بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Department of Plant Protection

In-Vitro Efficacy of two Plant Extracts and Amoxicillin against *Xanthomonas citri* pv. *citri* causing Citrus Bacterial Canker Disease in Lime (*Citrus aurantifolia)*

الفاعلية المختبرية لأثنين من المستخلصات النباتية واموكسيسلين ضد البكتيريا المسببة لمرض التقرح البكتيري علي الليمون (*Xanthomonas citri* pv. *citri*)

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Approval Page

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بسم الله الرحمن الرحيم

قال تعالي:

(وَآيَةٌ لَهُمُ الْأَرْضُ الْمَيْتَةُ أَحْيَيْناها وَأَخْرَجْنا مِنْها حَبًّا فَمِنْهُ يَأْكُلُونَ(33) وَجَعَلْنا فِيها جَنَّاتٍ مِنْ نَخِيلٍ وَأَعْنابٍ وَفَجَّرْنا فِيها مِنَ الْعُيُون (34) لِيَأْكُلُوا مِنْ ثَمَرِهِ وَما عَمِلَتْهُ أَيْدِيهِمْ أَفَلا يَشْكُرُونَ (35) سورة يس (الآيات 33- 35)

صدق الله العظيم

DEDICATION

To my Family with deep love

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ABSTRACT

This study was conducted under the laboratory condition at the Plant Pathology Laboratory of the Department of plant protection, college of agricultural studies, Sudan University of science and technology. Some experiments are conducted at the plant pathology laboratory, Shambat Research Station.

The aim of this experiment is to evaluate the effect of some plant aqueous extracts Roselle (*Hibiscus subdariffa* Linn) and the bark of Cinnamon (*Cinnamomum verum*) on the growth of the bacterium *Xanthomonas citri* pv.*citri* under laboratory conditions.

Different concentrations (25% , 12.5% and 6.25%) of each of the tested plants, in addition, to the Antibiotic, penamox (Amoxicillin 4%) were used and compared to the untreated control.

The results showed that the concentrations (25% and 12.5%) of the aqueous extracts of both tested plants in addition to antibiotic, penamox (Amoxicillin) recorded a high significant ($P<0.05$) inhibitory effect on the bacterial growth.

The isolated pathogen was proved to be a bacterium *Xanthomonas citri* pv.*citri* according to its properties morphological.

ملخص البحث

أجريت هذه الدراسة بمعمل أمراض النبات – قسم وقاية النبات ، كلية الدراسات الزراعية ، جامعة السودان للعلوم والتكنولوجيا. بعض التجارب الأخرى أجريت في معمل أمراض النبات بمحطة أبحاث شمبات.

هدفت هذه التجربة إلي معرفة ودراسة الأثر التثبيطي لبعض المستخلصات المائية لنباتات الكركدي والقرفة بالإضافة للمضاد الحيوي بيناموكس (اموكسيلين 4%) على نمو بكتريا التقرح *Xanthomonas citri* pv.*citri* ومن ثم قورنت النتائج بالشاهد غير المعامل.

استخدمت تراكيز مختلفة (25% , 12.5% و 6.25%) من كلا النباتين لأوراق نبات الكركدي ولحاء القرفة لتثبيط نمو البكتيريا *Xanthomonas citri* pv.*citri* , بالإضافة إلي الشاهد والمضاد الحيوي بيناموكس (Amoxicillin 4%) ضد نمو البكتيريا.

أظهرت النتائج أن تركيزات (25% و 12.5%) من المستخلصات المائية للنباتين المختبرين بالإضافة الي المضاد الحيوي ، بيناموكس (اموكسيسلين) سجلت تأثيرا مثبطا كبيرا ($P<0.05$) علي نمو البكتيريا .

وقد ثبت أن الممرض المعزول هو بكتيريا *Xanthomonas citri* pv.*citri* وفقا لخصائصه المورفلوجية.

CHAPTER ONE

INTRODUCTION

Citrus is one of the most important and commercial fruit crops world-wide (Kim *et al.*, 2005). It belongs to the family Rutaceae and it is thought to be native of the subtropical regions of Southeast Asia (Swingle, 1949). World production of citrus fruit has experienced continuous growth in the last decades of the 20th century, where the total annual citrus production was estimated as > 123 million tons in the period 2009 -2010 harvested from about 8.7 million hectors (FAOSTAT, 2012). Most citrus fruits are produced for fresh market consumption (UNCTAD, 2013). The largest citrus producers are Brazil (20 %), U.S.A. (14%), China (12%), Mexico (6%) and the countries of the Mediterranean Basin (15%) (FAOSTAT, 2006). Sudan lies within the citrus belt and appears to possess enormous potentialities in Citriculture. The commercial citrus production spreads all over the country including Gadaref State in eastern Sudan (Mahdi, 1979). The total area of citrus production is estimated over 170,000 ha producing annually around 2,400,000 metric tons with a sizable export, mainly to the Middle East countries and Turkey (National Horticulture Administration, 2013).

Citrus is subjected to pests and diseases in Sudan . Among the most important diseases of citruses are : citrus tristeza , citrus viroids and citrus bacterial canker (Abu Baker,2009., Elhassan *et al* ; 2014) . Citrus bacterial canker disease (CBCD) caused by *Xanthomonas citri* pv.*citri (Xcc*) is globally distributed and highly contagious bacterial disease of citrus species (Civerolo, 1984; Leite and Mohan 1990; Gottwald *et al*., 2002b). It has a substantial impact on the citrus industry, particularly fresh fruit production, processing and international trade (Stall and Seymour, 1983; Leite and mohan , 1990). The disease caused heavy losses in quality and quantity when infection occurs at

early stage of plant growth , the disease symptoms are characterized by raised circular necrotic lesions that develop on leaves, twigs, and fruits, and by manifestation of corky cankerous lesion on main branches and tree stems. Several infection result in defoliation , die-back , scabby fruit which are less valuable or entirely unmarketable leading to premature fruit drop (Stall and Seymour , 1983; Civerolo, 1984). Citrus canker has had a serious impact on local citrus industries whenever infections have been detected. As a result, millions of dollars are spent annually on prevention, quarantine, eradication programs and chemical control (Graham and Gottwald, 1991).

However, CBCD has not been reported in Sudan before autumn 2013 (Elhassan, *et al,* 2014).

The main objectives based on the above-mentioned points of this study were to

1- Isolate the Pathogen causing the Citrus Bacterial Canker Disease on lime.

2- Evaluate the *in -vitro* effect of two botanical extracts and antibiotic, penamox (Amoxicillin) against the causing bacteria.

CHAPTER TWO

LITERTURE REVIEW

2.1. The Citrus Industry

Citrus spp. is the largest genus in the family Rutaceae and citrus crops are first on value among fruit products in the international trade (Timmer *et al.,* 2000a). Taxonomic identification is difficult because there are many spontaneous and commercial hybrids but citrus can be generally classified into the following categories : sweet orange (most are *C.sinenisis* but also includes blood and acid less oranges), mandarins (such as Satsuma (*C. unshi*) , tangerines (*C. tangerina* ,and Clementine's (*C. Clementine*), sour/bitter oranges (such as Seville, *C. aurantium*), lemons (*C. Limón*) limes (*C.aurantifolia*) grapefruit (*C.paradisi*) and pummelo (*C. grandis*) , hybrids (e.g tangelos , tangors and citrons (*C. medica*) which has a rind that is used primarily for confectionary and is only commercially grown in limited areas). Citrus species originate from the Southeast Asia –India region. Today citrus is produced in more than 140 countries, mainly between 40° N and 40°S latitudes. Citrus production has been on the rise throughout the second half of the 20th century, and the total citrus production of the world is estimated as>123 million tons per year, harvested from about 8.7 million hectares (FAOSTAT,2012). Orange (*Citrus sinensis* L. Osb) represents more than half of the total citrus production (56%), followed by tangerine (*C.reticulata* Blanco) and mandarin *(C.unshi*) (17%, lemon (*C.limon* (L.) Burm.), and (*C.aurantifolia* L.) (11%), grapefruit (*C.paradisi* Macf.) and pummelo (*C.grandis*) (10%). (FAOSTAT, 2012). Citrus was introduced to Sudan in 1900's where it was established at beginning in some of the government orchards in the Northern states. Sudan, with all its vast area, wide range of soils, diverse climatic conditions and ample water resource possesses

great potential for citrus industry, nowadays the commercial citrus production in Sudan spreads all over the country, mainly along the narrow trips of alluvial soils of the main River Nile, Blue Nile and White Nile. (Mahdi, 1979). Citrus species grown commercially in different parts in the Sudan includes: small fruited acid lime (*C.aurantifolia* L.), grapefruit (*C.paradisi* Macf.), sweet orange (*C.reticulate Blanco*). Each groups

is composed of number of varieties and selections (Ali –Dinar), 1984).

Citrus fruits and juices have great demands due to their several beneficial health and nutritive properties and they are rich in vitamin C and folic acid, and also citrus fruit including a good source of fiber. They are free of fat, sodium and cholesterol. in addition , they contain potassium , calcium , thiamin, niacin, vitamin B6, phosphorus , magnesium and copper . They may help to reduce the risk of heart diseases and some types of cancer (Kim *et al.,* 2005). The current production cannot keep pace with the ever-increasing demand for citrus industry, but Sudan possesses great potentialities for citrus production, especially if the national strategy of citrus expansion is directed towards the large national schemes viz.. Gezira suki , Rahad Blue Nile schemes in the central clay plain (Sidahmed and Geneif,1984).

2.2. Citrus tree requirements

Citrus trees require a rich well-drained soil , periodical fertilization and irrigation of soil as well as pruning of the tree (Timmer *et al.*,2000b), in the modern commercial producing plants are grafted onto rootstocks which influence adaptation to soil type, tree size production characteristics and can provide cold hardiness and resistance to diseases, and insects. Some important rootstock species are sour orange (*C. aurantium* L.) , trifoliate orange (Graham *et al.,* 2003).

2.2.1. Taxonomy of the tree

Kingdom : Plantae

Phylum : Angoiospermea

Class : Dicotyledonae

Family : Rutaceae

Genus : *Citrus aurantifolia*

(FAOSTAT, (2012).

2.2.2. Uses of citruses

Citrus fruit contain a variety of vitamins, minerals, fiber and photochemical such as carotenoids', flavonoids, and limonoids, which appear to have biological activities and health benefits. They have antioxidant and anti mutagenic properties and positive association with bone, cardiovascular health system (Kim *et al.,* 2005).

2.3. Citrus Diseases

Citrus canker is serious disease of most commercial citrus varieties and some relatives. It is not present in all subtropical and tropical region of citriculture in the world , so considerable regulatory efforts are expended to prevent the introduction and spread of the citrus canker bacteria into areas with climates conductive to the disease development (Graham *et al* .,2004).

Unfortunately, citrus fruit suffer from different diseases that may considerably affect the fruit crop by destroying the tree and/ or the fruit. World-wide, citrus is known to be infected by many diseases including citrus canker caused by the bacterium *Xanthomonas citri* pv.*citri*, huanglungbing (HLB) caused by the bacterium *Candidates liberibacter asiaticus,* stubborn disease(*Spiroplasma citri*), citrus variegation chlorosis (CVC) caused by *Xylella fastidiosa*, citrus bacterium spot *Xanthomonas alfalfa pv .citrumelonis* (*Xanthomonas axonopodis pv.citrumelo*) ,citrus black spot (*Guignardia citricarpa),*anthracnose (*Colletotrichum gloeosporioides)*, brown spot (*Alternaria alternata*), citrus scab caused by the fungus *Elsinoe fawcettii* (*Sphaceloma citri*) . Other species of limited economic importance because they are more localized, include the sting nematode *(Belonolaimus longicaudatus*) and two species of lesion Nematode (*Pratylenchus coffeae* and *P.brachyurus*) (Duncan *et al.*, 2014).

2.3.1. Citrus diseases in Sudan

Citrus is attacked by many pests and diseases in Sudan. Among the most important diseases of citruses are citrus tristeza, citrus viroids and citrus bacterial canker (Abu Baker, 2009. Elhassan *et al* , 2014).

Citrus bacterial canker disease (CBCD) caused by *Xanthomonas citri* pv. *citri (Xcc*) is globally distributed and highly contagious bacterial disease of citrus species (Civerolo, 1984; Leite and Mohan 1990; Gottwald *etal*., 2002a). It has

a substantial impact on the citrus industry, particularly fresh fruit production, processing and international trade (Stall and Seymour , 1983; Leite and mohan , 1990). The disease caused heavy losses in quality and quantity when infection occurs at early stage of plant growth. All young above - ground tissues of citrus are susceptible to *Xcc*. the disease symptom are characterized by raised circular necrotic lesions that develop on leaves , twigs ,and fruit , and by manifestation of corky cankerous lesion on main branches and tree stems. Several infection result in defoliation, die-back, scabby fruit which are less valuable or entirely unmarketable leading to premature fruit drop (Stall and Seymour, 1983; Civerolo, 1984). Also, citrus canker has had a serious impact on local citrus industries whenever infections have been detected. As a result, millions of dollars are spent annually on prevention, quarantine, eradication programs and chemical control (Graham and Gottwald, 1991).

2.3.2. Fungal diseases

Citrus is susceptible to a number of fungal and fungal-like pathogens. *Fusarium spp., Phytophthora spp., Phoma tracheiphila*, and Postharvest *(Penicillium, Aspergillus, Galactomyces citri-aurantii, Alternaria citri, Glomerella cinguala , Botryotinia fuckeliana* and *Botryospaeria rhodin*a) are regarded as the more important pathogens of regions(OEPP/EPPO2004).

2.3.3. Virus and virus-like diseases

Citrus is subjected to over 40 viruses and virus-like and presumed virus pathogen (Timmer *et al*.2000). However, three pathogens, *Citrus tristeza virus* , *Citrus psorosis virus*, and *Citrus leprosies virus*, responsible for the diseases tristeza, psorosis and leprosies, respectively , are considered economically important and cause major losses in important citrus – growing regions. Tatterleaf, exocortis and cachexia are also quarantine risks.

2.3.4. Bacterial diseases

Xanthomonas axonopodis, Xyella fastidiosa , and *Candidatus Liberibacter* are listed as etiological agents of economically important citrus diseases . *Candidatus Liberibacter* ,by far , is the most destructive to citrus production globally various forms of the pathogen are situated in specific regions based on prevailing environmental conditions and insect vectors. Similarly, *Xanthomonas and Xyella* members of the same host, but induce different environment (Randall *et al*., 2007).

2.3.5. Arthropod pests

Insect species belonging to various orders are associated with the flowers, shoots, roots, of citrus. Insects –like Citrus leaf miner (*Phyllocnistis citrella),* Psyllids (*Diaphorina citri)* , Lime swallowtail butterfly (*Papilio* spp.) Root weevils, several species of root weevils (Coleoptera: Curculionidea) (Lacey and Shapiro-llan, 2003).

2.3.6. Nematode diseases

Eight genera of plant parasitic nematodes of destructive potential are associated with various citrus crops (Hutton *et al*. Duncan1999; 1982). However, two nematode species, *Tylenchulus semipenetrans,* commonly called the citrus nematode or the citrus root nematode, and *Radopholus citrophilus* (Loof ,1991).

The burrowing nematode of citrus, are considered to be predominantly responsible for damage to *Citrus* spp. While *T.semipenetrans* is a widely occurring nematode that is found at every location where *Citrus* spp. are grown. *T.semipenetrans* causes the condition referred to as slow decline of citrus (SDC), whereas *R.citrophilus* causes the condition called spreading decline (SD), from as far back as 1913, there is evidence that *T.semipenetrans* (Siddiqi

1973). SD has been known since 1928, but was not linked to the nematode until 25 years later (Orton Williams and Siddiqi 1973).

2.4. History and distribution of the canker disease

The geographical distribution of *X. citri* pv. *citri* differs from different types of citrus canker, canker A (Asiatic canker) is found in Asia, south America, Oceania and the USA (Carrera, 1933). Canker B (Cankerosis B) in South America (Carrera, 1933). Canker C (Mexican lime Cankerosis C) in Brazil (Namekata, 1971); and canker D (Citrus bacteriosis) Mexico (Rodriguez *et al.,* 1985).

Citrus bacterial canker disease (CBCD) is one of the most economically demanding and wide-spread disease of citrus globally. It is originated in south east Asia and now occurs in more than 30 countries in Asia , the pacific and Indian island , south America ,Africa and south eastern USA (Civerolo , 1984 ; Verniere *et al.,* 1998;Gottwald *et al ;*2002a) .

2.5. The pathogen

The bacterium *Xanthomonas citri. pv.citri* (*Xcc*) is rod shaped measuring 1.5-2.0 x 0.5- 0.75µm Gram-negative, and has a single polar flagellum. It is obligatory aerobic bacterium. The optimum temperature range for growth is 28-30C°, and maximum temperature for growth is 35C-39C . (EPPO,2006).

2.5.1. Taxonomic information of the pathogen

Xanthomonas citri pv. *citri* (Gabriel *et al.,* 1989; Schaad *et al.,* 2007).
Some of the known synonyms are: *Xanthomonas smithii* subsp.*citri* (Gbriel *et al.,* 1989; Schaad *et al*., 2007). , *Xanthomonas citri* pv.*citri (*Dye, 1978). , *Xanthomonas campestris pv.aurantifolii* (Gabriel *et al.,* 1989). , *Xanthomonas*

citri f.sp.aurantifoliae (Namekata oliveira, 1972), *Xanthomonas axonopodis pv. citri* (Gottwald*, et al*., 2002) and *Pseudomonas citri* (Hasse,1915).

2.5.2. Scientific classification of the pathogen

Domain : Bacteria

Phylum: proteobacteria

Class : Gammaproteobacteria

Ordre : Xanthomonadales

Genus : *Xanthomonas*

Species: *Xanthomonas citri*

Gabriel *et al.*, (1989).

2.6. Symptatology

All above-ground tissues of citrus are at maximum susceptibility to infection by *X. citri PV citri.* (*Xcc*) during the last half explanation phase of growth (Gottwald and Graham,1992). The diseased plants are characterized by the occurrence of conspicuous raised necrotic lesion that develop on leaves, twigs and fruits . Lesions can be detected by drawing the fingers over the surface of the infected tissues. On leaves, fruit appearance is as oily looking 2-10mm circular spots, usually on the biaxial surface (reflecting stomatal entry following rain dispersal (Das, 2003). Canker lesion begins as light yellow, raised, spongy eruption on the surface of leaves, twigs and fruits. The lesion can continuously enlarge from pin-point size over several months and can be of many different sizes based on the age of the lesion. As the lesion enlarge, the spongy eruptions begin to collapse, and brown depressions appear in their central portion. The edges of the lesion remain raised above the surface of the hot tissue and the area around the raised portion of the lesion may have greasy appearance. The lesions become surrounded by characteristic yellow halos (Reddy and Naidu , 1986).

Canker causes fruit losses ranging from premature fruit drop due to abscission to non marketable quality due to lesions. Severe infection results in defoliation, die-back, deformation of fruit and premature fruit drop (Rossetti, 1977|).

plate 1. Citrus Canker lesions on leaves from horticultural research shambat (ARC) nursery.

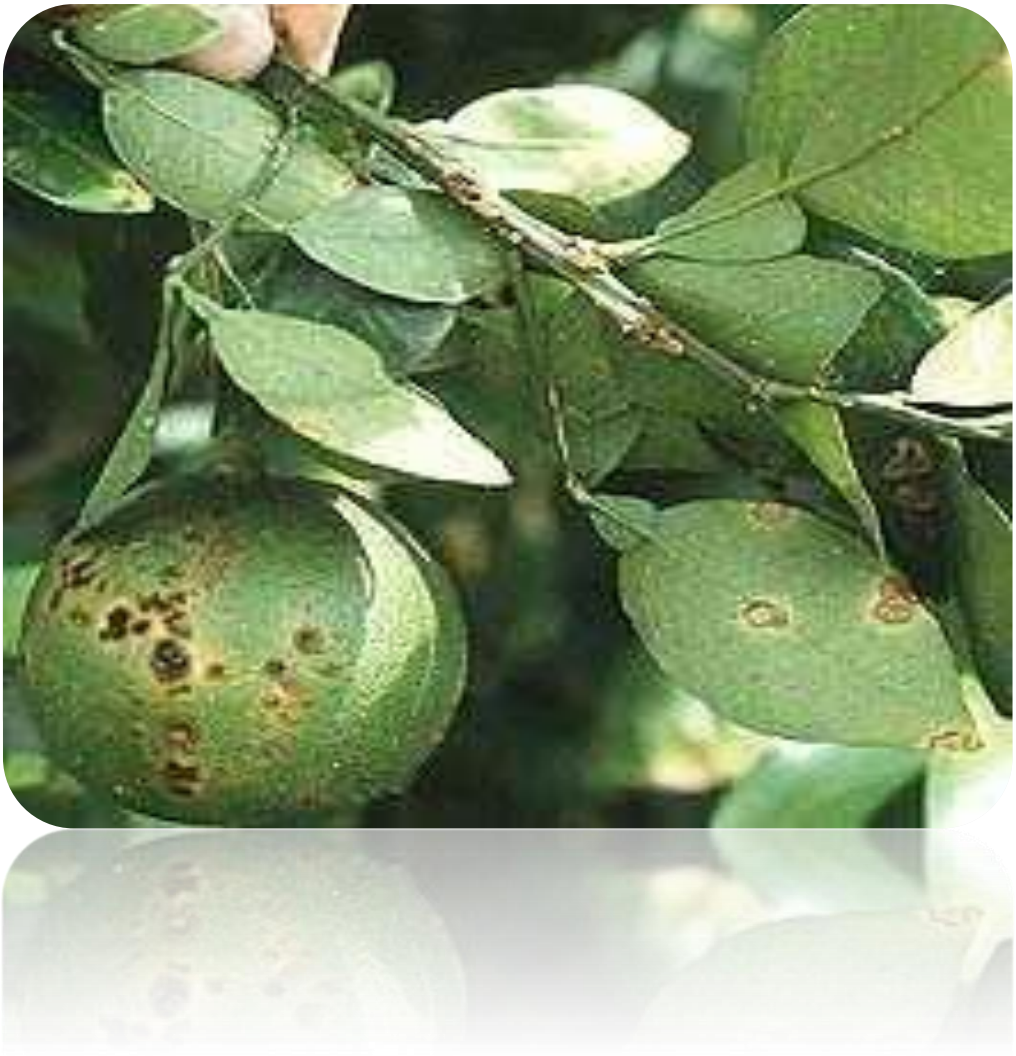


Plate 2. Symptoms of CBC on lime fruits and leaves from Rahad Kurdufan State

Plate 3. (A): canker lesion on secondary branches and thorns , (B): necrotic lesions on main branches and (C):sever leaves shedding and twig dieback from Gadaref State in eastern Sudan (Abu baker, *et al.*, 2016).

2.7. Isolation

For successful isolation of *X. citri* pv. *citri* from symptomatic plant material, plant tissue should be analyzed after collection or it may be stored at 4-8C° processing . When symptoms are very advanced or when environmental conditions are not favorable, the number of *X. citri* pv. *citri* cultural cell can be very low and isolation can result in plant being overcrowded with competing saprophytic or antagonistic bacteria. Isolation of the causal organism can be performed by streaking lesion extracts onto plant of suitable media, on which colonies of *X.citri* pv. *citri* have a characteristic appearance . However , there are no exclusively selective media available for (*X.cc*). lesions are macerated in 0.5-1.0ml saline (Distilled sterile water with 0.85% NaClO, ph 7.0), and when required they may be disinfected with 1% Naclo for 1min, rinsed three times with sterile distilled water , suitable general isolation media are nutrient agar (NA) . Filter-sterilized cycloheximide 100mg/liter) can be added when necessary as a fungicide after autoclaving the media. The colony morphology on the media is round colony is mucoid and creamy yellow. Growth is evaluated after incubation at 25-28C° for 24h. Pure culture of the pathogen is done by single colony streaking for several times (Graham *et al* ., 1996 ; Pruvost *et al* ., 2005).

2.8. Morphological and biochemical characteristics

Morphological characteristics of *X.cc* on nutrient Agar media (NA) are essential for identification that includes; round, convex and smooth-edged, mucoid and creamy yellow. It is gram negative and motile by means of a single polar flagellum. Some of the biochemical characteristics that identify *X.cc* are essential identification; include catalase test, oxides test, nitrate reduction test, starch hydrolysis test, casein hydrolysis, tween 80 lipolysis gelatin liquefaction test, pectate gel liquefaction test and utilization of asparagines. Other tests that

are commonly used to distinguish between isolates include geographical distribution and host range (Stall and Seymour, 1983; Vernier *et al.*, 1998).

2.9. Epidemiology

2.9.1. Disease cycle and spread

Like many other bacterial diseases, the pathogen enters host plant tissues through stomata (Gottwald and Graham, 1992 and wounds (Koizumi and Kuhara, 1982). The optimum temperature for infection falls between 20-30C° (Koizumi, 1977). The bacterium multiplies in lesions, in leaves, stems and fruits. Bacteria multiply 3-4 log units per lesion under optimum conditions and cells may emerges from stomata opening in as little as 5 days to provide inoculums for further disease development. When there is free moisture on the lesion surface, bacteria are released from an extracellular polysaccharide matrix and dispersed to new growth by rain droplets (Pruvost *et al*., 2002). Water congestion of leaf tissues can be seen during rainstorms, water congestion during inoculation with as few as 1-2 bacterial cells, forced through stomata opening, can lead to infection and lesion formation (Gottwald and Graham, 1992). The bacteria remain alive in the margins of the lesion in leaves and fruit until they fall. Bacteria also survive for up to several years in lesion on twigs and woody branch (Goto, 1992). Bacteria dispersed by windblown rain were detected up to 32m from infected trees in Argentina (Stall *et a*l., 1980). The pathogen is more efficiently spread in association with diseased rather than exposed plant material. Long-distance spread of *Xcc.* occurs through the movement of diseased or contaminated propagating material such as bud wood , rootstock seedlings budded trees including ornamental plants (Das,2003;Graham *et a*l .,2004). Workers can carry bacteria within and among planting on hands, clothes, vehicle and farm equipment/tools. This type of human-assisted dispersal will occur only within 24-72hours because survival on

inter surface is limited (Graham *et a*l., 2004). There is no record of seed transmission (Das, 2003; Sharma and Ram, 2009).

2.9.2. Pathogen survival

The bacterium survives primarily in naturally occurring on leaves, twigs, branches constitute the main source of the bacterium inoculums. The pathogen can survive for up 6 months in the infected leaves (Rao and Hingorani , 1963). Up to 76 months in diseased twigs (Chakravrti *et al.*, 1966). Found that the pathogen can survive for 52 days in the sterilized soil and in the unsterilized soils for 9 days only , but under desiccation at 30 C° the pathogen can survive for 11 or 12 days . The bacterium was resistance to drying and was killed 120 days in ordinary laboratory temperature (Paracer, 1961).

2.9.3. Host range

All cultivated species of Rutaceae are susceptible to citrus canker, such as citrus spp. *Fortunella spp.* and *poncirus spp*. Cultivars and hybrids of citrus and citrus relatives including orange , grapefruit , pummelo, mandarin, lemon, tangerine , sour orange , rough lemon, calamondin, trifoliate orange , and kumquat are hosts of citrus canker bacteria, among commercial citrus varieties and rootstock, citrus bacterial canker (CBC) is most sever on grapefruit (*C.paradisi*) and Trifoliate orange (*Poncirus trifoliate)* the cultivars have proven very challenging or impossible to grow profitably in the presence of citrus canker in most subtropical and tropical climates , Some citrus varieties are moderately resistance such as mandarin, tangerine and pummelo, and other highly resistance to *Xcc*. e.g citron , calamondin and kumquat . however , with plant tissues are disrupted by wounds or by feeding galleries of the Asian leaf miner (*Phyllocnistis citrella* stainton), internal leaf tissues are exposed, then all

cultivars and most citrus relatives that express some level of field resistance can become infected (Das,2003). Table 1 lists the citrus varieties susceptibility.

Table 1. susceptibility of several citrus varieties and rootstock to *Xcc*. (Das,2003)

Susceptibility		Host
Highly susceptible		*Citrus x paradisi* Macf.,grapefruit *C.aurantiifoli*a(Christ)Swingle,key lime *C.limattioides* Tan., Palestine, sweet lime *Poncirus trifoliate*(L.) Raf.,trifoliate orange
Moderately susceptible		*C.sinenisis* (L.) Osbeck, sweet orange *C.aurantium* L., sour orange *C.limon* (L.)Burm.Lemon *C. x tangelo* Moore,tangelo
Moderately resistance		*C.reticulata* Blanco, mandarin, tangerine *C.maxima* (Burm.) Merr., pummelo *C.aurantifolia* (Christ.)Swingle person or Tahiti lime
Highly resistance		*C.medica* L., citron Citrofotunella microcarpa (Bunge) Wijnands, calamondin *Fortunella* spp., kumquat

2.9.4 Citrus canker impact

Citrus canker impact on the citrus industry is of several levels. It reduced both fruit yield and quality. Loss assessment has not been determined clearly. When citrus infection occurs in the early growing stages, the fruit cracked or become malformed as they grow, and the heavily infected ones fall prematurely . Light infection in later growth stage may cause only scattered canker lesions on the surface of the fruit but makes fresh fruit unacceptable for market. (Goto, 1992b). A serious citrus canker infection can cause defoliation , die-back and premature fruit drop and under favorable environmental conditions may be associated with the death and destruction of the citrus tree as that which has been experienced in lime trees in Maldives islands (Roistacher *et al.*,1989). World-wide million of dollars are spent annually on prevention, quarantine, eradication programs and disease control (Gottwald *et al*., 2002a). In the Florida example ,during the years 1915- 33 , nearly 2,57,000 orchard trees and 3,000,000 nursery plant were cut and burnt at a cost of over $6 million and again during the years 1984-86 nearly 20 million citrus nursery plants were destroyed over $million however, the disease recorded for the first time in Gedarif State causing the destruction of citrus trees (Abu baker, *et al.*, 2016).

2.10. Control of Citrus Bacterial Canker Disease

Several strategies have been used to control the citrus canker disease, the basic strategies of the specific methods are to avoid or eradicate the pathogen, other methods are to reduce the amount of inoculums available for infection to minimize dissemination of the pathogen, and to protect susceptible tissue from infection (Civerolo, 1981). The use of canker-free nursery plants is the first essential step in the management of citrus canker.

2.10.1. Cultural practices

2.10.1.1. Resistant cultivars

The use of resistant cultivars is the most attractive economical and effective method to control this disease. The genetic engineering might be one of alternatives is complement classic citrus breeding programs in order to protect susceptible commercial varieties against pathogen. This technique allows the disease resistance, while preserving the genetic background of the original cultivar (Mendes *et al.,* 2010). For effective disease control and due to their high susceptible grapefruit, Mexican lime and several early to midseason sweet oranges (e.g Navel, Hamlin) are not recommended for planting unless very intensive control programs are to be undertaken (Leite and Mohan, 1990). Alternatively screening programs have recommended selected mid-and late season sweet orange, and Tahiti lime that have an a acceptable level of resistance to citrus canker (Leite and Mohan, 1984).

2.10.1.2. Windbreaks

The vast majority of the infection occurs by wind-blown rains that push the bacterial into tissues, and winds are needed to force bacterial into stomata on leaves and fruits (Dewdney and Graham,2014).

Windbreaks alone provide substantial control of canker and even more control in conjunctions with copper bactericides (Behlau *et al.,* 2008). In Argentina, (Gottwald and Timmer, 1995), demonstrated that windbreaks reduced spread of canker down nursery rows more effectively than copper sprays which were relatively ineffective. He also, conformed that windbreaks are an important component of integrated management in citrus production areas with endemic citrus canker (Leite and Mohan, 1990). Very susceptible cultivars will likely need a windbreak that surrounds each 5-10 are block, while, in less susceptible

cultivars, windbreak down the row about every 300 ft may be sufficient (Dewdney and Graham, 2014).

2.10.2. Quarantine

Countries that are free of citrus canker apply quarantine measures to prevent introduction of the pathogen. As a disease exclusion measure, they prohibit importation of fruit and plat material from canker infected areas (Gottwald *et al.,* 2001). World-wide, *Xcc.* is isolated as quarantine pest by the North America plant protection organization (NAPPO), the European and Mediterranean plant protection organization (EPPO), the inter-Africa phytosanitary commission (IAPSC), and the Board of the Cartagena Agreement (JUNAC),(EPPO) , citrus canker still doesn't exist in some countries or region of countries where climatic condition are favorable for pathogen establishment (Sharma and Ram, 2009).

2.10.3. Chemical control

Copper - based bactericides era standard control measure for citrus canker world-wide (Leite and Mohan 1990). Copper reduced bacterial population on leaf surface and multiple applications are needed to achieve adequate control on susceptible hosts (Stall *et al*., 1980).

Several formulations of copper products with bacterial activity have been evaluated for controlling CBC ; among the most effective are copper oxychloride (Leit,1990) , copper sulphate , copper hydroxide (Leite,1990), copper oxide and ammonia-copper carbonate (Gottwald and Timmer, 1995). These sprayers are used to protect the fruits from the infection and to reduce the inoculums on the new flushes. Environmental conditions, cultivars susceptibility and the implementation of other control methods are important factors that can affect positively or negatively, the suppression of the disease by copper spray. Control of citrus canker with copper sprays may achieved in the

case of resistance or moderately resistant cultivars, whereas in the case of susceptible cultivars using copper spray alone for controlling the canker is not effective (Kuhara,1978 ; Lleite,1990).

2.10.4. Integrated management

The most effective management of citrus bacterial canker is by supplementing the use of resistant cultivars with integrated systems of compatible cultural practices, chemical control and phytosanitary measures, including quarantine and regulatory programs.(Graham *et al.*, 2004).

2.11. Medicinal Uses of Roselle Plant (*Hibiscus subdariffa* L.)

The use of herbal extracts and nutritional supplements either as alternative or complementary medicine for treatment of diseases is well documented in various cultures such as Ayurveda in India and traditional Chinese medicine system. Medicinal plants as natural antimicrobial agents are gaining popularity. Roselle plant (*Hibiscus subdariffa* Linne (Malvaceae) has been used in folk medicine as a diuretic, mild laxative, and treatment for cardiac and nerve diseases. Herein we discussed some of the recent studies on its various activities Chao *et al*. (2008).

2.11.1. Scientific classification of Roselle

Kingdom : Plantae – Plants

Subkingdom : Tracheobionta- Vascular plants

Superdivition : Spermatophyta -Seed plants

Division :Magnoliophyta – Flowering plants

Class : Magnoliopsida – Dicotyledons

Subclass :Dilleniidae

Order: Malvales

Family: Malvaceae –Mallow family

Genus: *Hibiscus* L. - rosemallow

Species : *Hibiscus subdariffa* L. - Roselle

(Carvajal *et al.* 2012).

2.11.2. Nutritive value

The nutritional analysis of Roselle plant by proximate method (Luvonga *et al.* 2010) found the carbohydrate content (68.7%) was highest followed by crude fiber (14.6%) and ash content (12.2%) and others. The plant is also found to be rich in minerals especially potassium and magnesium. Vitamins (ascorbic acid, niacin and pyridoxine were also present in appreciable amounts. Various workers (Nnam and Onyeke 2003; Ojokoh 2006; Adanlawo and Ajibade 2006), reported variable content suggesting that the type of soil influences its ash and mineral content causing variations within the same species (Carvajal *et al.* 2012). It has long been used in herbal tea to treat hypertension, pyrexia and liver

damage although the pharmaceutical components are poorly defined (Hou *et a*l. 2005). Nutritional studies have indicated that low consumption of fruits and vegetables is consistently related to an increased incidence of cancer (Choi and Mason, 2000) reflecting dietary habits. The component in fruits and vegetables like polyphenol and anthocyanin may be responsible for the reduced risk of cancer (Weisburger and Chung 2002 ; Wang *et al.* 2003; Gao *et al.* 2002). Plants have the capacity of producing secondary metabolites like proteins, steroids, alkaloids, etc. (Sharaniah *et al*. 2013) that will enhance its nutritive value.

2.11.3. Antimicrobial properties

Roselle is widely used for the treatment of diseases. Olaleye (2007) used the aqueous methanolic extract of roselle to investigate its phytochemical constituents, antimicrobial activity and cytotoxicity, and reported that the extract contained cardiac glycosides, flavonoids, saponins and alkaloids. It exhibited antibacterial activities against *Staphylococcus aureus*, *Bacillus stearothermophilus*, *Micrococcus luteus*, *Serratia mascences*, *Clostridium sporogenes*, *Escherichia coli, Klebsiella, pneumoniae*, *Bacillus cereus* and *Pseudomonas fluorescence*. The results support the use of this plant in the treatment of diseases like abscesses, bilious conditions, cancer and coughs in traditional medicine, and also suggest the possibility of isolating antibacterial and anticancer agents while the antimicrobial activity on *E coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* isolates from food, veterinary, and clinical samples by Fullerton (2011) indicated that roselle extract was effective and suggest the application of extracts as potential antimicrobials in foods. The antibacterial effects of roselle calyx aqueous and ethanol extracts and protocatechuic acid against food spoilage bacteria Salmonella Typhimurium DT104, Escherichia coli O157:H7, Listeria monocytogenes, Staphylococcus

aureus and Bacillus cereus were examined by Chao *et al*. (2008) and shown the inhibitory activity in dose-dependent manner against test bacteria in ground beef and apple juice and suggested that it might be potent agents as food additives to bacteria in ground beef and apple juice and suggested that it might be potent agents as food additives to prevent contamination from these bacteria. However, other authors reported that the aqueous crude extracts of Roselle inhibited the infection of citrus canker bacteria (*X. citri* pv. *citri)* on lime leaves grown in pots in greenhouse (Leksomboon, *et al.*, 2001).

2.12. History of herbal medicine use of Cinnamon plant (*Cinnamomum verum* Presl.)

The use of herbal medicines has a long history, more than 50 % of clinical drugs come from herbal products or their derivatives (Kadir *et al.*, 2012). For example, metformin, which is a drug that lowers blood glucose by increasing the body's cell sensitivity to insulin in type 2 diabetes, is derived from French lilac, and was a herbal remedy used to treat diabetes in the Middle Ages similarly, ginger was used to treat sore throats, which is still used currently (Jungbauer and Medjakovic, 2012).

The use of herbal supplements has also been proved effective, for example in a double blind crossover study it was shown that consuming 1200 mg/day of curcumin as a supplement, plus the patient's medication (phenyibutazone 300 mg/day), for two weeks improved morning stiffness, walking time, and joint swelling in people with rheumatoid arthritis, while there was no changed in control (Deodhar *et al*., 1980). Moreover, herbs and spices such as cinnamon, cloves, tea, oregano, and nutmeg, have been shown to possess insulin-like biological activity (Broadhurst *et al.*, 2000).

Cinnamon has been used since ancient times and is the inner bark of the Cinnamomum tree, which belongs to Lauraceae family. The main types of cinnamon are C. zeylanicum (also known as C. verum, C. cylone) which is produced in Sri Lanka, and C. cassia which is mainly grown in China. The term 'Cinnamomum' is derived from Greek and means 'sweet wood' (Ravindran *et al.*, 2004).

Current research, both in vitro and in animals in vivo, suggests that cinnamon has antiinflammatory, anti-microbial, antibacterial, anti-oxidant, anti-diabetic, anti-obesity and immunomodulatory effects (Anderson *et al*., 2004; Wang *et al*., 2009; Dudonné *et al.*, 2009; Lee *et al*., 2005).

2.12.1.Scientific classification of Cinnamon

Kingdom: Plantae
Clade :Angiosperms
Clade: Magnoliids
Order : Laurales
Family : Lauraceae
Genus *: Cinnamomum*
Species : *C. verum*
(Deodhar *et al*., 1980).

CHAPTER THREE

MATERIALS AND METHODS

This study was conducted under laboratory conditions at Plant pathology Laboratory of the Plant Protection Department, College of Agricultural Studies (CAS), Sudan University of Science and Technology (SUST) and plant pathology lab Shambat Research Station (ARC) within the period November - April 2019.

3.1. Isolation and purification of the bacterium from infected Lime leaves

These procedures were conducted according to national diagnostic protocol (NDP) for Asiatic citrus canker (2011)

Selected plant material (leaves) were surface sterilized by rinsing the plant material in 0.1% sodium hypochlorite (NaClO) for two mins, and then thoroughly rinsing in sterilized distilled water several times to remove excess of sodium hypochlorite. Then the specimens were transferred to sterilized filter paper to dry the selected lesions, and were then placed on sterile slide or Petri dish. The selected lesion was excised, using sterile scalpel blade which was carefully used to cut the lesion into quarters and placed on sterilized slide. Then a drop of sterile 0.85% saline or sterile water was added using a sterile pipette and covered with slide cover. Bacterial ooze was examined within two minutes of preparing the slide, two to five minutes might be allowed for maximum efflux of bacteria ooze into saline. Then a sterile loop was used to collect small volume of exudates, inoculate agar plate; clean sterile loops were used to sterilely streak the plates. The plates were then incubated at 25-28C° and checked for bacterial growth after 24 hours as required thereafter. Colonies of bacteria were streaked several times, respectively onto the same medium until a

pure culture was obtained and maintained on slants of nutrient agar and kept in the refrigerator for further use.

3.2. Evaluation of the bio activity of some selected botanical extracts

3.2.1. Inoculum preparation

Pure isolates of *Xcc*. were grown on Nutrient Agar (NA) plates and incubated at 28C° for 24 h. bacterial cells were harvested in sterile distilled water (SDW) by using sterile glass rod and then bacterial suspension was adjusted to 1.0 at wavelength of 600 nm using a UV spectrophotometer (plant pathology lab-University of Khartoum) to give 1.0 x 10^8 CFU/ ml^{-1}. SDW was used to calibrate the spectrophotometer. The bacterial density was adjusted by either adding the concentrated bacterial suspension to the tubes or by diluting the inoculated tubes with SDW to obtain the desired concentration.

3.2.2. Preparation of plants aqueous extracts

Hibiscus subdariffa Linn "Rossle" and *Cinnamomum verum* "Cinnamon" were obtained from shambat local market , and used to prepare different concentrations (25% ,12% and 6.25%) extracts from each of the two plants (Cinnamon and Roselle) powders. The different information of the plants used was shown in Table (2).

Five grams of dried material of each plant species were placed in a 25ml conical flask, then 25ml of sterile distilled water added to each flask. The mixture was placed on a shaker (Orbital incubator S150) and left to extract for 18 h at a speed of about 133 rpm at room temperature (25°C). Extracts were expressed through 2 layers of cheesecloth. Filtered extracts were then collected in round bottom flasks and their antibacterial activity against citrus canker bacterium was tested in vitro. 100µI of bacterial suspension (1.0 x 10^8 CFU/ ml^{-1}) were

spread onto the surface of the NA plate using sterile cotton swabs(Casimiri and Burstein,1998). Sterile filter paper discs were dipped briefly in the respective Plant extracts and were then applied onto of the surface of the inoculated NA plates. Discs impregnated with Antibiotic 4% (Penamox 500m g) were used as positive controls , while sterile distilled water-treated discs were used as a negative control. The treated plates were incubated at 28°C for 48h and the developing inhibition zones were observed and measured to determine the relative efficacy of the plant extracts against *Xcc*. the recorded data were subjected to analysis of variance (ANOVA) according to the method described by Gomez and Gomez (1984). For a completely randomize design, applying Statisttx 8 Application. The least significant difference (LSD) test was used for the comparison between means.

Table2. Plant extracts used for the control of *Xanthomonas citri* PV. *citri*.

Common name	Botanicals name	Family	Plant parts used
Roselle	*Hibiscus subdariffa* Linn	Malvaceae	Dry flower
Cinnamon	*Cinnamomum verum* J. presl	Lauraceae	Inner bark

(A) (B)

Plate 4. (A,B) Inner bark of Cinnamon from Shabbat local market

(c)

Plate 5 (c) dry flower of Roselle from Shabbat local market

CHAPTER FOUR

RESULTS

This study was conducted under laboratory conditions at Plant pathology Laboratory of the Plant Protection Department, College of Agricultural Studies (CAS), Sudan University of Science and Technology (SUST) and plant pathology lab Shambat Research Station (ARC) within the period November - April 2019, to investigate the inhibitory effect of Roselle and Cinnamon leaves aqueous extracts against the test Bacterial canker.

4.1. Characterization of the presumptive causative agent of CBC

4.1.1. Cultural and morphological characteristics

They were inferred growth on nutrient Agar (NA). The result are shown in plat, 1. (A,B) The colony of the canker bacterium isolate appeared pale yellow on NA.

(A) (B)

Plate 6. (A,B) Cultural properties of the bacterium isolate, pale yellow colonies on NA. Media.

4.2. Effect of aqueous extract Roselle and Antibiotic, penamox (Amoxicillin) on the growth of *Xanthomonas citri* pv.*citri in vitro* .

The results (Tablte3,fig. 4) showed that the Roselle aqueous extract tested and Antibiotic, penamox "Amoxicillin 4%" effects against the bacterial growth after two days from inoculation, However, the Antibiotic , penamox (Amoxicillin 4%) and the highest concentration of the Roselle aqueous extract (25% , 12.5% ,6.25) gave the highest effectively on the bacterial growth percent (5.26% ,3.15% , 2.73%, 2.69%).

The Antibiotic, penamox "Amoxicillin 4%" was more effective to reduce growth of *Xcc.* than Roselle in vitro compared to the untreated control. This inhibitory effect from all concentrations tested was significantly ($P<0.05$) different from control. However, Roselle 6.25% was lowest inhibition zone percentage (2.69%), (Table, 3 and Fig, 4).

4.3. Statistical analyses

The obtained data was statistically analyzed according to analysis of variance (ANOVA); - LSD Range Test was used for mean separation.

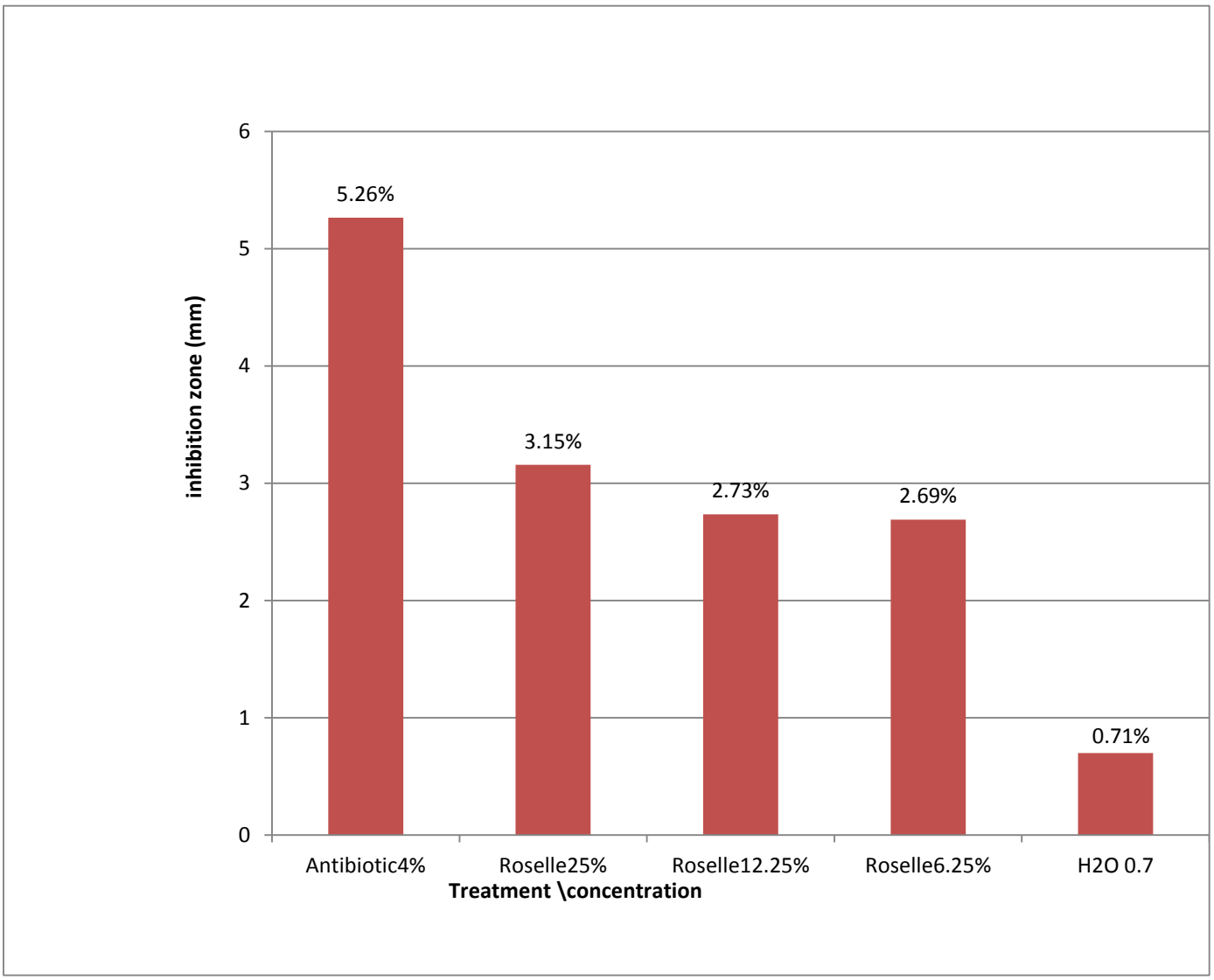
- Data in parentheses were transformed using square root transformation ($\sqrt{X+0.5}$) before analysis.

Table , 3: Effect of aqueous extracts of Roselle dry flower on growth of *Xanthomonas citri* pv. *citri* after two days after inoculation

Treatments	Concentration	Mean	Mean% relative to 4% Antibiotic
Roselle	25%	3.15	3.15^{b}
Roselle	12.5%	2.73	2.73^{c}
Roselle	6.25%	2.69	2.69^{d}
Untreated control (water only)		0.71	0.71^{e}
Antibiotic	4%	5.26	5.26^{a}
SE ±	0.08		
C.V. %	6.18		
LSD	0.27		

Dissimilar letters on the "Mean" column show significant differences at $P<0.05$. Data in the parentheses transformed using square root transformation $\sqrt{x}+0.05$ before analysis

Fig.1 Effect of aqueous extracts of Roselle dry flower on growth of *Xanthomonas citri* pv.*citri* two days after inoculation



4.4. Effect of aqueous extract Cinnamon and Antibiotic penamox "Amoxicillin 4%" on the growth of *Xanthomonas citri* pv.*citri in - vitro* .

The results (Table 4, fig. 5) showed that the Cinnamon aqueous extract tested and Antibiotic, penamox "Amoxicillin 4%" effects against the bacterial growth after two days from inoculation, However, the Antibiotic , penamox "Amoxicillin 4% " and the highest concentration of the Cinnamon aqueous extract (25% , 12.5% ,6.25%) gave the highest effectively on the bacterial growth percent (5.33% , 3.15% , 2.83% and 2.55%).

The Antibiotic penamox "Amoxicillin 4%" was more effective to reduce growth of *Xcc.* than Cinnamon in vitro compared to the untreated control. This inhibitory effect from all concentrations tested was significantly (P0.05) different from control. However, Cinnamon 6.25% was lowest inhibition zone percentage (2.55%). (Table, 4 and Fig, 5).

4.5. Statistical analyses

The obtained data was statistically analyzed according to analysis of variance (ANOVA); -LSD Range Test was used for mean separation.

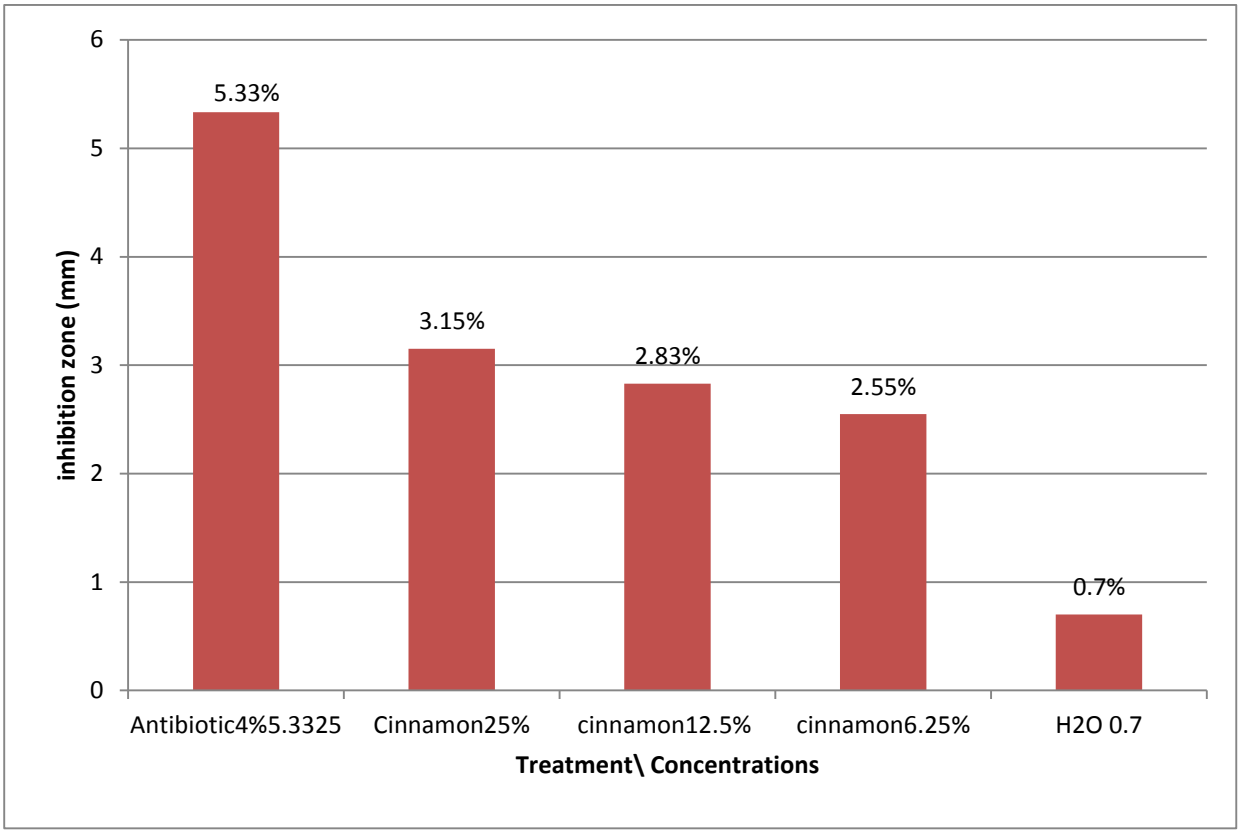
- Data in parentheses were transformed using square root transformation ($\sqrt{X + 0.5}$) before analysis.

Table, 4 Effect of aqueous extracts of Cinnamon inner bark on growth of *Xanthomonas citri* pv. *citri* two days after inoculation.

Treatment	Concentration	Mean	Mean% relative to 4% Antibiotic
Cinnamon	25%	3.15	3.15^{b}
Cinnamon	12.5%	2.83	2.83^{c}
Cinnamon	6.25%	2.55	2.55^{d}
Antibiotic	4%	5.33	5.33^{a}
Untreated control(water only)		0.7	0.7^{e}
SE±	0.0849		
C.V.%	5.83		
LSD	0.2561		

Dissimilar letters on the "Mean" column show significant differences at $P<0.05$. Data in the parentheses transformed using square root transformation $\sqrt{x}+0.05$ before analysis

Fig. 2 Effect of aqueous extracts of Cinnamon inner bark on growth of *Xanthomonas citri* pv. *citri* two days after inoculation.



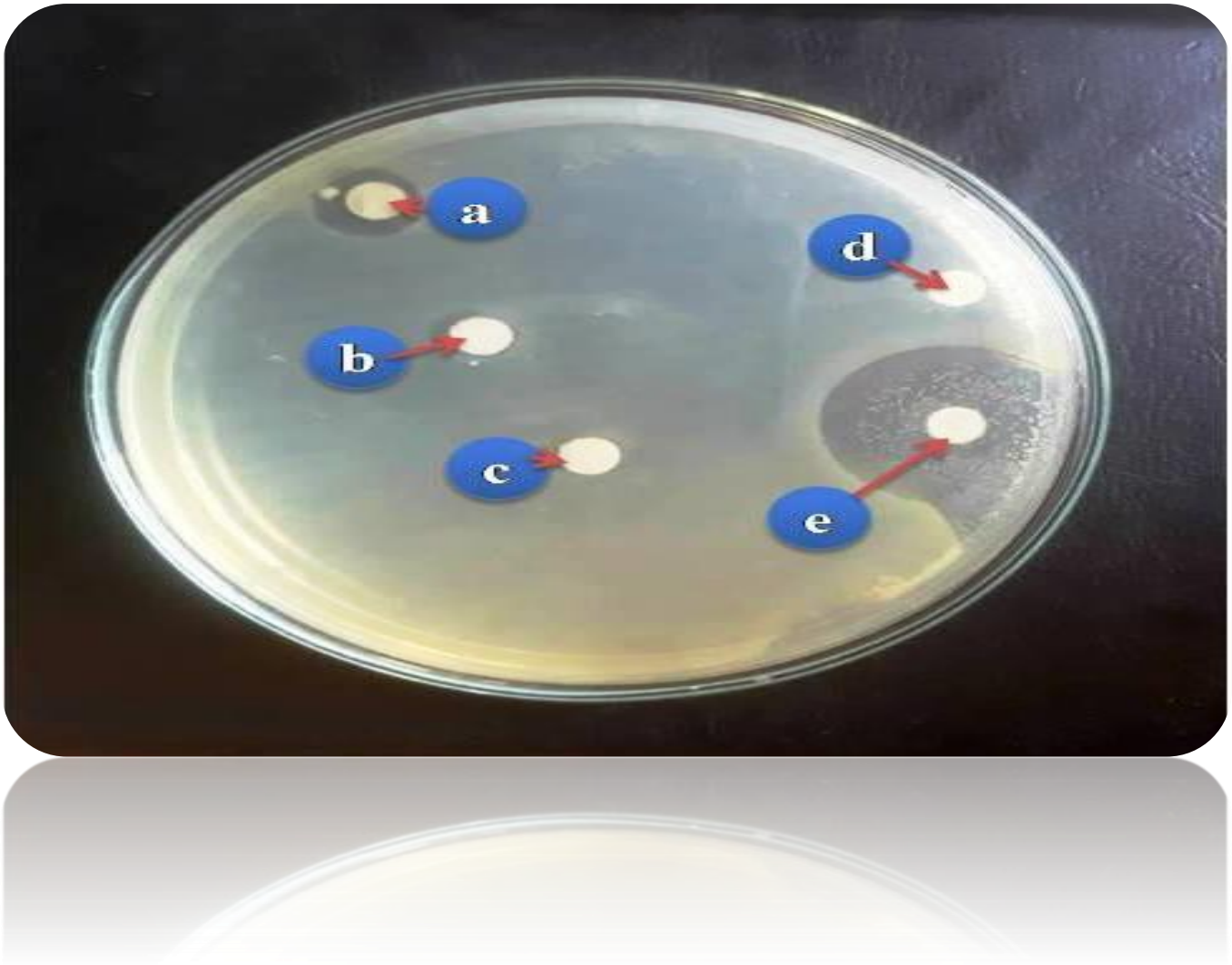


Plate 7. Effect of different concentrations of aqueous extract Roselle and Antibiotic, penamox "Amoxicillin4%" on growth of *Xanthomonas citri* pv *.citri in -vitro*

(A) **: 25% Roselle , (B): 12.5% Roselle, (C): 6.25% Roselle (D): Untreated Control (water only H_2O) and (E): Antibiotic, penamox "Amoxicillin 4%"**

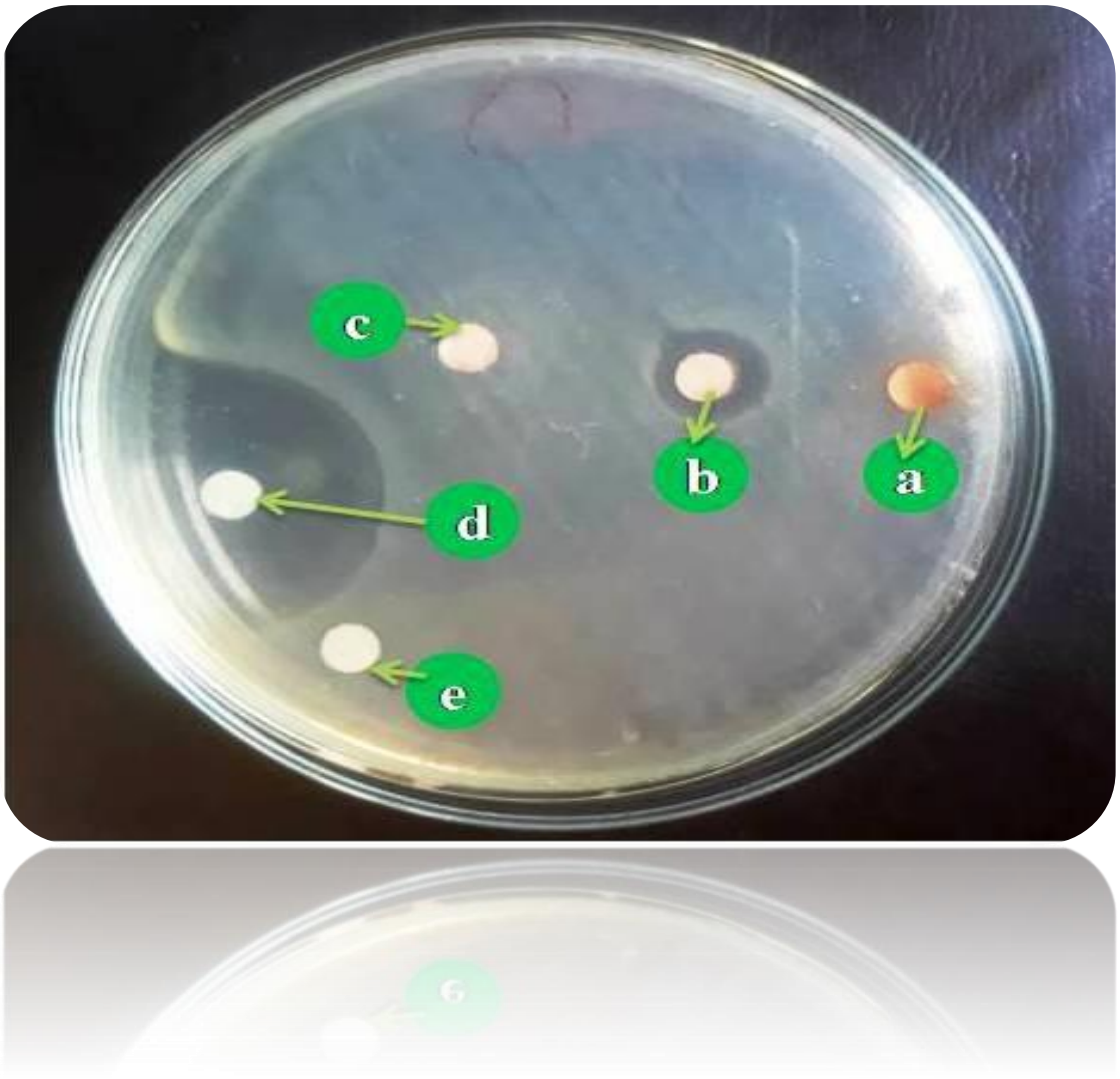


Plate 8. Effect of different concentrations of aqueous extract Cinnamon and Antibiotic Amoxicillin on growth of *Xanthomonas citri* pv .*citri in vitro*

(A) 6.25% , (B). 25% , (C) 12.5% , (D) Antibiotic, penamox " Amoxicillin 4%4% " and (E) Untreated Control (water only H_2O)

CHAPTER FIVE

DISCUSSION

Citrus (*Citrus aurantifolia* L.) is one of the most important fruit crops known by humans since antiquity and is a good source of vitamin "C" with high antioxidant potential , citrus originated from south-eastern Asia, China and the east of Indian Archipelago from at least 2000 BC Canker caused by *Xanthomonas citri* pv. *citri* an important bacterial disease of citrus spp. that affects the citrus production both quantitatively and qualitatively. It infects nursery and citrus trees with the symptoms on leaves, twigs, stems and fruits, while the lesions on fruits reduce its market value (Swingle, 1943).

Morphological characteristics of *X.cc* on Nutrient Agar media (NA) are essential for identification that includes; round, convex and smooth-edged, mucoid and creamy yellow. It is gram negative and motile by means of a single polar flagellum. Some of the biochemical characteristics that identify *X.cc* are essential identification; include catalase test, oxides test, nitrate reduction test, starch hydrolysis test, casein hydrolysis, tween 80 lipolysis gelatin liquefaction test, pectate gel liquefaction test and utilization of asparagines. Other tests that are commonly used to distinguish between isolates include geographical distribution and host range (Stall and Seymour, 1983; Vernier *et al.*, 1998).

This study investigates the efficacy of plant extracts for the effective control of citrus canker on lime plant under laboratory conditions, Comparatively, *Hibiscus subdariffa* (Roselle) and *Cinnamomum verum* (Cinnamon) demonstrated higher inhibitory effects with inhibition zones of 3.15% , 3.15% respectively compared to (Amoxicillin 4%) 5.33% .

The effectiveness of *Hibiscus subdariffa* was previously reported by Leksomoon (2001). He demonstrated that the spray with leaf extract of *Hibiscus subdariffa* could reduce the incidence of citrus canker to 78% compared to 100% in control plants. If there any efficacy against other results.

Conversely, the effectiveness of *Cinnamomum verum* (Cinnamon) was previously reported by (Akhtaret *et al.*, 1997). Showed either a mid inhibitory or did not show any noticeable effect, respectively. However, our results of Roselle showed higher inhibitory effect on the bacteria compared to Cinnamon treatment.

5.1. Conclusions

The present study indicated that the crude aqueous extracts of Roselle and Cinnamon have antimicrobial activity against the tested organism. The study also revealed that the highest inhibitory effects was recorded when Roselle was used compared to Cinnamon.

5.2. Recommendation

From the present work we recommend the use of Roselle extracts as antibacterial agent to manage the canker disease as natural antibacterial that inhibits the growth of the bacterium (*Xanthomonas citri* pv.*citri*) especially at concentration 25%.

Further work should also needed to investigate the specific compounds responsible for the affection. Moreover, we recommend repeating these experiments under field condition.

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APPENDIX

Statisttx 8.0

Completely Randomized AOV for Cinnamon

Source	**DF**	**SS**	**MS**	**F**	**P**
Treat	4	43.6127	10.9032	378	0.0000
Error	15	0.4330	0.0289		
Total	19	44.0457			

Grand Mean 2.9150 CV 5.83

At least one group variance is near zero,
variance-equality tests cannot be computed.

Component of variance for between groups 2.71858
Effective cell size 4.0

Treat	**Mean**
0	0.7100
6	2.5500
12	2.8300
25	3.1525
100	5.3325

Observations per Mean 4
Standard Error of a Mean 0.0849
Std Error (Diff of 2 Means) 0.1201

Completely Randomized AOV for Roselle

Source	**DF**	**SS**	**MS**	**F**	**P**
Treat	4	42.1052	10.5263	326	0.0000
Error	15	0.4849	0.0323		
Total	19	42.5901			

Grand Mean 2.9115 CV 6.18

At least one group variance is near zero,
variance-equality tests cannot be computed.

Component of variance for between groups 2.62349
Effective cell size 4.0

Treat	Mean
0	0.7100
6	2.6900
12	2.7350
25	3.1575
100	5.2650

Observations per Mean 4
Standard Error of a Mean 0.0899
Std Error (Diff of 2 Means) 0.1271

Statisttx 8.0

LSD All-Pairwise Comparisons Test of Cinnamon by Treat

Treat	Mean	Homogeneous Groups
100	5.3325	A
25	3.1525	B
12	2.8300	C
6	2.5500	D
0	0.7100	E

Alpha 0.05 Standard Error for Comparison 0.1201
Critical T Value 2.131 Critical Value for Comparison 0.2561
All 5 means are significantly different from one another.

LSD All-Pairwise Comparisons Test of Roselle by Treat

Treat	Mean	Homogeneous Groups
100	5.2650	A
25	3.1575	B
12	2.7350	C
6	2.6900	C
0	0.7100	D

Alpha 0.05 Standard Error for Comparison 0.1271
Critical T Value 2.131 Critical Value for Comparison 0.2710
There are 4 groups (A, B, etc.) in which the means are not significantly different from one another.