ANTIBACTERIAL EFFECTS OF *Gongronema latifolium* and *Vernonia amygdalina* LEAF EXTRACTS ON SOME BACTERIA IMPLICATED IN DIABETIC WOUNDS

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RESEARCH DISSERTATION SUBMITTED TO UNIVERSITY OF NIGERIA NSUKKA IN PARTIAL FULFILMENT OF THE REQUIREMETN FOR THE AWARD OF MASTERS DEGREE (M.Sc.) IN MEDICAL MICROBIOLOGY

AUGUST, 2018

CERTIFICATION

This is to certify that Onyemachi Peace Amarachi (PG/MSc./08/48282) a postgraduate student in the Department of Microbiology, Faculty of Biological Sciences University of Nigeria Nsukka has satisfactorily completed the requirements for research work for the degree of Master of Science in Medical Microbiology. The work embodied in this dissertation is original and has not been submitted in part or in full for any other degree of this or any other university. This dissertation has therefore been approved for the award of Master of Science degree in the Department of Microbiology University of Nigeria Nsukka.

BY

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SIGN	DATE	SIGN	DATE
REV.SR PROF. M. E. U. DIBUA (SUPERVISOR)		PROF. L. I. EZEOGU (HEAD OF DEPARTMENT)	

DEDICATION

This work is dedicated to God; the Father, Son and the Holy Spirit who were behind the inspiration, knowledge, wisdom and ability to carry on till the end of this program.

ACKNOWLEDGEMENT

I hereby acknowledge the help of my supervisor, Rev. Sis Dr M.E.U. Dibua whose guidance and assistance was a tremendous help during this research.

Also, I wish to express my profound gratitude to all my lecturers, graduate assistants, academic and non-academic staff of my beloved department for contributing immensely to the success of this research.

This write up is only possible because of the information gathered from various sources. The knowledge and experience of those who have written some books, journals on related issues helped me a lot. I wish to express my deep appreciation to the various authors: thanks to all of you.

My special thanks goes to my beloved biological parents Revd. and Revd. (Mrs.) Onyemachi William Ihemere, mostly for sound Christian and moral upbringing which helped to shape my life and preserved me in all my challenging undertaken. My lovely sister Ruth C. Okoye who was a great source of strength throughout my course of study.

I appreciate Revd. Aghachi Hyacinth Francis for his prayers during the course of this research.

I also recognize the effort of friends who in one way or the other have contributed to my life, and academic training. May my good God reward and bless you all immeasurably.

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ABSTRACT

The activities of leaf extracts of two Nigerian edible vegetables (*Gongronema latifolium* and *Vernonia amygdalina)* used in traditional medicine for the treatment of various infectious diseases, were evaluated *in vitro* for potential antibacterial activity and *in vivo* for efficacy of diabetic wound healing activity, the former was screened by agar well diffusion method while the later was carried out using the excision wound model in alloxan induced diabetic rats. The pattern of inhibition and the rate of wound area relative varied with the plant extract, the combination ratio and the organisms tested. Both aqueous and ethanolic extracts of these plants leaves were tested against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeroginosa* and *Streptococus pyogenes*, with the ethanolic extracts had significant antibacterial activity on the test isolates. The minimum inhibitory concentration (MIC) of both extracts ranged between 50mg/ml to 100mg/ml for aqueous extract and 6.25mg/ml to 12.5mg/ml for ethanolic extract. The combinations of the leaf extracts exhibited a higher zone of inhibition on the test bacterial species (16mm to 24mm) than any of the individual plant extracts (12mm to 16mm). For *in vivo* activity, wound surface area (WSA) and bacterial colony count (BCC) were used to measure the outcomes at different day interval. Results showed a significant reduction in wound contraction and bacterial colony count in extracts treated groups compared to control group. The wound size in animals of the treated group were significantly reduced ($P<0.05$) when compared with the control groups. Extract treated animals exhibited 90% reduction in the wound area when compared to controls which was 20%. Our present study revealed that the potency of the ethanol extract based on the zones of inhibition, MIC values, significant wound healing and accelerated wound closure promotes the great therapeutic potentials of these plants which might be used as adjunct in the treatment of diabetic wounds and associated infections. Topical application of *G. latifolium* and *V. amygdalina* singly and combined could be helpful in diabetics, in order to improve the wound healing process avoiding possible adverse effects from systemic medication. Further identification of the active constituents is suggested to exploit them in evaluating efficacy and safety *in vivo* against organisms implicated in diabetic wounds in humans.

INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND OF THE STUDY

The development of wounds is a serious complication for patients with diabetes. Diabetic wounds can be triggered by the slightest injury- a blister, bruise, small cut or scrape (Beard and Webster, 2004). Irrespective of the nature of the injury, acute wounds are expected to heal with a predictable time frame, although the treatment required to facilitate healing will vary according to the type, site and depth of the wound (Leaper, 1998). Exposure of subcutaneous tissue following a loss of skin integrity (i.e. a wound) provides a moist, warm and nutritious environment that is conducive to microbial colonization and proliferation. Since wound colonization is most frequently polymicrobial, involving numerous microorganisms that are potentially pathogenic, any wound is at some risk of becoming infected (Bowler *et al*., 2009).

Wound contaminants are likely to originate from three main sources; the environment (exogenous microorganism in the air or those introduced by traumatic injury); the surrounding skin (involving members of the normal skin microflora) and endogenous sources involving mucous membranes (primarily the gastrointestinal, oropharyngeal, and genitourinary mucosa). Aerobic pathogens such as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Streptococcus pyogenes* present the first line of attack and are the primary cause of delayed healing and infection in both acute and chronic wounds (Leaper, 1998; Fridkin *et al*., 2005; Bowler *et al.,* 2009). Numerous factors related to diabetes can impair wound healing, including wound hyposia (inadequate oxygen delivered to the wound), infection, nutritional deficiencies and the disease itself (Lavery *et al*., 2007).

1.2 STATEMENT OF THE PROBLEM

Diabetic patients are at increased risk of developing infection (Brace, 2007). This special vulnerability has been attributed to impaired leukocyte function, associated vascular diseases, poor glucose control and altered host response (McMahon and Bistrain 1995; Bhatia *et al*., 2003). Poorly controlled diabetes adversely affects the ability of leukocytes to prevent the harmful proliferation of commensals present on the healthy body (O'Dell, 1999). When infection occurs, it is difficult to treat since the clinical cause of the infection is severe and poses a greater threat to the glycemic status of the patient (Loiue *et al*., 1993; Beckert *et al.*, 2006). Fluctuating blood sugars and hypoxia from poor circulation may impair the ability of white blood cells to destroy invading pathogenic bacteria and fungi, increasing infection risk (Stabelmann *et al*., 1998). Therefore, the wound fails to heal, the patient suffers increased trauma, treatment costs rise and general wound management practices become more resource demanding (Bowler *et al.,* 2009). Sequel to this is the inaccessibility of many rural populations to antibiotic chemotherapy, the high cost of many antibiotics with their undesirable side effects, and the increasing resistance of pathogens to most commonly used antibiotics.

1.3 OBJECTIVE OF THE STUDY

In the present study, the research work aimed at;

- Determining the antibacterial effect and wound healing activities of the aqueous and ethanol extract of *G. latifolium* and *V. amygdalina*, against selected gram positive and negative bacteria implicated in diabetic wounds, evaluating their single and combined effects.

- Antibacterial efficacy of the different plant extracts (singly and combined) against selected bacterial pathogens were evaluated using the agar well diffusion method.
- Evaluation of bacterial colony count, comparison of wound surface area and percentage wound contraction of the different plant extracts (singly and in combination) against selected organisms. This was carried out using animal wound modelling method.

1.4 LITERATURE REVIEW

1.4.1 DEFINITION OF WOUND

The normal function of skin is to prevent colonization and invasion of underlying tissue by potential microbial pathogens. Loss of skin integrity (wound) provides moist and nutritious environment for microbial proliferation. Wound is a break in the continuity of any bodily tissue due to violence. Where violence is understood to encompass any action of external agency, including surgery. Foot wound is also defined as a full-skin thickness lesion involving any portion of the foot or ankle, excluding blisters and minor lacerations or abrasions (Oyibo, 2001). Wounds can be broadly categorized as having either an acute or chronic etiology. Acute wounds are caused by external damage to intact skin and include surgical wounds, bites, burns, minor cuts and abrasions. This wounds proceeds normally in a timely fashion along the healing pathway with at least external manifestation of healing apparent in the early post-operative period without complications (Lazarus *et al*., 1994). Chronic wounds involve endogenous mechanisms compromising epidermal and dermal tissue, impaired arterial supply or venous drainage, and sustained external skin pressure. A chronic wound is one that has failed to proceed through an orderly and timely process to produce anatomic and functional integrity or proceed through the

repair process without establishing a sustained and functional result. Chronic wounds are generally considered acute wounds that have failed to progress through the normal phases of wound healing.

1.4.2 BIOLOGY OF WOUND HEALING

It involves the sequence from injury to wound closure and finally organized scar tissue. The phases are defined as hemostasis, inflammation, proliferation, and epithelialization and finally scar formation. The biology of wound healing can be thought of as a succession of unique cellular and physiologic events. Normally at the time of injury, blood vessels rupture, exposing matrix proteins and leading to platelet aggregation, clot formation and hemostasis. Platelets release cytokines and growth factors that stimulate further proliferation of the clot and the recruitment and mitogenesis of cellular elements. Neutrophils are recruited within minutes to hours of the injury and more mediators and chemotactic substances are released. Subsequently, monocytes are activated to form tissue macrophages, which play a critical role in suppressing bacterial growth and clearing existent bacteria and necrotic tissue. Macrophages continue releasing cytokines and growth factors, which bring fibroblasts and endothelical cells to the wound. Reperfusion depends on angiogenesis, which in turn depends on the migration of endothelical cells to the site of injury. The clonal expansion of cells, particularly, fibroblasts, appears to define wound healing. Wound closure, occurring from the rim of the wound inward, is characterized by complete epithelialization. The end of healing is heralded by the migration of keratinocytes to the wound site. During this remodeling phase, tensile strength and cellular organization improve, skin integrity returns and the wound contracts (Inoguchi *et al*., 1992; Wolf *et al.*, 1991; Trengore *et al*., 2008; Yager and Nnomeh, 1999).

1.4.3 POOR WOUND HEALING IN DIABETIC PATIENTS

Chronic hyperglycemia in diabetic patient disrupts the normal cellular and inflammatory pathways involved in wound healing and increases susceptibility to infection. Individuals with diabetes have been found to have abnormal cellular function, particularly of fibroblasts and neutrophils (Inoguchi *et al.*, 1992; Wolf *et al.*, 1991). Hyperglycemia results in a decreased proliferation and differentiation of keratinocytes and fibroblast in the wound. Keratinocytes and fibroblasts exposed to an environment of high glucose levels do not migrate into the wound normally (Heherberger and Hansson 1997; Lerman *et al.*, 2003). Cells exposed to abnormal levels of glucose fail to produce normal levels of DNA and protein and eventually stop replicating (Terashi *et al.*, 2005). Healing resistance is thus a well-recognized element of frustration in their clinical care. In most of the above conditions, multiple factors play into healing resistance. Among them are circulatory impairments, neurological deficits, tissue injury and immunological compromise. Such cellular changes would predispose individuals to an increased risk of wound infection (Delware *et al.*, 1997; Blakytry and Jude, 2006; Spravchikov *et al.*, 2001).

1.4.4 MICROBIOLOGY OF DIABETIC WOUND

Microbial colonization precedes wound infection. If tissue is devitalized and/or host immunity compromised, conditions optimal for microbial growth is created, and invasion follows colonization. Source of microorganisms include; exogenous (environmental), surrounding skin, and endogenous (mucous membranes of gastrointestinal tract and genitourinary tract, oropharyngeal cavity). Poor blood perfusion with hypoxia inhibits granulation tissue response and wound repair. Cell death and tissue necrosis due to hypoxia creates ideal growth conditions for

wound microflora. Hypoxia compromises oxygen radical dependent killing of bacteria by polymorphonuclear neutrophils.

Factors important in determining delayed wound healing due to infection include the density of microorganisms which is the critical factor and the presence of specific microbial pathogens of primary significance Fundamental to healing is the chronic wound's "established microbiota" or biofilm (James *et al.,* 2008). In particular, the more diverse the microbiology of the chronic wound, the more recalcitrant the microbial populations are to therapeutics and the greater the risk of infection developing.

Many factors affect the progress of microorganisms in a wound from colonization to infection: the number of organisms, the virulence factors they produce, and the resistance of the host to infection (Infection = dose X virulence/host resistance). Host resistance is the single most important determinant in wound infection. Local and Systemic factors both play a role in increasing the chances a wound will become infected. Local factors that increase chances of wound infection; large wound area, increased wound depth, degree of chronicity, anatomic location (distal extremity, perineal), foreign body, necrotic tissue, mechanism of injury (bites, perforated viscus), reduced perfusion (Dow *et al.,* 1999).

Clinical indicators for wound infections include; heat, delayed healing malodor, epithelial bridging tissue breakdown and necrosis, systemic illness, increased wound size, painful spreading erythema, failure to heal, increased exudate (purulent, serous, or serosanguinous), increased redness swelling, increased pain, increased local temperature, periwound cellulitis, ascending infection and change in appearance of granulation tissue (discoloration, prone to bleed, highly friable) (Bowler *et al.,* 2001).

Robson and Heggers (1969) and Bowler *et al.,* (2001) reported that wound healing progressed only when bacterial counts in wound fluid were below 10^6 CFU/ml. According to the authors, above 10^6 CFU/ml wound healing was inhibited. Robson and Heggers (1969) also found that infection risk increased when the microbial load of a wound was $>10^5$ CFU/g of tissue. Healing of decubitus ulcers occurs only when bacterial load $<10^6$ CFU/ml of wound fluid. Acute and chronic wound infection occurs with microbial load $\geq 10^4$ (complex extremity wounds) or $>10^5$ CFU/g of wound tissue. Critical microbial load for wound infection appears to be 10^4-10^6CFU/g wound tissue or ml wound fluid, and 10^6CFU/wound swab specimen. Based on research papers and additional studies, Robson and Heggers (1969), Robson *et al*., (1968), Krizek *et al.,* (1967) and Pruitt *et al.,* (1998) concluded that in quantitative microbiology, principally levels of bacteria below 10^5CFU/g or ml, could be useful to help predict wound healing and risk of infection. In addition to types of bacteria, levels of colony forming units (CFU) per gram of tissue, or ml of wound exudates, specifically above 10^5 CFU, are traditionally used as predictors of nonhealing /infection in a wound (Gardner and Frantz, 2008).

1.4.5 ORGANISMS IMPLICATED IN DIABETIC WOUND

Bacteria that have been involved in wound infections have included *Mycobacterium fortuitum, Acinetobacter sp,* and, of growing importance the fungi, *Candida albicans* (Verghese *et al.,* 2007; Sebeny *et al.,* 2008; Lima *et al.,* 2008; Giamarellou *et al.,* 2008; Elston *et al.,* 2008). *Candida albicans* is not generally screened for in wounds but represent potent highly virulent opportunistic pathogens that are known to interact synergistically with bacteria (Yener *et al.,* 2009; Ballard *et al.,* 2008). In addition to the microorganisms mentioned above, an array of other bacteria have been isolated from burns (Polavarapu *et al.,* 2008; Oncul and Acar, 2008) and infected diabetic foot ulcers. Without doubt, meticillin-resistant *S. aureus* (MRSA) are major concerns in both acute

and chronic wounds, and as a result, screening in hospitals is becoming routine practice to prevent the spread of these bacteria (Bansal *et al.,* 2008; Hirsch *et al.,* 2008; Edris and Reed, 2008).

The most frequently isolated pathogens observed in surgical site infections based upon clinical culture methods, as proposed by Mangram *et al.,* (1999) have included *Enterococcus* spp., *S. aureus*, coagulase-negative s*taphylococci* (CNS), *E. coli*, *Enterobacter* spp., and *P. aeruginosa*. In a recent study of 390 patients, comprising 280 surgical wounds, 92 ulcers, and 32 sinuses and lacerations, coliforms (63.5%) were found to be the most predominant microorganisms isolated followed by *Proteus* sp (37.2%). Multiple species infections have been reported in 22.7% of surgical wounds and 24.6% in ulcers (Ozumba, 2007).

Other opportunistic pathogens have also been associated with delayed wound healing and infections and have included CNS such as *S. epidermidis* as well as *Enterococcus*, *Micrococcus*, and *Corynebacterium* spp. (often referred to as diphtheroides). *Streptococcus* sp are also cultured frequently in wounds (Schraibman, 1990; Madsen *et al.,* 1996). Gram-negative bacteria that have been proposed as a cause of infection in wounds have included *Pseudomonas* sp, *Acinetobacter* spp (a bacteria now becoming multidrug resistant), and *Stenotrophomonas* spp. Other bacteria predominately found in wounds and significant to infections in both acute and chronic wounds have also included *E. coli*, *Enterobacter cloacae*, *Aeromonas*, *Klebsiella*, *Enterococcus*, *Bacteriodes* and *Proteus* species (Brook, 1996; Schmidt *et al*., 2000; Fisher *et al.*,2009; Flattau *et al.*,2008; Tena *et al*.,2009). Anaerobic bacteria such as *Petostreptococcus*, *Fingoldia magna*, *Peptoniphilus* spp, *Anaerococcus*, *Prevotella sp* and *Bacteroides* are highly ubiquitous in chronic wounds. These organisms are associated with mono microbial and poly microbial wound infection; *S. pyogenes* (capable of wound infection $<10^5$ CFU/g wound tissue), *S. aureus*, *P. aeruginosa*, anaerobic bacteria that have been isolated from many chronic wounds: *Peptostreptococcus* and

Bacteroides spp. (Hansson *et al.*,1995; Halbert *et al.*,1992; Murdoch *et al.*,1994) and other pyogenic β-hemolytic streptococci.

Oxygen consumption by aerobic bacteria induces tissue hypoxia and favorable growth conditions for anaerobic bacteria. Nutrients produced by one organism supports the growth of other fastidious and potentially pathogenic organisms. For instance in a polymicrobial wound infection mechanisms; Vitamin K production by *S. aureus* supports growth of vitamin K-dependent *Prevotella melaninogenica* ; succinate produced by *Klebsiella pneumoniae* is a critical growth factor for *Prevotella melaninogenica* .

The pus in closed, drained soft tissue abscesses frequently contains only one organism as the causative agent, most commonly staphylococci, streptococci, and enteric gram-negative rods. The proliferation of these infectious microorganisms by the variety of their families, their toxin-producing capacities and their resistance to antibiotics, offer daunting obstacles to standard treatment regimens (Brooks *et al*.,1995). However, the abundance and diversity of microorganisms in any wound will be influenced by factors such as wound type, depth, location and quality, the level of tissue perfusion and antimicrobial efficacy of the host immune response (Bowler *et al*., 2009). Widespread opinion among wound care practitioners is that aerobic or facultative pathogens such as *S. aureus, P. aeruginosa, E. coli* and *S. pyogenes* are the primary causes of delayed healing and infection in diabetic wound (Leaper 1998).

1.5 Staphylococci (*Staphylococcus aureus*)

The *staphylococci* are gram-positive spherical cells, usually arranged in grape-like irregular clusters. They grow readily on many types of media and are active metabolically, fermenting carbohydrates and producing pigments that vary from white to deep yellow. Some are members of

the normal flora of the skin and mucous membranes of humans; others cause suppuration, abscess formation, a variety of pyogenic infections, and even fatal septicemia. The pathogenic staphylococci often hemolyze blood, coagulate plasma, and produce a variety of extracellular enzymes and toxins. The staphylococcus has at least 30 species. The three main species of clinical importance are *Staphylococcus aureus*, *Staphylococcus epidermidis and Staphylococcus saprophyticus*. *S. aureus* is coagulase positive, which differentiates it from the other species. It is considered to be the most problematic bacterium in surgical and burn wound infections and has also been identified, as the most predominant bacteria found in chronic wounds (Brook *et al.,* 1981; Meislin *et al.,* 1977; Page and Beattie, 1992; Regev *et al.,* 1998; Frank *et al.,* 2009; Chen *et al.,* 2009). *S. aureus* has also been reported to be the single most commonly isolated bacteria responsible for a high percentage of cutaneous abscesses and necrotizing fasciitis (Brook and Finegold, 1981; Reger *et al.,* 1998; Sotto *et al.,* 2008). In numerous studies, *S. aureus* has been found in association with other bacteria such as coliforms, *Enterococcus* sp, *S. epidermidis*, *Streptococcus* spp, and *P. aeruginosa* (Armstrong *et al.,* 1995; Pathare *et al.,* 1998; Gardner *et al.,* 2004). Increasing concern to wound care is the growing incidence and prevalence of vancomycin-resistant and methicillin-resistant *S. aureus* (Hong *et al.,* 2008).

1.5.1 Streptococci (beta-hemolytic *Streptococcus pyogenes*)

The streptococci are gram-positive spherical bacteria that characteristically form pairs or chains during growth. They are widely distributed in nature. Many streptococci are members of the normal flora of the human body. They produce disease only when established in parts of the body where they do not normally occur. *Streptococcus pyogenes* contain the group A antigen and are β-hemolytic. It is the main human pathogen associated with local or systemic invasions. More than 20 extracellular products that are antigenic are elaborated by group A streptococci including the

following streptokinase (fibronolysis), streptodomase, hyaluronidase, pyrogenic exotoxins, hemolysins and diphosphopyrodine nucleotidase (Brooks *et al*., 1995). Wright and colleagues (Wright *et al.,* 1981) reported that a surgical wound could not be successfully closed if a hemolytic *S. pyogenes* strain was present. In addition, a study by Robson and Heggers (Robson and Heggers, 1970) highlighted that beta-hemolytic streptococcus was the only bacterium that caused infections in tissue. Further studies have revealed that *S. pyogenes* is the sole pathogen in some cases of necrotizing fasciitis (Reger *et al*., 1998). In bite wounds, for example, beta hemolytic streptococci have been proposed as the main cause of infection (Edris and Reed, 2008; Dutta, 1998). Based on the evidence to date, beta-hemolytic streptococci are significant to wound healing and have been reported to often require guided therapies to eradicate it from wounds (Akgulle *et al.,* 2009).

1.5.2 Enterobacteriaceae (*Escherichia coli*)

The enterobactericeae are a large, heterogeneous group of gram-negative rods whose natural habitat is the intestinal tract of humans and animals. Some enteric organism e.g. *Escherichia coli* are part of the normal flora and incidentally cause diseases. *E. coli* typically produces positive tests for indole, lysine decarboxylase and mannitol fermentation and produces gas from glucose. Some strains of *E. coli* hemolyze blood agar (Brooks *et al*., 1995).

1.5.3 Pseudomonads (*Pseudomonas aeruginosa*)

Pseudomonads are gram-negative, motile aerobic rods which are widely distributed in soil, water, plants and animals. *Pseudomonas aeruginosa* is frequently present in small numbers in the normal intestinal flora and on the skin of humans. It is the major pathogen of the group. *P. aeruginosa* is invasive and toxigenic, produces infectious diseases in humans with abnormal host defenses. It is an important nosocomial pathogen which produces infectious wounds giving rise to blue-green pus (Brooks *et al.,* 1995). *P. aeruginosa* has been implicated as the primary cause of infection in

a chronic wound (Madsen *et al.*, 1996). They are opportunistic pathogens and associated with approximately 10% to 20% of all hospital-acquired infections (Ikeno *et al.,* 2007; Trautmann *et al.,* 2009). *P. aeruginosa* have been isolated frequently from chronic wounds, based solely on culturable studies (Howell-Jones *et al.,* 2005; Schmidt *et al.,* 2000; Aufiero *et al.,* 2004). *P. aeruginosa* are intrinsically resistant to antimicrobial agents and are known to produce an array of virulence factors which include adhesions, fimbriae (pili), polysaccharide capsules, lipopolysaccharide, extracellular enzymes (elastases, proteases, hemolysis), alginate, flagella, iron-binding proteins, leukocidins, phospholipase C, hydrogen cyanide, exotoxin A, and exoenzyme S (Tredget *et al*., 2004). Lipopolysaccharide (LPS) and other products derived from *P. aeruginosa* have been shown to have a dose-dependent inhibition of keratinocytes, which effects their migration. Preventing the migration of keratinocytes is considered significant to wound healing (Loryman and Mansbridge, 2007). In addition, *P. aeruginosa* are avid biofilm formers. Removal of biofilms containing *P. aeruginosa* would be beneficial to wound healing. Essentially, all virulence factors produced by *P. aeruginosa* have a role to play in delaying wound healing (Danielsen *et al.,* 1996).

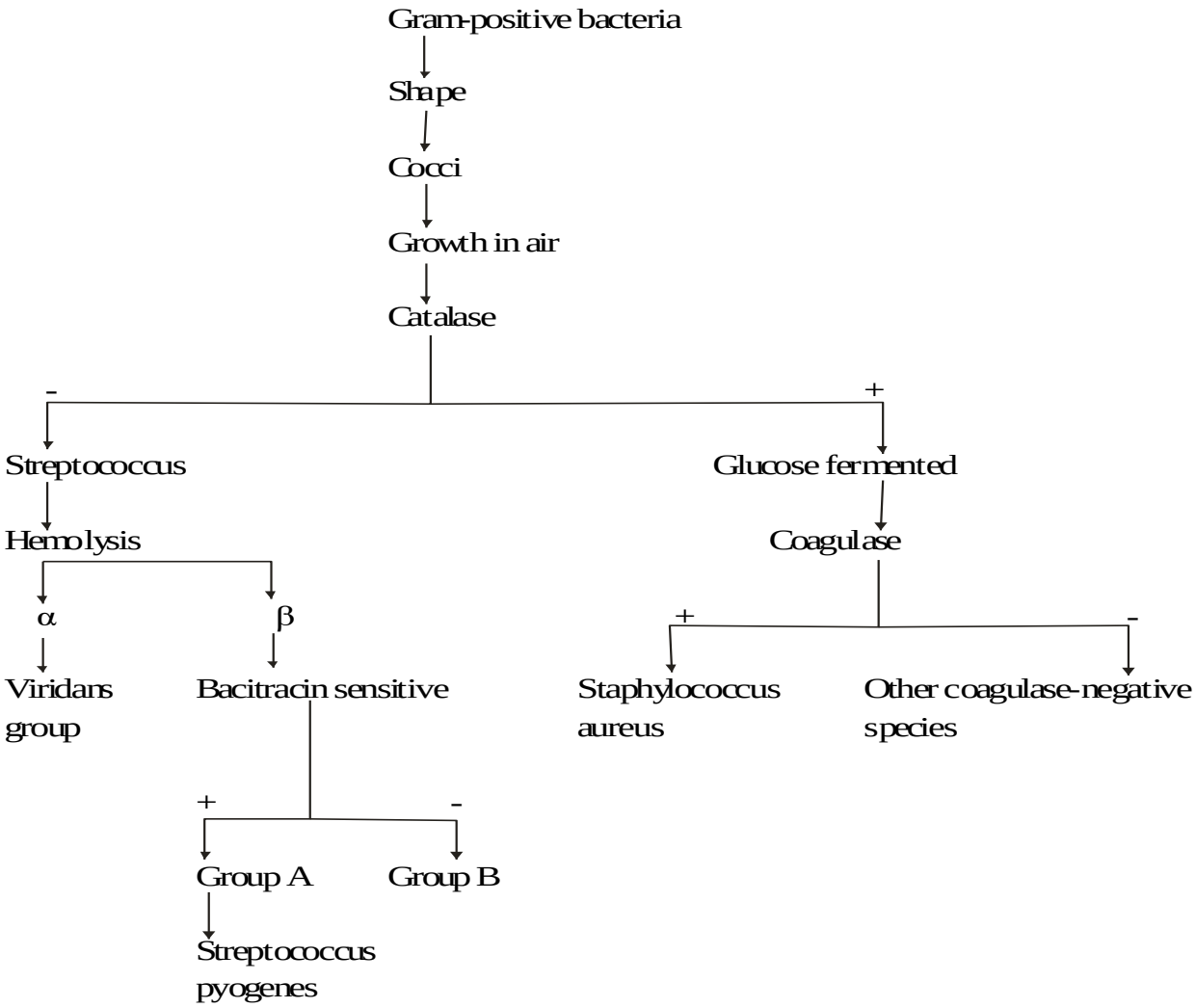

Figure 1: Schematic outline for the identification of Gram-positive bacteria

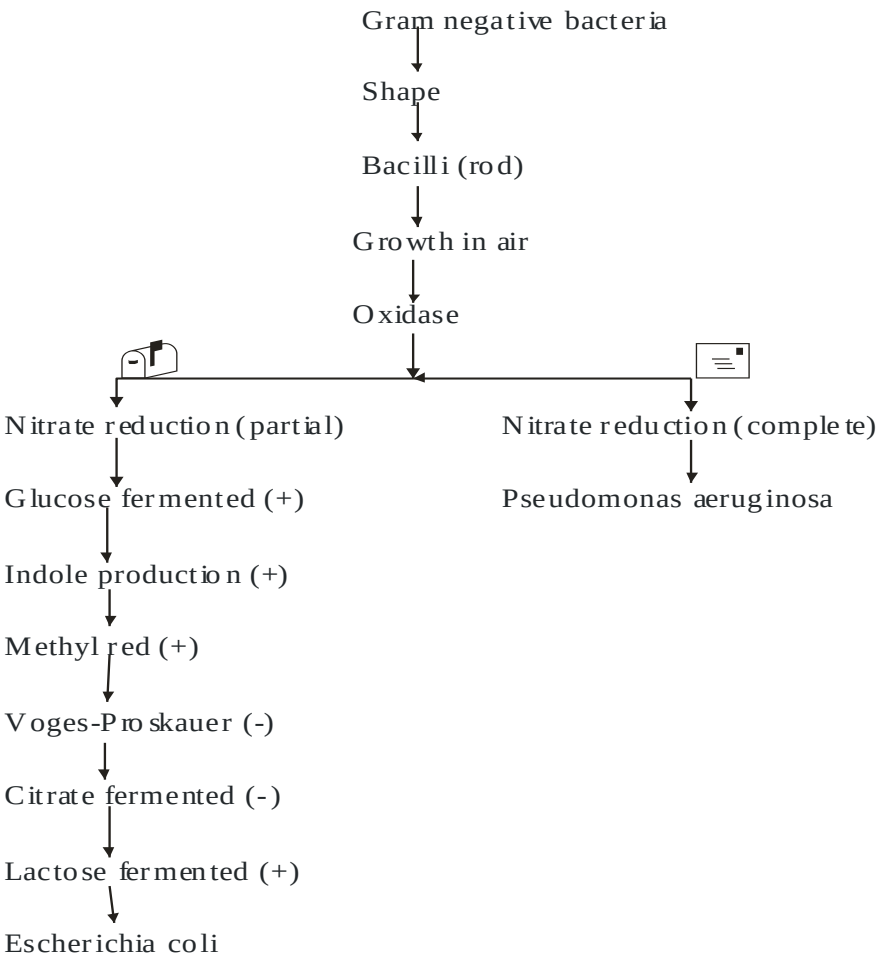

Figure 2: Schematic outline for the identification of Gram-negative bacteria

1.6 TREATMENT

Microscopic study of smears, and culture of specimens from wounds or abscesses, may often give early and important indicators of the nature of the infecting organism. This helps in the choice of antimicrobial drugs.

1.6.1 Localized care

The choice of antimicrobial agent is based on the suspected bacterial flora, appearance of the infected site, history of the lesion, and general condition of the patient. It is important to use drugs that are bactericidal, since diabetic patients have poor immune defenses, and if the pathogen is not

eliminated, the infection will recur. Localized care of diabetic foot ulcers consists of topical antimicrobial therapy, appropriate dressing application, foot elevation, debriding agents, and limitation of weight bearing activities (Joseph and Lefrock, 1987). The success of localized care depends on a high degree on compliance by the patient and caregiver. Topical antimicrobial agents, such as silver sulfadiazine (e.g. silvadene cream, thermazene) and mupirocin (bactrobar), can stimulate wound healing by eliminating bacteria on the wound surface. They should be used, however only as adjunctive therapy. The use of povidine-iodine solutions (betadine) and ointments (betadine ointment) on healing tissue remains controversial. Foot soaks and lubricating lotions have been recommended in the past, but their efficacy has not been proven in controlled trials. Chemical or enzymatic debriding agents are reserved for patients with mild ulcers or those who refuse surgical debridement. Options for topical debriding agents include collagenase (santyl) or papain – urea (accuzyme, panafil). Becaplermin gel (regranex) has been found to significantly increase the incidence of complete wound closure and significantly reduce the time to complete closure of diabetic foot ulcers (Wierma *et al*., 1998). A moist environment is most conducive to formation of a nascent tissue matrix. Several existing products promote maintenance of a moist wound. A recent Cochrane review examining three randomized controlled trials of hydrogel dressing products, found a significant improvement over saline dressing alone (Smith, 2002). Larval therapy with sterile maggots has been described, in patients failing other types of therapy for debriding tissue (Stoddard *et al*., 1997). Ultimately the length of treatment depends on the severity of the infection, the causative organism, and the clinical response to the chosen medication. It is important to continue antibiotic therapy until an infection has been eliminated.

1.7 Medicinal Plants and their Importance

The use of medicinal plants in curing illnesses is as old as man (Grably and Thierickee, 1999; Abim *et al*., 2007). Large populations of people, especially in the developing world, rely on folk medicines for the treatment of common infections as well as persistent diseases. The plants are usually ingested as decoctions and teas or used as spices in the preparation of local delicacies (Okafor, 1975). Medicinal plants contain some bioactive substances or compounds which produce definite physiological action on the human body and they include tannins, alkaloids, carbohydrate, terprenoids, steroids and flavoids (Edoga *et al*., 2005; Mann, 1998). These plants are of great importance to the health of individuals and communities (Edeoga *et al.,* 2005). Many of these indigenous medicinal plants are used as spices and food plants. They are sometimes added to foods meant for pregnant and nursing mothers for medicinal purposes (Okwu, 2001). Medicinal plants : *Carica papaya, Occinum grattissimum, Adenia cissampeloides, Vernonia amygladina, Gongronema latifolium and Cymbopogan* citrates amongst others are generally used in traditional medicine for the treatment of various ailment especially in south eastern Nigeria (Njoku and Ezeibe, 2007 ; Oguke *et al.,* 2004). Despite extensive applications of these plants in traditional medicine, there are little or no scientific data on some of them.

1.7.1 Herbal treatment for diabetes.

Even from early civilization, herbs have been considered to be powerful tool in treating illnesses. In places where physicians cannot reach, people have invented their own concoction of herbs and plants to deal with the common afflictions of daily life. Sometimes, these herbal treatments are far more superior to and effective than their orthodox counterparts. They are generally less expensive. Nowadays, because of the expensive treatment for diabetes as well as the contraindications that these medications have, a lot of people recourse to discover the wonders of these herbal treatments in diabetes mellitus. Their use and dosage are unregulated and not standardized and this poses a

risk for their use. Although some argue that natural ingredients are not harmful to health unless taken in significantly large amounts. Any medication, be it herbal or orthodox, should always be taken in moderation.

Plant drugs and herbal formulation are frequently considered to be less toxic and more free from side effects than synthetic ones (Halberstein, 2005; Mitra *et al.*, 1996)

Table 1: Diabetic Foot Infection Classification Schemes

Clinical description	Infectious diseases society of America	International working group on the diabetic foot
Wound without purulence or any manifestations of inflammation	Uninfected	1
Manifestations of inflammation (purulence or erythema pain, tenderness, warmth or induration) ;any cellulitis or erythema extends 2cm around ulcer, and infection is limited to skin or superficial subcutaneous tissues, no local complications or systemic illness	Mild	2
Infection in a patient who is systematically well and metabolically stable but has one of the following: cellulitis extending 12cm, lymphangitis, spread beneath fascial, deep tissue abscess, gangrene, muscle tendon, joint or bone involvement	Moderate	3
Infection in a patient with systemic toxicity or metabolic instability (e.g., fever, chills, tachycardia, hypotension, confusion, vomiting, leukocytosis, acidosis hyperglycemia or azotemia)	Severe	4

1.8 Review of Plants under study

1.8.1*Vernonia amygdalina*

Vernonia amygdalina popularly known as bitter leaf is a shrub of 2.5m tall with petiolate green leaves of about 6mm diameter and elliptic shape (compositae). It is known as *ewuro* in Yoruba, *olugbo* in Igbo, and *chusar doki* in Hausa land. The leaves are characteristically bitter. The stem and root divested of the bark are used as chew sticks in Nigeria. More importantly the leaves are

very popular soup vegetable. All parts of the plant are pharmacologically useful. The roots and leaves are used in ethno medicine to treat fever, hiccups, kidney problem and stomach discomfort among several other uses. Both aqueous and alcoholic extracts of the stem, bark, roots and leaves are reported to be extensively used as a purgative, antimalarial, antitumorigenic, antihelminthic, and in the treatment of eczema. The active components of the plant have been shown to be mainly sesquiterpene lactones like vernodalin, and vernoamygdalin and steroid glycosides like venonioside B1 and vernoniol B1.

1.8.2 *Gongronema latifolium*

Gongronema latifolium is a climbing perennial plant that belongs to the family of asclepidecea (Okafor and Ejiofor, 1996; Eleyinmi *et al.*, 2006). It is a rainforest plant which has been traditionally used in the south-eastern part of Nigeria for the management of disease such as diabetes, high blood pressure etc (Ugochukwu *et al.*, 2003). Commonly called *utazi* and *arokeke* in the southeastern part of Nigeria, it is used for a number of medicinal and nutritional purposes such as spice and vegetables (Dalziel, 1932), control of weight gain in lactating women and promotes fertility in women (Schneider *et al.*, 2003). It is also used to treat malaria, cough, ulcers and cancers. Various parts of the plant, particularly the stem and leaves, are used as chew sticks. The liquor, usually obtained after the plant is sliced and boiled with lime juice or infused in water for over three days, is frequently taken as a purge for colic and stomach pains as well as to treat symptoms connected with worm infection (Okafor,1975). Photochemical analysis of the leaves extract revealed it is rich in proteins (27.2% DM) and the presence of saponins (asterglycosides), alkaloids, minerals like calcium, phosphorus, magnesium, copper and potassium (Schneider *et al.*, 2003; Eleyinmi and Bressler 2007).

Experiments have showed that many parts of these herbs used as spices in preparing local delicacies have significant antibacterial activity. Most of the plants are known to contain aromatic oils from which they derive their main flavoring character. Phytochemical analysis have also disclosed that the antibacterial properties of these plants depend on certain active ingredients, especially the oils such as saponins, tannins and flavonoids (Morebise and Fafunso, 1998; Morebise *et al*., 2002). Research studies revealed that ethanolic extracts of the plants exhibited greater inhibitory effects on the organisms. This is attributed to the ability of ethanol to extract essential oils inhibitory to the organisms. Alkaloids are the lead molecules of therapeutic importance from these herbal species. These are heterocyclic compounds which have proved to have pharmacological properties such as hypotensive activity, anticonvulsant activity, antiprotozoal, antimicrobial and antimalarial activities (Quentin *et al.*, 1995; Frederich *et al.*, 2002).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plant Sample Collection

Fresh leaves of *Gongronema latifolium and Vernonia amygdalina* were obtained from farms in Nsukka, and where identified. The plants where air dried under a shade for seven days.

2.2 Preparation of Plant Extracts

Dried leaves of the plants (*G. latifolium* and *V. amygdalina)* were crushed to fine powder using a manual grinding machine, so as to increase surface exposure to solvent. The two solvents used for extraction were ethanol, and hot water. Two hundred milliliters (200ml) of water and 200ml of ethanol (60% vol) respectively were used to extract 250g of each of the dried powdered leaf. This extraction was done for 24 hours with manual agitation of the container after every 6 hours. The extracts where filtered using Whatman No.1 filter paper and concentrated by air drying. The concentrated extracts (dried paste) where stored in air-tight containers and refrigerated at 4^0c.

2.3 Wound Sample Collection

Wound swab samples were collected from hospitalized diabetic patients and the swab sticks introduced into freshly prepared nutrient broth.

2.4 Culture and Identification of test organisms

Tenfold serial dilution was carried out to obtain varying (10^{-2}, 10^{-3} and 10^{-4}) concentrations. The pour plate method was used to culture the organisms, were freshly prepared nutrient agar was poured into petri dishes containing 1ml of each dilution. Each petri dish containing the mixture was gently rotated to increase uniform dispersion of the colonies and incubated at 37^0c. After 24 hours, suspected colonies of *Staphylococcus aureus, Eschericha coli, Pseudomonas aeruginosa*

and Streptococus pyogenes were subcultured into their respective selective media and identified using microscopy and the necessary biochemical tests. Pure cultures of the test bacteria were each inoculated into bijiou bottles containing nutrient agar and stored in the refrigerator at 4^0c. The methods used in the identification of each organism are described below.

2.4.1 *Escherichia coli* (*E. coli*)

Culture: *E. coli* grows at 37^0c to form circular, convex, smooth colonies with distinct edges on Nutrient agar. On Eosin Methylene Blue agar the colonies appear flat, nonviscous, green with metallic shiny edges.

Microscopy (Wet mount and Gram stain): They were seen as motile rod-shaped cells, singly and in clusters. They are Gram negative rod-shaped cells with flagella.

Biochemical tests used in the identification include: the IMViC test which is an acronym that stands for Indole, Methyl red, Voges-Proskauer, and Citrate tests, others are Sucrose utilization and Oxidase.

Indole test: A single inoculum of *E. coli* was inoculated into a test tube containing tryptophan broth and inoculated for 24hrs at room temperature. A drop of Kovac's reagent was added to the broth and the development of a red colour confirmed a positive indole test.

Methyl red: *E. coli* was inoculated into a tube containing glucose and peptone. After 48 hrs, the tube was divided into; one was marked for use in the MR test while the other for the VP test. Several drops of methyl red solution was added to the MR broth to ascertain the production of acid. A bright red colour was indicative of a positive test.

VP test: The reagents- Barritt' A (alpha-napthol) and Barritt's B (potassium hydroxide) were added to the VP broth. No visible reaction was seen. This was recorded as a negative test.

Citrate fermentation: This was carried out by inoculation of *E. coli* into Simmon's citrate media. Growth with no development of a blue colour confirmed *E. coli*.

Sugar fermentation: Two grams of glucose and lactose where added into two test tubes containing sterilized water respectively. After an overnight incubation at room temperature, the gas was seen trapped in the Durham tube inside the glucose test tube while the test tube containing lactose showed yellowish colouration due to acid formation.

2.4.2 *Pseudomonas aeruginosa* (*P. aeruginosa)*

Culture: *P. aeruginosa* grows on Nutrient agar at 37-42^{0}c forming smooth round colonies with fluorescent greenish colour. This colonies have a grape-like odour. It also grows on *Pseudomonas* agar supplemented with glycerol and C-N complements to form smooth round colonies with brown pigmentation.

Microscopy (Wet mount and Gram stain): They organisms were seen as motile and Gram negative cells.

Biochemical tests used in the identification of *Pseudomonas aeruginosa* include; Nitrate reduction and Oxidase tests.

Nitrate reduction test: *P. aeruginosa* was inoculated into a test tube containing nitrate broth and incubated at room temperature for 24 hrs. After incubation, a drop of sulphanilic acid and α-naphthylamine where added, no visible reaction was seen. The broth also remained clear after a pinch of zinc dust was added. This showed a positive complete result (reduction of nitrate completely to nitrogen gas) for *Pseudomonas aeruginosa*.

Oxidase test: A filter paper was moistened with a few drops of 1% oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride). A wooden applicator was used to smear growth from an

agar medium onto the paper. The development of a purple colour within 10 seconds was considered a positive test for *P. aeruginosa.*

2.4.3 *Streptococcus pyogenes* (*S. pyogenes)*

Culture: Streptococci grew in blood agar showing discoid colonies at 37^0c.

Microscopy (Wet mount and Gram stain): It showed non-motile cocci and Gram positive cells in chains.

Biochemical tests include coagulase, catalase, hemolysis and bacitracin tests.

Catalase: A wooden applicator stick was used to pick up a colony of Streptococcus from an agar medium and placed in a drop of hydrogen peroxide on a glass slide. No formation of gas bubbles was observed, this is a negative catalase reaction.

Hemolysis: The sample was inoculated into plates containing freshly prepared blood agar media and incubated at 37^0c for 24 hours. The appearance of clear zones of hemolysis around the growing organism was indicative of β-hemolysis.

Bacitracin test: The bacteria were inoculated into a blood agar plate and an antibiotic bacitracin disk placed in the inoculated plate. This was incubated at 37^0c for 24 hours. The bacteria as presumptively identified as group A *Streptococci* because of inhibition by the antibiotic bacitracin.

2.4.4 *Staphylococcus aureus* (*S. aureus*)

Culture: Staphylococcus grows at 37^oc on nutrient agar supplemented with 9% NaCl. They form round, smooth, raised golden yellow colonies. On Mannitol Salt agar, its colonies are round, bright yellow with smooth edges.

Microscopy (Wet mount and Gram stain): They are non-motile cocci cells in irregular clusters and Gram positive cocci with uniform spherical cells.

Biochemical tests include coagulase, catalase and carbohydrate fermentation.

Slide catalase test: A wooden applicator stick was used to pick up a colony from a culture plate and placed in a drop of hydrogen peroxide on a glass slide. Positive catalase reaction was interpreted as the formation of gas bubbles.

2.5 Determination of Antibacterial activity

Antibacterial activities of the aqueous and ethanol extracts of the plants were evaluated by the agar well diffusion method on Muller-Hinton agar plates. In a test tube, 0.5g of each extract was dissolved in 2ml of sterile distilled water to obtain a stock solution of 500mg/ml strength. This stock solution was subsequently diluted serially into various concentrations (250, 125, 62.5, 31.25mg/ml) in test tubes containing 1ml of sterile distilled water. Prior to antimicrobial assay, the respective test organisms where inoculated into agar broth and left overnight. The overnight broth cultures where then adjusted to turbidity equivalent of 0.5 McFarland standards, each inoculated into already prepared Muller-Hinton agar plates and evenly distributed using a sterilized spreader; it was allowed to set for 20mins. Five wells where bored equidistance in each of the plates with a sterile 6mm cork borer. Using a sterile pipette, 0.1ml of each concentration of the extracts were introduced into the wells and as a control, 0.1ml of the diluent (sterile water) was pipetted into a well and all were allowed to diffuse at room temperature for two hours. The plates were then incubated at 37^{o}c for 24 hours. For each extract, three replicate tries were conducted against test organisms. Antibacterial activity was determined by the measurement of zone of inhibition in millimeters around each well.

2.6 Animal Modelling

The rat model was considered appropriate for simulating wound healing in humans. The animal model was selected for this study to provide better control over confounding variables such as diet,

exercise, environmental stress. In the study we standardized the animal samples in such a way that the variables which might interfere with the healing process would be the same. All animals had approximately the same age, were fed the same diet, operated on by the same researcher, and exposed to the same quantity and quality of inoculum. Healthy inbred gender-matched (male) albino rats weighing 200–270g were used. They were individually housed in clean cages and maintained by allowing free access to standard diet, water, temperature, humidity and light period. Animals were periodically weighed during the experiment. The animals were acclimatized for a period of 5 days in the new environment before initiation of experiment. Seventy two (72) adult male Albino Wistar rats were included in this study to determine the healing effect of the extracts in the treatment of diabetic wound. The use of different topical herbal agents to clean the wounds was the only variable under study.

2.7 Induction of Diabetes

Animals were weighed and their fasting blood glucose levels were determined before inducing diabetes. Those showing very low or high glucose levels were replaced. The animals were injected with a single dose of Alloxan monohydrate (100 mg/kg of body weight, Sigma) in freshly prepared phosphate buffer by i.p. route (Appendix: Fig. 1 and 2). Applying the method of Cristiano *et al.*, 30 minutes after injection, food and water were offered again. Animals with similar age and weight were controls. Fasting blood glucose level was measured 3 days later using Glucometer (LifeScan Inc. Milpitas U.S.A.) to confirm the diabetic status of the animals, the blood was drawn through the tail vein. Rats were considered diabetic if blood glucose concentrations increased to 200 or more mg/dl according to Nagy *et al*., 1961. Wounds were made on the rats showing hyperglycemia.

2.8 Creation of Wound

The skin of the rats were shaved to remove hair and cleaned with 70% ethanol. Superficial wound was created on the dorsal skin of the rats using a shaving stick and scalpel. The depth of the wound was made just to the point where oozing of fluid into the abraded tissue will be seen. Every animal was submitted to a 3cm length by 1cm width incision (area- $3cm^2$) in the median dorsal region. Surgical procedures were carried out with aseptic technique. Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound was left open.

Figures show researcher administering Alloxan to rats.

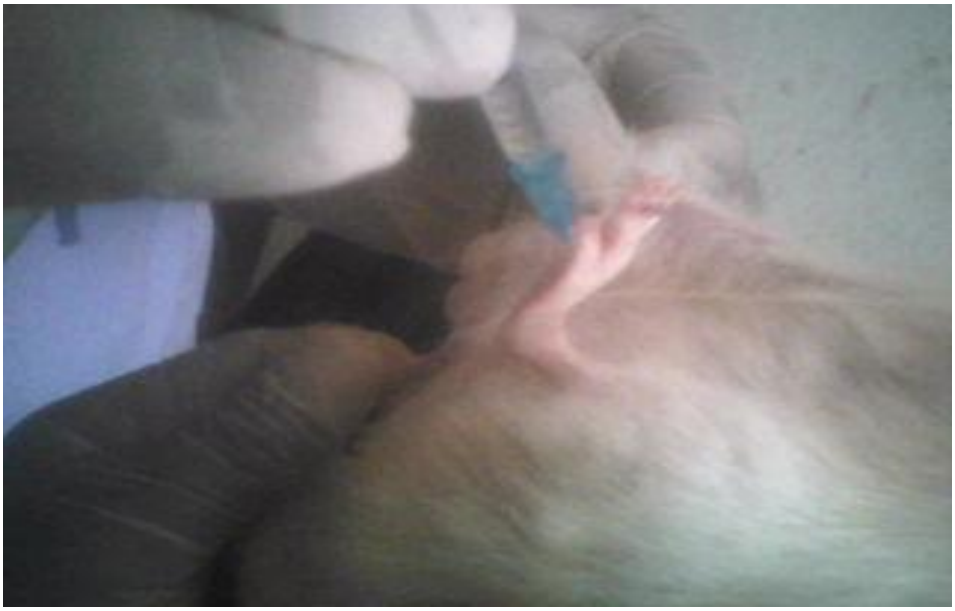

Figure 3

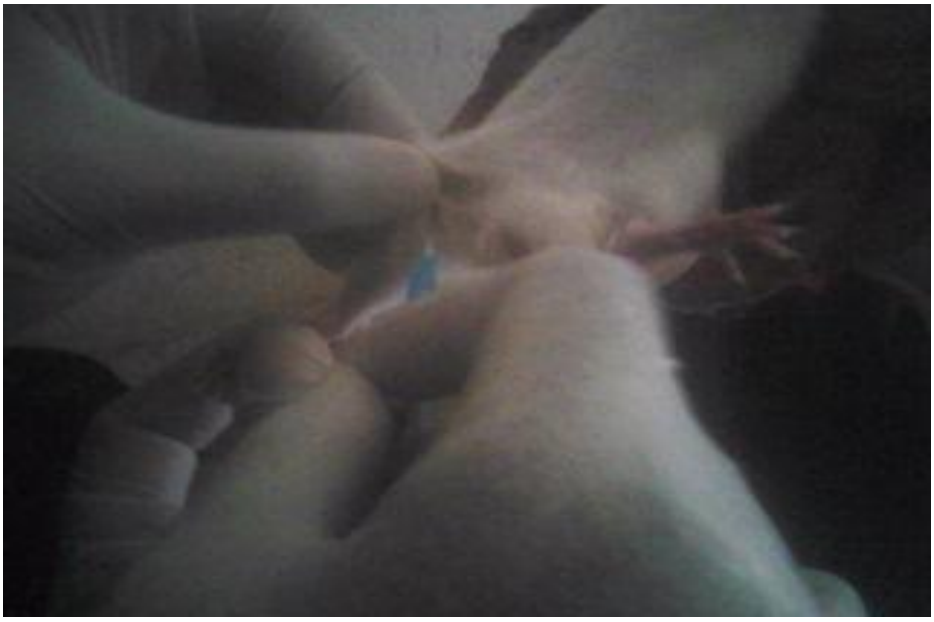

Figure 4

2.9 Evaluation of the area of wound contraction

Determination of the area of wound contraction which contributed to wound closure, excision

wounds were studied by tracing the raw wound and the change in wound size was calculated as the percentage of wound area that had healed.

2.10 Wound healing activity and Wound area measurement

The measurements of the wound areas for all the groups were taken on day 5, 10 and 15. During the wound healing period and at time intervals, the wounded areas were traced manually and photographed. Percentage of wound closure on these days was calculated using the formula (Chah *et al*., 2006) below.

$$\% \text{ Wound closure} = \frac{\text{Wound area on day (0)} - \text{Wound area on day (n)}}{\text{Wound area on day 0}} \times 100$$

KEYS: Where, n= numbers of days (5th, 10th and 15^{th}); 0= the day of wound creation.

The wounded areas in the first control groups where treated with sterile physiological saline solution. Other groups, had their wounds treated with herbal extracts of *Gongronema latifolium* and *Vernonia amygdalina* singly and in combination. The treatment was done topically in all the cases, once a day for 15 days. The animals were treated topically with the extracts (500 mg kg^{-1} body weight) which was applied as a single layer thickness to the wound for 15 days.

Animals were divided randomly into groups having six (6) rats each as follows:

Group I (n = 6): Diabetic wound + normal saline (positive control)

Group III (n = 6): Diabetic wound + *Gonglonema latifolium* treated group

Group IV (n = 6): Diabetic wound + *Vernonia amygdalina* treated group

Group V (n=6): Diabetic wound + *Gonglonema latifolium and Vernonia amygdalina* in equal combination (1:1) treated group

Group VI (n=6): Diabetic wound + *Gonglonema latifolium and Vernonia amygdalina* in combination (2:1) treated group

No analgesia and no dressing where used in the post-operatory of the animals being studied.

2.11 Preparation of bacterial inoculum

A clinical isolate of *Esherichia coli* and *Staphylococcus aureus* were used, because they are often detected in human chronic wounds. The bacteria isolates used in the present study were subcultured into nutrient broth, incubated overnight and diluted to MacFarland standard before use.

2.12 Inoculation Routes

The dorsal wound model method was used, which involved topical application to the epidermis. In groups having six rats per group, three rats each were inoculated with 0.1ml of *Esherichia coli* and *Staphylococcus aureus* respectively before the commencement of the herbal treatment. The rats where then placed in separate cages to prevent the animals from licking the inoculated area of the epidermis and to protect the excision wounds.

2.13 Wound Infection Assessment

Quantitative bacteria culturing of wounds was performed, so as not to disturb the wound environment, on days 5, 10, and 15 post-wounding. The Moist-swab technique was used to collect sample (wound swab). The method described by Georgiade *et al*. was used with slight modification. A sterile swab stick moistened with sterile normal saline was rubbed over the dorsal wound, across and back, advancing and rotating the applicator with each stroke.

2.14 Laboratory Analysis

The sterile swab stick was aseptically placed in a tube containing 5ml of sterile normal saline and agitated to mix the bacterial cells. Using a sterile syringe, 1ml of the solution was inoculated into 9ml of sterile distilled water and subsequently diluted to 10^{-5} (100-fold dilution). The pour plate method was used to culture the bacteria, where 0.1mL were pipetted and inoculated on nutrient Agar plate and grown aerobically at 37°C for 24hrs. Bacterial colonies were counted at10^{-5} dilution factor ($x10^5$ bacterial organism/ mL, Paul and Gordon, 1978).

2.15 Topical Application of Herbal Treatment

In a test tube, 0.5g of each extract was dissolved in 2ml of sterile distilled water to obtain a stock solution of 500mg/ml strength. The animals were topically treated with the stock herbal preparation singly and in combination.

Wounds of Group 1 animals were dressed with sterilized normal saline given as a placebo to the control group daily. However, wounds of other groups were dressed topically with 0.2 ml of the

herbal extracts daily, singly and in combination. Moreover, the wound was observed daily for 15 days to determine the rate of wound-healing enclosure.

2.16 Parameters Evaluated

For the duration of the treatment parameters checked where;

1. Rate of Infection: This was determined by swab sticks used to collect wound swabs from each rat on day 5, 10, and 15 then inoculated into normal saline with the cell biomass determined by plate count method.
2. The rate of wound closure: It was assessed on days 1, 5, 10 and 15 post-wounding.
3. Percentage of wound closure.
4. Appearance of wounded area on day 1,5,10 and 15 were noted.

2.17 Data Analysis

Statistical analyses were calculated using the two-way ANOVA. The results are expressed as mean values of triplicate numbers. Groups were compared for differences at different day interval. The level of significance was set at 0.05 for all statistical tests.

2.18 Ethical Considerations

The study protocol was approved by the University. During the study, animals were fed appropriately for the species, having free access to pelleted food and tap water throughout the experiment, being kept under ambient temperature, light and noise, maintaining the day-night cycle in cages. Each cage contained only one animal, without any contact with its physiological secretions (urine and feces).

Figures showing rats treated topically with herbal extracts in paste form.

Figure 5: Herbal extract applied singly.

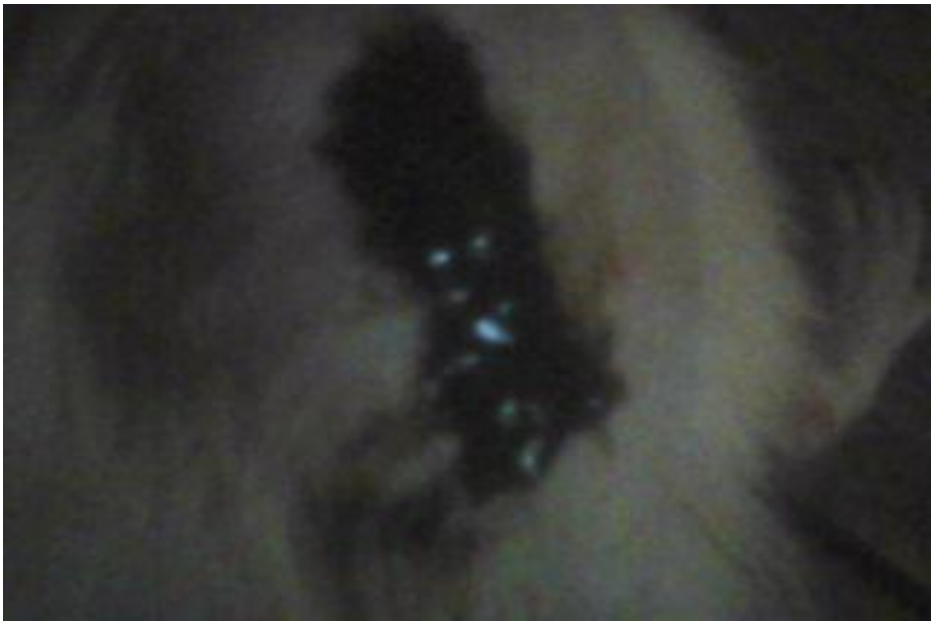

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Figure 6: Herbal extract applied in combination.

CHAPTER THREE

RESULTS

3.1 ANTIBACTERIAL ACTIVITIES

The results of the antibacterial activity of the aqueous and ethanolic extracts against the test organisms, *Escherichia coli, Pseudomonas aeroginosa, Staphylococcus aureus* and *Streptococcus pyogenes* are shown in Tables 2- 5. The antimicrobial activities were determined by the width of the zone of growth inhibition (Bauer, 1996).The zone of inhibition of the growth of the isolates is a function of the relative antibacterial activity of the extracts. The antibacterial activities of the individual extract and combination of extract of plant parts (*Gongronema latifolium* and *Vernonia amygdalina*) were very encouraging. Broad antimicrobial activities against both Gram positive and negative test bacteria were demonstrated. *V. amygdalina* showed more antibacterial activity against the organisms compared to *G. latifolium*. Higher growth inhibitory activity was obtained with the ethanolic extracts.

3.1.1 Effects of *Vernonia amygdalina* extracts on test bacterial species.

Results obtained with the ethanol and aqueous extracts as shown in (Table 2) implicated *Staphylococcus aureus* as the organism with the highest zone of growth inhibition ranging from 7.3mm (15.6mg/ml) to 20.3mm (250mg/ml) and 9.7mm (62.5mg/ml) to 16mm (250mg/ml) for ethanolic and aqueous extracts respectively. This is closely followed by *Escherichia coli* with zones of growth inhibition ranging from 9.7mm (62.5mg/ml) – 16.7mm (250mg/ml) for both extracts. The antibacterial effect decreased with decrease in concentration. There were no significant differences when the antibacterial effect of the aqueous extracts of *Pseudomonas*

aeroginosa was compared with that of its ethanol extract as shown in Table 2. All the bacteria were more sensitive to ethanol extract than the aqueous extract. Activities were observed against both Gram positive and negative bacteria. This showed that the extracts had broad spectrum activities.

3.1.2 Effects of *Gongronema latifolium* extracts on test bacterial species.

Ethanol and aqueous extraction of *Gongronema latifolium* showed that all the test bacteria were sensitive at 250mg/ml (Table 3). The highest zone of growth inhibition was recorded with *Escherichia coli* 15.7mm (250mg/ml) and 13.7mm (250mg/ml) for ethanolic and aqueous extracts respectively. The aqueous and ethanolic extraction showed *Streptococcus pyogenes* responded by presenting with similar growth inhibition pattern (Table 3). The test bacteria *S. pyogenes, S. aureus* and *P. aeroginosa* were observed to show no susceptibility to the extract at 31.2mg/ml and 15.6mg/ml for both aqueous and ethanolic extracts.

Table 2: Antibacterial Activity of Aqueous and Ethanolic Extract of *Vernonia. amygdalina* at varying concentrations (mg\ml) on test organisms with zones of inhibition in millimeters (mm)

Concentration	**AQUEOUS**					**ETHANOLIC**				
Organism	**250**	**125**	**62.5**	**31.2**	**15.6**	**250**	**125**	**62.5**	**31.2**	**15.6**
Esherichia coli	*12.7	11	9.7	0	0	16.7	14.3	11.3	0	0
Pseudomonas aeroginosa	8.3	7	0	0	0	*8	7	0	0	0
Staphylococcus aureus	16	12	9.7	10	0	20.3	15.3	13	10	7.3
Streptococcus pyogenes	10	9.7	8.3	0	0	11.7	8.7	6	0	0

***Figures are expressed as statistical means of triplicate numbers**

Table 3: Antibacterial Activity of Aqueous and Ethanolic Extract of *Gongronema latifolium* at varying concentrations (mg\ml) on test organisms with zones of inhibition in millimeters (mm)

Concentration	**AQUEOUS**					**ETHANOLIC**				
Test Organism	**250**	**125**	**62.5**	**31.2**	**15.6**	**250**	**125**	**62.5**	**31.2**	**15.6**
Esherichia coli	13.7	11	9.7	8.3	0	15.7	13.7	9.7	8.7	7
Pseudomonas aeroginosa	6.7	0	0	0	0	7	7	7	0	0
Staphylococcus aureus	11.7	9.7	8.3	0	0	14	9.7	8	0	0
Streptococcus pyogenes	8.3	7	0	0	0	8.3	7	0	0	0

3.2 Synergistic Relationship

Pairwise relationships between the plant extracts were evaluated using the method of Andy *et al.*, (2008). To obtain 1 in 1 dilution, equal volumes of *Vernonia amygdalina* (0.5g), and *Gongronema latifolium* (0.5g) each were dissolved in a test tube containing 4mls of sterilized water to form a stock solution of 250mg/ml strength, the mixture was used to test the individual bacteria. For 1 in 2 and 2 in 1 dilutions, *Va* (0.5g): *Gl* (1g) and *Va* (1g): *Gl* (0.5g) respectively were measured and dissolved using the method described above. These stock solutions (1:1, 1:2, 2:1) were subsequently diluted serially into various concentrations (125, 62.5, 31.25mg/ml) in test tubes containing 1ml of sterile distilled water. Dilutions described above were carried out with both aqueous and ethanolic extracts. Results are shown in tables 4 and 5.

3.2.1 Effect of the pairwise combination of Aqueous *Vernonia amygdalina* and *Gongronema latifolium* extracts on test bacterial species.

The combination of the aqueous extracted at A, B and C, ratios (Table 4) revealed *Staphylococcus aureus* as the most susceptible 14.7mm (250mg/ml, A and B ratios) to 8.3mm (15.6mg/ml, B ratio) followed by *E. coli* 8.3mm (15.6mg/ml, B ratio) to 14.7mm (250mg/ml, B ratio) while *Pseudomonas aeroginosa* was the least susceptible. No activity was recorded at extracts concentration of 31.25mg/ml and 15.62mg/ml for *Streptococcus pyogenes* across all the ratios A, B, and C (Table 4)

Table 4: Pairwise effect of Aqueous *V. amygdalina* and *G. latifolium* on test organisms

Concentration (mg/ml)	***A**					***B**					***C**				
Test Organism	**250**	**125**	**62.5**	**31.2**	**15.6**	**250**	**125**	**62.5**	**31.2**	**15.6**	**250**	**125**	**62.5**	**31.2**	**15.6**
Esherichia coli	13.7	11.7	9.7	9.7	0	14.7	11.7	9.7	8.3	8.3	12.7	11.7	8.3	0	0
Pseudomonas aeroginosa	* 0	0	0	0	0	8.3	8.3	6.7	0	0	8.7	8.3	6.7	0	0
Staphylococcus aureus	14.7	12.7	10	1 0	0	14.7	12.7	10	8.7	8.3	13.7	11.7	10	8.3	0
Streptococcus pyogenes	9.7	8.3	8.3	0	0	8.3	8.3	6.7	0	0	11.3	9.3	8.3	0	0

***Keys: *A (1 in 1 extract combination; Va1Gl1),**

***B (1 in 2 extract combination; Va1Gl2) and**

***C (2 in 1 extract combination; Va2Gl1).**

***0- no visible zone of inhibition.**

3.2.2 Effect of pairwise combination of ethanolic *Vernonia amygdalina* and *Gongronema. latifolium* on test bacterial species.

As shown in (Table 5) the combination of the ethanol extracts of *Gongronema latifolium* and *Vernonia amygdalina* at A, B and C pairs exerted a much higher activity on both *Staphylococcus aureus* (8.3mm to 20.3mm, Figure. 8) and *Esherichia coli* (9.7mm to 20.3mm) than *Streptococcus pyogenes* (8.3mm- 12.7mm). *Pseudomonas aeroginosa* was the least susceptible 8.0mm (62.5mg/ml, A and B pairs), however, at 31.25mg/ml and 15.62mg/ml concentrations, there was no zone of inhibition (Figure 9).

3.3 MINIMUM INHIBITORY CONCENTRATION (MIC)

The test organisms on which the plant samples showed good antibacterial activity were chosen to assay for the minimum inhibitory concentration with the agar dilution method. A serial dilution of the stock solution of each extract was obtained with the method described above. One (1ml) of nutrient broth was added and then a loopful of the test organism previously diluted to 0.5 turbidity standard was introduced. A tube containing nutrient broth only was seeded with the test organism to serve as control. All culture tubes were then incubated at 37^{o}c for 24hours. After incubation, they were examined for bacterial growth by observing the level of turbidity. Results of the minimum inhibitory concentration (MIC) are summarized in (Table 6). Generally, higher values were obtained for the ethanolic extracts. Concentrations of the respective extracts needed to inhibit *E. coli* and *S. aureus* were almost the same. The MIC of the aqueous extract of *G. latifolium* was 100 mg ml^{-1} for both *E. coli* and *S. aureus* whereas that of the ethanolic extract was 12.5 mg ml^{-1} and 6.25 mg ml-1 for *S. aureus* and *E. coli* respectively. The MIC of the aqueous extract of *V.*

amygdalina was 50.0 mg ml^{-1} for both *E. coli* and *S. aureus* while that of the ethanolic extract was 6.25 mg ml-1 and 12.5 mg ml-1 for *S. aureus* and *E. coli* respectively.

Table 5: Pairwise effect of ethanolic *Vernonia amygdalina* and *Gongronema latifolium* on test organisms

Concentration	*** A**					***B**					***C**				
Test Organism	**250**	**125**	**62.5**	**31.2**	**15.6**	**250**	**125**	**62.5**	**31.2**	**15.6**	**250**	**125**	**62.5**	**31.2**	**15.6**
Esherichia coli	17.7	14.7	13.0	0	0	20.3	17.7	14.7	9.7	0	16	11.7	10.7	9.7	0
Pseudomonas aeroginosa	9.3	8.7	8. 0	0	0	9.3	8.7	8.0	0	0	9.3	8.3	0	0	0
Staphylococcus aureus	20.3	9.7	8.7	0	0	20.3	13.0	9.7	9.7	8.3	20.3	9.7	9.3	8.3	0
Streptococcus pyogenes	11.7	10	9.7	0	0	12.7	9.7	8.3	0	0	11.7	9.7	8.3	0	0

Keys: *A (1 in 1 extract combination; Va1Gl1),

***B (1 in 2 extract combination; Va1Gl2) and**

***C (2 in 1 extract combination; Va2Gl1)**

Table 6: Minimum Inhibitory Concentration (MIC) of the aqueous and ethanolic extracts of *G. latifolium* and *V. amygdalina*

Test organisms	MIC Concentration (mg/ml)			
	*EU	*EO	*AU	*AO
S. aureus	12.5	6.25	100	50
E. coli	6.25	12.5	100	50

*EU- Ethanolic Utazi,

*EO-Ethanolic Olugbo.

*AU- Aquoeus Utazi,

*AO- Aqueous Olugbo

3.4 Rat Modelling

The aim of the study was to investigate the healing area of wounds treated with two topically applied extracts (*Gongronema latifolium and Vernonia amygdalina*, singly and in combination), in diabetic rat model. Wounds were induced as previously described, diabetic rats were treated with the topical application of the extracts (as a paste) singly and in combination daily. Rats were divided into groups, each group treated with a different volume of the extracts for study periods (15 days). The 5 groups were evenly balanced according to age, wound surface area created, standard daily care, and diabetes level. The time from the moment the excision wound was made to the beginning of repair processes in the wound was different depending on the applied remedy. Sampling for bacterial culture was taken at specific day intervals, and the results are shown in (Figure 8-12).

3.5 Evaluation of Bacterial Colony Count (BCC)

Data in Figures (9-12) showed that the herbal extracts of *Gongronema latifolium* and *Vernonia amygdalina* used, significantly reduced Bacterial Colony Count (BCC), as compared to normal saline (NS) treated control group in diabetic rats. The Bacterial Colony Count (BCC) values were more significantly reduced compared to the groups treated with the herbal extracts singly and the saline solution treated group. Interestingly, throughout the duration of the experimental treatment (15 days), the extracts in varying combinations showed masked, antagonistic, additive and synergistic effects against the infected diabetic wounded rats (Figures 7-12).

3.5.1 Viable count for Diabetic treated rats infected with *Escherichia coli.*

Bacterial colony count (BCC) at day 15 (D15) shows that the herbal extracts singly and in combination significantly reduced the count. Additive effect between the plant extracts Va and Gl significantly reduced the BCC value at combinations Va1Gl1 (1.6) and Va2Gl1 (1.6) compared to their singular effects (1.7).

3.5.2 Viable count for diabetic rats infected with *Stapylococus aureus*

By D15, Va1Gl2 (1.2) extract combination resulted in a much less BCC value compared to either Va (1.6) or Gl (1.7) singly because the combination had a synergistic effect against the *S. aureus* infected diabetic wounded rats. It's important to note that the herbal extract singly Va and in combination Va2Gl1 significantly reduced the BCC on day 15 the same way and therefore had the same values (1.6); Va completely masked the effect of Gl. At the extract combination Va1Gl1 (1.7) for the duration of the treatment, Gl (1.7) completely antagonised Va (1.6) resulting to the more BCC value compared to the herbal extracts applied singly.

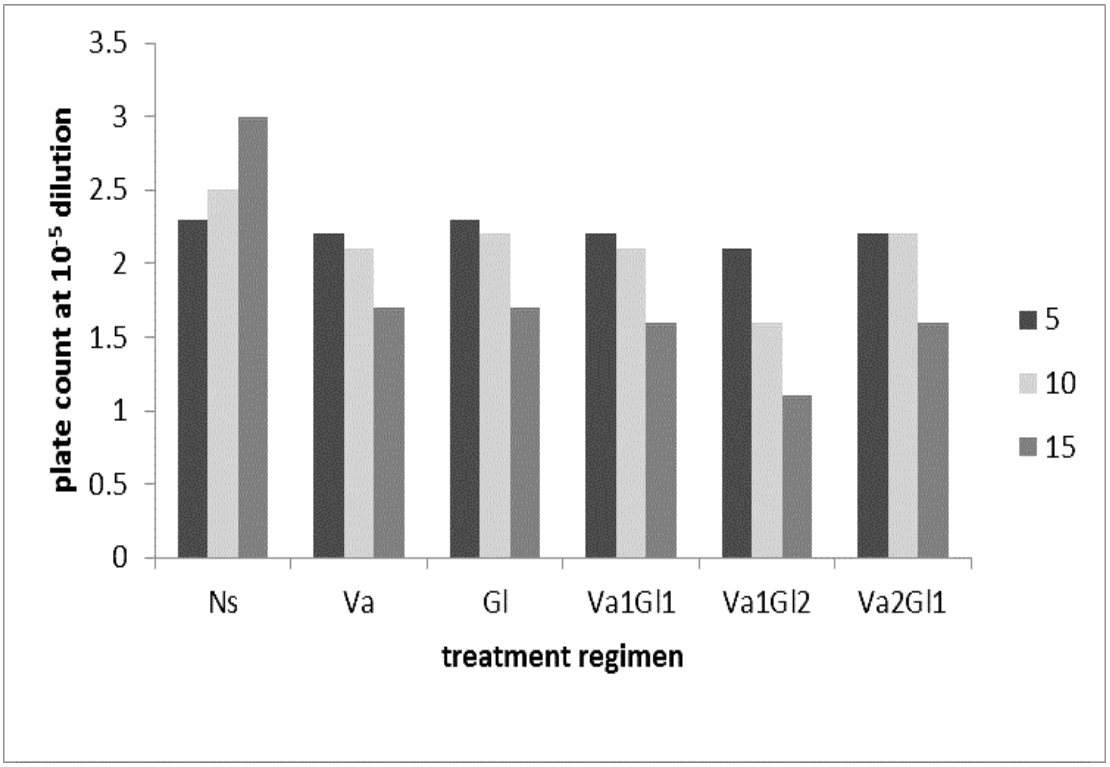

Figure 7: Viable count for Diabetic treated rats infected with *Escherichia coli.*

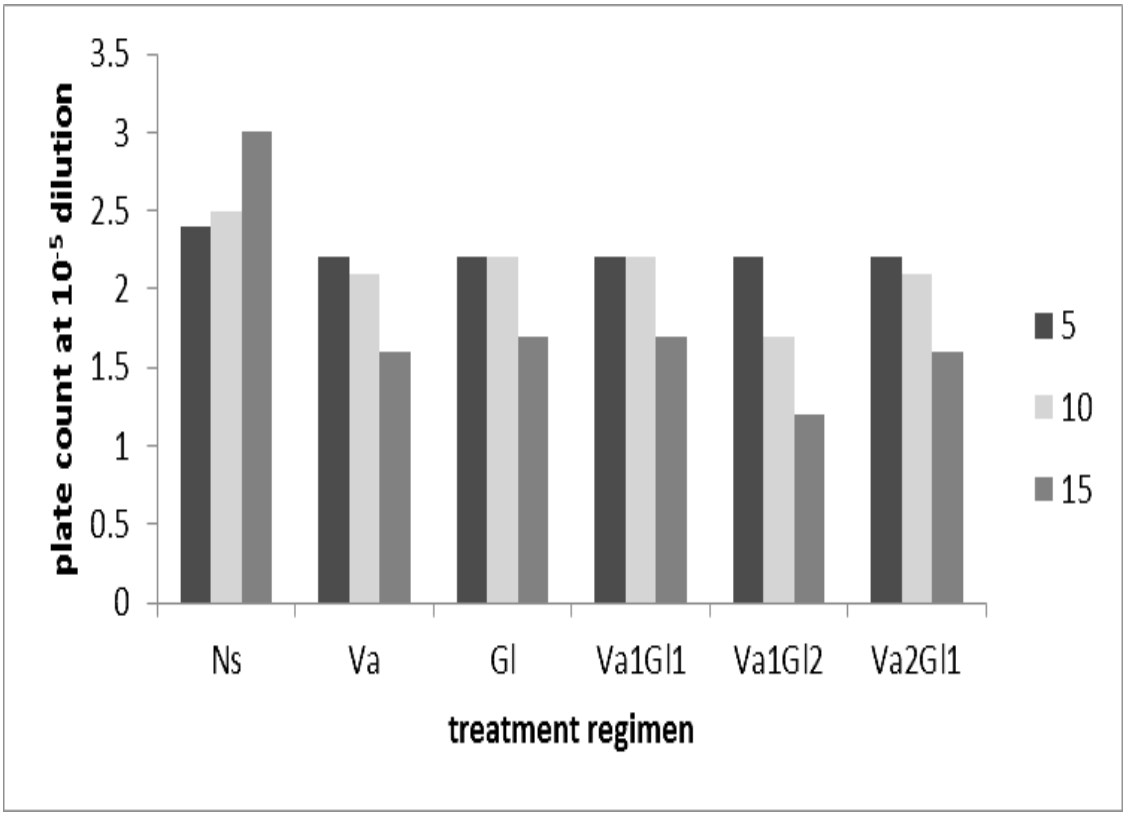

Figure 8: Viable count for diabetic rats infected with *Staphylococcus aureus*

3.6 COMPARISON OF WOUND SURFACE AREA (WSA)

Wound surface area (WSA) was measured at specific day intervals as explained in the figures. Data shows that all the extracts used significantly reduced WSA as compared to normal saline (NS) treated control group. The wound area relative of herbal extracts *Gongronema latifolium* and *Vernonia amygdalina* paired-combinations-treated groups were found to be significant ($p < 0.05$) and significantly reduced WSA at D5 - D15 compared to the herbal extracts singly treated group.

The duration of the treatment was significantly effective in the reducing WSA in extract treated groups, but increased WSA in normal saline (NS) treated group (fig. 9 and 10). NS treated diabetic groups infected with *E. coli* and *S. aureus* respectively showed dramatic increase in wound area (no significant area of wound contraction).

3.6.1 Area of wound contraction for diabetic treated rats infected with *E. coli*

The extract combination Va1Gl2 had the least wound size (1.9) at D15 but increased dramatically for the normal saline (NS) treated group (3.8).The herbal extracts of *Gongronema latifolium* and *Vernonia amygdalina* in paired combination Va1Gl2 and Va2Gl1 significantly reduced wound surface area (1.9, 2.4 respectively) at D15 compared to the herbal extracts singly treated groups Va (2.6) and Gl (2.6); they had a synergistic effect on the area of wound closure. At the paired combination Va1Gl1 there was no significant reduction of the wound area (2.6).

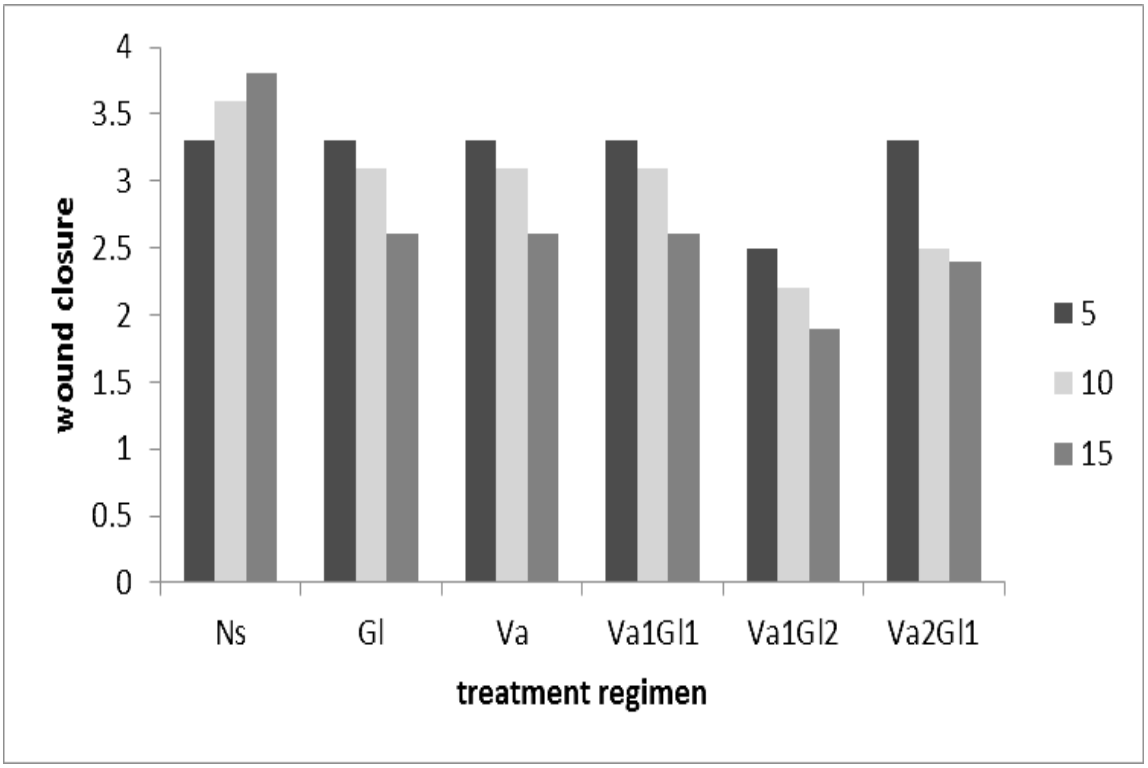

Figure 9: Area of wound contraction for diabetic treated rats infected with *E. coli*

3.6.2 Area of wound contraction for diabetic treated rats infected with *Staphylococcus aureus*

The least wound area was the value of Va1Gl2 (2.0) at D15 while NS treated group at D15 had the highest wound area (3.7). Plant extracts *Gongronema latifolium* and *Vernonia amygdalina* in combination Va1Gl2and Va2Gl1had a synergistic effect on the rate of wound closure, hence,

significantly reduced wound surface area (2.0 and 2.2 respectively) by D15 compared to the herbal extracts singly treated group Va (2.6) and Gl (2.6). At the extract combination Va1Gl1 there was no significant reduction of the wound area (2.6) compared to the singular effect of Va (2.6) and Gl (2.6).

3.7 GRAPHIC REPRESENTATION OF PERCENTAGE WOUND CONTRACTION (PWC)

Topical applications of the paste of the extracts showed effect on the healing process on the rats (fig. 11-12). In diabetic animals, percentage wound contraction (PWC) was greater in extract treated group than in normal saline control group animals. From these animal studies it can be concluded that significant increase in the wound healing activity was observed in the herbal extracts of *Gongronema latifolium* and *Vernonia amygdalina* singly and in combination treated rats. Throughout the experiment, the percentage wound healing in control treated group was significantly lower than those of extracts treated groups.

In NS treated group (control) the rats' excision type of wound does not heal almost completely unlike that obtained with the herbal extracts treated group. Diabetic rats treated with herbal extracts of *G. latifolium* and *V. amygdalina* in combination healed 40% by D15, while the group treated with the herbal extracts administered singly showed 20% PWC.

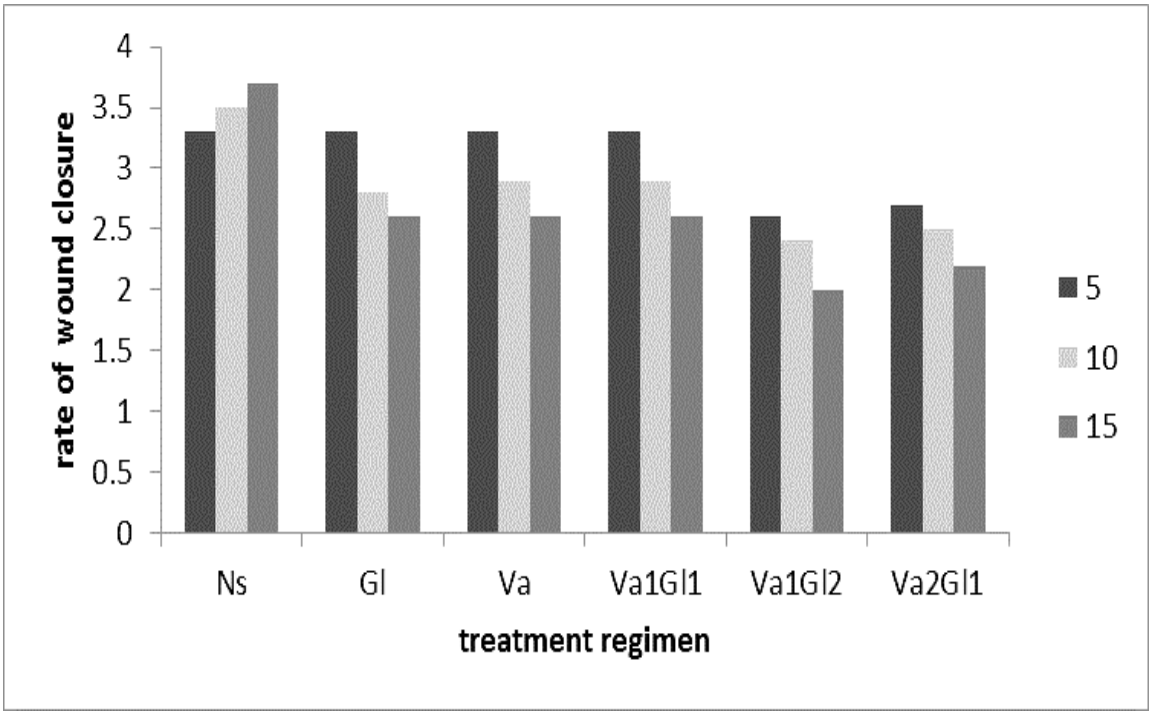

Figure 10: Area of wound contraction for diabetic treated rats infected with *Staphylococcus aureus*

3.7.1 Percentage Wound Contraction for diabetic treated rats infected with *Staphylococcus aureus*

From the above data, we deduce that 33% of the wound had closed by D15 for rats treated with extract combination Va1Gl2 while for NS treated group, at D15 the wound size expanded by 23%. Va, Gl applied singly and Va1Gl1 combined at D15 the rate of wound closure was 13% but for Va2Gl1 it was 27%.

3.7.2 Percentage Wound Contraction for diabetic treated rats infected with *Escherichia coli*

The wounded rats treated with the extract at combination Va1Gl2 had the highest percentage wound closure 40% by D15, while the least percentage was the NS treated group which expanded 27% by D15. Va and Gl singly and Va1Gl1 combined had percentage wound closure 13% at D15. Extract combination Va2Gl1 used had PWC of 20% by D15.

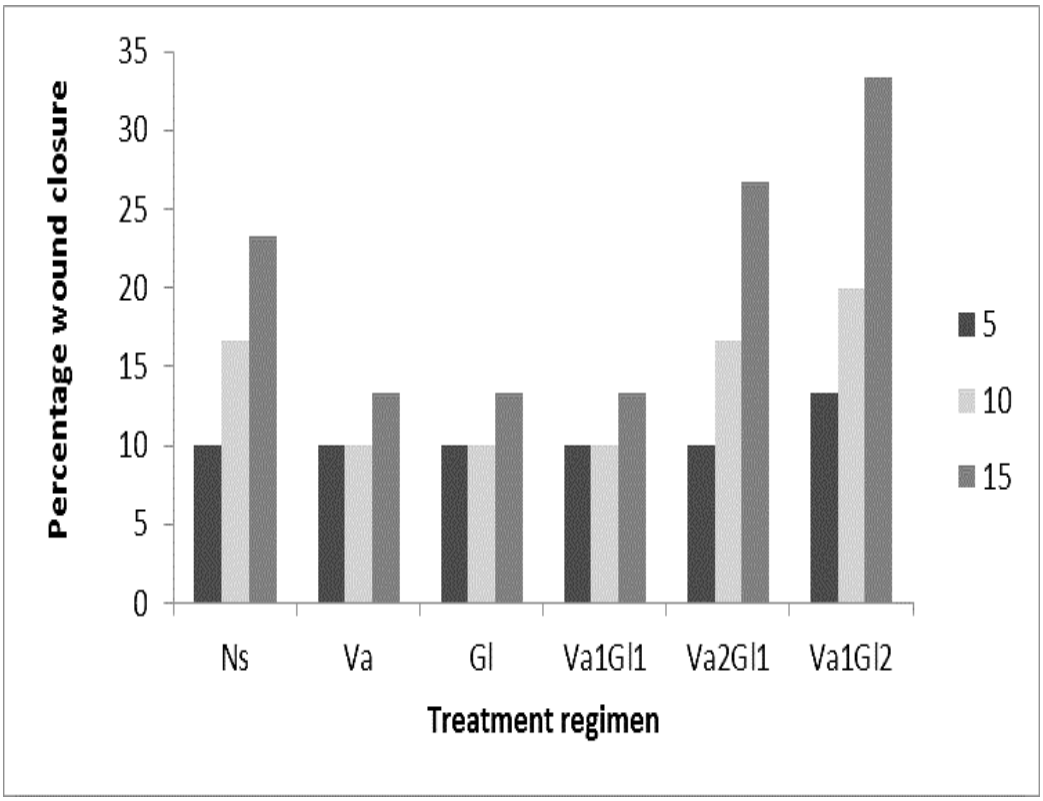

Figure 11: Percentage wound closure for diabetic treated rats infected with *Staphylococcus aureus*

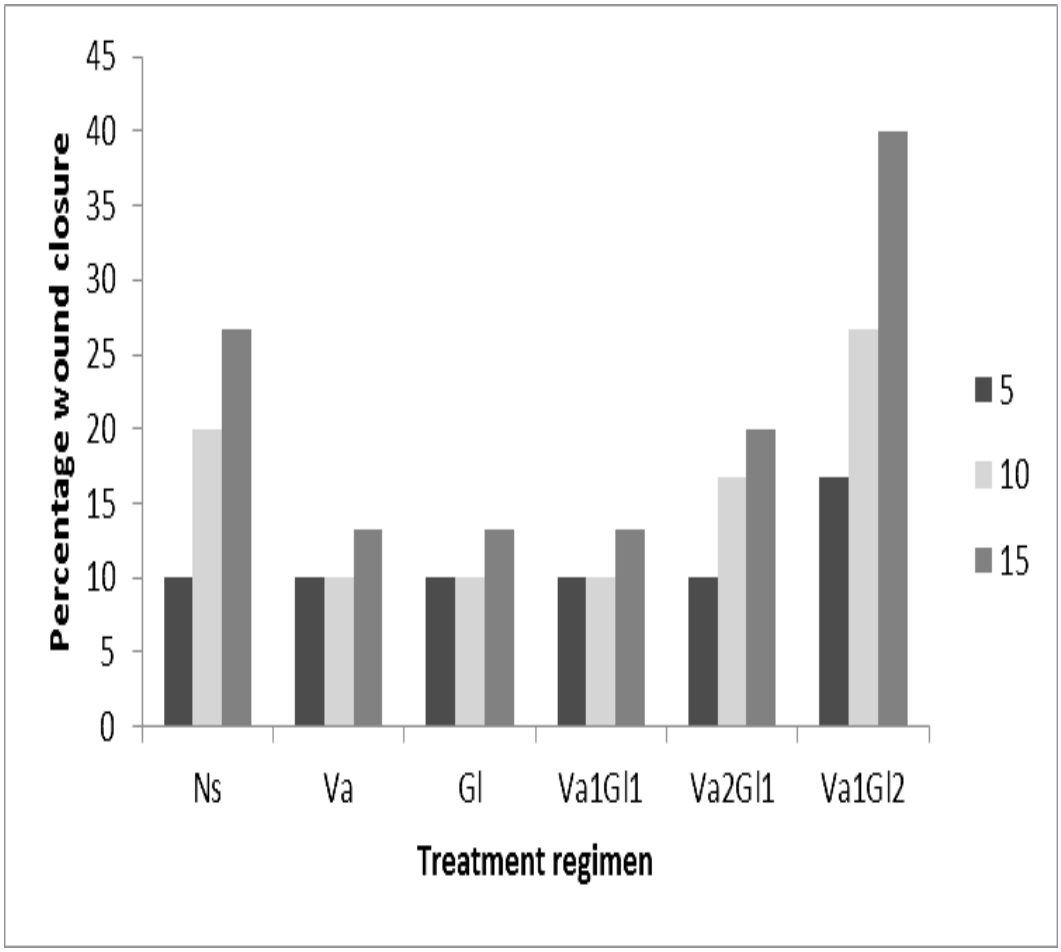

Figure 12: Percentage of Wound Closure for diabetic treated rats infected with *Escherichia coli*

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 Discussion

In the present study, *Escherichia coli* and *Pseudomonas aeroginosa* represent the group of gram negative bacterial pathogens, while *Staphylococcus aureus* and *Streptococcus pyogenes* represent the group of gram positive bacterial pathogens. The pattern of inhibition varied with the plant extract, the solvent used for extraction and the organisms tested. Ethanol and aqueous extracts of the leaves of *Vernonia. amygdalina* (Va) and *Gongronema latifolium* (Gl) possessed appreciable antimicrobial activity at 250mg/ml (tables 2 and 3) against *Escherichia coli* (Va, 16.7; Gl,15.7), *Staphylococcus aureus* (Va, 20.3; Gl, 14) and *Streptococcus pyogenes* (Va,11.7; Gl, 8.3). Antimicrobial activities of the plant extracts appeared to be broad spectrum since both Gram positive and negative bacteria were sensitive to the extracts. This study revealed that aqueous and ethanolic extracts of *V. amygdalina* and *G. latifolium* have very high concentration-dependent inhibitory antimicrobial activity (mean zone of inhibition > 16 mm) against the test organisms. Nwinyi *et al*., 2008 obtained a concentration-dependent growth inhibition of *S. aureus* by aqueous extract of olugbo and utazi leaf. Aqueous and methanol leaf extracts of *G. latifolium* has been reported to exhibit antibacterial effects on a number of bacteria including *E. coli* and *S. aureus* (Eleyinmi, 2007; Oshodi *et al*., 2004).

Among the extracts applied singly, *V. amygdalina* (Table 2) had the most impressive activities gainst test organisms and compares favorably with that of the combination of *V. amygdalina* and *G. latifolium* (Table 5)**.** The single effect of the extracts on the test bacterial species revealed that *V. amygdalina* (Table 2) was more active on the test organisms than *G. latifolium* (Table 3). The sensitivity of the test organisms to *V. amygdalina* may be due to the presence of active saponins

and essential oils (Desta, 1993). The susceptibility of the test organisms to the leaf extract of *V. amygdalina* agreed with the findings of Scalbert (1991) that demonstrated the antimicrobial activity of some medicinal plants against bacteria by using the extract of *V. amygdalina* as one of the samples. In similar studies, Aina and Uko, (1990), reported that *V. amygdalina* possess bacteriostatic activity but has little or no effect on yeast. Phytochemical composition of *G. latifolium* has been reported to consist of about 0.5% flavonoids, 2% tannins, 0.66% saponins, 0.33% polyphenols, 1.97% alkaloids and 13.2% hydrogen cyanide (Atangwho *et al*., 2009). Studies have also shown that the antibacterial properties of these plants depend on certain active ingredients, especially the oils such as saponins, tannins and flavonoids. *G. latifolium* contain saponins and these have been known to be responsible for its antioxidant and antimicrobial properties (Morebise and Fafunso, 1998; Morebise *et al.*, 2002). Flavonoids are said to be inhibitory to *S. aureus* and it has been used in treatment of inflamed tissues (Ali *et al*., 1996).

The ethanol extract of the leaves appeared to be more effective in inhibiting the growth of the test organisms than the aqueous extracts. Antibacterial activities of the ethanol extracts (Va, 20.3; Gl, 15.7) were significantly higher, than the antibacterial activities of the aqueous extracts (Va, 16; Gl, 13.7) of the two plants (Tables 2 and 3). This confirmed the work of Cowan (1999) who stated that the reason for the effectiveness of ethanol extraction could be that most of the identified active components from plants, aromatic or saturated organic compounds, are most often obtained through ethanol or methanol extraction.

The very low antibacterial activities observed with *P. aeroginosa* (Tables 2-5) is not surprising since multiple antibiotic resistant strains exist in clinical settings worldwide as well as Nigeria (Karen and Edzard, 2003; Kesah *et al*., 2003). The absence of activity against the test organism as observed with *G. latifolium* (Table 3) when used singly and in combination may suggest that the

concentration of active constituents in the extracts are too low for any appreciable antimicrobial activity. Furthermore, this may be due to factors such as time of collection of plant material and climate which might in turn affect the concentration of constituents in the plant material. In addition, low concentration of diffusible water soluble active constituents which often affect biologically active substances such as flavonoid, essential oils and other heterogeneous phyto-constituents present in the extracts might also influence their respective activity.

In the paired combinations (Va1Gl1, Va1Gl2, Va2Gl1; Tables 4 and 5) of the extracts *V. amygdalina* (Va) and *G. latifolium* (Gl), it is apparent that they are more effective than when used singly (Tables 2 and 3) for the treatment of infections associated with the test organisms. Results showed that when the leaf extracts were used in paired combinations, they had wider antibacterial activities than when used singly. The combinations of the leaf extracts exhibited a higher effect on the test bacterial species (16mm to 24mm) than any of the individual plant extracts (12mm to 16mm). The result obtained indicated that the activity against test bacteria is enhanced when *Vernonia amygdalina* and *Gongronema latifolium* are paired in the combination 1 in 1, 1 in 2 and 2 in 1 respectively. Results of this kind herald the interesting promise of designing a potentially active antibacterial synergized agent of plant origin. The reports on the combined effects of *V. amygdalina, G. kola* and *G. latifolium* on some microbial species, including *E. coli* and *S. aureus* in a study of the antimicrobial activity of Nigerian medicinal plants potentially usable as hop substitute (Oshodi *et al*., 2004) concurs with the findings of the present study. In the report (Oshodi *et al.*, 2004), *G. latifolium* appeared to have little or no effect on *E. coli*, but the combination of G. *latifolium, G. kola* and *V. amygdalina* had high effect on *E. coli, S. aureus* and *Streptococcus salivarus*. Apparently, high concentrations were used besides combining three plant extracts. Even

then, the report of Oshodi *et al*. (2004) supports and did not deviate much from the findings of this study.

From this study, we can also deduce that the combination of olugbo (*Vernonia amygdalina*) and utazi (*G. latifolium*) may not always yield the desired effect on both Gram positive and Gram negative bacteria, as there is evidence of some antagonism among the herbal extracts when paired at particular concentration (Tables 4 and 5) against some test organisms. There is an indication therefore, that though when used singly the antimicrobial effect of a medicinal plant extract might be highly active (Table 2 and 3), that it could be suppressed (Tables 4) or masked (Table 5) when taken together with another medicinal plant. Antagonistic or masked effects are more evident when the combination is used on Gram negative organisms, *E. coli* (Table 4 and 5). There was promise of desired effect when the combinations were used on Gram positive bacteria, *S. aureus*, in which case, the effect was either additive (Table 4) or synergistic (Table 5). Additive effect as applied in this study means an enhanced effect produced by the combination of two or more plant extracts (Table 4) but the effect is masked when the combination of the plant extracts and their singly effect are the same giving a constant value (Table 5). The effect is antagonistic when one plant suppresses the effect of the other resulting in a diminished effect (Tables 4 and 5), and synergistic when the elevated effect potentially destroys a resistant test organism. This agrees with the findings of Andy *et al.,* (2008).

4.2 RAT MODELLING (*INVIVO* EXPERIMENT)

The present investigation clearly deduced that the topical application of ethanolic plant extract of *Gongronema latifolium and Vernonia amygdalina* produced significant wound healing. Preliminary phytochemical analysis of the herbal extracts showed the presence of tannins,

flavonoids, saponins, polyphenols, alkaloids and hydrogen cyanide. Any one of the observed phytochemical constituents present in the extracts may be responsible for the wound healing activity. The wound-healing property of the herbal extracts of *G. latifolium* and *V. amygdalina* singly and in combination may be attributed to the phytoconstituents present in the plant, and the quicker process of wound healing could be a function of either the individual or the additive effects of the phytoconstituents. Recent studies have shown that phytochemical constituents like flavonoid and triterpenoids are known to promote the wound-healing process mainly due to their astringent and antimicrobial properties, which appear to be responsible for wound contraction and increased rate of percentage wound closure (Atangwho *et al.,* 2009, Morebise and Fafunso, 1998; Morebise *et al.,* 2002).

Topical application of *G. latifolium and V. amygdalina*, singly and in paired combinations (Figure 4-7) significantly reduced bacteria colony count. After the diabetic rats were wounded and then micro-organisms inoculated into their wounds, the formation of purulent exudate persisted in rats being treated with normal saline unlike their extract treated counterpart. Abscess formation in the animals treated with *G. latifolium and V. amygdalina* in combination was not verified under unarmed eye on the tenth postoperative day (D10). This suggests that the group treated with *G. latifolium and V. amygdalina* in combination (Va1Gl1, Va1Gl2, and Va2Gl1) had greater protection against bacteria or had a slower inflammatory process. Available results further showed that by day 15, single administration of plant extracts moderately reduced bacterial colony count of both *Staphylococcus aureus*, and *Eschericha coli*, in the infected diabetic rats (1.6/ml,), compared with the combined group (1.2/ml,1.1/ml respectively) at B dilutions. The efficacy of this plant extracts in wound healing may be due to its action against bacteria and the effect on antioxidant enzymes. This was also reported by Tachi and colleagues who stated; conversely, in

the healing process of pressure-induced ischemic wounds with bacterial inoculation (bacterial-inoculation group), bacterial proliferation was noted, and immunological reactions against the infection resulted in abscess formation in several sites from the epidermis and wound healing was delayed. As to the relationship between bacteria and wound contraction, it is reported that contraction in acute wounds is delayed in the presence of bacterial infection (Manish and Chandra, 2009)

Wound healing activity results showed that upon application of the extracts (paste) of *G. latifolium* and *V. amygdalina* singly and in combination (Figure 9-10) there was a visibly decreased scar area. The extracts of *G. latifolium* and *V. amygdalina* singly and in combination significantly stimulated wound contraction. No healing effect was observed with saline treated diabetic rats. The area of wound healing activities were evaluated, wounds treated with extracts of *G. latifolium* and *V. amygdalina* singly (Gl, Va) and in combinations (Va1Gl1, Va1Gl2, Va2Gl1) showed considerable signs of dermal healing in diabetic rats and significantly healed earlier (reduction in the wound area) compared to wounds in the normal saline (NS) treated (control) group (Figure 9-10). On the 5^{th} day, animals of groups treated with the extracts combined showed greater percentage of wound contraction when compared with the animals treated with the herbal extracts singly. The wounds after 15 days treatment exhibited marked dryness of wound edges and the wound area was also considerably reduced compared to controls indicating the healing potential of the herbal extracts. Diabetic wounded rats treated with the herbal extracts of *G. latifolium* and *V. amygdalina* singly and in combination were clean and showed bright red healthy surface. The pairwise combinations (A, B, and C) on the otherhand, was more effective in the reduction of the wound surface area of both *S. aureus,* and *E. coli* infected diabetic rats to 2.0cm^2 and 1.9cm^2 respectively at B dilutions, compared with the group with single extract administration 2.6 cm^2 for both test organisms.

Therefore, the results in this study can be used to support the concept that wound healing and repair are accelerated by applying *G. latifolium* and *V. amygdalina* extracts. Moreover there was a significant reduction in WSA in the herbal extract (in combination) treated group when compared to either remedy alone especially at D15. Enhanced capacity of wound healing with *G. latifolium* and *V. amygdalina* could be explained on the basis of the anti-inflammatory effects of the plants that are well documented in literature (Ghorbani, 2005; Ghasemi and Herba, 2009).

The present study demonstrates that *G. latifolium and V. amygdalina* extracts applied topically promotes healing by wound contraction in alloxan induced diabetic rats where healing is delayed. Furthermore, pairwise combination of plant extracts significantly increased percentage wound contraction of both *S. aureus,* and *E. coli* infected diabetic rats to 33% and 40% respectively at B dilutions, compared with the single administration of plant extract 13% for both test organisms. The study underscores the efficacy of pairwise administration of test plant extract in diabetic wound healing. These results further suggest that *G. latifolium and V. amygdalina* facilitate healing by increasing the area and extent of wound closure. Interestingly, it was found that the complementary of both agents (*G. latifolium and V. amygdalina*, in pairwise combinations) led to superior results in comparison with strict adherence to either extract alone. Result of the present study demonstrates that tissue repair and healing took place more rapidly in the diabetic group treated with *G. latifolium and V. amygdalina* in paired combinations, then singly and lastly the group treated with normal saline. Animals with induced diabetes had reduced wound size as early as day 5 and till the end of the experiment compared with the wounds in control animals. Several authors also reported this type of pro-healing action with the extract of many indigenous medicinal plants (Haque *et al.,* 2003; Manjunatha *et al.,* 2005).

4.3 CONCLUSION AND RECOMMENDATION

The work aimed to evaluate the antibacterial activity of these two Nigerian edible vegetables on selected organisms of medical importance, implicated in diabetic wound as the results will increase the knowledge on the tradomedical use of these plants. The antibacterial effect of *G. latifolium* and *V. amygdalina* which is evident from this study explains the long history of the use of these plants in traditional medicine for the treatment of different bacterial infections. Leaf extracts of *V. amygdalina* and *G. latifoilium* showed antibacterial activity against *E. coli* and *S. aureus.* This study has provided the basis for the use of *V. amygdalina* and *G. latifoilium* in the treatment of diabetic wound caused by *E. coli* and *S. aureus*. The potential antibacterial effects of the plants could be enhanced by extracting with ethanol instead of water as applied in the traditional practice. The information provided by this study of the extracts will make it easier for dosage determination and chemotherapeutic index of the extract if they were to be processed into drugs.

V. amygdalina (Va) and *G. latifolium* (Gl) individually possess relatively high antimicrobial activity against *E. coli* and *S. aureus*. However, the combined effects of the plant extracts on organisms like *P. aeruginosa* and *S. pyogenes* are in doubt especially as the factors of antagonism and or additive effects are not yet properly elucidated. What is apparent is the synergism between *V. amygdalina* (Va) and *G. latifolium* (Gl) on *S. aureus* and *E. coli* which was noted. The synergism observed in this study is attractive in view of the current problem of drug resistance by microorganisms and the fact that the modes of action of *V. amygdalina* (Va) and *G. latifolium* (Gl) that is inhibition of cell wall synthesis are similar (Eja *et al*., 2007). Therefore the fact that the extracts of these medicinal plants inhibited some medically important bacteria proves that these plants might have some potential as an alternative source of antibacterial substances.

Results of the study also revealed that herbal extracts of *G. latifolium and V. amygdalina* (both in paste form) effectively stimulate wound contraction as compared to control groups. Parameters used to determine the speed of healing were low bacteria cell count value and high rate of percentage wound closure. Their absence was considered to be a delay in the healing process. These finding could justify the inclusion of this plant in the management of wound healing. Present study has demonstrated that the ethanol extracts of *G. latifolium and V. amygdalina* has properties that render it capable of promoting accelerated wound healing activity compared with controls. High rate of wound contraction, and antimicrobial activities of these plants recorded, support further evaluation of their herbal extracts in the topical treatment and management of diabetic wounds.

Thus, the plant extracts might be useful as a wound healing agent. The potent wound healing capacity as shown from the wound contraction thus validated the ethno therapeutic claim. Meanwhile, it was found that the complementary of both agents *G. latifolium and V. amygdalina*, in paired combination (Va1Gl1, Va2Gl1, Va1Gl2) led to superior results in comparison with strict adherence to either extract alone. Our results allow us to conclude that: diabetic animals really show a deficiency of healing, compared to controls; the use of *G. latifolium and V. amygdalina* could be a helper way to quicken the healing process in patients diabetic wounds without the inconvenience of adverse side effects; and the observed benefic effect of *G. latifolium and V. amygdalina* on the healing process of non-diabetic could justify its use in normal rats, or be it, those that doesn't present deficiency of healing.

In summary, the method of using both *G. latifolium and V. amygdalina*, in pairwise combination have offered a renewed hope to patients with impaired wound healing. The results obtained

encourage us to carry out a wider and more profound study of this plant to obtain better knowledge of its therapeutic possibilities.

We therefore recommend that more research be carried out to standardize the usage of these plant extracts for the management of diabetic wounds.

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APPENDIX 1

GENERAL ANALYSIS OF DATA BY TWO-WAY ANOVA

Antibacterial activity of Aqueous *Vernonia amygdalina* (Zone of inhibition)

******* Analysis of variance ***** Variate: Aqueous *Vernonia amygdalina***

Organism	*E. coli*	*P. aeroginosa*	*S. aureus*	*S. pyogenes*
	6.67	3.07	9.53	5.60

Volume	15.60	31.25	62.50	125.00	250.00
	0.00	2.50	6.92	9.92	11.75

Organism	Volume	15.60	31.25	62.50	125.00	250.00
E. coli		0.00	0.00	9.67	11.00	12.67
P. aeroginosa		0.00	0.00	0.00	7.00	8.33
S. aureus		0.00	10.00	9.67	12.00	16.00
S. pyogenes		0.00	0.00	8.33	9.67	10.00

Antibacterial activity of Aqueous *Gongronema latifolium*

Organism	*E. coli*	*P. aeroginosa*	*S. aureus*	*S. pyogenes*
	8.53	1.33	5.93	3.07

Volume	15.60	31.25	62.50	125.00	250.00
	0.00	2.08	4.50	6.92	10.08

Organism	Volume	15.60	31.25	62.50	125.00	250.00
E. coli		0.00	8.33	9.67	11.00	13.67
P. aeroginosa		0.00	0.00	0.00	0.00	6.67
S. aureus		0.00	0.00	8.33	9.67	11.67
S. pyogenes		0.00	0.00	0.00	7.00	8.33

Antibacterial activity of Ethanolic *Vernonia amygdalina*

***** Analysis of variance *****

Variate: Ethanolic *Vernonia amygdalina*

Organism	*E. coli*	*P. aeroginosa*	*S. aureus*	*S. pyogenes*
	8.467	3.000	13.200	5.267

Volume	15.60	31.25	62.50	125.00	250.00
	1.833	2.500	7.583	11.333	14.167

Organism	Volume	15.60	31.25	62.50	125.00	250.00
E. coli		0.000	0.000	11.333	14.333	16.667
P. aeroginosa		0.000	0.000	0.000	7.000	8.000
S. aureus		7.333	10.000	13.000	15.333	20.333
S. pyogenes		0.000	0.000	6.000	8.667	11.667

Antibacterial activity of Ethanolic *Gongronema latifolium*

***** Analysis of variance *****

Variate: Ethanolic *Gongronema latifolium*

Organism	*E. coli*	*P. aeroginosa*	*S. aureus*	*S. pyogenes*
	10.93	4.20	6.33	3.07

Volume	15.60	31.25	62.50	125.00	250.00
	1.75	2.17	6.17	9.33	11.25

Organism	Volume	15.60	31.25	62.50	125.00	250.00
E. coli		7.00	8.67	9.67	13.67	15.67
P. aroginosa		0.00	0.00	7.00	7.00	7.00
S. aureus		0.00	0.00	8.00	9.67	14.00
S. pyogenes		0.00	0.00	0.00	7.00	8.33

APPENDIX 2

Synergistic relationship between Aqueous *Vernonia amygdalina* and Aqueous *Gongronema latifolium* on selected bacteria

Variate: Aqueous *Vernonia amygdalina* and Aqueous *Gongronema latifolium*

Organism	*E. coli*	*P. aeroginosa*	*S. aureus*	*S. pyogenes*
	8.667	3.133	9.689	5.244

Paired	A	B	C
	5.917	7.683	6.450

Volume	15.50	31.50	62.50	125.00	250.00
	1.389	3.750	7.861	9.556	10.861

Organism	Ratio	A	B	C
E. coli		8.933	10.533	6.533
P. aeroginosa		0.000	4.667	4.733
S. aureus		9.467	10.867	8.733
S. pyogenes		5.267	4.667	5.800

Organism	Volume	15.50	31.50	62.50	125.00	250.00
E. coli		2.778	6.000	9.222	11.667	13.667
P. aroginosa		0.000	0.000	4.444	5.556	5.667
S. aureus		2.778	9.000	10.000	12.333	14.333
S. pyogenes		0.000	0.000	7.778	8.667	9.778

Paired	Volume	15.50	31.50	62.50	125.00	250.00
A		0.000	4.917	7.000	8.167	9.500
B		4.167	4.250	8.250	10.250	11.500
C		0.000	2.083	8.333	10.250	11.583

Organism	Ratio	Volume	15.50	31.50	62.50	125.00	250.00
E. coli	A		0.000	9.667	9.667	11.667	13.667
	B		8.333	8.333	9.667	11.667	14.667
	C		0.000	0.000	8.333	11.667	12.667
P. aeroginosa	A		0.000	0.000	0.000	0.000	0.000
	B		0.000	0.000	6.667	8.333	8.333
	C		0.000	0.000	6.667	8.333	8.667
S. aureus	A		0.000	10.000	10.000	12.667	14.667
	B		8.333	8.667	10.000	12.667	14.667
	C		0.000	8.333	10.000	11.667	13.667
S. pyogenes	A		0.000	0.000	8.333	8.333	9.667
	B		0.000	0.000	6.667	8.333	8.333
	C		0.000	0.000	8.333	9.333	11.366

"General Analysis of Variance."

Synergistic relationship between Ethanolic *Vernonia amygdalina* and *Gongronema latifolium* on selected bacteria

Variate: Ethanolic_*Vernonia amygdalina* and *Gongronema latifolium*

Grand mean 7.878

Organism	*E. coli*	*P. aeroginosa*	*S. aureus*	*S. pyogenes*
	10.378	5.200	9.822	6.111

Paired	A	B	C
	7.067	9.000	7.567

Volume	15.50	31.50	62.50	125.00	250.00
	0.694	3.111	9.722	10.944	14.917

Organism	Ratio	A	B	C
E. coli		9.067	12.467	9.600
P. aeroginosa		5.200	5.200	5.200
S. aureus		7.733	12.200	9.533
S. pyogenes		6.267	6.133	5.933

Organism	Volume	15.50	31.50	62.50	125.00	250.00
E.coli		0.000	6.444	12.778	14.667	18.000
P. aeroginosa		0.000	0.000	8.111	8.556	9.333
S. aureus		2.778	6.000	9.222	10.778	20.333
S. pyogenes		0.000	0.000	8.778	9.778	12.000

Ratio	Volume	15.50	31.50	62.50	125.00	250.00
A		0.000	0.000	9.833	10.750	14.750
B		2.083	4.833	10.167	12.250	15.667
C		0.000	4.500	9.167	9.833	14.333

Organism	Ratio	Volume	15.50	31.50	62.50	125.00	250.00
E.coli	A		0.000	0.000	13.000	14.667	17.667
	B		0.000	9.667	14.667	17.667	20.333
	C		0.000	9.667	10.667	11.667	16.000
P. aeroginosa	A		0.000	0.000	8.000	8.667	9.333
	B		0.000	0.000	8.000	8.667	9.333
	C		0.000	0.000	8.333	8.333	9.333
S. aureus	A		0.000	0.000	8.667	9.667	20.333

	B	8.333	9.667	9.667	13.000	20.333
	C	0.000	8.333	9.333	9.667	20.333
S. pyogenes	A	0.000	0.000	9.667	10.000	11.667
	B	0.000	0.000	8.333	9.667	12.667
	C	0.000	0.000	8.333	9.667	11.667

APPENDIX 3

Plate count for diabetic rats infected with *Escherichia coli*

Variate: *E_coli*

Grand mean 2.0437

Days	5.00	10.00	15.00
	2.2406	2.1078	1.7828

Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1	Va1Gl2
	2.0278	2.5978	2.0111	1.9911	1.6233

Groups_of_diabetic_treated rats	Va2Gl1
	2.0111

Days	Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1
5.00		2.2600	2.3533	2.2400	2.2233
10.00		2.1567	2.4700	2.1333	2.1133
15.00		1.6667	2.9700	1.6600	1.6367

Days	Groups_of_diabetic_treated rats	Va1Gl2	Va2Gl1
5.00		2.1300	2.2367
10.00		1.6233	2.1500
15.00		1.1167	1.6467

Plate count for diabetic rats infected with *Staphylococcus aureus*

***** Analysis of variance *****

Variate: *S.aureus*

Days	5.00	10.00	15.00
	2.2483	2.1189	1.7917

Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1	Va1Gl2
	2.0433	2.6289	1.9811	2.0133	1.6811

Groups_of_diabetic_treated rats	Va2Gl1
	1.9700

Days	Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1
5.00		2.2533	2.4167	2.2067	2.2233
10.00		2.1900	2.5000	2.1067	2.1600
15.00		1.6867	2.9700	1.6300	1.6567

Days	Groups_of_diabetic_treated rats	Va1Gl2	Va2Gl1
5.00		2.1767	2.2133
10.00		1.6667	2.0900
15.00		1.2000	1.6067

APPENDIX 4

Area of wound closure for diabetic rats infected with *E. coli*

***** Analysis of variance *****

Variate: *E. coli****** Tables of means

Grand mean 2.917

Days	5.00	10.00	15.00
	3.167	2.933	2.650

Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1	Va1Gl2
	3.000	3.567	3.000	3.000	2.200

Groups_of_diabetic treated rats	Va2Gl1
	2.733

Days	Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1
5.00		3.300	3.300	3.300	3.300
10.00		3.100	3.600	3.100	3.100
15.00		2.600	3.800	2.600	2.600

Days	Groups_of_diabetic_treated rats	Va1Gl2	Va2Gl1
5.00		2.500	3.300
10.00		2.200	2.500
15.00		1.900	2.400

Area of wound closure for diabetic rats infected with *Staphlococcus aureus*

***** Analysis of variance *****

***** Tables of means *****

Variate: *S. aureus*

Grand mean 2.844

Days	5.00	10.00	15.00
	3.083	2.833	2.617

Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1	Va1Gl2
	2.900	3.500	2.933	2.933	2.333

Groups_of_diabetic_treated rats	Va2Gl1
	2.467

Days	Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1
5.00		3.300	3.300	3.300	3.300
10.00		2.800	3.500	2.900	2.900
15.00		2.600	3.700	2.600	2.600

Days	Groups_of_diabetic_treated rats	Va1Gl2	Va2Gl1
5.00		2.600	2.700
10.00		2.400	2.500
15.00		2.000	2.200

APPENDIX 5

Percentage wound closure for diabetic rats infected with *Escherichia coli*

Grand mean 15.93

Days	5.00	10.00	15.00
	11.11	15.56	21.11

Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1	Va1Gl2
	11.11	18.89	11.11	11.11	27.78

Groups_of_diabetic_treated rats	Va2Gl1
	15.56

Days	Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1
5.00		10.00	10.00	10.00	10.00
10.00		10.00	20.00	10.00	10.00
15.00		13.33	26.67	13.33	13.33

Days	Groups_of_diabetic_treated rats	Va1Gl2	Va2Gl1
5.00		16.67	10.00
10.00		26.67	16.67
15.00		40.00	20.00

Percentage closure for wounds infected with *Staphylococcus aureus*

Grand mean 15.00

Days	5.00	10.00	15.00
	10.56	13.89	20.56

Groups_of_diabetic_treated rats	Gl	Ns	Va	Va1Gl1	Va1Gl2
	11.11	16.67	11.11	11.11	22.22

Groups_of_diabetic_treated	VAbGLa
	17.78

Days	Groups_of_diabetic_treated	GC	NS	VA	VAaGLa
5.00		10.00	10.00	10.00	10.00
10.00		10.00	16.67	10.00	10.00
15.00		13.33	23.33	13.33	13.33

Days	Groups_of_diabetic_treated	VAaGLb	VAbGLa
5.00		13.33	10.00
10.00		20.00	16.67
15.00		33.33	