ANTIBACTERIAL PROPERTIES OF *CROTON* SPECIES

Prince Yaw Dadson
Master's thesis
Institute of Public Health and Clinical Nutrition
School of Medicine
Faculty of Health Sciences
University of Eastern Finland
April 2012

UNIVERSITY OF EASTERN FINLAND, Faculty of Health Sciences, Institute of Public Health and Clinical Nutrition
DADSON YAW PRINCE: ANTIBACTERIAL PROPERTIES OF CROTON SPECIES
Master's thesis, 50 pages
Supervisors: Carina Tikkanen-Kaukanen, PhD, Adjunct Professor, Research Director, Ruralia Institute, University of Helsinki,
Juhani Miettola, MD, PhD Assistant Professor, Department of International Health
University of Eastern Finland, Kuopio Campus
April 2012

Keywords: Croton, extracts, antibacterial, antimicrobial, bacteria, zone of inhibition, minimum inhibitory concentration, minimum inhibitory dose.

ANTIBACTERIAL PROPERTIES OF CROTON SPECIES

ABSTRACT

Natural substances of botanical origin have been important in African traditional medical practice. They have been used for various illnesses such as infections. Infectious diseases caused by pathogenic bacteria affect many communities and the treatment is made difficult partly because of antibiotic resistant strains. Phytochemicals isolated from medicinal plants are known to be effective in treating bacterial infections. Species under *Croton* genus are found in the different parts of the world and are widely used for the treatment of bacterial infections. The objectives of the study were to evaluate antibacterial properties and synergistic effects on antibiotic treatments of *Croton* species.

Electronic database MEDLINE was searched for studies from January 1990 to November 2011 on antibacterial properties of extracts of *Croton* species against different bacterial strains. Ten articles, with twelve species of *Croton*, filled the inclusion and exclusion criteria. The following *Croton* species were used in the studies: *C. megalobotrys C. steenkapianus, C. silvaticus*, *C. pseudopulchellus*, *C. zambesicus*, *C. macrostachyus*, *C. tiglium*, *C. campestris*, *C. zehntneri*, *C. cajucara*, *C. urucurana* and *C. sonderianus*.

In these articles organic solvents (methanol, ethanol, acetone and hexane), inorganic solvents (water) and hydrodistillation were described for extraction. From different *Croton* species different methods were used to extract the active contents from roots, leaves, stem-bark and seeds. The resulting extracts and fractions of the extracts were tested against nine Gram negative and eleven Gram positive bacteria in addition to sensitive and resistant strains of *Mycobacterium tuberculosis* using different antibacterial tests. The antibacterial properties were quantitatively evaluated by the minimum inhibitory concentration (MIC), the minimum inhibitory dose (MID) and the zone of inhibition (ZH).

Organic extracts were effective growth inhibitors of Gram negative and Gram positive bacteria. In addition, they enhanced the effectiveness of specific antibiotics. Water extracts were inactive against *M. tuberculosis* strain, which was sensitive to streptomycin, isoniazid, ethambutol and rifampin. Essential oils from certain species of *Croton* were not only effective against tested bacterial strains, but they enhanced the antibiotic activities of the drugs used in the studies.

Extracts of *Croton* species can be used as an alternative means of treating bacterial infections and could be possible to use as an adjuvant in antibiotic therapy against pathogenic bacterial infections.

Further studies including the use of animal models are required to investigate the activities of the active compounds. Toxicological evaluation of the *Croton* species is also needed.

ACKNOWLEDGEMENTS

I am truly indebted and thankful to my principle supervisor, Research Director, Adjunct Professor, Carina Tikkanen-Kaukanen, PhD, for her patience, guidance and support throughout my dissertation writing. I would also like to thank Assistant Professor Juhani Miettola, MD, PhD, for agreeing to be my second supervisor as well my examiner. I also wish to express my gratitude to Senior Scientist Marko Toivanen, PhD, for being my examiner. This dissertation was made possible by the moral support and pieces of advice from my family, friends and classmates.

ABBREVIATIONS

BHI	Brain Heart Infusion
C	*Croton*
C. spp	*Croton species*
DMSO	Dimethyl sulfoxide
EOCC	Essential oil of *Croton cajucara*
EOCZ	Essential oil of *Croton zehntneri*
GI	Growth Index
HECC	Hexane extracts of *Croton campestris*
MDR-TB	Multidrug resistant tuberculosis
MECC	Methanol extracts of *Croton campestris*
MIC	Minimum Inhibitory Concentration
MID	Minimum Inhibitory Dose
TB	Tuberculosis
ZH	Zone of Inhibitory

TABLE OF CONTENTS

1. INTRODUCTION

Many communities in Asia, Africa and South America have used medicinal plants for the treatment of diseases for centuries. These substances have been used for various illnesses such as infections. Microorganisms especially bacteria can be found in almost everywhere and have the tendency to adapt quickly to their immediate environment. Infections caused by bacteria are responsible for considerable mortality and morbidity worldwide especially in developing countries due to poor sanitation, unhygienic and overcrowded living conditions. Drugs for treating bacterial infections may lose their effectiveness with time, because the targets of these drugs keep shifting their forms. The time period for developing new drugs are often long and hence drug resistance take place (Theuretzbacher 2011).

The increasing global trend of resistance to drugs among Gram-positive and Gram negative bacteria pose major challenges to health care workers (Bassetti et al. 2011). Multidrug resistant bacteria are resistant to several different antibiotics. The management of multi-drug resistant bacterial strains is difficult because treatment options are limited and if available are beyond the reach of the poor. This may increase risks of death, increase length and the cost of hospitalization and increase the cost on healthcare systems (Miyakis et al. 2011). There is urgent need to explore new effective areas for the treatment of infectious diseases (Aiyegoro et al. 2011).

Currently, studies on herbal medicines appear under different names, such as plant medicines, phytomedicines, natural products and under pharmacognosy usually referring to products processed from living organisms: plants, animals, insects, microorganisms and marine organisms. Atropine, morphine, quinine, ephedrine, warfarin, salicin, digoxin, vincristine, taxol, and hyosine are some examples of extracts from traditional plants currently used in modern medicines. Findings from ethnobotanical and ethnomedicinal studies have shown correlation between medicinal use and laboratory results. Natural sources are usually the starting points for most pharmacological agents (Liu 2011).

Owing to the continuous development of antibiotic resistant strains, screening of plant materials and plant extracts for new antimicrobial compounds represent a significant source of new and effective medicine.

Reasons for the use of traditional medicines may vary, beside the fact that the plant preparations are relatively cheaper, adverse drug reactions (ADR) are rarely observed when compared with synthetically produced pharmaceuticals (Chariandy et al. 1999). Antibacterial effects against certain resistant infectious pathogens are profound and effective if combinations of different plant extracts are used (Nascimento et al. 2000). Various studies have established that herbal medicines can be developed as safe, effective and less costly alternatives to the current medicines to the treat certain bacterial infections (Vermani and Garg 2002).

Croton can be a tree, shrub or herbaceous plant which grows in tropical and warm regions. Some of the most popular uses include treatment of cancer, constipation, diabetes, digestive problem, dysentery, external wounds, intestinal worm, pain, ulcers and weight loss. Several *Croton* species are characterized by the presence of red sap (Salatino et al. 2007).

2. JUSTIFICATION OF THIS STUDY

There are limited reviews on the antibacterial properties of *Croton* species: the efficacy of the extracts, pharmacology and phytochemistry. The *Croton* species are found in different parts of the world and are known for their medicinal properties. Popular uses of Croton include treatment of malaria, fever, external wounds, dysentery, digestive problems, hypercholesterolemia, hypertension, intestinal worms, pains, ulcers and weight loss. Some species of Croton are known to contain proanthocyanidins or alkaloids. These alkaloids may be in the form of taspine or some of the several benzylisoquinoline-like compounds. Diterpenes a common compound in *Croton* may be represented as clerodanes, cembranoid, halimanes, kauranes, labdanes, phorbol esters, trachylobanes and sarcapetalanes. Volatile oils are present in some of the *Croton* species (Salatino et al. 2007). Some of these compounds present in *Croton* species have been shown to have antibacterial properties (Peres et al. 1997, Abo et al. 1999). Evidence from *in vitro* studies point to the use of essential oils as antibacterial agents for wide range of pathogenic bacteria strains such as *Listeria monocytogenes, Listeria innocua, Salmonella typhimurium, E. coli, Shigella dysenteria, Bacillus cereus, Staphylococcus aureus* and *Salmonella typhimurium* (Burt 2004, Nguefact et

al. 2004, Schmidt et al. 2005). Biologically active compounds such as sonderianin, korberin A and B isolated from the genus *Croton* with specific antibacterial activities against *Mycobacterium smegmatis*, *Staphylococcus aureus*, *Bacillus subtilis*, *E. coli* (McChesney et al. 1991) are however well known. Compilations of information on the antibacterial activity and synergistic effects of extracts and essential oils from *Croton* species and the biologically active compounds responsible for these antibacterial activities will be justified.

3. OBJECTIVES

- To evaluate the antibacterial properties of the extracts from different *Croton* species.
- To assess the synergistic effects of the extracts from *Croton* species on antibiotic activities.
- To identify the biologically active compounds present in the different parts of the *Croton* plants used.

4. METHODS

4.1. Search strategy

The search was done on November 2011. PubMed was used as the sole electronic database for searching the articles. The language was limited to English and the years from 1990 to 2011. The following key words were used; "*Croton*", "*Croton* and antibacteria". The articles were examined either they met the inclusion criteria or not.

The first selection was made based on the titles of the articles and the articles with the relevant titles were selected. Subsequently, the abstracts were read and those that were in line with the inclusion criteria were selected.

4.2. Inclusion criteria

The studies included were either *in vivo* or *in vitro* studies carried out with a human bacterial pathogen. All the species under the genus *Croton* were selected. Studies on the *Croton* extracts tested against viruses, fungi, protozoa and parasitic infections were not included. Extracts from the *Croton* species should have been used alone or in combination with other plants. The studies should be based on experiments, any reviews or meta-analyses were rejected.

4.3. Data analysis

The results are described in the narrative and collected in the tables, because of the various methodologies used in the studies.

5. RESULTS

5.1. Outline of the included studies

Using Pubmed as the sole electronic data base, 1795 articles on *Croton* were identified. Further searches on *Croton spp*. and antibacterial agents generated 53 articles. When the studies were limited from 1990 to 2011, 27 articles were produced. All of the 27 articles were read. After excluding those studies that did not fulfill the inclusion criteria, 10 studies were included in this review.

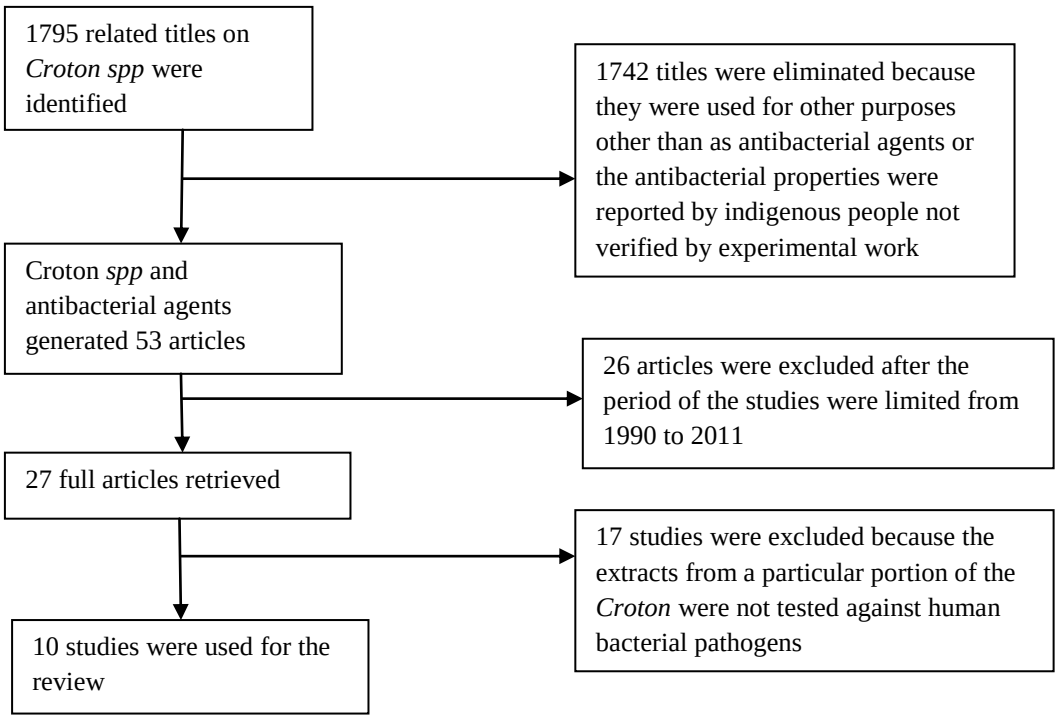

Fig. 1. Flow chart of literature search

5.2. Characteristics of the included studies

5.2.1. *Croton* species

The genus *Croton* belongs to the subfamily Crotonoideae of family Euphorbiaceae, one of the largest families of plants, often characterized by being monoecious. The predominant genera under the Euphorbiaceae family are *Drypetes, Jatropha, Macaranga, Croton, Euphorbia, Acalypha, Glochidion* and *Macaranga*.

The genus *Croton* has about 1300 species of trees, shrubs and herbs and is found in the tropical and subtropical regions of North and South Hemispheres. A number of the *Croton* species are known for their medicinal qualities especially in Africa, Asia and South America. *Croton* has been found to possess secondary metabolites such as alkaloids, terpenoids, flavanoids and compounds such as diterpenoids. *Croton spp*. are commonly used for the treatment of non communicable diseases such as diabetes, cancers and other ailments such as digestive problems, dysentery, wounds, fevers, constipation, diarrhea, intestinal worms, malaria, pain ulcers, inflammation. The parts that are used for the treatments of the different kinds of disease are the leaves, the roots, the stem barks, the fruit and the seeds (Yibralign 2007).

The *Croton* spp. used for this review are found in different regions; *C. megalobotrys C. steenkapianus, C. silvaticus (*Matias et al. 2011) and *C. pseudopulchellus* in South Africa; *C. zambesicus* (Abo et al. 1999) in Nigeria; *C. macrostachyus* (Wagate et al. 2010), in Kenya; *C. tiglium* (Shalid et al. 2008) Pakistan; *C. campestris* (Matias et al. 2011), *C. zehntneri* (Rodrigues et al. 2009), *C. cajucara (*Alviano et al. 2005), *C. urucurana* (Peres et al. 1997); and *C. sonderianus* (McChesney et al. 1991) in Brazil.

Fig. 2. The picture above is *C. macrostachyus* one of the *Croton* species. Photographied by Jackie Obey in “Nature Preserve” of the campuses of University of Eastern Africa, Baraton Kenya.

Table 1. Information on the various species of *Croton*

Author(s)	*Croton spp*.	Location of the plant species	Plant identification	Local name(s)/ vernacular name(s)	Traditional use
Matias et al. 2011	*C. campestris*	Municipality of Crato, Ceara, Brazil	Herbario da Universidade Federal do Rio Grande do Norte	Velame do campo	Depurative against scrophulosis, venereal diseases, skin diseases, rheumatism, ulcers, tumors
Selowa et al. 2010	*C. megalobotrys* *C. steenkapianus* *C. silvaticus*	Lowveld National Botanical Garden in Nelspruit, South Africa.	Lowveld national Botanical Garden staff	N/A	Use as purgative
Rodrigues et al. 2009	*C. zehntneri*	Cranto county, Ceara State, Brazil	Da'rdano Andrade Lima Herbarium, University of Regionaldo Cariri - URCA. Brazil	N/A	Sedative, appetite stimulating antianorexigen, relief of gastrointestinal disturbances
Shalid et al. 2008	*C. tiglium*	Punjab Agriculture College	Department of Botany, University of Agriculture, Faisalabad, Pakistan	N/A	Use as purgative and antispasmodic
Alviano et al. 2005	*C. cajucara*	Embrapa Experimental Farm, Amazonas, Brazil	Embrapa Experimental Farm, Amazonas, Brazil	N/A	Antileishmanial activity

Table 1 continued

Reference	*Croton spp*.	Location of the plant species	Plant identification	Local name(s)/ vernacular name(s)	Traditional uses
Lall and Meyer 1999	*C. pseudopulchellus*	South and Central parts of South Africa (Lady Grey, Aliwal North, Elliot, Berkley East, Durban, Umalazi)	HGWJ Schweicherdt Herbarium of the University of Pretoria and the Herbarium of the National Botanical Institute, Pretoria, South Africa	N/A	TB symptoms such as coughs, fever, blood in sputum
Abo et al. 1999	*C. zambesicus*	Collection was done in Ibadan, Nigeria	Forestry Research Institute of Nigeria	'Iyeye', 'Ajekofol e'	Typhoid, diarrhoea, dysentery
Peres et al. 1997	*C. urucurana*	Dourados MS, Brazil	Herbarium of the Centro de Ciencias Biologicas e da Saude, Campo Grande MS Brazil	Sangra d'agua (Dragon' d blood)	Wound infections, celearte wound healing, rheumatism, cancer
McChesney et al. 1991	*C. sonderianus*	Sobral, Ceara, Brazil	Herbarium of the Botanica, University of Ceara. Brazil	Marmelei ro preto	Gastric diseases
Wagate et al. 2010	*C. macrostachyus*	Machakos and Kitui regions of Eastern Kenya	Department of Land and Resource management and Agricultural Technology, University of Nairobi, Kenya	Mukambi /Kitundu	Typhoid and measles

NA: Not available

5.2.2. Parts of the *Croton* plants used for the experiments

Efficacy of biologically active compounds in plant extracts against bacterial pathogen depends on factors such as region where the species is found, the time period within which the plants parts were collected and the storage condition (Taniguchi and Kubo 1993). Different parts of plants *Croton* spp. plants have their typical compounds; saponins and resins are usually found in the seeds (PROTA 2011) crotepoxide and crotomacrine in fruits (Tane et al. 2004). Stem barks and twigs contain fatty acids, lupeol, betulin (PROTA 2011), roots contain chalcone and secondary metabolites such as 3β-acetoxy tetraxer-14-en-28-oic acid, trachyloban-19-oic acid, trachyloban-18-oic acid, neoclerodan-5,10-en-19,6β; 20,12-diolide, 3α,19-dihydroxy trachylobane, 3α,18,19-trihydroxy trachylobane (Kapingu et al. 2000).

Table 2. Parts of *Croton* species used in the studies

Croton species	Part of the plant used in the studies
C. macrostachyus	Whole plant Leaves Roots Bark Tuber
C. sonderianus	Roots
C. zambesicus	Leaves Stem bark
C. pseudopulchellus	Whole plant, Stem bark Roots Leaves
C. cajucara *C. zehntneri* *C. steenkapianus* *C. silvaticus* *C. campestris*	Leaves
C. tiglium	Seeds
C. urucurana	Stem bark

5.2.3. Preparations of the various parts of the *Croton* species in the studies

Different methods have been used for the preparation of the *Croton* plant material before the process of extraction takes place. Plant parts (whole plant, bark, root, leaves, tubers or a mixture of different parts) were chopped into smaller pieces air-dried at room temperature under shade and pulverized (Wagate et al. 2010). Similarly, stem bark of *C. urucurana was* air-dried at room temperature and pulverized (Peres et al. 1997). Roots of *C. sonderianus* were air-dried at room temperature and ground into powder (McChesney et al. 1991), in the case of *C. cajucara* leaves were coarsely ground into power after being dried at room temperature (Shalid et al. 2008). *C. zambesicus* was however oven-dried (Abo et al. 1999).

5.2.4. Extraction

Extraction with organic solvents

Various extracts, organic and inorganic, at different concentrations, have been used to obtain the biologically active compounds present in the various parts of *Croton* plants. Most often organic solvents have been used for the extraction. In a study by McChesney et al. (1991), plant material was extracted using hexane, acid and neutral solvents. The extracted compound *ent*-beyer-15-en-18-oic acid and its derivatives such as ent-*beyer-15-en-18-oic acid methyl ester*; ent-*beyer-15-en-18-ol*; ent-*beyer-15-en-18-al*; and *dihydro*-ent-*beyer-15-en-18-ol* exhibited antibacterial properties. In the study done by Peres et al. (1997), both methanol as the primary extract and *n*-hexane, *n*-hexane/dichloromethane, ethyl acetate and methanol as fractions of the primary extract exhibited antibacterial activities. Studies by Shalid et al. (2008) used ethanol but at different concentrations for the extraction process. Experiment by Lall and Meyer, 1999, used acetone to extract plant material. In cases of Abo et al. (1990), Selowa et al. (2010), Matias et al. (2011), Wagate et al. (2010) methanol was used for the extraction processes.

Extraction with inorganic solvents

In one study the use of water for extraction was reported. The water extract was prepared by boiling 20 grams of the aerial part of the plant material in 500 ml of distilled water under reflux and the extracts were dried. The residue was dissolved in water to a final concentration of 500 mg/ml (Lall and Meyer 1999).

Extraction with other methods

Two studies use hydrodistillation by the Clevenger apparatus to obtain oil from the leaves of *C. cajucara* (Alviano et al. 2005) and *C. zehntneri* (Rodrigues et al. 2009). Hydrodistillation in the case of *C. zehntneri* was followed by drying with anhydrous sodium sulphate to produce gaseous oil.

Table 3. Extracts and extraction methods used for the *Croton* species

Reference	Preparation if the plant material (into a powder)	Primary extracts	Extraction process	Secondary extracts	Isolated compounds
C. campestris (Matias et al. 2011)	N/A	Methanol Hexane	N/A	N/A	N/A
C. megalobotrys (Selowa et al. 2010)	N/A	Methanol n-Hexane	N/A	Chloroform *n*-Hexane	N/A
C. steenkapianus (Selowa et al. 2010)	N/A	Dichloromethane Ethyl acetate Acetone	N/A	Carbon tetrachloride Butanol	N/A
C. silvaticus (Selowa et al. 2010)	N/A	N/A	N/A		
C. zehntneri (Rodrigues et al. 2009)	N/A	N/A	Hydrodistillation	N/A	N/A
C. tiglium (Shalid et al. 2008)	N/A	Ethanol	N/A	N/A	Ct-50 (purified protein)
C. cajucara (Alviano et al. 2005)	Coarsely grounded into a powder, dried at RT	N/A	Hydrodistillation	N/A	Linalool Purified linalool

N/A: Not available, RT: Room temperature

Table 3 continued

Reference	Preparation if the plant material (into a powder)	Primary extracts	Extraction process	Secondary extracts	Isolated compounds
C. pseudopulchellus (Lall and Meyer, 1999)	N/A	Acetone Water	N/A	N/A	N/A
C. zambesicus (Abo et al. 1999)	N/A	Methanol	N/A	N/A	N/A
C. urucurana (Peres et al. 1997)	Air-dried at RT and pulverized.	Aqueous ethanol	N/A	n-Hexane n-Hexane/ dichloro methane Ethylacetate Methanol	Acetyl-aleuri-tolic acid β-Sitosterol-O-gasslucoside Sonderianin β-Sitosterol Campesterol Catechin Gallocatechin
C. sonderianus (McChesney et al. 1991)	Air-dried at RT, ground into a powder	Hexane	N/A	Neutral fraction Acidic fraction	N/A
C. macrostachyus (Wagate et al. 2010)	Chopped, air-dried at RT under shade, pulverized	Methanol	N/A	N/A	N/A

N/A: Not available, RT: Room temperature

5.3. Studied bacteria used for the experiments

Different bacteria were used in the referred studies they were: *Bacillus subtilis, Staphylococcus aureus, E. coli, Pseudomonas aeruginosa, Mycobacterium smegmatis, Salmonella typhimurium, Mycobacterium tuberculosis, Salmonella typhosa, Shigella dysenteriae, Klebsiella pneumoniae, Proteus mirabilis, Bacillus megaterium, Lactobacilus casei, Streptococcus sobrinus, Streptococcus mutans, Porphyromonas gingivalis, Pasturella multocida, Enterococcus faecalis, Micrococcus lutea, Bacillus cereus.*

For infections to occur, bacterial adhesion is required as well as the host cell surface carbohydrates, which serve as receptors for adhesion. Adherence of bacterial cells to specific receptors such as glycoconjugates and extracellular matrix molecules prevents dislodgement by the host mucociliary defense mechanisms and aid bacterial invasion into cells (Kouki et al. 2011). *E. coli* P fimbrial adhesins bind to the galactosyl-1–4-galactose (Galα1-4Gal) in the host receptor cells. Thus *E. coli* causes many infectious diseases in human such as urinary tract infections (Dodson et al. 2001) and common intestinal diseases (Matias et al. 2011).

The genus *Staphylococcus* is distributed in the environment and is often etiological agent for opportunistic infections in humans and animals. *S. aureus, S. epidermidis, S. saprophyticus* and *S. haemolyticus* are on top of the list of causative organisms of human and hospital infections. *S. aureus* is known to be the common etiological agent for purulent infections. *E. coli, E. faecalis, S. aureus* and *P. aeruginosa* as species of bacterial often involved in nosocomial infections (Verhoeff et al. 1999).

According to Alviano and colleagues (2005), *L. casei, S. aureus, S. sobrinus, P. gingivalis* and *S. mutans* are generally associated with oral cavity disease. These microorganisms occupy surfaces of teeth and below the gingival margins (Parsek *et al*., 2003; Wu *et al*., 2002). The pathological states such as dental carries, periodontal diseases and tooth loss may eventually affect the overall health of the individuals (Socransky et al. 2002). *Staphylococcus aureus* and *P. aeruginosa* are known to be respiration tract bacterial pathogens. Sputum from patients with cystic fibrosis has been found to contain substantial amount of *P. aureginosa* and *S. aureus* (Coutinho et al. 2008, Valenza et al. 2008).

Tuberculosis caused by *Mycobacterium tuberculosis* (TB) affects close to 33% of the world's population. Individuals infected by human immunodeficiency virus (HIV) are susceptible to TB. Majority of the incidents occur in Sub-Saharan Africa where in 2009 1.7 million people were estimated to have died of the tuberculosis disease. 5-10% of individuals infected with TB bacilli develop the infection at a point in their lives. The treatment and control of tuberculosis have been increasingly difficult because the bacilli have developed resistance to antibiotics such as isoniazid and rifampicin (WHO 2011).

5.4. Methods for testing the antibacterial properties

For the antibacterial tests different methods have been used. McChesney et al. 1991 used the two-fold serial broth dilution assay for quantification of the antimicrobial activity. Lall and Meyer (1999) used the agar plate and radiometric methods. This method utilized 7H12 Middlebrook TB medium with ^{14}C-labelled substrate (palmitic acid) as a source of carbon. Abo et al. (1999) used the agar disc diffusion method to determine the antimicrobial activity of the *Croton* plant extracts. Shalid et al. (2008) reported that they used the disc diffusion method to determine the antimicrobial activity. Selowa et al. (2010) tested antibacterial activity of the *Croton* species employing the micro-dilution techniques on 96 well micro-plates.

In order to determine the antibacterial activity of the essential oil isolated from *C. zehnteri*, the two-fold serial dilution method was used. In the plate method bacterial organisms were innoculated into Petri dishes, which contained the nutrient agar. The antibiotic modifying activity of the volatile component of the essential oil was also assessed using the plate method. Antibiotic disks containing gentamycin and tetracycline were used to determine alterations in inhibition zone diameter against *P. auriginosa* (Rodrigues et al. 2009).

In studies carried out by Mathias et al. 2011, the antibacterial activity was assessed by using a microdilution assay. In the method certain amount of each bacterial strain was suspended in 96-well microtiter plate and titrated by using two-fold serial dilution method.

Antibacterial activity of the plant extracts against the reference bacterial strain was carried out using the broth dilution method in studies by Wagate et al. 2010. The test organisms and the different methods used for the antibacterial tests have been collected in Table 4.

Table 4**.** Test organisms and methods for antibacterial tests

Reference	Test organisms		Method for antibacterial test
	Gram-negative bacteria	Gram-positive bacteria	
McChesney et al. 1991	*E. coli* *P. aeruginosa*	*S. aureus* *B. subtilis* *M. smegmatis*	Two-fold serial broth dilution assay
Lall and Meyer, 1999	N/A	**M. tuberculosis*	Plate method Radiometric method
Abo et al. 1999	*E. coli* *S. typhosa* *S. dysenteriae* *K. pneumonia* *P. mirabilis* *P. aeruginosa*	*S. aureus* *B. subtilis* *B. megaterium*	Agar disc diffusion method
Shalid et al. 2008	*P. multocida*	*B. subtilis*	The disc diffusion method
Selowas et al. 2010	*E. coli* *P. aureginosa*	*E. faecalis* *S. aures*	Micro-dilution technique with 96 well micro – plates
Rodrigues et al. 2009	*P. aureginosa*	*S. aureus*	Serial dilution (two fold)
Matias et al. 2011	*E. coli*	*S. aureus*	Serial dilution (two fold)
Wagate et al. 2010	*E. coli* *P. aeruginosa*	*M. lutea* *B. cereus*	Broth dilution method

Mycobacterium tuberculosis is neither Gram positive nor Gram negative bacteria, N/A: Not available.

The minimum inhibitory concentration (MIC), zone of inhibition (ZH) and minimum inhibitory dose (MID) were the commonly used measures of the antibacterial activity of the extracts and pure compounds against the selected bacterial organisms. Lall and Meyer (1999) simply defined MIC as the lowest concentration of drug that inhibited 99% of the growth of the bacterial population. Matias et al. (2011) defined MIC as the lowest concentration, where no growth was observed. One study determined the antibacterial activity visually. MIC value was therefore taken as the lowest concentration of each of the substance at which turbidity was absent (Alviano et al. 2005).

The appearance of a clear zone around the growth bacteria indicated antimicrobial activity. The zone of inhibition (Shalid et al. 2008) was measured using a zone reader (Huynh et al. 1999). Studies by Abo et al. (1999) used the zone of inhibition measured manually in millimeters to express the antimicrobial activities of the plant extracts.

McChesney et al. (1991) recorded the antimicrobial activity against *B. subtilis* as the width (millimeters) of the inhibitory zone (average radius) after incubating the bacteria for 24 and 48 hours measured from the edge of the agar well to the edge of the inhibitory zone. The concentration of the compound in the first of the test tube that showed no visible growth after 24- and 48-hours incubation was taken as the MIC.

The antibacterial and the synergistic effects of the volatile component of the essential oil extracted from *C. zehntneri* were expressed as the minimum inhibitory dose (MID). It was defined as the minimum inhibitory dose per unit space required to suppress the growth of microorganisms in a closed system (Rodrigues et al. 2009). MID values were given as the weight per volume of air (mg/L) (Inouye et al. 2001). Three studies (Wagate et al. 2010, Selowa et al. 2010, Peres et al. 1997) did not give specific definitions for the MIC but provided the general experimental procedures and values for the antibacterial activity of the plant extracts.

5.5. Antibacterial activity of organic extracts

5.5.1. Antibacterial activity of the methanol extracts

Wagate and colleagues (2010) found MICs between 15-250 mg/ml after testing methanol extracts of *C. macrostachyus* against four bacterial organisms. *M. lutea* was, however, not sensitive to methanolic extracts from the plant. Inoculum of 0.1mL in a test tube containing Muller Hilton broth was used as a negative control. As the positive controls, benzylpenicillin with MIC of 0.6mg/mL and streptomycin with MIC of 0.25mg/ml were used for Gram-positive and Gram negative bacteria, respectively (Wagate et al. 2010).

When gentamicin, kanamycin and amikacin were used as antibiotics and tested against *E. coli*, MICs were 0.091, 0.157 and 0.157 mg/ml, respectively. When the methanolic *Croton* extracts were used alone, MIC value was 0.512mg/ml. Against *S. aureus*, gentamicin, kanamycin and amikacin gave MICs of 0.039, 0.317 and 0.078 mg/ml respectively. When methanolic *Croton* extracts were used alone, MIC of ≥1.024 mg/ml was achieved. Values shown in the table 7 indicate the MICs of the various combinations of drug-hexane extracts decreased MIC values for both *S. aureus* and *E. coli* (Matias et al. 2011).

Results from Selowa et al. (2010) showed that, when tested against the different bacterial organisms, methanol extracts from the three *Croton* species: *C. megalobotrys, C. steenkapiamus, and C. silvaticus* produced different results. *C. megalobotrys* inhibited only *E. coli* and at higher concentrations, *C. steenkapiamus* inhibited *P. auruginosa*, but was inactive against *E. coli*, *S. aureus* and *E. faecalis*. In comparison, *C. salvaticus* inhibited weakly all the test organisms at the constant concentration of 1.25 mg/ml.

Table 5. Antibacterial activity of the methanol extracts from different *Croton* species expressed as MIC

Reference	*Croton* species	Bacterial strain	Antibacterial activity MIC (mg/ml)
Wagate et al. 2010	*C. macrostachyus*	*M. lutea*	N/A
		B. cereus	15.6
		E. coli	250
		P. aeruginosa	250
Selowa et el. 2010	*C. megalobotrys*	*E. coli*	1.25
		S. aureus	0.625
		E. faecalis	0.02
		P. aeruginosa	0.313
	C. steenkapianus	*E. coli*	N/A
		S. aureus	N/A
		E. faecalis	N/A
		P. aeruginosa	0.625
	C. silvaticus	*E. coli*	1.25
		S. aureus	1.25
		E. faecalis	1.25
		P. aeruginosa	1.25

MIC: Minimum inhibitory concentration, N/A: Not active

The figures in the table 6 show the zones of inhibition in millimeters of the methanol extracts of *C. zambesicus* against the tested bacterial organisms. Dilutions of each dried extract were prepared in 70% methanol to give final test concentrations of 100mg/ml, 50mg/ml and 25mg/ml. For Gram positive and Gram negative bacteria 10µg/ml of gentamycin and 10µg/ml of ampicillin both in 70% methanol were used as the positive controls, respectively. When 70% of methanol was used as the negative control, no inhibition was observed. Methanol extracts from the stem bark had an overall better antibacterial activity values than methanol extracts from the leaves against *P. aeruginosa* and *K. pneumonia*, but had no inhibitory activities on *S. typhosa* and *E. coli*. The measured antibacterial activity of methanol extract of *C. zambesicus* against *P. mirabillis*, *S. aureus*,

B. megaterium and *B. subtilis* were comparable to that of ampicillin at 10µg/ml. Methanol extracts from the stem back of *C. zambesicus* produced similar results as gentamycin at the concentration of 10µg/ml (Abo et al. 1999)

For *C. campestris*, the antibiotic activities were assessed using their respective MICs in the presence or absence of methanol and hexane extracts at the subinhibitory concentration of 8 µg/ml. As the control, dimethyl sulfoxide (DMSO) was used. Table 7 shows the MICs of the extracts and extract-antibiotic combinations.

