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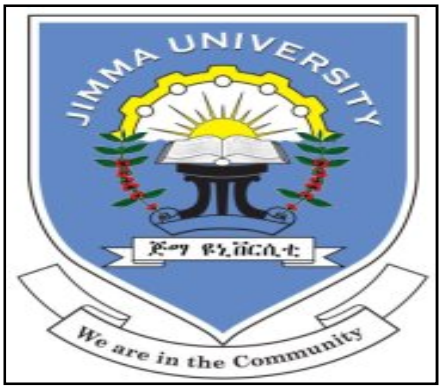

ANTIBACTERIAL EFFICACY OF SELECTED PLANTS TRADITIONALLY USED AS DETERGENT IN RURAL ETHIOPIA

BY

GUTEMA TARESSA (B.Sc.)

A THESIS SUBMITTED TO THE DEPARTMENT OF ENVIRONMENTAL HEALTH SCIENCES & TECHNOLOGY, COLLEGE OF PUBLIC HEALTH AND MEDICAL SCIENCES, JIMMA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ENVIRONMENTAL SCIENCE AND TECHNOLOGY

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BY:

GUTEMA TARESSA (B.Sc.)

ADVISORS: GUDINA TEREFE (MSc, PhD Candidate)

WONDWOSSEN BIRKE (MSc, Ass. Professor)

SEPTEMBER, 2013

JIMMA, ETHIOPIA

APPROVAL SHEET

JIMMA UNIVERSITY

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As thesis research advisor, I herby certify that I have read and evaluated this thesis prepared under my guidance by Gutema Taressa entitled "Antibacterial efficacy of traditional plants used as detergent in rural Ethiopia" I recommend it would be submitted as fulfilling the thesis requirement.

Name of advisor	**Signature**	**Date**
Gudina Terefe (MSc, PhD Candidate)	----------	----------
Wondwossen Birke (MSc, Ass. Professor)	----------	----------

As members of the examining board of the final M.Sc. thesis open defense examination, we certify that we have read and evaluated the thesis prepared by Gutema Taressa and examined the candidate. We recommend that it would be accepted as fulfilling the thesis requirement for the degree of Master of Science in Environmental Science and Technology.

_____	_____	_____
Name of Chairperson	Signature	Date
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I declare that this piece of work is my own and all sources of materials used for this thesis work have been duly acknowledged. The thesis has been submitted in partial fulfillment of the requirements for the degree of Master of Science at Jimma University.

Name:Gutema Taressa

Signature _____

Place: Jimma, Ethiopia

Date of Submission _____

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ABSTRACT

Antibacterial activities of traditional plants were commonly used all over the world including Ethiopia. Among those plants that are used as traditional detergents like for personal hygiene in most developing countries were well practiced. Hand hygiene is basic part of personal hygiene which is a fundamental practice in the prevention, control, and reduction of communicable diseases. The prevalence of hand hygiene related diseases including diarrhea and the current emerging bacterial resistance are the big global health challenges but the degree of the problem is greater in developing countries. Use of traditional plants for hand hygiene were not given due consideration as detergent due to the fact that their antibacterial activity for cleaning has not been yet well understood. The purpose of this study is therefore to determine the antibacterial efficacy of traditional plant used as detergent in most rural and some peri urban areas of Ethiopia. Selected plants traditionally used as detergents (*Phytolacca dodecandra fruit, Rumex nepalensis* leaf, *Grewia ferruginea* bark and *Grewia ferruginea* leaf) were collected from surroundings of Jimma town based on ethnobotanical information. An acetone extract of these plants were tested against reference and isolated bacteria for their antibacterial efficacy using disc diffusion and macro dilution methods. The preliminary evaluations of the extracts were exhibited appreciable inhibitory activities against the tested bacteria isolates except for *P. aeroginosa* at concentration of 200mg/mL .The diameter of inhibition zone ranged from 8.0±1.0 mm to 11.0±1.0 mm.The MIC values of the extracts were ranged from 6.25 mg/ml to 50 mg/ml whereas the MBC values were ranged from 12.5 mg/ml to 100 mg/ml against isolated bacteria except *P. aeroginosa*. Among these plants, *Rumex nepalensis leaf, G.ferruginea leaf and G.ferruginea bark* have shown the best antibacterial activity against salmonella species. Phytochemical qualitative analyses were also made to identify the level of antibacterial constituents of the extracts. The variation on antibacterial activity could be attributed to the level of phytochemical constituents of the extracts like saponins, tannins, flavinoids, terpinoids and reducing sugar. This finding suggests the use of the plants extract for formulation of hand sanitizers and as active ingredients for production of antibacterial soaps to improve public health problems.

Keywords: Plant extracts, inhibition, antibacterial effect, minimum inhibitory concentration, minimum bactericidal concentration

LIST OF ABBREVIATIONS

AMR	Global antimicrobial
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CFU	Colony forming unit
CSA	Central Statistical Agency
DCA	Deoxycholate citrate
DCLS	Desoxycholate Citrate Lactose Sucrose Agar
DHS	Demographic Health survey
DMSO	Dimethyl sulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E.coli	Escherichia coli
EHNRI	Ethiopian Health and Nutrition Research Institute
MBC	Minimum Bactericidal Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
MIC	Minimum Inhibitory Concentration
P. aeruginosa	*Pseudomonas aeruginosa*
S. aurues	*Staphylococcus aureus*
UTM	Universal Transverse Mercator
WaSH	Water supply sanitation and hygiene
WHO	World Health Organization
XLDA	Xylose lysine desoxycholate

CHAPTER ONE: INTRODUCTION

1.1 **Background**

The beginning of personal cleanliness is in prehistoric time when people started to live around the water bodies. The cleaning action of soap is due to its detergent properties and can be used to remove foreign materials from hands (Simonne, 2011).The people used the cleansing properties of water to rinse and mud off their hands (SDA, 1994). Use of soaps for personal cleanness has been considered during the past centuries and addition of antiseptic agents in soaps for its antimicrobial effect was emerged in 19th century (Wolf and Wolf, 2001; Chauhan, 2006).

Microbes present on the skin can be broadly classified into two distinct categories such as resident and transient. Healthy human hands can carry millions of resident and transient bacteria (Simonne, 2011). Resident microflora has been found to be localized in the skin crevices, where skin oils and hardened skin make their removal difficult. Bacteria that are found on skin resident flora are coagulase negative Staphylococci, members of Corynebacterium, Propionibacterium and Acinetobacter species (Bojar and Holland, 2002; Chauhan, 2006).

S. aureus is the pathogenic organism included in both the resident and transient microflora of skin and is a common bacterium found up to 25% on skin of healthy people (Kluytmans, 2010). About 35% of normal adults carry *S. aureus* in the nose and are susceptible to infection when skin barrier is broken (Chauhan, 2006). Residence species of human micro flora capable of continued growth on the skin that are found on all human being where as transient microbes from the environment may not continuously grow in the skin (Bojar and Holland, 2002).

Good hygiene is not only protecting human health but also aids to beauty, comfort, and social interactions. Our hands, from the body parts perform majority of activities through which it can be easily contaminated. Unless proper hand hygiene takes place, it creates routes for disease transmission. Hand hygiene is a broad term that applies to hand washing, antiseptic hand wash,

and antiseptic hand rub. Hand hygiene using hand sanitizers is the current best tool in preventing hand related communicable diseases caused by different pathogenic micro organisms (Vyas *et al*., 2011).

Clean hands stop the spread of germs; therefore hand washing is often emphasized as the single most important measure in any infection control programme for preventing cross transmission of microorganisms between patients. Hand washing is the act of cleaning the hands with or without the use of soap or other detergent to remove dirt or loose transient flora thus preventing cross-infection (Adzam, 2012).

Hand washing is the simplest, cost effective and easily applicable measure that can be followed by people to reduce the risk of spread of infectious diseases. Hand washing is the act of cleaning the hands with or without the use of soap or other detergent to remove any foreign from hands. Hand hygiene is a general term that applies to hand washing, antiseptic hand wash, antiseptic hand rub, or surgical hand antisepsis. Hand hygiene is the first line of defense against the spread of pathogenic microbes and in conjunction with stringent sanitation, can greatly reduce the risks of nosocomial and community acquired diseases (CDC, 1995).

Plant extracts have antibacterial activity against bacterial species such as *S.aurues* and *E.coli* with no or little side effect on skin (Cown, 1999; Handali *et al*., 2012). Neem kernel oil obtained from mechanically pressed neem seed is used for production of neem soap which has favorable medicinal properties (Mak-Mensah and Firempong, 2011). Thus, currently there is considerable interest in natural products from plants with antimicrobial activity for controlling micro-organisms due to increase bacteria resistance (Wallace, 2004).

Different plants are now traditionally used as detergents for cleaning purposes such as for washing hands, hairs and clothes. *Phytolacca dodecandra* and *Grewia ferruginea* are the most commonly traditionally used plants in different rural areas of Ethiopia (Esser *et al*., 2003; Kumbi, 2007). Also, *Rumex nepalensis* leaf used as traditional medicine and as detergents to wash hairs (Suleman and Alemu, 2012). The uses of plant extracts with known antibacterial properties have great significance for antibacterial hand hygiene to prevent or stop chain of disease transmission. There are very few studies that use isolated human pathogenic bacteria from real environment for evaluation of antibacterial efficacy of traditional plants extracts.

Therefore, this study used plants that are traditionally used as detergents to determine invitro bacteriostatic and bactericidal concentration to be used to combat pathogenic bacteria transmitted through hands.

1.2 Statement of the problems

Hands perform majority of functions of the human's body for our day to day life and are exposed to pathogenic bacteria from the environment. Practice of anal ablution is implemented by hands through which it is risk for contaminating food, drinking water and even transmission to other people through direct hand contact which can be potential source for diarrheal and other diseases (Asafu Maradufu, 2012; Chauhan, 2006). Among disease causing microorganisms, bacterial infection is one of the most serious global health problems in 21st century (Morris and Masterton, 2002).

Diarrhea is one of the leading public health problems in developing countries. Simple hand washing can reduce diarrhea disease by about 48% compared to source water treatment which is only 11 % when appropriate and efficient hand washing products are used (Fewtrell *et al*., 2005). According to Ethiopian Demographic and Health survey, 38 percent of households have no toilet facility, which means Ethiopia is categorized among countries with low sanitation coverage. This low sanitation may increase contamination of hands from the environment with pathogenic bacteria that able to cause diarrheal diseases. In Ethiopia, the two weeks preceding survey of prevalence of diarrheal diseases was 13% among children under five years of age and this figure was 23% in Benishangul Gumuz and Gambella region (CSA, 2012). Global antimicrobial Resistance (AMR) is indiscriminant, impacting every region and country. Antimicrobial resistance is undermining every clinical and public health program designed to contain infectious diseases worldwide. International policy overview on antibiotic resistance was given attention to hygienic measures which are effective in combating transmission of the antibiotic resistance bacteria (Marieke Gerards, 2011).

In developing countries, antimicrobial resistance has increased drastically in recent years, which becomes the leading public health problems. The prevalence of antimicrobial resistance varies between and within countries and between different pathogenic microorganisms. Limited access to medical care and effective treatments, the common practice of self-medicating, inappropriate antibiotic use are main causes for antibacterial resistance in the developing countries (Vila and

Pal, 2010). Currently, microbes are getting resistance to antimicrobial agents for occurrence of diseases which have great impact on development. Triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol) is a phenolic compound which is added to antimicrobial soap due to its broad spectrum activity and its action is by inhibiting of fatty acid synthesis by binding to bacterial. In U.S.A, triclosan has been used for personal hygiene products such as soap and underarm deodorant since 1960s and also the first used in the clinical environment as an ingredient in hand sanitizers (Aiello A and Larson E, 2003). Hygiene and cosmetic products used in developing countries including Ethiopia also contain triclosan. Due to wide and longer duration use of triclosan containing antibacterial soap and hand sanitizers, MRSA, *E. coli* and *P. aerugninosa* were common in general community as well as Health facilities(Allison *et al*., 2005 ; Aiello *et al*.,2008). *S. aureus* (MRSA) is one of the predominant bacteria in both health facilities and community. The efficacy of current antimicrobial agents has been reduced due to the continuing emergence of antimicrobial resistant organisms. Different researches also showed that use of triclosan increase resistant bacteria in our environment. 96% of products that used for personal care and pharmaceutical activity contain triclosan which eventually disposed and drained down to our immediate environment ,water and marine ecosystem not only increase bacterial resistance but also toxic to marine ecosystem (Diego, 2005).

Alcohol based hand rubs were active against gram negative and gram positive bacteria in both in hospital and community setting. Despite some of the limitations like due to its short time residual effect, appears a quick and ready for decontaminating hands in both hospital and community settings (Guide, 2010). Also, continued use of alcohol based hand sanitizers may lead to skin irritation, cracking or flaking which expose the skin to infections (Larson *et al*., 2006). Hand hygiene is still the single most important to stop or minimize the spread of infectious disease in both health care setting and general community that can be achieved though implementation of hand hygiene (Revelas, 2012). Since 2008, inception of celebration of global hand washing day, there is mass mobilization for hand hygiene by WaSH sectors. Since the beginning of the celebration day, a lot of hand washing products was distributed without knowing their antibacterial efficacy and traditionally available plant detergent materials were not given attention.

Therefore, the present study verifies invitro antibacterial efficacy of the extract of traditional plant detergent for their minimum inhibitory concentration and minimum bactericidal concentration. The study also identified the phytochemical characteristics of these plant extracts for their antibacterial activity.

1.3 Significance of the study

- The study could serve as one component of medicated soap ingredient in commercial soaps and detergents production.
- It may also be used as an alternative for antibacterial hand washing agent that substitute antibacterial commercial soaps and detergents in rural and urban areas.
- It has contribution for improvement of the health status of the community through combating pathogenic bacteria including the current emerging antibacterial resistant.
- It has big contribution and helps for achievement of goal of global hand washing days through providing effective hand washing products.
- Also the study findings could serve as base line information for further investigation of antibacterial activity from traditional plant detergent.

CHAPTER TWO: LITERATURE REVIEW

2.1 Traditional plants used for hygiene purposes

Plant parts including its ash were used as cleaning agents by the earliest civilizations at least 4000 years ago. Also as recently as 100 years ago, Europeans were using plant ashes to wash their clothes. Soap nuts plants are used as cleaning agents due to the presence of saponins, chemical compounds that produce a soapy lather (Spitz, 2004).

Camellia oleifera found in tropical and sub-tropical regions of Asia, especially in China contain saponins which exhibits foaming and detergent property (Yu-Fen Chen *et al*., 2010). The dried and powdered fruits of *P. dodecandra* yield a foaming detergent when mixed with water which is traditionally used in Ethiopia, Somalia and Uganda for washing clothes and body. Soap has also been made from the ashes of burnt plant of *P. dodecandra*. The unripe fruits of *P. dodecandra* contain saponins which are used to control bilharzia-transmitting Snail (Esser *et al*., 2003). In Ethiopia, Nilotica plant species used for soap making due to its antibacterial activity which grows at an altitude of 600 m.a.s.l, growing on very poor soil types in Districts of the Gambella National Regional state. The Oil/butter produced from the seeds/kernels of Nilotica plant species is for skin moisturizer and the bark extracts are taken as a drink or bath as antimicrobial to *Sarcina lutea* and *Staphyllococus aureus* (Benti, 2009).

Herbaceous plant with cleansing properties for hand hygiene are accessible and inexpensive means of preventing health care associated communicable diseases and spread of the growing antimicrobial resistance. Study done on antimicrobial activity of Ayurvedic hand Sanitizers contains, Panchavalkala, the known herbal combination showed multi dimensional antimicrobial activities (Vyas *et al*., 2011). Evaluation of aqueous extracts of peppermint against *Staphylococcus aureus revealed to have* good preventive treatment with regard to pathogen when using it as hand sanitizer on hand with higher antimicrobial activity compared to water washes (Al-Hadi, 2011).

2.2 Description of the studied plants used as detergent

2.2.1 *Phytolacca dodecandra*

Phytolacca dodecandra is a perennial climbing plant with hanging branches growing up to 10 meters. Usually, in a year twice the plant is used to provide fruits and it is found in highlands of

Ethiopia from 1600-3000 m above sea level. Parts of the *Phytolacca dodecandra* plant have been used as a detergent and traditional medicine in Ethiopia. The plant is known for centuries as traditional soap mainly in rural areas of Ethiopia. Also, in different parts of Ethiopia, there is different opinion in use of *Phytolacca dodecandra* for various purposes. For example, young leaves are cooked and eaten as a vegetable and also its leaf chopped and mixed with crop (barley) to increase alcoholic content in local areki (local barley liquor), in different parts of Ethiopia. In Shewa, Chopped endod leaves mixed with injera (teff pancake) serve as medicine for cows. Also cattle, goat and sheep use leaves of *Phytolacca dodecandra*. Berry and ground leaves of is known as a detergent in most parts of the country (Esser *et al*., 2003).

2.2.2 *Rumex nepalensis*

Rumex nepalensis is widespread throughout Africa, Mediterranean and eastern Asia. Also the plant is distributed in Eritrea, throughout Africa and in all regions of Ethiopia. The plant is a stout, grow upward up to 2 m tall, perennial herb, but usually less than 1m tall. It grows best in altitudes of ranges from 700-400 m.a.s.l and common in disturbed habitats as a weed (Bosch *et al*., 2005). Its medicinal uses are widely known in Ethiopia and the countries abroad. The injured body part is covered with an intact leaf blade. The leaves and young shoots are locally eaten as a cooked vegetable, but often only in times of scarcity and mixed with other vegetables similar to *Rumex absynnica* (Bussmann *et al*., 2011). Also, in the lowlands of Konta Special Woreda, southern nations, nationalities and peoples regional state, Ethiopia the plant used as traditional medicines (Bekalo *et al*., 2009). In Wollega, some communities use the plant leaves for washing hairs , cleaning of their hands after farming activities (Suleman and Alemu, 2012).

2.2.3 *Grewia ferruginea*

Grewia ferruginea is an indigenous plant growing in different parts of Ethiopia in reverine forest and along rivers in open. Climatic zone favorable for its growing are moist Weyna Dega, Moist and Dry Kolla. Community is benefitted from the plant through using it for edible (fruit), firewood, timber (local construction), farm tools, fodder (leaves), and rope (bark).When it grows in farm land, it considered as a weed (Azene Bekele ,2007). In rifty valley areas, *G. ferruginea* is used for drop of retained placental for cows that have faced such problems (Shenkute *et al*., 2012), leaf and bark of *Grewia ferruginea* used for worm expulsion among rural communities of

Ethiopia (Assefa *et al*., 2010), in West Wollega, its bark and leaves used as commercial shampoo and jell to wash hair (Etana, 2007).

2.3 Choice of solvent and extraction of plant parts

Extraction is separation of portions of active parts of plant tissues from the inactive or inert components by using selective solvents in standard extraction procedures. These solvents diffuse in to the powdered plant solid and solubilize compounds of similar polarity. The products so obtained from plants are relatively semisolids or powder. Extraction of biologically active compounds from plant parts depends on the type of solvent used in extraction. Solvents which have rapid absorption of extract, low toxicity and ease of evaporation at low heat and pressure are their good properties. The compounds to be extracted for antimicrobial activity largely depend on choice of solvents. Water is universal solvent and used to extract plant products which have no antimicrobial significance but the compounds extracted are less active than the extract from others organic solvents like acetone (Das *et al*., 2010; Igbinosa *et al*., 2009). Plant extract for antimicrobial activity test can be suspended in acetone solvent for determination of minimum inhibitory concentration and minimum bactericidal concentration, because acetone solvent has no activity against test microorganisms (Sharma and Kumar, 2008).

Solvent strengths are measured in terms of polarity. Polar solvents have a strong power of elution and a non-polar solvent with a lower ability to elute a component from a sorbent. Common solvents used for extraction have different polarity index. Heptane and Hexane is zero where as water has the highest polar with 9.0. Acetone is organic solvent with intermediate polarity index with 5.2 and its boiling point of is 56^{o}c which is less than boiling point of water (Cannell, 1998).

Efficacy of extracts of medicinal plants against some pathogenic bacteria also showed that acetone solvent extract has active substance for antibacterial activity (Bhattacharjee *et al*., 2013). There are different factors affecting choice of solvents such as quantity of phytochemical to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process and potential health hazard of the solvents (Tiwari *et al*., 2011).

From organic solvent used for extraction of plant parts, acetone dissolves many hydrophilic and lipophilic components from most plant parts and the extracts are easily miscible with water, are

volatile and have a low toxicity. Acetone is capable of extracting active antimicrobial compounds from plant materials (Eloff, 1999). Acetone solvent is useful for study of antimicrobial activities due to its ability to extract tannins, saponin and phenolic compounds, cost and availability. 100% acetone extracts had higher antibacterial potency compared to the lower percentage of acetone extracts and the lowest antibacterial activity was reported in the water extract (Tiwari *et al*., 2011).

Extracts of medicinean plant like *M. communis, Eucalyptus,* and *N. oleander* have antimicrobial activity showed efficient disinfection against pathogenic bacteria (Ihsan and Thuraya, 2010). Also, comparison of acetone and methanol extract of five medicinal plants for their antimicrobial activity revealed that acetone extract showed higher activity. Acetone extracts of *M.oleifera leaf* was active against *Salmonella typhi* and *Salmonella paratyphi* A when compared to methanol extracts (Bharat Gami, 2011). Dimethylsulfoxide (DMSO) is a widely used non toxic chemical solvent because of its high polarity to dissolve others solvents of plant extracts (Arivuselvan *et al*., 2011).

2.4 Phytochemical nature and antimicrobial activity of plant extracts

Plants are natural chemical factories providing different source of organic chemicals. The non nutritive biologically active chemical compounds which occur naturally in plants are called Phytochemicals. Phytochemical word came from Greek word *Phyto*–plant and chemicals. Phytochemical constituents of similar plant parts are affected by geographical location, season and time of collection and different climatic conditions of the area. Most of these phytochemical classes which have antimicrobial activity are saponin, tannin, alkaloids, steroid, flavinoids, terpenoid and carbohydrates as shown by different studies (Kamaraj *et al*., 2012). Plant compounds inhibit growth of microorganisms mainly important to resistance pathogens due to production of different compounds by different plants which make variation of their antimicrobial activity result different in their sensitivity test. Plant extract is also added to shampoos, liquid detergents, toothpastes and beverages as emulsifier and long-lasting foaming agent (Chen *et al*., 2010). Different geographic zone and varying soil minerals have effect on phythochemical parameters which greatly affect also susceptibility and inhibitory concentration of pathogenic microorganisms (Ubani *et al*., 2012).

Crude extracts of *Achillea biebersteinii Afan, Ecbolium viride* (Forssk) and *Rumex nervosus* exhibited a significant antimicrobial activity and properties that support folkloric use in the treatment of some diseases as broad-spectrum antimicrobial agents (Essam *et al.,* 2008). Antibacterial soaps and detergents kill the bacteria at a specific concentration. They also have bacteristatic activity and ability to inhibit the growth of bacteria. For example, beauty soaps contain plant extracted ingredients in their composition which can inhibit or kill the bacteria (Bhat *et al*., 2011). A natural hand sanitizer could come from extracts of *Senecio lyratipartitus*. *S. lyratipartitus* grows widely in East Africa.

Research done in Kenya showed that contamination of drinking water ,food and utensils by hands after anal abolition can be prevented by using of natural hand disinfectants with extracts of *Senecio lyratipartitus* plant which also known as *Senecio lyratus*. Organic solvent extraction of these plants showed that bacterial activity with zones of inhibition of 15mm for *E. coli*, 14mm for *Salmonella* species, and 14mm for enterobacter and 13 mm for Klebsiella. MIC test on bacterial species also showed result at concentration range between 1.95mg/ml to 62.5mg/ml (Asafu maradufu *et al.,* 2012).

Hand sanitizer prepared from known combination of herbal plants showed antibacterial activities. Culture sensitivity test using gel hand wash these plants also showed promising results (Vyas *et al*., 2011).Due to the difference of Standard criteria used among authors for in vitro evaluation of antimicrobial activity of plant extracts and environmental factors that affect constituents of phytochemical compounds that have antimicrobial activity, it is difficult to use published result for their comparison (Das *et al*.,2010).

In Africa, even though soap industry is in growing, people are still using traditional soap which is cheap and specific function (Siendou *et al*., 2012). In Ethiopia, Study conducted in Amhara National regional sate showed that nearly three-quarters of the respondents reported using different types of cleansing agents like plant leaves, shrubs, etc. other than soap for cleansing purposes (Dejene, 2008). Study done on nutrition baseline survey report for the national nutrition program of Ethiopia for prevention of diarrhea showed that more than half of the disease could be prevented by hand washing. *Phytolacca dodecandra* plant is one of the substances used for hand washing in urban and rural areas of the country (EHNRI**,** 2009).Due to the increase of

resistance bacteria to conventional antibacterial, finding natural antibacterial is the best option to control infectious diseases (Green *et al., 20*10).

2.4.1 Antimicrobial active phythochemical compounds

Antimicrobial activity plant extract is due to the presence of natural active chemical compounds in plant parts. These chermical compounds like: Saponins, tannis, alkaloids, Steroid, flavinoids, terpenoid, anthraquinine and Phlobatannins (Wani *et al*., 2013) Saponins are naturally occurring surface-active glycosides widely found in the plant kingdom and giving stable foam when dissolved in water. Also, saponins have detergent properties and exhibit anti inflammatory properties. Biological action of saponin compounds has also observed to kill protozoans and molluscs (Francis *et al*., 2002). Tannins compounds are located in the cell vacuoles of plant parts, which serve as a defensive role. Tannin is capable of tanning leather or precipitating gelatin from solution, a property known as astringency. Flavonoids contain diverse family of aromatic molecules which have roles in defence against pathogens microorganisms and pests. It is a 15 carbon (15C) which is composed of a 6C-3C-6C is the basic characteristic structural feature common to all flavonoids. Terpenoids, are naturally occurring organic chemicals which is similar to terpenes, it derived from five-carbon isoprene units assembled and arranged in thousands of ways. Terpenoids are used widely for their aromatic qualities play a role in conventional herbal medicine for their antimicrobial activities. A reducing sugar is any sugar that either has an aldehyde group or is capable of forming one in solution through isomerism (Harborne, 1998). Seed and leaf extract of azadirachta indica showed presence of reducing sugars but not bark extracts (Daniel *et al., 2012)*.

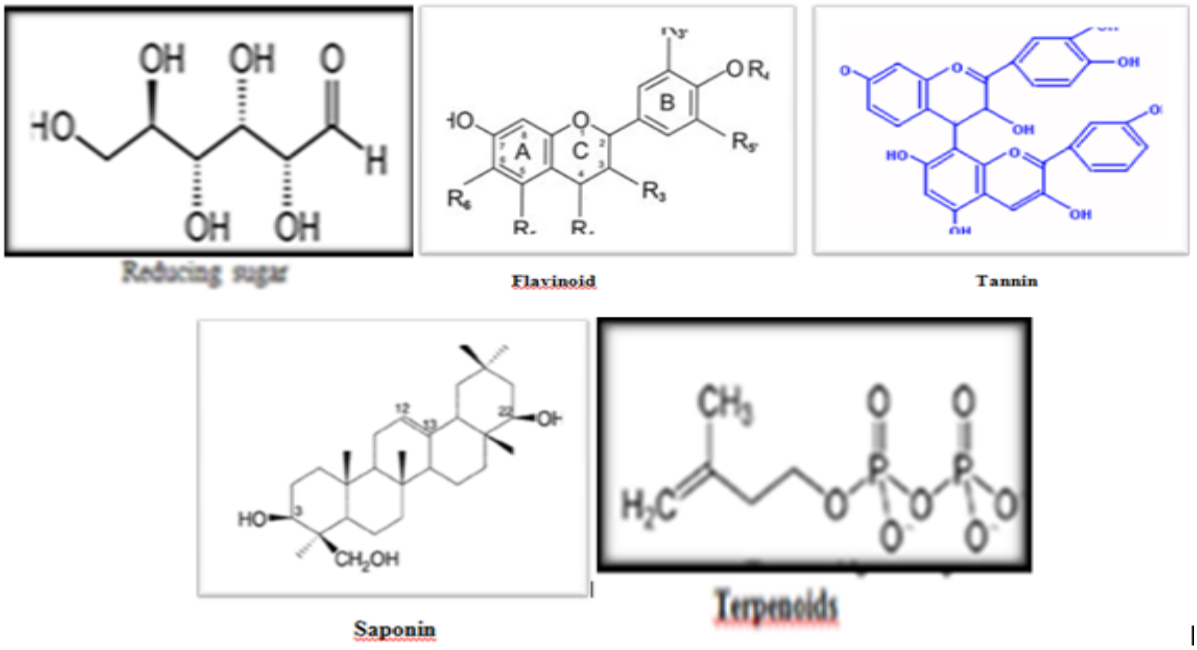

Figure 1 **Chemical structure of phytochemical compounds**

2.5 Culturing and identification of bacterial species

2.5.1 Media for growth of bacteria

Identification of bacteria can be accomplished by growing them first on the surface of solid nutrient media. Such a medium consists of a mixture of protein digests such as, peptone, tryptone, inorganic salts and can be solidified by addition of agar. For successful growing of bacteria, the medium should contain all essential nutrients, ions, and moisture, maintain the correct pH and osmotic pressure, and neutralize any toxic materials produced. Differential and selective media are used for isolation and identification of particular organisms. Differential media are media to which dyes are added to distinguish between bacteria by incorporating an indicator which changes color when acid is produced following fermentation of a specific carbohydrate .Many media used to isolate pathogens are both selective and enrichment or both selective and differential. Differential media contain compounds that allow groups of microorganisms to be distinguished visually by the appearance and color of the colony due to the constituent of dyes or substrates. Indicators which are added to the media used to identify microorganisms by color change of the media due to acid production followed by fermentation.

MacConkey agar is media used to isolate pathogenic bacteria (Cain *et al.,* 2013). Selective media contain ingredients that inhibit the growth of some organisms but allow others to grow. For example, mannitol salt agar contains a high concentration of sodium chloride that inhibits the growth of most organisms but permits pathogenic staphylococci to grow. Mannitol salt agar able to screen Staphylococcus aureus based growth characteristics (Hemraj *et al*., 2013). Enrichment media is usually a fluid selective media which contain substances that inhibit the growth of unwanted organisms, that means enrichment media are used to enrich (selectively grow) different microorganisms based on their specific characteristics (Saskatchewan, 2010).

Microbes grow on solid media as colonies. A colony is defined as a visible mass of microorganisms all originating from a single mother cell; therefore a colony constitutes a clone of bacteria all genetically alike. Different bacteria species can produce very different colonies morphology and color. Media also prepared in liquid called broth, such as nutrient broth, tryptic soy broth or glucose broth can be used in studies of growth of microorganisms when microbes are unable to grow on solid media. The only difference between broth and agar media is that broths do not contain an agar component. We use broth tubes primarily for specific assays, or (rarely) for bacteria that will not form colonies on a solid surface (Saskatchewan, 2010).

2.5.2 Gram stain reaction

An initial step in identifying a bacterial species is determining whether the cells are Gram positive or Gram-negative. The Gram stain is one of the most important and widely used tools in the identification of unknown bacteria. The Gram stain reaction is dependent on the cell wall structure of the bacteria. The cell wall of Gram-positive bacteria is composed of a thick layer of peptidoglycan that surrounds the plasma or inner membrane. In contrast, a thin layer of peptidoglycan and a second phospholipid bilayer, known as the outer membrane, surround the plasma or inner membrane of Gram-negative bacteria. These characteristically different cell wall structures permit microbiologists to classify bacteria based on the color of the stain retained by cells treated with the Gram stain. The Gram stain is a differential stain because it divides bacteria into two groups: Gram-positive and Gram-negative, where Gram-positive bacteria stain purple and Gram-negative bacteria stain pink (Christopher and Bruno, 2003; Cain *et al*., 2013).

2.5.3 Biochemical test

Bacteria species can be identified based on their different biochemical properties. Combination of different biochemical test used for identification of bacteria species. Citrate utilization test is used to identify based on the ability of bacteria to use citrate as the sole source of carbon and assist in the identification of enterobacteria. Citrate agar slant composed of bromthymol blue (pH indicator), sodium citrate, sodium and water. Urease test is used to identify bacteria based on hydrolysis of urea using the enzyme urease to ammonia, CO_2 and H_2O. The media contains urea as a substrate and phenol red as a pH indicator. Urease-positive bacteria produce ammonia which raises the pH of the media above 8.4 and the pH indicator, phenol red, turns from yellow to pink. Urease test identifies species of genus *Proteus* from other lactose non fermenting enteric microbes such as *salmonella typhi*. Motility agar is a differential medium used to differentiate an organism having flagella or form turbidity away from the stab line. Non-motile organisms do not grow out from the line of inoculation. Indole test is used to distinguish bacteria species such as *E. coli* and *E. aerogenes* in combination with other biochemical test. It screens bacteria that degrade the amino acid tryptophan and produce indole. Degradation of the amino acid tryptophan by microbial produce indole. Hydrolysis of tryptophanis produces three possible end products such as indole, pyruvic acid and ammonia. Kligler iron agar (KIA) is the medium prepared in a tube as a slant contains agar base, dextrose, lactose phenol red and has a hydrogen sulfide indicator. Uninoculated medium is red in color. They are inoculated by stabbing the butt and streaking the surface of the Slant and can be used for the reaction of typical salmonella. S. typhi produces an alkaline slant an acid butt and a small amount (weak) blackening of the agar due to production of H_2S. Triple sugar iron agar (TSIA) also similar property as KIA and the medium is prepared in tube as a slant and contains agar base, dextrose, lactose and sucrose phenol red and ferrous sulfate. Used to identify the ability of an organism to ferment glucose, lactose and sucrose, and produce hydrogen sulfide. Lysine Iron agar (LIA) is medium prepared in tube as a slant contains agar base, lysine, small amount of glucose, ferric ammonium citrate, sodium thiosulfate and bromcresol purple (pH indicator). Uninoculated Medium is purple in color. It is used to differentiate organisms that produce lysine decarboxylase (LDC) or not in lysine iron agar. Bacteria that produce LDC Cause an alkaline reaction (purple color) in the butt of the medium and also on the slant and H_2S production is indicated by a blackening of the

medium. Organisms not produce LDC typically produce an alkaline slant (purple), an acid butt (yellow), no gas, and no H_2S. (Hemraj *et al*., 2013).

Oxidase test is used to detect the presence of oxidase enzyme which assists in the identification bacteria species like Pseudomonas of which produce the enzyme cytochrome oxidase. The reagent contains phenylenediamine which react with the oxidase producing organisms to give a Blue-purple colour within 10 seconds taken as positive oxidase test. No blue-purple colour with in ten seconds taken as negative oxidase test. coagulase test is used to identify *S. aureus* which produces the enzyme coagulase. Coagulase causes plasma to clot by converting fibrinogen to fibrin. Coagulase test can be produced by free Coagulase test. Free coagulase which converts fibrinogen to fibrin by activating a coagulase reacting factor present in fresh plasma. Catalase test is used to differentiate bacteria that produce the enzyme catalase. Staphylococci aureus which is catalase positive can be differenciated from non-catalase producing bacteria such as streptococci. Breakdown of H_2O_2 to O_2+ H_2O takes place by catalase producing bacteria. Catalase producing bacteria release bubbles of oxygen (Vashist *et al*., 2013).

2.6 **Pathogenic bacteria related to Hand hygiene**

Our environment is full of microorganisms that cannot be seen by our naked eyes. Some of these can cause illness and disease and others can be essential to our environment and wellbeing. Different types of wastes generated from different activities are considered as a reservoir of pathogenic microorganisms which mainly transmitted by direct and indirect contact by our hands (Simonne, 2011).The study done on a comparison of the bacteria found on the hands of homemakers and neonatal nurses from intensive health care unit showed that existence of bacterial species include Enterococcus faecalis , Pseudomonas aeruginosa ,Enterobacter cloacae and Staphylococcus aureus (Aielloa *et al*., 2003). Human skin is home to about 10^{12} microbes and the densities and pathogenicity of the microbes vary considerably based on their residence area. Some of bacteria species isolated from skin of health personnel hands are *Staphylococcu*s species, and *Pseudomonas* sp., *Streptococci* and *Klebsiella-Enterobacter* (Snyder, 1997). Also, *Escherichia Coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Staphylococcus aureus* are some of bacterial pathogens isolated from hand swab of health setting workers and students of different categories (Tambekar *et al*., 2007).These bacteria species can be removed by hand washing for reduction of spread of diarrheal disease (Tambekar and Shirsat, 2009).

2.6.1 S. aureus

Staphylococcus aureus is a common bacterium found up to 25% on skin of healthy people and can cause illnesses. Hand is the main reservoir of *S. aureus* can be introduced into food during preparation (Kluytmans, 2010). Methicillin Resistant *Staphylococcus aureus* (MRSA) has been isolated from health care environment as well as from community. Hands of health care providers are the primary mode of transmission of MRSA and other pathogenic microorganisms to new patients as well as in community. The microorganisms are easily sheded from the patients or health workers to the immediate environment (Vineeta, 2006; Esrakocoglu *et al*., 2007). Staphylococcus aureus cause skin infections which leads to development of pneumonia in both community and health care setting. The degree of infection is greater in a person whose immunity is compromised (Bloomfield *et al*., 2007). Invitro antibacterial efficacy of antibacterial soap was evaluated that there was reduction of both reference and isolated *S.aureus* with greater reduction of reference bacteria than due to resistance development of isolated *S.aurues* (Geraldo *et al*., 2008).

2.6.2 E.Coli

E. coli are gram negative bacteria that normally live in the intestines of humans and animals. Although most strains of *E. coli* are indicator of other pathogenic microorganisms and they are harmless but several are known to produce toxins that can cause diarrheal diseases. Enteroinvasive *E. coli* (EIEC), Enterohaemorrhagic *E. coli* (EHEC) and Enteroaggregative *E. coli* (EAEC) are *E.coli* strains that cause different types of diarrheal diseases. These diarrhea diseases causing pathogens are usually transmitted through the faeco-oral route in which hands play great role. *E.coli* is lactose fermenting bacteria and produce pink to red colonies with good to excellent growth on macconkey agar. *Biochemical reaction of E.coli strains produce* Urease negative, Citrate negative, not produce H_2S, motile and produce gas during fermentation(Cheesbrough, 2006). Infectious doses of *E.coli* for gastrointestinal pathogens through Oral may be as little as 10 cells and medium dose 100 organisms (Bloomfield *et al*., 2007).

2.6.3 Salmonella species

Salmonella is a genus of rod-shaped, Gram-negative, non-spore-forming and predominantly motile enterobacteria. *Salmonella* is similar to the *Escherichia* genus and are found worldwide

in both cold- and warm-blooded animals including human being and in the environment (Ekdahl *et al*., 2005). *S. Typhi* is the etiologic agent that causes typhoid fever and it only infects human being. Globally, an estimated 16.6 million cases and 600,000 deaths reported each year. The major cause is food borne illness, typhoid fever, also comes from poor personal hygiene, particularly a lack of proper hand hygiene. Media such as XLD agar, DCA agar and DCLS agar are able to grow salmonella species. On MacConkey agar, the colonies have colorless and gram negative rod shape under microscope (Cheesbrough, 2006). Research conducted on Prevalence of Salmonella species specifically on Salmonella typhi and intestinal parasites among food handlers in Bahir Dar Town showed that 1.6 % *S. typhi* carrier rate from which majority of them inexperienced with poor personal hygienic practices (Abera *e al*., 2010).

2.6.4 P. aeruginosa

Is a Gram-negative, aerobic, rod shape, motile by single polar flagellum, non spore forming, oxidase and catalase positive belonging to the family Pseudomonadaceae. Large and rough colonial morphology formation of pigmentation, typically and odor are characteristics of *P. aeruginosa* strains on specified media and many members of the genus are pathogenic. *P. aeruginosa* is cause for opportunistic pathogenic of variety of infection. Human in contact with the agent from where the agent frequently found in moist environment such as: in health care setting and in the intestinal tract, water, soil and sewage (Cheesbrough, 2006).

2.6.5 Others bacteria species related to hand hygiene.

Others bacteria species isolated from finger nail cultures of food handlers are Coagulase negative staphylococci, Citrobacter spp., Klebsiella species Entrobacter species Dagnew *et al*., (2013), also Moulds and Yeasts isolated from hands of health workers. The study done on contamination rate of mobile phone of health worker showed that similar bacteria species were isolated from hands of heath workers from different working environment in hospital and mobile phone which is important contamination with nosocomial pathogens (Ulger *et al*., 2009). Hands are the medium most common mode of transmission of pathogens to patients at health setting and community and proper hand hygiene can prevent poor hand hygiene related infection and the spread of antimicrobial resistance (Wani *et al*., 2013).

2.7 Antimicrobial Resistance

Bacteria acquire resistance to different antibiotics when a structure is altered so as to prevent antibiotic binding activities or the cell acquires genes (plasmid) encoding enzymes that inactivate the antibiotic. Bacteria become resistant to antimicrobial agents through a number of mechanisms. The commonest way forming resistant are: enzymes are produced to inactivate or modify antibiotics, changing in the bacterial cell membrane, modification of the target so that it no longer interacts with the antimicrobial. Also, bacteria develop metabolic pathways by which it enables the site of antimicrobial action to be by passed (Vila and Pal, 2010). Some bacteria like *Pseudomonas aeruginosa* have inherent resistance to certain antibiotics, while others acquire resistance to antibiotics from new pieces of genetic information. Acquired resistance is the type of resistance that is of great health problems due to the continuous change and development of bacteria to further resistance. In recent, there are factors for the increase emergence of resistance bacteria to antibiotics like; heavy use of antibiotics both in medicine and in agriculture. As a result there is dramatic increase of antibiotic resistant bacteria in both health care setting and community (Soothill *et al*., 2013).

CHAPTER THREE: OBJECTIVES

3.1 General:

- To determine antibacterial efficacy of the extracts of plants traditionally used as detergent in rural areas.

3.2 Specific:

1. To determine the susceptibility of bacterial strains against the extracts of *Phytolacca dodecandra* fruit, *Rumex nepalensis* leaf, *Grewia ferruginea* leaf *and Grewia ferruginea* bark.
2. To determine minimum inhibitory concentration and minimum bactericidal concentration of the extracts.
3. To characterize phytochemical constituent of the plant extracts.

3.3 Hypothesis

Extract of plants traditionally used as detergents could have potential antibacterial efficacy against pathogenic bacteria species isolated from hands.

CHAPTER FOUR: MATERIALS AND METHODS

4.1 Study period and description of the plant sampling sites

The plants samples were collected from the surroundings of Jimma town, local name called Duro Awura Godana (about 5 kilometers from Jimma town), up side of Boye pond, South West Ethiopia. Geographical location of the plants were at elevation of 1749 m, 37N 0264974, UTM 0848313 and 1729m, 37N 0264920, UTM 0848259. The study was conducted from February to May 2013.

4.2 Plant sample collection

Different parts of healthy and uninfected test plants of "Andoodee" or *Phytolacca dodecandra* (Fruit), "Timijii" or *Rumex nepalensis* (leaf)," Dhoqonu" or Grewia ferruginea (leaf and bark) were collected from the sampling sites (Table 1). The selection of the herbal plants was based on ethno-botanical survey and relevant information of traditional use of the plants as detergents (Kumbi, 2010; Suleman and Alemu, 2012).

Table 1 Ethnobotanical and relevant information of the plants used in this study.

Name			Parts used	Traditional use	Picture of the plant part
Scientific	**Family**	**Local (Oromic,Amharic)**			
Phytolacca dodecandra	Phytolaccaceae	Andoodee (Indodi)	Fruit	Washing of clothes, hands and body	
Rumex nepalensis	Polygonaceae	Timijii (Tult)	Leaf	Washing of hands, hairs using freshly squeezed leaf.	
Grewia ferruginea	Tiliaceae	Dhoqonu (Lenkoata)	Leaf	The leaf is used to wash the hair thoroughly.	
Grewia ferruginea	Tiliaceae	Dhoqonu (Lenkoata)	Bark	The bark is used as soap to wash the hair thoroughly.	

4.3. Sample preparation

The collected plants were washed under running clean tap water to eliminate dust and any foreign particle. The collected plant materials were shade dried at room temperature for about seven to fourteen days without exposure to direct sunlight. The plant materials were then ground using a grinder to make it powder and sieved using 2.5mm sieve to homogenize the sample extraction. The samples were stored in airtight container at 4^{o}c in dry area until for extraction (Cannell, 1998).

4.4 Extraction

The powdered plant materials, 100g each was subjected to 700ml of acetone solvent for extraction using maceration technique for 72 hrs with constant speed (300 rpm) and continuous shaking using HY-5A Manoeuvre style vibrator shaker. Acetone solvent was selected based on its properties; like low toxicity, easily absorbed to plant material easy for extraction, ease for

evaporation and useful than other solvents especially for antimicrobial studies (Tiwari *et al*., 2011). The extracted materials were filtered using Whatmann (No.1) filter paper and the filtrates were concentrated using rotary evaporator. Finally, the extracted sticky-masses were dried in desiccators and the dried materials were stored in a refrigerator below 4^0C in a air tight materials until used for microbial assay.

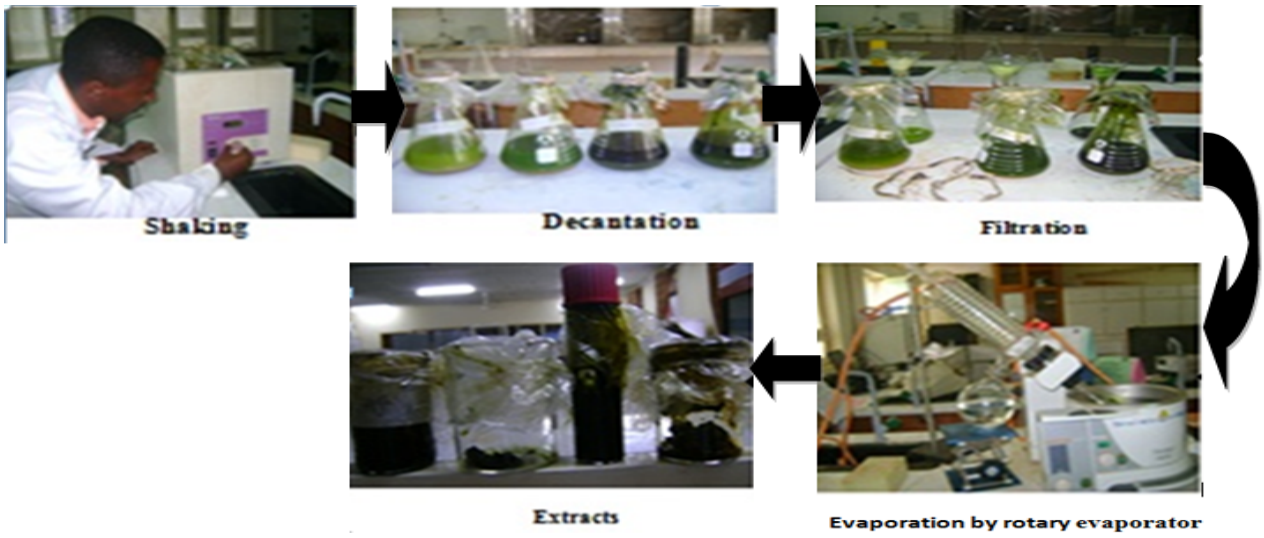

Figure 2 Extraction procedure

4.5 Qualitative phytochemical analysis

Standard qualitative methods were used for phytochemical analysis of the extracts (Evans, 2009; Chandrashekar *et al*., 2013).The extracts were tested for phytochemical constituents using the following tests and reagents.

4.5.1 Saponins (foam and emulsion test)

The foam test was performed by adding 5 ml of distilled water to 0.5 g of extract. The solution was shaken vigorously and observed for frothing. Again three drops of olive oil was added and shaken vigorously and emulsion was observed.

4.5.2 Tannins (ferric chloride test)

0.5g of the extracts were mixed with distilled water and heated on water bath until the extracts were fully dissolved. The mixtures were filtered and 0.1% ferric chloride was added to the filtrates. A dark green or blue-black colour solution indicates the presence of tannins.

4.5.3 Flavinoids

About 0.2 g of the acetone extracts were dissolved in diluted NaOH and HCl was added. A yellow solution that turns colourless, indicates the presence of flavonoids.

4.5.4 Reducing sugars

0.2g of acetone extracts were shaken with distilled water and filtered. Then the filtrate was boiled with drops of Fehling's solution A and B for minutes. Formation of red precipitation indicates the presence of reducing sugars.

4.5.5 Terpenoids (Salkowski test)

0.2 g of the extract was mixed with 2ml of chloroform ($CHCl_3$) and 3 ml of concentrated sulfuric acid (HSO_4) was carefully added to form a layer. Formation of a reddish brown color of the interface was indicated positive results for the presence of terpenoids.

4.6 Microorganisms

4.6.1 Standard strains

Reference Bacteria strains such as Escherichia coli (*E. coli*) *ATCC 25922, Pseudomonas aeruginosa (P. aeruginosa*) *DSMZ 1117, Salmonella typhimurium (S.typhimurium) ATCC 13311 and Staphylococcus aureus* (*S.aureus*) *ATCC25923* were obtained from the Department of Biology, Jimma University.

4.6.2 Isolation and identification of selected bacteria from hands

4.6.2.1 Sample collection.

Swab samples were taken using sterile cotton swabs being soaked in 0.85% sterile saline solution from palm , fingers and fingernails of left and right hands of selected 29 health workers, cleaners and food handlers in Jimma specialized Hospital. The swabs taken were added into the saline solution kept at below 4^oC and immediately transported to laboratory for analyses.

4.6.2.2 Preparation of culture media

Macconkey agar, Manitol salt agar, Xylose Lysine Desoxycholate agar (XLD agar) and selenite F broth were prepared according to the instruction of manufacturer and the operating standard procedures (ANNEX-I).

4.6.2.3 Spread plating

The swab sample soaked in saline solution was vigorously shaked and 0.2ml of the solution was cultured on the media and incubated in an inverted position at 37 ºC for 24hours. The petridishes were then observed for the formation of distinct isolated colonies for further sub culture. Primarily, the bacteria species were screened based on the colony morphology of culture positive samples. Lactose fermenting colonies (LFCs) and non lactose fermenting colonies (NLFCs) were characterized by pink color and pale color respectively on Mac Conkey agar. Small colonies surrounded by yellow zones or colonies changed color of manitol salt agar (MSA) to yellow and White creamy colonies that did not change color of MSA were also identified from Mannitol salt agar (Tambekar *et al*., 2007). Selenite broth was used as an enrichment broth for identification of Salmonella species prior to culture on XLD (Cheesbrough, 2006).

Table 2 Colony characteristics using spread plate.

Bacteria strain	**Agar media used**	**Colony morphology and characteristics**
E.coli	Macconkey agar	lactose fermenting, flat dry pink, irregular colonies
Salmonella spp.	XLD	Colonies with black center
P. aeruginosa	Nutrient agar	Large, irregular opaque colonies, with bluish green pigment
	Macconkey agar	Non lactose fermenting with colorless colonies.
S. aureus	Manitol salt agar	Yellow/golden/color

4.6.2.4 Streak Plating

Isolated LFCs and NLFs colonies were inoculated by streaking on agar plate using a sterile wire loop to get well separated surface colonies of pure culture. Then petridishes were incubated in inverted position at 37^{o}C for 24 hours.

4.6.2.5 *Preservation* of Cultures

Isolated colonies were preserved by inoculating pure culture bacteria on nutrient agar slants. Inoculated slants were incubated overnight at 37^{o}C then preserved at 4^{0}C being sealed using paraffin tape for further analysis (Kumar *et al*., 2012).

4.6.2.6 Gram staining

Gram staining method was used to classify bacteria species in to two broad categories, such as gram positive and gram negative based on the properties of cell walls. Pure culture colonies were spread on smear on a clean slide then air dried and fixed by heating over a flame. The purple dye crystal violet was used for staining. Then, the smears were treated with iodine followed by decolonization with alcohol. Bacteria that are not decolorized and decolorized (lost the crystal violet stain) are by the 95% alcohol are Gram-positive and negative respectively. Counter stain was done by flooding the slide with safranine and allowed to react for one minute then rinsed using water. Finally, the slide was dried and observed under oil immersion objective microscope (Cheesbrough, 2006).

4.6.2.7 Biochemical test

Different biochemical tests such as oxidase test, catalase test, Klinger iron agar (KIA), lysine iron agar (LIA), citrate utilization test, urease Test and Motility test were done using standard methods to identify the bacterial species (Cheesbrough, 2006).

4.7 Preparation of inoculums

Pure bacteria strains grown on nutrient agar at 37°C for 18 h were suspended in 5 ml a sterile physiological saline solution (0.85% NaCl w/v) and the suspended turbidity was adjusted to 0.5 MacFarland standards which corresponds to 1.5 x 10^8 CFU/mL.0.5 McFarland standard was prepared by mixing 0.05 mL of 1% barium chloride with 9.95 mL of 1% sulfuric acid for comparison of microbial suspension in test tubes. 1% barium chloride was prepared by dissolving 1g of barium chloride in 100ml of distilled water and 1% of sulfuric acid was prepared by mixing 1ml of concentrated sulfuric acid in 100ml of distilled water. The standard was Vigorsily vortexed prior to use and distributed in to the tubes of the same size as those of for the cultures for comparative with McFarland 0.5 standard (Kiehlbauch *et al*., 2000).

4.8 Antimicrobial activity screening by disc diffusion method

The acetone extract of the plants detergents were tested for antibacterial activities by disc diffusion method. Mueller Hinton agar (MHA) media was prepared according to its instruction for the test. The standardized suspension of bacteria strains 1.5x 10^8 CFU/ml was prepared and diffused on the agar using swab. Sterile filter paper discs of 6 mm diameter were impregnated with two different concentrations such as 200 and 100 mg of the plant extracts separately

dissolved in 1ml of DMSO being placed on swabbed agar. Then the plates were incubated at 37 ^{o} C for 24h. The diameters of the inhibition zones were measured in millimeters using ruler and the average result of the triplicate was used for analysis.1% phenol solution and DMSO were used as positive and negative control respectively (Farzana *et al*.,2011 ; Sharma and Sharma,2011).

Figure 3 Antimicrobial activity using disc diffusion method

4.9 Determination of minimum inhibitory concentration and minimum Bactericidal concentration of the plant extracts

Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC) were determined according to the National Committee for Clinical Standard. Stock solution with concentration of the plant extracts 200mg of plant extract each in 1ml of DMSO was diluted using double fold serial dilution. Therefore, test concentrations of 100mg/l, 50mg/ml, 25mg/ml,12.5mg/ml, 6.25mg/ml and 3.125mg/l were obtained (Sule and Agbabiaka, 2008). From the standardized inoculums, 0.1 ml of microbial cell was inoculated to the different concentrations then incubated at 37^{0}C for 24 hours (Jagessar *et al*., 2008; Das *et al*.,2010.).The presence of growth of the inoculums in the broth indicated by turbidity or cloudiness of the broth and the lowest concentration of the extract which inhibit the growth of the test organism was taken as the Minimum Inhibitory Concentration (MIC).1% Phenol was taken as positive control and nutrient broth with DMSO (without plant extracts) was used as negative control (Farzana *et al*.,2011).

Minimum bactericidal concentration (MBC) of the plant extracts on those selected microbes was determined by streaking using a sterile loop of inoculums from MIC tubes and the one with no visible growth on freshly prepared nutrient agar (Aliyu *et al*., 2008).

Figure 4: Minimum inhibitory concentration using macrodilition method.

4.10 Study Variables

4.10.1 Dependent

- ✓ Antibacterial efficacy of plant extract.

4.10.2 Independent

- ✓ Concentration of plant extracts
- ✓ Phytochemical constituents of the extracts
- ✓ Isolated and standard bacteria strains

4.11 Statistical analysis

The data were analyzed using Microsoft Excel 2007. Also, SPSS version 16 was used for statistical comparison of the mean ± SD value for inhibition zone obtained from the plant extracts and positive control through using analysis of variance (ANOVA) followed by post hoc Tukey test and P values < 0.05 were taken as statistically significant.

4.12 Quality control

All activities were done based on standard procedures. Shelf life and quality of all media and quality control during laboratory activities were kept. Controls were used for antibiotic agents and test bacteria. Temperature and time during sample transportation and incubation was closely monitored. Each analysis was replicated.

4.13 Ethical consideration

Before the start of data collection, ethical clearance was obtained from Ethical clearance committee of Jimma University. A formal letter was written to all concerned bodies and permission was secured at all levels. Also, informed verbal consent was obtained from each participant during data collection.

CHAPTER FIVE: RESULT

5.1 Percentage of extract Yield of the plants

Plants that are used as traditional detergents were extracted maceration method using acetone solvent. Solvents that are intermediate in polarity like acetone are selective in dissolving bioactive active chemical compounds from plant parts when compared with water which is highly polar. The extract yields from the four plant parts were varied from 6.4 - 15.47(Table.2).The Percentage yield extract value of *P. dodecandra* fruit is the highest where as *G.ferruginea* bark is smallest, 7.735% and 3.2% respectively. The percentage extractive yield was calculated as below:

Percentage of Extractive yield (w/ w) = $\frac{\text{Weight of dried extract}}{\text{Weight of dried powder}} \times 100$

Table 3 Percentage of extract Yield of the plants

S.No.	Plant parts	Weight of powder in gram	Weight of Extract in gram	Percentage (%) yield extract in gram
1	*P.dodecandra fruit*	200	15.47	7.74
2	*R.nepalensis leaf*	200	7.1	3.55
3	*G.ferruginea bark*	200	6.4	3.20
4	*G.ferruginea leaf*	200	11.14	5.57

5.2 Phytochemical screening of the selected plant extracts

All the evaluated traditional plant extracts had saponins, tannins and flavinoids in varying concentration. Terpinoids and reducing sugar were absent in *P.dodecandra fruit and R.nepalensis* leaf but present in different concentration in *G.ferruginea bark* and *G.ferruginea* leaf (Table 4) as shown in below.

Table 4 Phytochemical constituents of the plants extract.

S. No.	**Phytochemical components**	***P.dodecandra* fruit**	***R.nepalensis* leaf**	***G.ferruginea* bark**	***G.ferruginea* leaf**
1	Saponins	++	+	+++	+++
2	Tannins	++	+++	++	+
3	Flavonoid s	+++	+	+++	++
4	Terpenoids	-	-	++	+++
5	Reducing sugar	-	++	+++	+++

- = absent, + = present in less amount; ++ = Present in Moderate amount;

+++ = present in Rich amount

5.3 Biochemical and enzymatic reactions test

The bacteria species were identified /isolated by cultural characteristics, Colony morphology, gram staining, biochemical and enzymatic reaction test as shown in table 5 below:

Table 5 Biochemical and enzymatic reactions test

Gram stain		**Biochemical and enzymatic reactions test**												
+ve	**-ve**	**Citrate**	**Urease**	**KIA**	**TSI**	**LDC**	**H_2S**	**G**	**I**	**M**	**Oxidase**	**Catalase**	**Cuagulase**	**Organism**
	X	-ve	-ve	Y/Y	Y/Y	+ve	-ve	+ve	+ve	+ve	-ve	+ve		*E. coli*
	X	+ve	+ve	R/R	K/K		-ve	-ve		+ve	+ve	+ve		*P. aeruginosa*
	X	V	-ve	R/Y (+,-)	R/Y (+,-)	R/R (+)	W /S	-ve/+ve	-ve	+ve				*Salmonella Spp.*
X		+ve	+ve					-ve	+ve	- ve	-ve	+ve	+ve	*S. aureus*

Y = Yellow (Acid), R = Red-pink (Alkaline), KIA= Kligler iron agar, G = gas, I = Indole, M = Motility, -Ve =Negative, +Ve = Positive, V= Variable in strains, LDC= Lysine decarboxylase, TSIA= Triple sugar iron Agar, (+) = weak reaction W = Weak H_2S reaction, S = strong H_2S reaction.

5.4 Antimicrobial activity of the plants extract

Antimicrobial activity of all the plant extract was conducted in replicates and the mean value of the result was considered for analyses. The extract of the four plant parts: *P. dodecandra fruit, G. ferruginea leaf, G.ferruginea bark* and *R. nepalensis* showed varying degree of antibacterial activity against human pathogenic bacteria (isolated and reference) such as *E.coli, S. aureus, P. aeroginosa and Salmonella* species using agar disc diffusion method as shown below(Table 5). All plant extracts showed greater activity than 1% phenol solution. The fruit extract of *P. dodecandra* inhibited the growth of all the tested pathogens with highest and lowest activity or zone of inhibition against *S. aureus* and *E.coli* respectively. The inhibition zone for salmonella spp. was higher when compared to other bacteria species to the extracts of *R. nepalensis* leaf, *G. ferruginea leaf* and *P.dodecandra fruit*. Leaf extract of *G. ferruginea* showed higher activity against all the tested pathogens than bark extract of *G. ferruginea.*

Table 6 Growth inhibition zones in mm (means ± SD, n = 3) of various plant extracts and control using disc diffusion method

Extracts (antibacteri)	**Bacteria strains**							
	E.coli		*S. aureus*		*P. aeroginosa*		*Salmonella spp.*	
	Isolate	Ref.	Isolate	Ref.	Isolate	Ref.	Isolate	Ref.
P.dodecandra fruit	9.7±0.6	11.3±1.5	10.3±2.1	12.3±2.3	9.3±2.5	12.0±1.0	11.0±1.0	16.3±0.6
G. ferruginea leaf	10.0±1.0	12.0±0.0	9.7±0.6	11.7±0.6	9.0±1.0	10.7±1.5	11.0±1.0	14.3±0.6
G. ferruginea leaf bark	8.0±1.0	12.0±2.6	9.0±1.0	11.3±2.9	9.7±2.5	10.3±1.2	9.3±0.6	16.7±1.2
R.nepalensis leaf	9.0±1.0	10.3±0.6	9.0±1.0	12.0±3.5	NA	NA	10.0±1.0	20.7±5.5
Phenol	7.3±0.6	8.7±1.5	8.0±1.0	8.7±1.2	7.7 ±0.6	8.0±1.7	5.0±4.4	5.0± 4.4
DMSO	0	0	0	0	0	0	0	0

Key: ± SD = Standard Deviation, Ref. = Reference bacteria strain,

NA = No Zone of inhibition, n = number of replicates

5.5. MIC and MBC values for plant extracts against the test organisms using macro dilution method.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extracts are shown below (Table 6).The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts for the tested bacteria ranged between 3.125, 6.25mg/l and 50, 100mg/l respectively. The minimum MIC and MBC was achieved by the extracts of *G.ferruginea* leaf and *G.ferruginea* bark against salmonella salmonella species. *R. nepalensis* leaf extract also showed minimum MIC and MBC against *E.coli*. The maximum value was obtained by the extract of *G.ferruginea* and *R. nepalensis* leaf againt *S.aurues* and *P. aurogenosa.*

Table 7 MIC and MBC mean values of selected plant extracts against the test organisms using micro dilution method.

Plant species	MIC and MBC of the plant extracts															
	E.coli				*S. aureus*				*P. aeroginosa*				*Salmonella spp.*			
	Isolate		Standard		Isolate		Standard		Isolate		Standard		Isolate		Standard	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
P.dodecandra fruit	25	50	12.5	25	12.5	25	25	50	25	50	25	50	25	50	12.5	25
G.ferruginea leaf	25	50	12.5	25	50	100	25	50	25	50	25	50	6.25	12.5	12.5	25
G.ferruginea bark	25	50	12.5	25	50	100	12.5	25	50	100	25	50	6.25	12.5	6.25	12.5
R.nepalensis leaf	6.25	12.5	3.125	6.25	12.5	25	6.25	12.5	-	-	-	-	12.5	25	3.125	6.25
Positive control	12.5	50	25	50	25	50	12.5	25	25	50	12.5	25	6.25	12.5	25	50

CHAPTER SIX: DISCUSSION

6.1 Extraction and phytochemical constituents of the extracts

Ethnobotanical investigations have been found to provide relevant and important information in identification and development of plant traditionally used as detergent into active antibacterial hand hygiene products .In ethnobotanical literatures, the plants were used as detergents for personal hygiene (Kumbi, 2010; Suleman and Alemu, 2012). Evaluation of antibacterial activity of selected medicinal plants traditionally used in Ethiopian against wound infecting bacteria was based on information gained from ethno botanical investigation (Taye *et al*., 2011).

The extract using maceration method of the four plant parts namely: *P.dodecandra fruit, R.nepalensis leaf ,G.ferruginea bark and G.ferruginea* leaf were evaluated for antibacterial activity against human pathogenic bacteria isolated from hands (palm, finger and fingernails) such as; *E.coli , S. aureus, P. aeroginosa* and *Salmonella* species. The solvent extractive yield of the four plant parts in this study was varied in concentration. The percentage extractive yield ranged (3.2% -7.74%) which is similar with the value of the study finding obtained by Bharat Gami, (2011), with highest yield of extracts was 7.11 % using acetone as extractive solvent. From modern methods of extraction, maceration is effective in extracting bioactive compounds at room temperature and development of traditional herbal remedies for herbal formulation (Gupta *et al*., 2012).

Phytochemical constituent that found in all extracts were; saponin, tannins and flavanoids but terpenoids were obtained from *G.ferruginea bark* and leaf only and reducing sugar and was found in all extracts except *P.dodecandra fruit* extact. These analyzed phytochemical compounds in the extracts are bioactive broad spectrum responsible for antibacterial activity against both isolated and reference bacteria strains. Several study findings in agreement with this study in identifying similar phytochemical compounds using the same solvent for extraction. Doughari *et al*., (2008) have identified saponin, tannin, alkaloids and flavinoids from leafs of *Senna obtusifolia (*Zimudzi et al., 2013), have identified and reported the phytochemical constituents of acetone extract of *E. memoralis* stems to be terpenoids, flavinoids and tannin (in moderate amount) and absence of saponin and reducing sugar in the same plant. *Terminalia catappa* Linn. (Indian Almond Tree) leaves which is locally used as herbal hand wash contains

antibacterial active compounds such as flavonoids, polyphenols, alkaloids and tannin (Joshi et al., 2008). From the study findings there was variation in the types of phytochemical compounds and concentration in the extrats which may be due to different plant species and different plant part from the same species like that of *G.ferruginea* bark and leaf.

6.2 Isolation of bacteria species.

Bacteria that are pathogenic to human being such as *E.coli, S. aureus , P. aeroginosa and Salmonella species* were isolated from the hands (palm, finger and fingernails) of health workers, cleaners from different health care units and food handlers from Jimma University specialized hospital. During the study time, Salmonella species were not isolated from hands at health care units but few salmonella species were isolated from the samples taken from food handlers. Several study findings revealed similar result like that of (Tambekar and Shirsat, 2009), reported 13(2%) salmonella species were isolated from hands using similar method. Similarly, among 35 hotel workers 23 of them (78.9%) showed positive to salmonella species from the sample obtained from finger swabs (Francis et al., 2012). Absence of salmonella species from hands health workers may due to frequent hand hygiene practiced by health workers and cleaners at health care units which is in agreement with the study done by Tambekar and Shirsat, (2009) showed that 100% removal of salmonella species from hands by simple hand washing with water and soap but other bacteria do not removed completely. The study done on bacteria isolation among food Handlers at Gondar University Cafeteria, North West Ethiopia showed that *S.aurues* 16%, *E.coli* 2.67% and *P.aurogenosa* 0.67% were isolated from fingernail swab culture.

Hands of health workers were contaminated by different bacteria strains during health care activities at different health care units. *S.aurues* is the predominat bacteria accounts 59.1% at entry and 90.9% at exit isolated from hands of doctors. Also, gram negative bacteria like *Escherichia coli* (4.5%) and *Pseudomonas* (4.5%) isolated from doctors' hands swab culture methods (Paul *et al*., 2011). Similarly, this study revealed that high load of *S.aureues* followed by *E.coli* and *P. aurogenosa* were isolated from hands of health workers including cleaners. Also, there are studies that have shown that majority of healthcare workers are colonized by MRSA. Similarly, MRSA strains reported 40.1% of total bacterial isolates from the study done in Nepal (Tiwari *et al*., 2011).

6.3 Evaluation of antibacterial activity of the plant extract

The four plants (*P.dodecandra* fruit, *G. ferruginea* leaf, *G.ferruginea* bark and *R. nepalensis* leaf) extracts using acetone were screened for antibacterial activities against some selected human pathogenic bacteria strains namely; *E.coli*, *S. aureus*, *P. aeroginosa* and *Salmonella species*. The result showed that the extracts have shown antibacterial activities in varying degree against both gram negative and gram positive bacteria. The antibacterial activity observed may be due to the presence of phytochemical constituents in the acetone extracts. Similarly; antibacterial activity of acetone leaf extracts of *Senna obtusifolia (L)* against clinical and laboratory isolates of bacteria using similar method (disc diffusion) showed the highest activity than other solvents used for extraction (Doughari *et al*., 2008). Also, antibacterial activity done using soap containing active ingredient of plant extracts (*Azadirachta indica* and *Sida cordifolia*) had susceptible to both gram positive and negative bacteria (Joy *et al*., *2012*).

All the extracts had shown the highest zone of inhibition against salmonella species except for *G.ferruginea* bark (isolate and reference). Among the extracts, *R. nepalensis* leaf had the highest antibacterial activity with zone of inhibition 20.7±5.5 mm (mean±SD) diameter against reference Salmonella strain (*S. typhimerium*). Also, *P.dodecandra* fruit and *G. ferruginea* leaf extracts had showed the best antibacterial activity against the isolates of salmonella spp. Except the isolates and references of *P. aeroginosa,* the extract of *R. nepalensis* had showed anti bacterial activity ranged between 9.0±0 - 12.0 ±3.5mm *(*mean±SD).This relatively best activities obtained may be due to the presence of tannins in rich amount in extract of *R.nepalensis* and presence saponins, terpinoids and reducing sugar in rich amount in *G. ferruginea* leaf extract than other extracts. Also, presence of flavinoids, saponin and reducing sugar present in moderate and less concentration in the extract may have contribution for the activities. Similarly, the study findings by Natarajan *et al*.,(2012), showed that acetone extract of *Barleria montana* nees had better antibacterial activity against clinically isolated gram negative bacteria such as *E.coli*, *Salmonella spp*. and *P.aurogenosa* than gram positive bacteria like *S. aurues* and DMSO used as negative control had showed no activity against the test organisms which is in agreement with this study finding. Maatalah *et al*., (2012) reported that antibacterial activity of the saponin rich extract of *Anabasis articulata* using similar method against *S.aurues* showed less zone of inhibition when compared with other gram negative bacteria like *E.coli* and *P.aurogenosa.*

Generally, the extracts had shown antibacterial activity against the isolated bacteria strains from hands. Most of bacteria isolated from Hospital workers were resistant to antibiotic agents (Aiello *et al*., 2003) . Pure isolated *S.aureus* and *E.coli* strains from palm washes health care givers were found to be resistant to antimicrobial agents. Surprisingly, 81% of all *S.aureus* strains detected were as resistant to multi antimicrobial agents (Chauhan, 2006). Therefore, the extracts used in this study may have antibacterial activity against resistant bacteria . Study done by Ahmad and Beg, (2001), showed that different species of plant extracts having phytochemical constitunts like tannins,flavinoids and saponins had antibacterial activity against resiatant bacteria.

Mechanism of action of tannins on bacteria cell is also through formation of Complex with cell wall, bind to proteins, Membrane disruption and Enzyme inhibition. Flavinoids act on bacteria through formation complex with the bacteria cell wall and inactivating the enzymes and disruption membrane where as reducing sugar affect the cell membrane of the bacteria through osmotic pressure. Also, antibacterial effect of saponin is through inactivating extracellular medium and membrane of the bacteria cell (Cowan, 1999).

6.4 Determination of minimum inhibitory concentration and minimum bactericidal Concentration of the plant extracts.

All plant extracts in the study had shown antibacterial activity against the isolated and reference bacteria strains. The plant extracts had broad spectrum against both gram positive and gram negative bacteria with varying degree of activities. The study findings of broad spectrum antibacterial activity of the plant extracts agree with the study done by Basri and Fan, (2005). Most of the plant extracts with high zone of inhibition showed low MIC and MBC values due to the sensitivity of the macro dilution of the extracts. The extract of *R.nepalensis* had the lowest MIC and MBC value of 3.125 mg/ml and 6.25mg/ml respectively against *Salmonella* species. The extract of *G. furredinea* leaf and bark showed highest MIC and MBC against *S.aurues* with 50mg/ml and 100mg/ml respectively.

Except *P.aurogenosa*, all isolated bacteria and reference bacteria had showed antibacterial activity with higher zone of inhibition was obtained in most reference bacteria than isolated bacteria. Similarly, MIC and MBC values of the most extracts against isolated bacteria were higher than the value obtained by reference bacteria. The greater MIC and MBC value obtained

by isolated bacteria than reference bacteria may be due to development of resistant of isolated bacteria to antibacterial agents.

Presence of flavinoids and saponins in all extracts, reducing sugars in all extracts except *P.dodecandra* and terpinoids in *G.ferruginea* bark and *G. ferruginea* leaf in varying concentration had showed antibacterial activity with minimum inhibitory effect at different concentration against those bacteria strains. The extract of *G. ferruginea* leaf and bark showed highest MIC and MBC against *S.aurues* at 50mg/ml and 100mg/ml respectively. On the other hand, *G.furreginea* bark showed minimum MIC and MBC with equal value against isolated salmonella species and reference bacteria at 6.25mg/l and 12.5mg/l respectively. MBC values were found higher than the MIC values of the plant extracts against the test bacteria indicated that the bactericidal and bacteriostatic effect of the extracts. According to Njume *et al*., (2011),organic Solvent-Extracts of Selected South African Medicinal Plants Possess antimicrobial activity with high susceptibility patterns and Low MIC and MBC values could also an indication of high efficacy of the extract.

CHAPTER SEVEN: CONCLUSION AND RECOMMENDATIONS

7.1 CONCLUSION

Plants traditionally used as detergent in the community have significant contribution to achieve optimum sanitation since they have antibacterial activities. As shown in the results, all the studied plant extracts have antibacterial activities with slightly varying inhibitory and bactericidal concentration. The variation in anibacterial activity could be attributed to the plants phytochemical constituents like saponins, tannins, flavinoids, terpinoids and reducing sugar. Among the plant extracts, *Rumex nepalensis leaf has* shown highest antibacterial activity with minimum inhibitory concentration against *E.coli* and salmonella species. *P.dodecandra fruit and G. ferruginea leaf* also the next antibacterial activity against the tested bacteria species. This finding suggests the use of the plants extract for formulation of hand sanitizers and as active ingredients for production of antibacterial soaps which is environmentally friendly to improve public health problems.

7.2 RECOMMENDATIONS

- The plant extract can be used for formulation of hand sanitizers and as active ingredient for antibacterial soap production.
- Further studies should be needed to isolate, identify and characterize the structure of the bioactive pure chemical compounds from the crude extract. In this study, only crude extract of antibacterial efficacy is covered, which require further in vivo antibacterial efficacy from potential health risk environments (materials).

REFRENCES

Abera, B., Biadegelgen, F. & Bezabih, B.(2010). Prevalence of Salmonella typhi and intestinal parasites among food handlers in Bahir Dar Town, Northwest Ethiopia. *Ethiop J Health Dev,* 24,46-50.

Adam, E. (2012). The Efficacy of cleansing agents in hand washing. Kwame Nkrumah University of Science and Technology, Ashanti Region,Ashanti.

Adegoke,A.,Iberi, P., Akinpelu, D., Aiyegoro, O. & Mboto, C.(2010). Studies on phytochemical screening and antimicrobial potentials of Phyllanthus amarus against multiple antibiotic resistant bacteria. *International journal of applied research in natural products,* 3**,** 6-12.

Ahmad, I. & Beg, A. Z. (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *Journal of ethnopharmacology,* 74**,** 113-123.

Aielloa, A. E., Cimiottib, J., Della-Lattac, P. & Larsonb, E. L. (2003). A comparison of the bacteria found on the hands of 'homemakers' and neonatal intensive care unit nurses *J Hosp Infect.,* 54**,** 310-315.

Aiello, A. E., CimiottI, J., Della-Latta, P. & Larson, E. (2003). A comparison of the bacteria found on the hands of homemakers and neonatal intensive care unit nurses. *Journal of Hospital Infection,* 54**,** 310-315.

Al-Hadi, L. M. (2011). The antibacterial activity of aqueous extract of peppermint and Bay leaf against *Staphylococcus aureus*. *J Bagh College Dentistry* 23.

Aliyu, A., Musa, A., Abdullahi, M., Oyewale, A. & Gwarzo, U. (2008). Activity of plant extracts used in northern Nigerian traditional medicine against methicillin-resistant Staphylococcus aureus (MRSA). *Nig. Journ. Pharm. Sci.,* 7(1)**,** 1-8.

Arivuselvan, N., Silambarasan, D., Govindan, T. & Kathiresan, K. (2011). Antibacterial Activity of Mangrove Leaf and Bark Extracts Against Human Pathogens. *Advan. Biol. Res.,* 5 (5): 251-254.

Asafu maradufu, J. K. o., Betsy c. Sang, and John e. Khang'ati (2012).Using *senecio lyratipartitus* as a hand disinfectant after anal ablution University of Eastern Africa, Baraton, Eldoret, Kenya.

Assefa, B., Glatzel, G. & Buchmann, C. (2010). Ethnomedicinal uses of Hagenia abyssinica (Bruce) J.F. Gmel. among rural communities of Ethiopia. *Journal of Ethnobiology and Ethnomedicine* , 6**,** 1-6.

BASRI, D. F. & FAN, S. H. (2005) The potential of aqueous and acetone extracts of galls of Quer Quercus cus infectoria as antibacterial agents *Indian J Pharmacol* 37**,** 26-29.

Benti, D. G. (2009). Ecology, composition and population structure of *vitellaria paradoxa* subspecies nilotica (kotschy), a.n. Henry et.al.), in Ethiopia: implication for sustainability of production. World agroforestry center.

Bharat Gami, F. P. (2011). Screening of methanol & acetone extract for antimicrobial activity of some medicinal plants species of Indian folklore. *Int. J. Res. Pharm. Sci.,* 2, 69-75.

Bhat, R., Prajna, P. S., Menezez, V. P. & Shetty, P. (2011). Antimicrobial Activities of Soap and Detergents. *Adv. Biores*., 2, 52-62.

Bhattacharjee, I., Chatterjee, S. K., Mukherjee, S. & Chandra, G. (2013). Efficacy of extracts of six medicinal plants of india against some pathogenic bacteria. *Intern. J. Appl. Bio. and Pharm Techn.,* 4(1).

Bloomfield, S. F., Aiello, A. E., Cookson, B., O'boyle, C. & Larson, E. L. (2007).The effectiveness of hand hygiene procedures in reducing the risks of infections in home and

community settings including handwashing and alcohol-based hand sanitizers. *American journal of infection control,* 35**,** S27-S64.
Bojar, R. & Holland, K. (2002) Review: the human cutaneous microflora and factors controlling colonisation. *World Journal of Microbiology and Biotechnology,* 18**,** 889-903.
Bosch, C., Borus, D. & Siemonsma, J. (2005). Vegetables Tropical Africa: Conclusions and Recommendations Based on PROTA 2:'Vegetables', PROTA Foundation.
Bussmann, R. W., Swartzinsky, P., Worede, A. & Evangelista, P. (2011). Plant use in Odo-Bulu and Demaro, Bale region, Ethiopia. *Journal of Ethnobiology and Ethnomedicine,* 7**,** 1-21.
Cain, D., Hanks, H., Weis, M., Bottoms,C. & Lawson, J. M. (2013). Microbiology laboratory manual cambridge university press.
Cannell, R. J. (1998). Natural products isolation, Springer.
Chauhan, V. (2006). *In vitro assessment of indigenous herbal and commercial antiseptic soaps for their antimicrobial activity.* M. Sc. Dissertation in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering & Technology, Deemed University, Patiala, India.
Cheesbrough, M. (2006). District laboratory practice in tropical countries, Cambridge university press.
Chen, Y.-F., Yang, C.-H., Chang, M.-S., Ciou, Y.-P. & Huang, Y.-C. (2010).Foam Properties and Detergent Abilities of the Saponins from *Camellia oleifera. Int. J. Mol. Sci., 11,* 4417-4425.
Christopher, K. & Bruno, E. (2003). