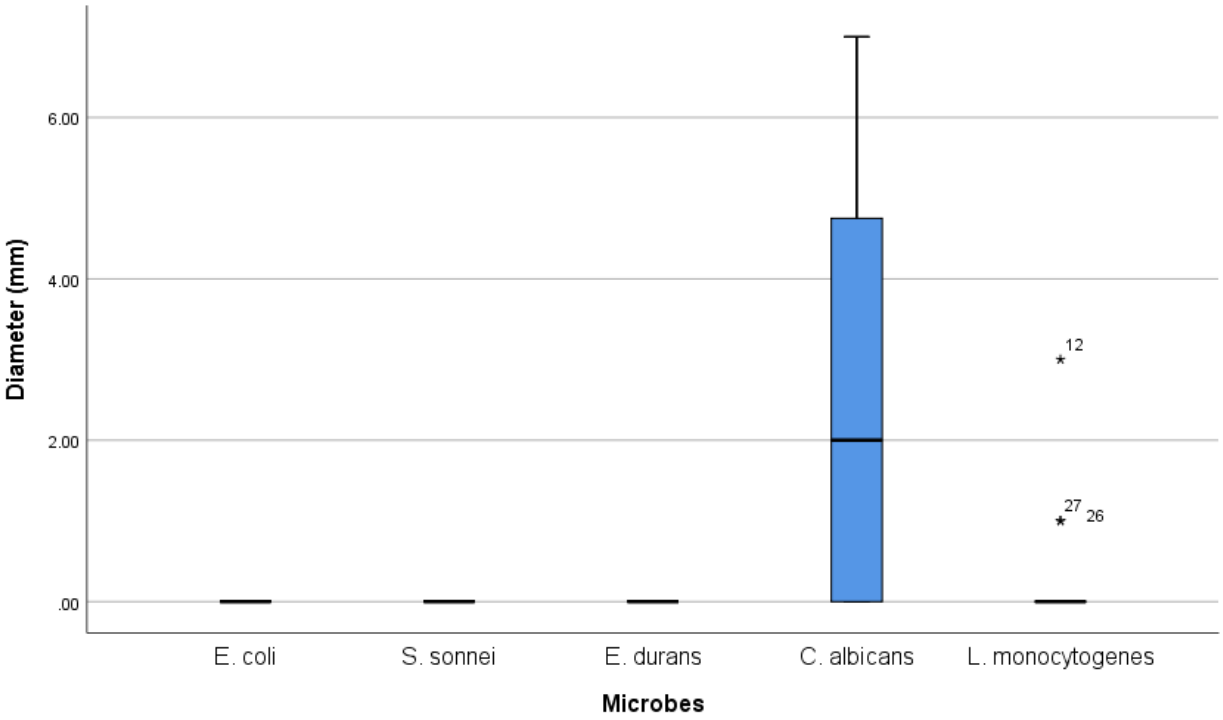


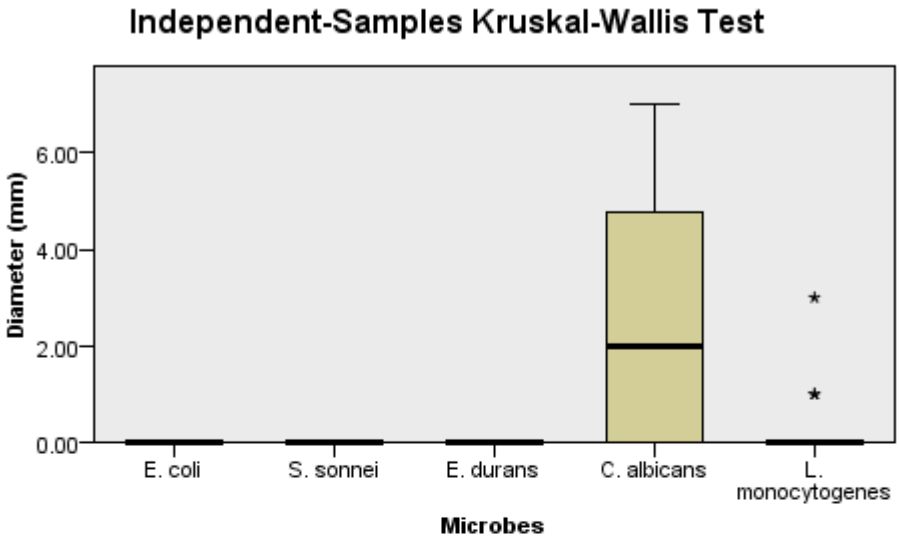
After removing the outliers

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Microbes.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

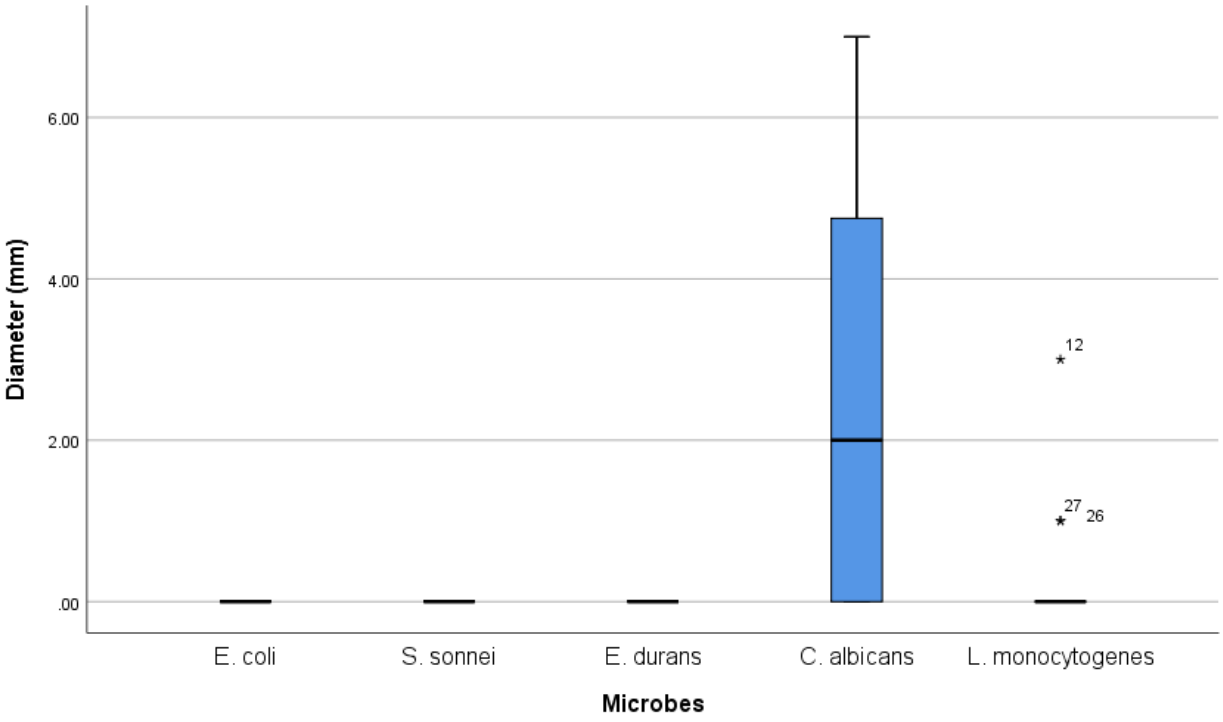
Asymptotic significances are displayed. The significance level is .05.


Diameter (mm)
6.00
4.00
2.00
.00
12
27
26
E. coli
S. sonnei
E. durans
C. albicans
L. monocytogenes
Microbes



Total N	71
Test Statistic	33.889
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

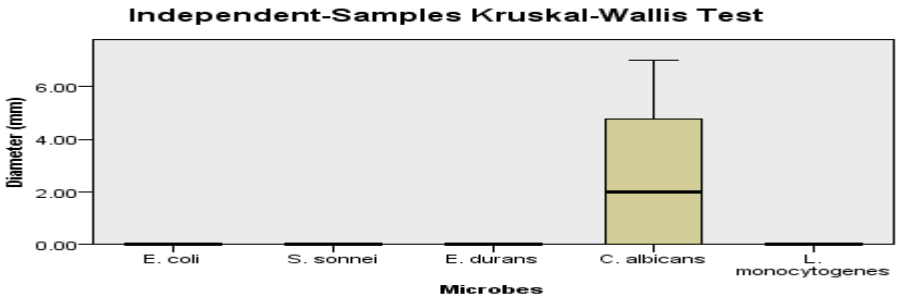
1. The test statistic is adjusted for ties.



Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameter (mm) is the same across categories of Microbes.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

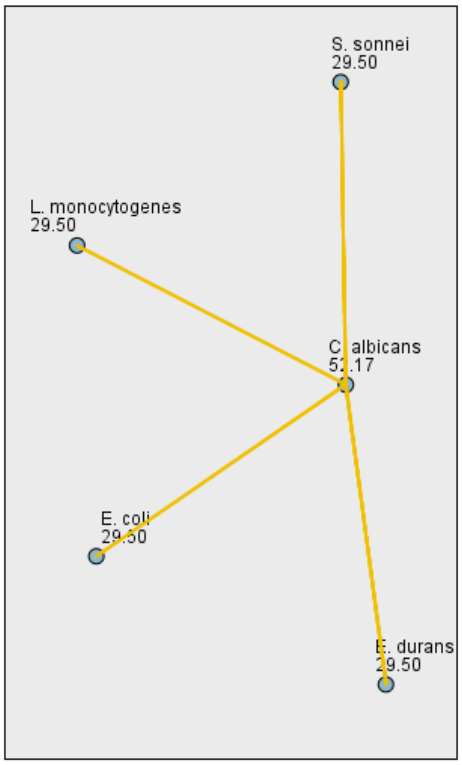
Asymptotic significances are displayed. The significance level is .05.



Total N	68
Test Statistic	40.484
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

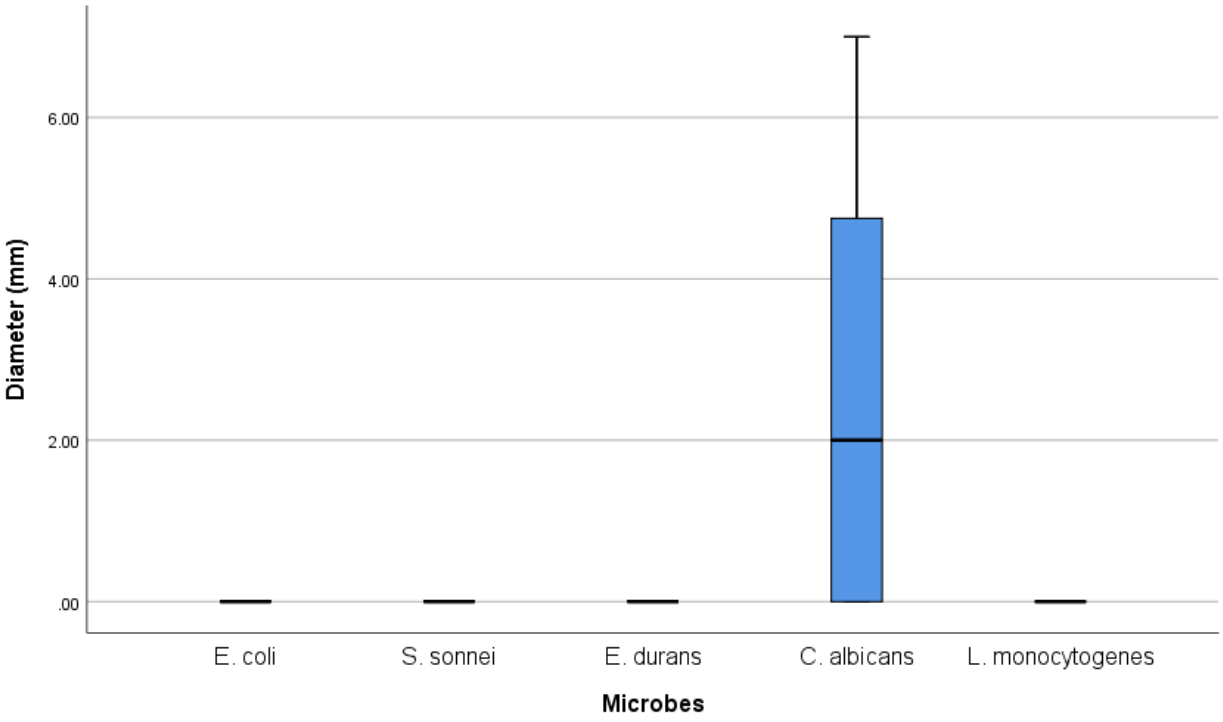
Pairwise Comparisons of Microbes



Each node shows the sample average rank of Microbes.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
E. coli-S. sonnei	.000	4.448	.000	1.000	1.000
E. coli-E. durans	.000	4.616	.000	1.000	1.000
E. coli-L. monocytogenes	.000	4.973	.000	1.000	1.000
E. coli-C. albicans	-22.667	4.448	-5.096	.000	.000
S. sonnei-E. durans	.000	4.616	.000	1.000	1.000
S. sonnei-L. monocytogenes	.000	4.973	.000	1.000	1.000
S. sonnei-C. albicans	-22.667	4.448	-5.096	.000	.000
E. durans-L. monocytogenes	.000	5.124	.000	1.000	1.000
E. durans-C. albicans	-22.667	4.616	-4.911	.000	.000
L. monocytogenes-C. albicans	22.667	4.973	4.558	.000	.000

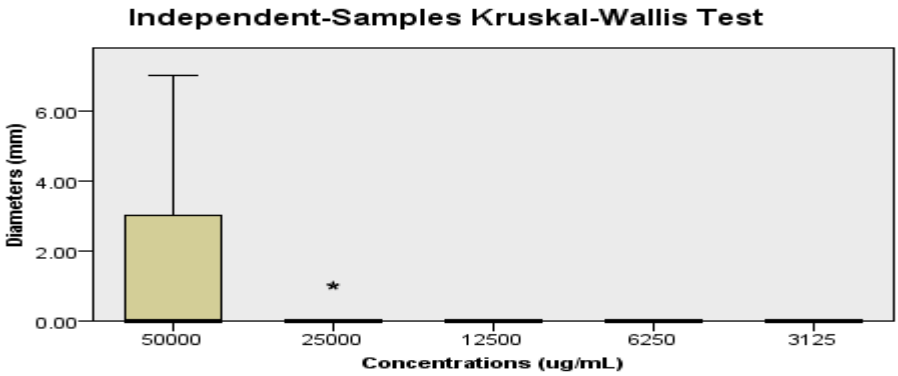
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.



Hypothesis Test Summary

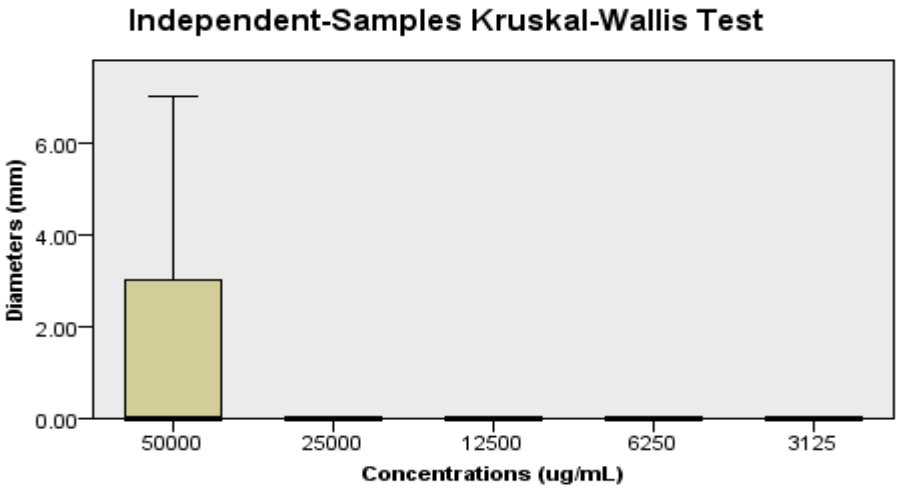
	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Diameters (mm) is the same across categories of Concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.



Total N	68
Test Statistic	20.450
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

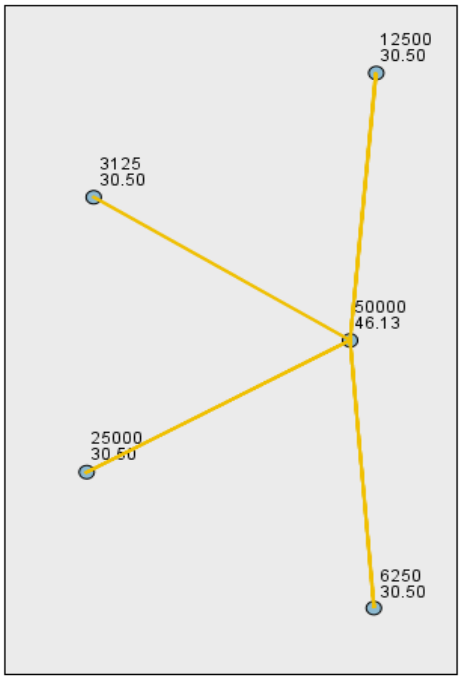
1. The test statistic is adjusted for ties.



Total N	67
Test Statistic	26.595
Degrees of Freedom	4
Asymptotic Sig. (2-sided test)	.000

1. The test statistic is adjusted for ties.

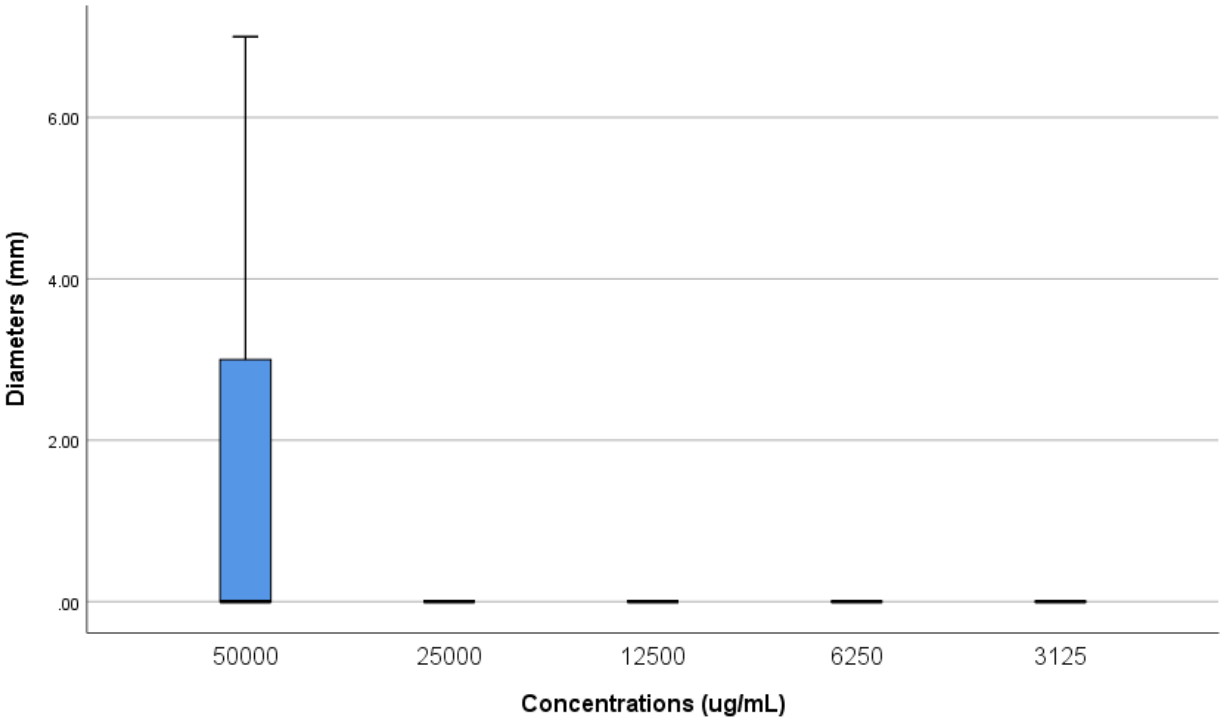
Pairwise Comparisons of Concentrations (ug/mL)



Each node shows the sample average rank of Concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
25000-12500	.000	4.351	.000	1.000	1.000
25000-6250	.000	4.283	.000	1.000	1.000
25000-3125	.000	4.223	.000	1.000	1.000
25000-50000	15.633	4.223	3.702	.000	.002
12500-6250	.000	3.984	.000	1.000	1.000
12500-3125	.000	3.919	.000	1.000	1.000
12500-50000	15.633	3.919	3.989	.000	.001
6250-3125	.000	3.844	.000	1.000	1.000
6250-50000	15.633	3.844	4.067	.000	.000
3125-50000	15.633	3.777	4.139	.000	.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.


Diameters (mm)
6.00
4.00
2.00
.00
50000
25000
12500
6250
3125
Concentrations (ug/mL)

APPENDIX J: Cytotoxicity statistical analysis

Tests of Normality

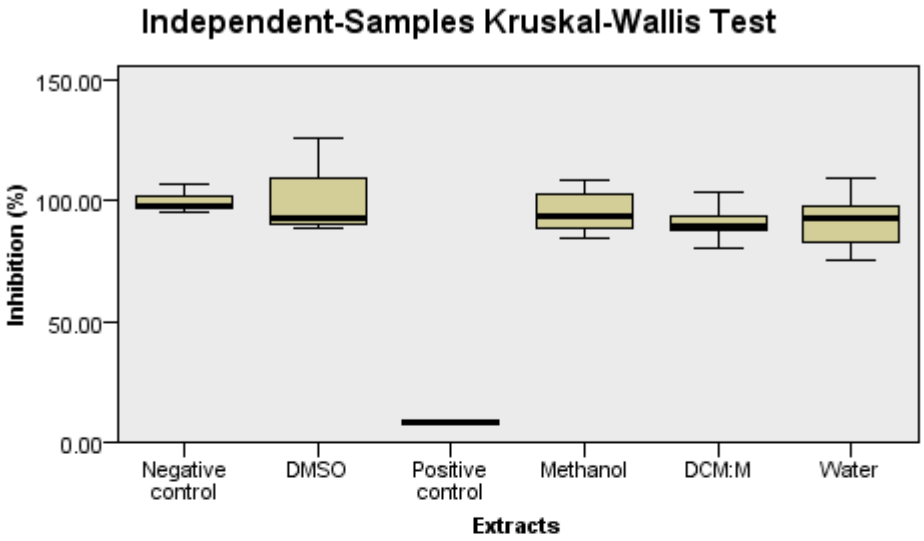
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Inhibition (%)	.257	81	.000	.627	81	.000

a. Lilliefors Significance Correction

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Inhibition (%) is the same across categories of Extracts.	Independent-Samples Kruskal-Wallis Test	.003	Reject the null hypothesis.

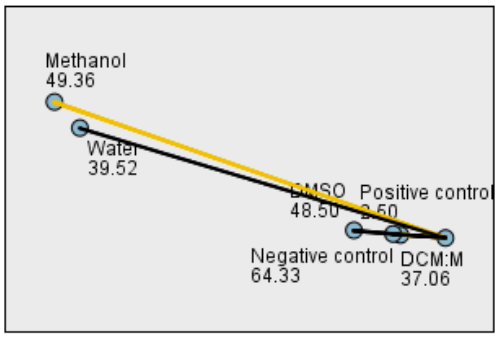
Asymptotic significances are displayed. The significance level is .05.



Total N	81
Test Statistic	17.617
Degrees of Freedom	5
Asymptotic Sig. (2-sided test)	.003

1. The test statistic is adjusted for ties.

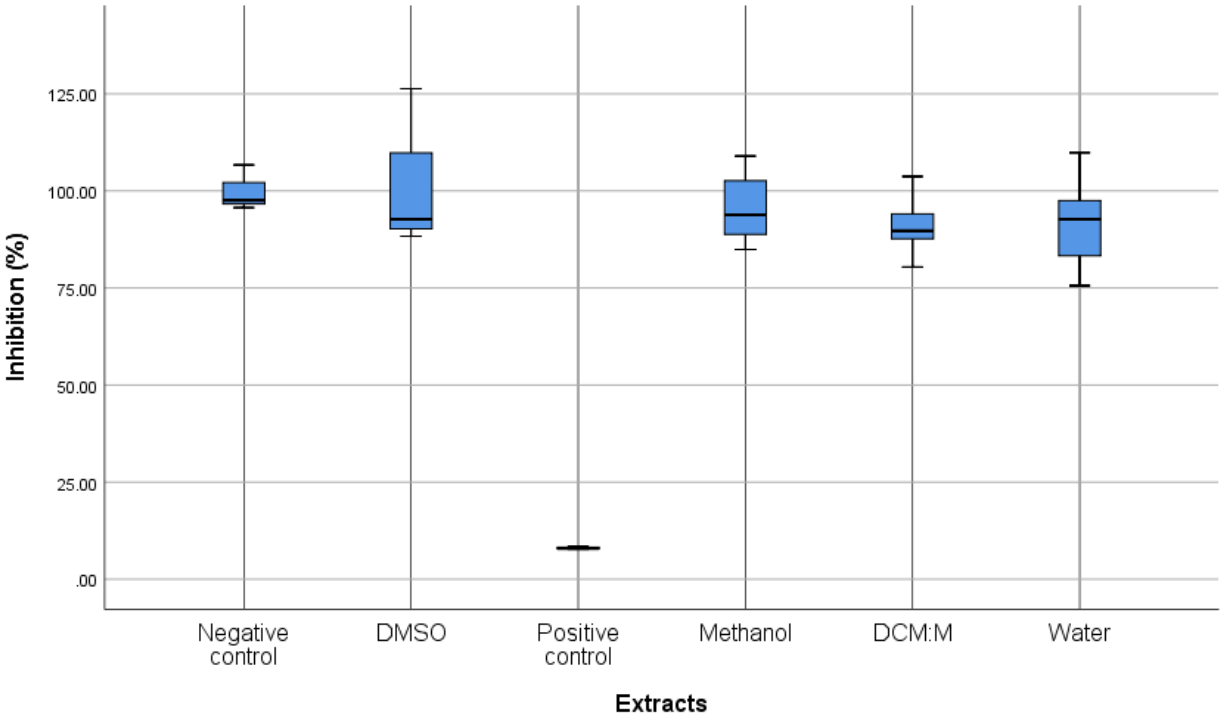
Pairwise Comparisons of Extracts



Each node shows the sample average rank of Extracts.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
Positive control-DCM:M	-34.562	12.706	-2.720	.007	.098
Positive control-Water	-37.021	12.706	-2.914	.004	.054
Positive control-DMSO	46.000	16.636	2.765	.006	.085
Positive control-Methanol	-46.864	12.788	-3.665	.000	.004
Positive control-Negative control	61.833	17.969	3.441	.001	.009
DCM:M-Water	-2.458	6.791	-.362	.717	1.000
DCM:M-DMSO	11.438	12.706	.900	.368	1.000
DCM:M-Methanol	12.301	6.944	1.771	.076	1.000
DCM:M-Negative control	27.271	14.407	1.893	.058	.876
Water-DMSO	8.979	12.706	.707	.480	1.000
Water-Methanol	9.843	6.944	1.417	.156	1.000
Water-Negative control	24.812	14.407	1.722	.085	1.000
DMSO-Methanol	-.864	12.788	-.068	.946	1.000
DMSO-Negative control	15.833	17.969	.881	.378	1.000
Methanol-Negative control	14.970	14.479	1.034	.301	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.

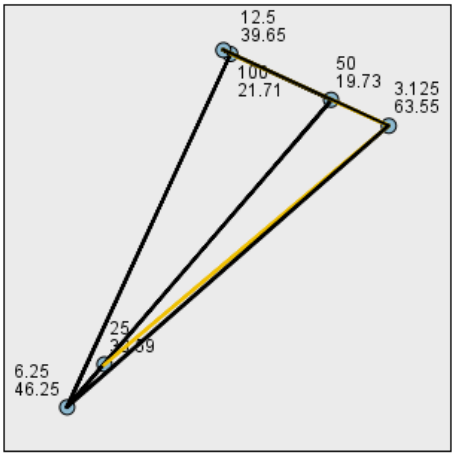


Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Inhibition (%) is the same across categories of Concentrations (ug/mL).	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

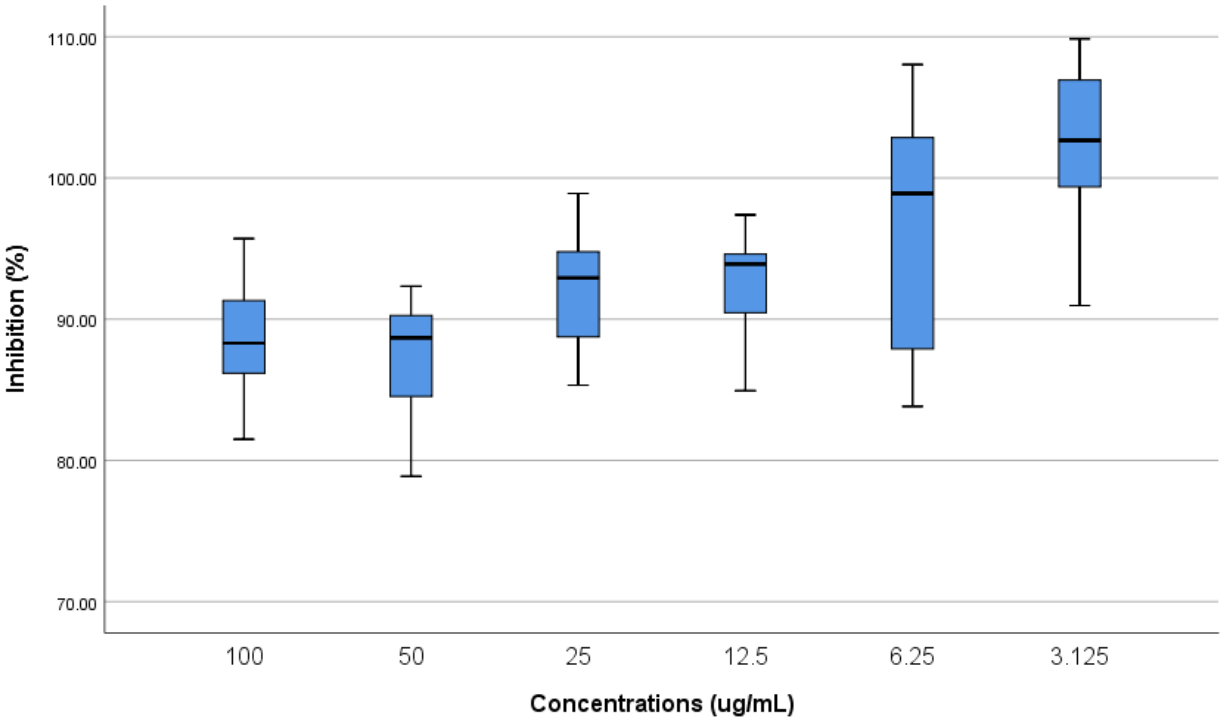
Pairwise Comparisons of Concentrations (ug/mL)



Each node shows the sample average rank of Concentrations (ug/mL).

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
50-100	1.987	9.361	.212	.832	1.000
50-25	-13.864	9.907	-1.399	.162	1.000
50-12.5	-19.923	10.152	-1.962	.050	.746
50-6.25	-26.523	9.699	-2.735	.006	.094
50-3.125	-43.818	8.580	-5.107	.000	.000
100-25	-11.877	9.361	-1.269	.205	1.000
100-12.5	-17.936	9.620	-1.864	.062	.934
100-6.25	-24.536	9.140	-2.684	.007	.109
100-3.125	-41.831	7.943	-5.266	.000	.000
25-12.5	-6.059	10.152	-.597	.551	1.000
25-6.25	-12.659	9.699	-1.305	.192	1.000
25-3.125	-29.955	8.580	-3.491	.000	.007
12.5-6.25	-6.600	9.948	-.663	.507	1.000
12.5-3.125	-23.895	8.861	-2.697	.007	.105
6.25-3.125	-17.295	8.338	-2.074	.038	.571

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.


110.00
100.00
90.00
80.00
70.00
Inhibition (%)
100
50
25
12.5
6.25
3.125
Concentrations (ug/mL)