For *E. coli*, MECC-antibiotic combination produced lower MIC values with the exception of gentamicin-MECC, and for HECC-antibiotic combination, the MICs were generally lower for all the antibiotics. In the case of *S. aureus* HECC-antibiotic combinations had MICs lower than antibiotics alone. *E. coli* showed greater sensitivity to the extracts than *S. aureus* but *S. aureus* exhibited greater sensitivity when antibiotics were combined with the extracts. The DMSO control produced the MIC of ≥1024 µg/ml and exhibited no antibiotic modifying activities (Matias et al. 2010).

Table 6. Antibacterial activity of *C. zambesicus* and reference antibiotics (10µg/ml) measured as zone of inhibition (ZH) (Abo et al. 1999)

Bacteria	Dose (mg/ml)	ZH (mm)		Antibiotic
		Leaf	Stem bark	
P. aeruginosa	100	0	12	0 (ampicillin)
	50	0	10	14 (gentamycin)
	25	0	0	
S. dysenteriae	100	10	15	
	50	7	13	15 (gentamycin)
	25	0	10	
S. typhosa	100	17	14	
	50	14	10	18 (gentamycin)
	25	10	0	
E. coli	100	16	15	
	50	13	12	18 (gentamycin)
	25	10	0	
K. pneumonia	100	0	12	
	50	0	10	18 (gentamycin)
	25	0	9	
P. mirabilis	100	9	13	
	50	7	11	12 (ampicillin)
	25	7	0	
S. aureus	100	14	16	
	50	11	14	12 (ampicillin)
	25	9	10	
B. megaterium	100	12	12	
	50	10	10	10 (ampicillin)
	25	7	8	
B. subtilis	100	10	11	
	50	0	9	9 (ampicillin)
	25	0	7	

0 - no inhibition

Table 7. MIC values (µg/ml) of aminoglycosides in the absence and presence of 8 µg/ml of MECC and HECC against *E. coli* and *S. aureus* (Matias et al. 2010)

Antibiotic	*E. coli*			*S. aureus*		
	MIC alone	MIC combined		MIC alone	MIC combined	
		MECC	HECC		MECC	HECC
Gentamicin	19	9	2.2	39	2.2	2.2
Kanamycin	157	157	19	317	78	4.5
Amikacin	157	9	39	78	9	4.5
MECC	512	-	-	≥1,024	-	-
HECC	256	-	-	≥1,024	-	-

MECC: Methanol extracts of *C. campestris*, HECC: Hexane extracts of *C. campestris*, MIC: Minimum inhibitory concentration

5.5.2. Antibacterial activity of the ethanol extracts

In the ethanol extracts the appearance of clear zone was an indication of antibacterial activity. Table 8 shows different figures used to represent the extent of the activity measured in zone size: 0 mm, 1-5 mm, 6-15 mm and 16-25 mm, for no or poor activity, moderate activity, strong activity and very strong activity, respectively. Ciprofloxacin was used as the positive control and the negative control was autoclaved water. The ethanol extracts of *C. tiglium* strongly inhibited both *P. multocida* and *B. subtilis*. Ciprofloxacin inhibited strongly *P. multocida* and *B. subtilis* compared with autoclaved water, which had no antibacterial activity (Shalid et al. 2008).

Table 8. Antibacterial activity of the ethanol extracts and controls expressed as zone of inhibition (ZH) (Shalid et al. 2008)

Croton species	Bacterial strain	ZH (mm)	Control	
			Positive	Negative
C. tiglium	*P. multocida*	6-15	16-25	NA
	B. subtilis	6-15	16-25	NA

NA: no activity, ZH: Zone of inhibition

According to Peres et al. (1997), the aqueous ethanolic, *n*-hexane and *n*- hexane/ dichloromethane extracts of *C. urucurana* exhibited better antibacterial activity against *S. aureus* than against *S. typhimurium* (table 9). The *n*-hexane/dichloromethane extracts showed the highest inhibitory activity against *S. aureus* with MIC value of 0.8mg/ml followed by aqueous ethanol extracts with MIC value of 2mg/ml and *n*-hexane with MIC value of 3.5 mg/ml. *S. typhimurium* was resistant to the aqueous ethanolic extracts and all activity with MIC values of 4-6 mg/ml.

Table 9. Antibacterial activity of the ethanol extracts and fractions of *C. urucurana* expressed as MIC (Peres et al. 1997) against *S. aureus* and *Salmonella typhimurium*

Extracts	MIC (mg/ml)	
	S. aureus	*Salmonella typhimurium*
Aqueous ethanolic	2	5
n-Hexane	3.5	6
n-Hexane/dichloromethane	0.8	4
Ethyl acetate	4.0	4
Methanol	5.0	5

MIC: Minimum inhibitory concentration

5.5.3. Antibacterial activity of hexane extracts

McChesney et al. (1991) tested hexane extracts from *C. sonderianus* against five bacterial strains to determine its antimicrobial activity. The zones of inhibition were put into different categories: no activity, 1-2 mm, 3-6 mm, 7-12 mm and greater than 13 mm of inhibition. The positive control was streptomycin sulfate at the concentration of 1mg/ml. Hexane extracts produced no inhibitory activity against *E. coli* but against *B. subtilis* the highest activity level of >13 mm was recorded. Against *P. aeruginosa, S. aureus* and *M. smegmatis* had activity of 7-12 mm. Comparable results were obtained when streptomycin sulfate was used, however, *M. smegmatis* was the most sensitive strain.

Table 10. Antibacterial activity of the hexane extracts and streptomycin sulfate expressed as the zone of inhibition (ZH) (McChesney et al. 1991)

Croton species	Bacterial strain	ZH (mm)	
		Hexane extract	Positive control
C. sonderianus	*E. coli*	N/O	7-12
	P. aeruginosa	7-12	7-12
	S. aureus	7-12	7-12
	B. subtilis	>13	7-12
	M. smegmatis	7-12	> 13

ZH: Zone of inhibition, NO: No observed zone of inhibition.

5.5.4. Antibacterial activity of the acetone extracts

The proportion method referred also as the plate method proposed by Middlebrook and Cohn (1958) allows the determination of the proportion of bacterial population resistant to the plant extracts. A population is considered resistant when 1% or more of the microorganisms are resistance to a drug. The *M. tuberculosis* H37Rv strain was susceptible to several antibiotics: streptomycin, isoniazid, ethambutol and rifampin. Two dilutions from the sensitive strain (H37Rv) suspensions were made (1×10^{-2} mg/ml and 1×10^{-4} mg/ml). 0.2ml of 1×10^{-2} mg/ml H37Rv suspension was added to the plant extracts (acetone and water). For the two controls (medium + 1% DMSO), 0.2ml of 1×10^{2} mg/ml dilution and 0.2ml of 1×10^{4} mg/ml dilution were added separately.

The antibacterial activities were determined by comparing growth of bacteria in the medium with plant extract (marked as N^{-2} for 1×10^{-2} mg/ml dilution) to bacterial growth in the control (NO^{-2} for 1×10^{-2} mg/ml and NO^{-4} for 1×10^{4} mg/ml). The strain was considered resistant when $N^{-2} \geq NO^{-2}$ sparingly susceptible when $NO^{-4} \leq N^{-2} \leq NO^{-2}$ and at $N^{-2} \leq NO^{-4}$ the strain was considered sensitive (less than 1% growth). As shown in the table 10, the MIC of acetone extracts of the areal parts of *C. pseudopulchellus* was 0.5mg/ml (Lall and Meyer 1999).

Table 10. Antibacterial activity of the acetone and water extracts of *C. pseudopulchellus* against H37Rv strain of *Mycobacterium tuberculosis* (MIC) using the agar plate method (Lall and Meyer 1999)

Croton species	Bacterial strain	MIC (mg/ml)	
		Acetone extract	Water extract
C. pseudopulchellus	H37Rv strain	0.5	NA

MIC - Minimum inhibitory concentration, NA - not active, H37Rv - Antibiotic sensitive strain of *M .tuberculosis*.

The radiometric method was used to confirm the results achieved from the agar plate method. This method is fast, reliable and convenient method for the determination of levels of antibacterial activities of drugs used against *M. tuberculosis* (Heifets et al. 1985). The plant extracts were tested against *M. tuberculosis* strain (CCKO28469V), which is resistant to two drugs (isoniazid and rifampin) and against the sensitive strain H37Rv susceptible to several antibiotics (streptomycin, isoniazid, ethambutol and rifampin). Antibacterial activities of the plant extracts were assessed at the concentrations of 1.0, 0.5 and 0.1 mg/ml. Two vials without plant extracts were used as the controls. Values in the table 11 indicate that, MICs for sensitive and resistant strains were 0.1 and 0.5 mg/ml, respectively. The changes in growth index (ΔGI) values, given as mean±SD, of the control vials were 29±4.04 and 24±4.04 for the sensitive and resistant strains of *M. tuberculosis,* respectively (Lall and Meyer, 1999).

Table 11. Antimicrobial activity of the acetone extracts from *C. pseudopulchellus* on the growth of the strains H37Rv and CCKO28469V of *Mycobacterium tuberculosis* (Lall and Meyer, 1999)

Bacterial strain	MIC (mg/ml)	ΔGI values (mean±SD) of plant extracts (mg/ml)
Sensitive strain (H37Rv)	0.1	2±0.5
Resistant strain (CCKO28469V)	0.5	1±1.1

MIC: minimum inhibitory concentration; ΔGI: Differences in the growth index values; SD: standard deviation.

5.6. Antibacterial activity of the inorganic extracts

Lall and Meyer (1999) tested water extract of *C. pseudopulchellus* against a sensitive strain (H37Rv) of *Mycobacterium tuberculosis*. As seen in the table 10, water extract was inactive against the bacteria.

5.7. Antibacterial activity measured by other extraction methods

Hydrodistillation was used to extract oil from the leaves of *C. zehntneri*. The antibacterial activity of the essential oil was expressed as minimum inhibitory dose (mg/l air). From Table 12, *P. aeruginosa* showed resistance against the essential oil of *C. zehntneri* at all concentration used. *S. aureus* showed susceptibility at the concentrations 1 and 0.5 mg/l air (Rodrigues et al. 2009).

Table 12. Antibacterial activity of *C. zehntneri* essential oil assessed as MID (Rodrigues et al. 2009)

Bacterial strain	MID (mg/L air)					
	1	0.5	0.25	0.125	0.0625	0.03125
S. aureus	-	-	+	+	+	+
P. aeruginosa	+	+	+	+	+	+

+: growth observed; - : no growth observed; MID: minimum inhibitory dose.

Using the plate method, the antibiotic modifying activity of volatile compound of *C. zehntneri* essential oil (CZEO) was determined. Two antibiotics, gentamicin and tetracycline were used against *P. aeruginosa*. There were two set of controls used: plates with antibiotic discs without essential oil and plates with DMSO alone (Rodrigues et al. 2009). As seen from table 13, no significant changes in inhibition were observed for the controls. *C. zehntneri* essential oil (CZEO) enhanced the antibiotic activity of gentamicin by 42.8%; antibiotic activity of tetracycline plus CZEO remained unchanged (Rodrigues et al. 2009).

Table 13. Antibacterial modifying activity of *C. zehntneri* essential oil (CZEO) volatile compound assessed as mimimum inhibitory dose (MID 1 mg/L air) against *P. aeruginosa* (Rodrigues et al. 2009)

Treatment	ZH			
	Gentamicin ± SD	Enhancement (%)	Tetracyclin ±SD	Enhancement (%)
No treatment	14±1.6	-	32±1.3	-
DMSO	14±1.3	-	32.5±1.6	-
CZEO	20±1.6	42.8	32±1.6	0

CZEO: *C. zehntneri* essential oil, SD: Standard deviation, DMSO: dimethyl sulfoxide, ZH: Zone of Inhibition, - : no enhancement value provided.

Hydrodistillation was also used to extract linalool-rich essential oil from *C. cajucara* (EOCC). The extracted essential oil inhibited the growth of the all tested bacterial organisms. Table 14 shows the effects of EOCC on *L. casei, S. aureus, S. sobrinus, P. gingivalis* and *S. mutans*. The EOCC had higher activity than the standard drug chlorhexidine based on the MIC values. *S. sobrinus* was the most sensitive bacterial to EOCC (Alviano et al. 2005).

Table 14. Antibacterial activity of linalool rich essential oil from *C. cajucara* represented as MIC values (mg/ml) (Alviano et al. 2005)

	MIC	
Bacterial strain	Linalool-rich EOCC	*Chlorhexidine
L. casei	22.3	36.5
S. aureus	33.4	40.5
S. sobrinus	13.8	65
P. gingivalis	31.2	48
S. mutans	40.1	55

*Chlorhexidine was used as the standard drug, EOCC: Essential oil of *Croton cajucara*, MIC: Minimum inhibitory concentration.

5.8. Chemical composition of the various *Croton* species

Three studies carried out by Wagate et al. 2010, Lall and Meyer, 1999 and Selowa et al. 2010, did not provide information on the biologically active compounds responsible for the antibacterial activity. They mentioned, however, further studies on phytochemical analysis of the bioactive components of the extracts will be required. Information on the active compounds provided by Rodrigues et al. (2009) was based on literature. In addition to *ent*-beyer-15-en-18-oic acid, derivatives such as ent-*beyer-15-en-18-oic acid methyl ester*; ent-*beyer-15-en-18-ol*; ent-*beyer-15-en-18-al*; and *dihydro*-ent-*beyer-15-en-18-ol* were mentioned (McChesney et al. 1991). Table 15 shows the various compounds present in the different *Croton* species.

Table 15. Compounds present in the various *Croton* species

Studies	*Croton* species	Compound(s) present
Peres et al. 1999	*C. urucurana*	Acetyl aleuritic acid *β*-sitosterol-*O*-glucoside Sonderianin Steroids (stigmasterol, *β*-sitosterol, campesterol) Catechin Gallacatechin
Abo et al. 1999	*C. zambesicus*	Tannins Saponins Anthraquinones Alkaloids
McChesney et al. 1991	*C. sonderianus*	*ent*-Beyer-15-en-18-oic acid
Alviano et al. 2005	*C. cajucara*	Linalool
Shalid et al. 2008	*C. tiglium*	Proteins
Rodrigues et al. 2009	*C. zehntneri*	Sesquiterpens *Trans*-Anethol *Trans*-Caryophyllen Myrcene,α-Pinene 1,8-Ceneole, Estragole Thymol, Carvacrol
Matias et al. 2011	*C. campestris*	Tannin phlobaphenes Flavones Flavonols Xanthones Chalcones Aurones Flavononols Catechins Flavonones Alkaloids Terpenes

6. DISCUSSION

Methanol was the most commonly used extraction solvent of *Croton* species against the various pathogenic bacteria. *C. zambesicus, C. megalobotrys*, *C. steenkapiamus*, *C. silvaticus*, *C. macrostachyus* and *C. campestris* were extracted with methanol. Ethanol was used with *C. urucurana* and *C. tiglium* and to extract *C. sonderianus* hexane was used. *Croton pseudopulchellus* was extracted using acetone and water. Essential oils were extracted from *C. zehnteri* and *C. cajucara* using hydrodistillation.

Gram positive bacteria were the most commonly tested bacteria in the various experiments: *S. aureus* appeared in most of the studies. The gram negative *E .coli* was reported in five out of the ten studies used. Essential oils of *C. zehntneri* and hexane and methanol extracts of *C. campestris* enhanced antibiotic activity against certain Gram positive and Gram negative bacteria.

The sensitivity of the studied bacteria was dependent on the *Croton* species used, the origin of the plant species, time of the collection, the storage conditions, the part of the plant used, the kind of the extracts used, the dose and the bacterial strain. In addition, the methods of the antibacterial assessment may influence on the outcome of the tests (Wagate et al. 2010).

6.1. Gram negative bacteria and *Croton* extracts

When hexane extracts of *C. sonderianus* was tested against *E. coli,* no activity was recorded. Standard antibacterial, streptomycin sulfate, produced appreciable zone of inhibition of 7-13 mm. In addition to acidic and neutral fractions of the hexane extracts as well as *ent*-beyer-15-en-18-oic acid obtained from acidic portion demonstrated no antibacterial activity against *E. coli*. In one study, methanol extracts of leaves and roots of *C. macrostachyus* were active against *E. coli* having MIC of 250 mg/ml. *Salmonella typhimurium* was resistant to aqueous extracts of *C. urucurana* at the MIC value of 5mg/ml. In addition, *S. typhimurium* was resistant to all extracts with the MIC values between 4-6 mg/ml. When *S. typhimurium* was tested against catechin isolated from ethyl acetate fraction and acetyl aleuritolic fraction derived from hexane/dichloromethane

fraction, MICs of 1.0 mg/ml and 0.1 mg/ml respectively were obtained. This indicated the inhibitory potencies of the tested compounds.

When methanol extract of *C. zambesicus* was tested against *Shigella dysenterium* and *Proteus mirabilis* antibacterial activity of 10µg/ml was achieved. This activity was similar to the MICs produced by gentamycin and ampecillin. Tannins, saponins, anthraquinones and alkaloids present in the leaves and stem bark of *C. zambesicus* may possess the antibacterial activity. Essential oil of *C. cajucara* inhibited the growth of *Porphyromonas gingivalis* owing to the presence of linalool, although purified fraction of linalool did not have any inhibitory effects against *P. gingivalis*.

Certain proteins (Ct-50) with molecular mass of 50 kDa in the crude hexane extracts and fractions of *C. tiglium* may have had inhibitory activity against *Pasteurella multocida*. While the methanol extracts of *C. steenkapianus* had no activity, those of *C. megalobotrys* and *C. slivaticus* weakly inhibited *E. coli* at the concentrations of 1.25mg/ml. Dichloromethane, ethyl acetate, acetone and methanol fractions of *C. megalobotrys* strongly inhibited *E. coli* at MICs ranging from 0.332-0.705 mg/ml. The *n*-hexane extracts inhibited weakly *E. coli*. The active compounds responsible for the inhibition *E. coli* had not been identified.

6.2. Gram positive bacteria and *Croton* extracts

Staphylococcus aureus was sensitive to aqueous ethanol extracts of *C. urucurana* at the MIC of 2.0 mg/ml. Hexane/dichloromethane fraction isolated from methanol extract had the MIC of 0.8mg/ml. When *S. aureus* was tested either to catechin purified from ethyl acetate fraction or to acetyl aleuritolic fraction derived from hexane/dichloromethane fraction, MICs of 1.0 mg/ml and 0.1 mg/ml were obtained respectively.
Tannins, saponins, anthraquinones, glycosides and alkaloids may have been the active compounds in the methanol extracts of *C. zambesicus* against *S. aureus, M. mageterium* and *B. subtilis*. This antibacterial effect was similar to that of ampicillin (10µg/ml).

Methanol extracts of roots and leaves of *C. macrostachyus* produced some antibacterial activity against *Bacillus cereus* and *Pseudomonas aeruginosa* at MICs of 15.6 mg/ml and

250 mg/ml, respectively, but no activity was recorded against *Micrococcus lutea*. Hexane extract of *C. sonderianus* and its acidic and neutral fractions broadly showed inhibitory activities against *B. subtilis, S. aureus* and *M. smegmatis*. Hexane extracts and the acidic fraction of the hexane extracts showed inhibitory activity against *Pseudomonas aeruginosa,* but not the neutral fraction and *ent*-beyer-15-en-18-oic acid. The compound *ent*-beyer-15-en-18-oic acid obtained from acidic portion of *C. sonderianus* exhibited strong antibacterial activity against *Bacillus subtilis* at the MIC value of 6.25µg/ml. Streptomycin had lower MIC of 3.12µg/ml, amphotericin B exhibited no activity. *Croton cajucara* essential oil inhibited all the tested bacteria: *Lactobacillus casei, S. aureus, Streptococcus sobrinus* and *S. mutans*. The purified linalool fraction was not effective against any of these bacteria.

Crude hexane extracts, which were fractionated by gel filtration and ion-exchange chromatography form *C. tiglium* showed antibacterial activities of 16-25mm, 6-15mm and 6-15mm zones of inhibition, respectively, against *B. subtilis.* This was due to the presence of antimicrobial protein (Ct-50) with estimated molecular mass of 50 kDa. Methanol extracts of *C. steenkapianus* were inactive against *S. aureus* and *Enterococcus faecalis,* but active against *P. aeruginosa* at the MIC of 0.625 mg/ml. *C. silvaticus* methanol extracts weakly inhibited all the tested Gram positive bacteria: *S. aureus, E. faecalis, P. aeruginosa* at the constant MIC of 1.25 mg/ml. *C. megalobotrys* methanol extracts inhibited *S. aureus* and *P. aeruginosa* at the MICs of 0.625 mg/ml and 0.313 mg/ml, respectively. *C. megalobotrys* methanol extracts were effective inhibitors against *E. faecalis* at the MIC of 0.02 mg/ml.

Dichloromethane, ethyl acetate, acetone, methanol and *n*-hexane fractions of *C. megalobotrys* inhibited strongly *E. faecalis* and *P. aeruginosa* at the MICs between 0.120-0.276 mg/ml and 0.060-0322mg/ml, respectively. Dichloromethane, ethyl acetate, acetone and methanol fractions of *C. megalobotrys* had remarkable inhibitory activity against *S. aureus* at MICs ranging from 0.236-0.468 mg/ml but *n*-hexane extracts were weak against *S. aureus* at the MIC of 1.562 mg/ml.

Volatile compounds of *Croton zehntneri* essential oils (CZEO) were tested against *P. aeruginosa.* The bacteria were less susceptible owing to growth of the bacteria at all the concentrations ranging from 1-0.031 mg/L air. CZEO inhibited the growth of *S. aureus* at

the concentrations of 1 and 0.5 mg/L air. One possible mechanism of action of CZEO is the disruption of the enzymatic activity of the bacterial cell (Wendakoon and Sakaguchi 1995).

6.3. Other forms of bacteria and extracts of *Croton*

Acetone extracts of *Croton pseudopulchellus* were tested against sensitive strain (H37Rv) of *Mycobacterium tuberculosis*. The MIC value of the acetone extract of *C. pseudopulchellus* was 0.5 mg/ml in the agar plate method, but the water extract of *C. pseudopulchellus* did not produce any activity. Nonpolar active compounds may not have been present in the water extracts of *C. pseudopulchellus*. In addition, instead of using the aerial portions of the plant, the roots should have been taken for the study. Acetone extracts of *C. pseudopulchellus* inhibited both the sensitive (H37Rv) and resistant (CCKO28469V) strains of *M. tuberculosis* at MICs of 0.1mg/ml and 0.5mg/ml, respectively when the radiometric method was used. Radiometric method produces results faster than plate method because cell-to-drug interactions occur faster. In addition, test compounds are unlikely to breakdown because of the reduced incubation time (Lall and Meyer 1999).

6.4. Antibiotic modifying activity of *Croton* extracts

Two antibiotics, gentamicin and tetracycline, were tested against *P. aeruginosa* alone or together with a volatile component of the essential oil of *C. zehntneri* (EOCZ). No significant activity was recorded for the tetracycline EOCZ combination. EOCZ enhanced the activity of gentamicin by 42.8%. The hydrophobic nature of essential oils disrupted respiratory activities and energy production line of the bacterial cells. They affected on the plasma membrane in general making the bacterial cells more permeable to antibiotics. Essential oils can be used in combination with antibiotics for effective treatment of certain bacterial infections (Burt 2004, Juven et al. 1998).

Hexane extracts of *C. campestris* (HECC) and methanol extracts of *C. campestris* (MECC) enhanced the antibiotic activity of gentamicin, kanamycin and amikacin when tested against *E. coli* and *S. aureus* at subinhibitory concentrations of 8µg/ml. HECC exhibited

synergy when combined with gentamicin, kanamycin and amikacin against *E. coli*. MECC was effective against *E. coli*, when combined with gentamicin and amikacin, but combination with kanamycin was not effective. Against *S. aureus*, both extracts and all the antibiotics proved effective (Matias et al. 2010). The mechanism behind the synergistic effect may due to the presence of tannins, flavonols and terpenes in *C. campestris* non polar extracts (methanol and hexane). Tannins are known to provide natural defense against microbial infections (Ho et al. 2001), and they can be used to prevent bacterial infections. In response to microbial infections, plant flavonoids form complexes with bacterial cell proteins and interfere with the cell's activities in the process of bacterial adhesion. Actions of some flavanoids may result in the rupture of bacterial plasma membrane (Tsuchiya et al. 1996). Terpene antibacterial activities appear in different forms: sesquiterpenes, tetraterpenes, diterpenes or triterpenes (Ahamd et al. 1993).

6.5. Further research implications

In order to carry out detailed characterization of the bioactive compounds from the *C. megalobotrys*, *C. steenkapiannus*, *C. silvaticus*, *C. pseudopulchellus* and *C. macrostachyus* species, further work will be needed. Animal models will be required to study *in vivo* activities of the extracts of the active *Croton* plants. The active biological agents should be tested as regards to their pharmacological applicability. Toxicity studies should also be carried out for all the extracts and fractions. Further, studies on the synergistic relationship between other *Croton* extracts and synthetic antibiotic drugs will be required in the future.

7. CONCLUSIONS

This review evaluated the antibacterial properties of *Croton* species based on relevant research reports from 1990 to 2011. Organic extracts of *Croton* species inhibited both Gram positive and Gram negative bacteria. Inorganic extracts were ineffective but acetone extracts inhibited the growth of the antibiotic sensitive strain of *M. tuberculosis.* When the radiometric method was used both the sensitive and resistant strains of *M. tuberculosis* were inhibited by the acetone extracts. Essential oil obtained by hydro-distillation inhibited some strains of the studied bacteria and enhanced the effect of some of the antibiotics used in the studies. The results show that the extracts of *Croton* species could be used as alternative means in pharmacotherapy of bacterial infections. They could be also used as adjuvant agents in antibiotic therapy against pathogenic bacterial infections.

REFERENCES

Abo KA, Ogunleye VO, Ashidi JS. Antimicrobial potential of *Spondias mombin, Croton zambesicus* and *Zygotritonia crocea*. Phytother Res. 1999; 13(6):494-7.

Ahamd AA, Mahmoud AA, Williams HJ, Scott AI, Reibebspies JH, Mabry TJ: New sesquiterpene alpha-methylene lactones from the Egyptian plants *Jasonia candicans*. J Nat Prod 1993; 56: 1276-80.

Aiyegoro O, Adewusi A, Oyedemi S, Akinpelu D, Okoh A. Interactions of antibiotics and methanolic crude extracts of *Afzelia africana* (smith.) against drug resistance bacterial isolates. Int J Mol Sci. 2011; 12(7): 4477-503.

Alviano WS, Mendonça-Filho RR, Alviano DS, Bizzo HR, Souto-Padrón T, Rodrigues ML, Bolognese AM, Alviano CS, Souza MM. Antimicrobial activity of *Croton cajucara* Benth linalool-rich essential oil on artificial biofilms and planktonic microorganisms. Oral Microbiol Immunol. 2005;20(2):101-5.

Bassetti M, Ginocchio F, Mikulska M, Taramasso L, Giacobbe DR. Will new antimicrobials overcome resistance among Gram-negatives? Expert Rev Anti Infect Ther. 2011; 9(10):909-22.

Burt S. Essential oils: their antibacterial properties and potential applications in foods– A review. Int J Food Microbiol. 2004; 94:223-53.

Chariandy CM, Seaforth CE, Phelps RH, Pollard GV, Khambay BP. Screening of medicinal plants from Trinidad and Tobago for antimicrobial and insecticidal properties. J Ethnopharmacol. 1999; 64(3):265-70.

Dodson KW, Pinkner JS, Rose T, Magnusson G, Hultgren SJ, Waksman G. Structural basis of the interaction of the pyelonephritic *E. coli* adhesin to its human kidney receptor. Cell. 2001; 105(6):733-43.

Heifets LB, Iseman MD, Cook JL, Lindholm-Levy PJ, Drupa I. Determination of in vitro susceptibility of *Mycobacterium tuberculosis* to cephalosporins by radiometric and conventional methods. Antimicrob Agents Chemother.1985;27(1):11-5.

Ho KY, Tsai CC, Huang JS, Chen CP, Lin TC, Lin CC: Antimicrobial activity of tannin components from *Vaccinium vitisidaea* L. J Pharm Pharmacol 2001; 53: 187-91.

Inouye S, Takizawa T, Yamguchi H. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. J. Antimicrob. Chemother. 1992; 47: 565-573.

Juven J, Kanner J, Schved F, Weisslowicz H. Factors that interact with antimicrobial action of thyme essential oil and its active constituents. J Appl. Bacteriol. 1994; 76: 626-631.

Kapingu MC, Guillaume D, Mbwambo ZH, Moshi MJ, Uliso FC, Mahunnah RL. Diterpenoids from the roots of *Croton macrostachys*. Phytochemistry. 2000; 54(8):767-70.

Kouki A, Haataja S, Loimaranta V, Pulliainen AT, Nilsson UJ, Finne J. Identification of a novel streptococcal adhesin P (SadP) protein recognizing galactosyl-α1-4-galactose-containing glycoconjugates: convergent evolution of bacterial pathogens to binding of the same host receptor. J Biol Chem. 2011; 286(45):38854-64.

Lall N, Meyer JJ. In vitro inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by ethnobotanically selected South African plants. J Ethnopharmacol. 1999; 66(3):347-54.

Liu WJH. Introduction to Traditional Herbal Medicines and their Study, in Traditional Herbal Medicine Research Methods: Identification, Analysis, Bioassay, and Pharmaceutical and Clinical Studies. John Wiley & Sons 2011.

Matias EF, Santos KK, Almeida TS, Costa JG, Coutinho HD. Phytochemical prospection and modulation of aminoglycoside antibiotic activity by *Croton campestris* A. Chemotherapy. 2011; 57(4):305-9.

McChesney JD, Clark AM, Silveira ER. Antimicrobial diterpenes of *Croton sonderianus*. II. ent-Beyer-15-en-18-oic acid. Pharm Res. 1991; 8(10):1243-7.

Middlebrook G, Cohn ML. Bacteriology of tuberculosis: laboratory methods. Am J Public Health Nations Health. 1958; 48(7):844-53.

Miyakis S, Pefanis A, Tsakris A. The challenges of antimicrobial drug resistance in Greece. Clin Infect Dis. 2011; 53(2):177-84.

Nascimento GGF, Locatelli JPC, Freitas PC, Silva GL. Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. Braz. J. Microbiol. 2000; 31: 247-56.

Nguefack J, Budde B, Jakobsen M. Five essential oils from aromatic plants of Cameroon: their antibacterial activity and ability to permeabilize the cytoplasmic membrane of Listeria innocua examined by flow cytometry. Lett. Appl.Microbiol. 2004; 39,395-400

Parsek M, Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 2003; 57: 677-701.

Peres MT, Monache DF, Cruz AB, Pizzolatti MG, Yunes RA. Chemical composition and antimicrobial activity of *Croton urucurana* Baillon (Euphorbiaceae). J Ethnopharmacol. 1997; 56(3):223-6.

PROTA, *Croton macrostachyus* Hochst. ex Delile. 2011 (accessed on 07.06.2011) http://www.prota4u.org/protav8.asp?h=M4&t=Croton&p=Croton+macrostachyus#Synonyms.

Rodrigues FF, Costa JG, Coutinho HD. Synergy effects of the antibiotics gentamicin and the essential oil of *Croton zehntneri*. Phytomedicine. 2009; 16(11):1052-5.

Salatino A, Salatino MLF, Negri G. Traditional uses, Chemistry and Pharmacology of Croton species (Euphorbiaceae). J. Braz. Chem. Soc. 2007;18:11-33.

Schmidt E, Jirovetz L, Buchbauer G, Denkova Z, Stoyanova, A, Murgov I. Antimicrobial testing and gas chromatographic analysis of aroma chemicals.J. Essential Oil Bearing Plants 2005; 8:99-106.

Selowa SC, Shai LJ, Masoko P, Mokgotho MP, Magano SR. Antibacterial activity of extracts of three *Croton* species collected in Mpumalanga region in South Africa. Afr J Tradit Complement Altern Med. 2009; 30:7(2):98-103.

Shalid M, Tayyab M, Naz F, Jamil A, Ashraf M, Gilani AH. Activity-guided isolation of a novel protein from *Croton tiglium* with antifungal and antibacterial activities. Phytother Res. 2008; 22(12):1646-9.

Socransky SS, Haffajee AD. Dental biofilms: difficulty therapeutic targets. Periodontal 2000 2002; 28: 12-55.

Tane P, Akam MT, Tsopmo A, Ndi CP, Sterner O. Two labdane diterpenoids and a seco-tetranortriterpenoid from *Turreanthus africanus*. Phytochemistry. 2004; 65(23):3083-7.

Taniguchi M, Kubo I. Ethnobotanical drug discovery based on medicine men's trials in the African savanna: screening of east African plants for antimicrobial activity II. J Nat Prod. 1993; 56(9):1539-46.

Theuretzbacher U. Resistance drives antibacterial drug development. Curr Opin Pharmacol. 2011; 11(5):433-8.

Tsuchiya H, Sato M, Miyazaki T, Fujiwara S, Tanigaki S, Ohyama M, Tanaka T, Jinuma M. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant *Staphylococcus aureus*. J Ethnopharmacol. 1996; 50: 27-34.

Tuberculosis. World Health Organization 2011. (Accessed on 7.1.2012) http://www.who.int/mediacentre/factsheets/fs104/en/index.html

Valenza G, Tappe D, Turnwald D, Frosch M, König C, Hebestreit H. Prevalence and antimicrobial susceptibility of microorganisms isolated from sputa of patients with cystic fibrosis. J. Cyst. Fibrosis. 2008; 7:123-127.

Verhoeff J, Beaujean D, Vlok H, Baars A, Meyler A, Werkwn VDC: A Dutch approach to methicillin-resistant *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis. 1999; 18: 461-6.

Vermani K, Garg S. Herbal medicines for sexually transmitted diseases and AIDS.J Ethnopharmacol. 2002; 80(1):49-66.

Wagate CG, Mbaria JM, Gakuya DW, Nanyingi MO, Kareru PG, Njuguna A, Gitahi N, Macharia JK, Njonge FK. Screening of some Kenyan medicinal plants for antibacterial activity. Phytother Res. 2010; 24(1):150-3.

Wendakoon C, Sakaguchi M. Inhibition of amino acid decarboxylase activity of Enterobacter aerogenes by active components in species. J Food Prot. 58; 280-283.

Wu CD, Wei GX. Tea as a functional food for oral health. Nutrition 2002; 18: 443-444.

Yibralign Z. Master's thesis on Phytochemical Investigation on the stem bark of *Croton* macrostachyus (Bisana). Addis Ababa University 2007.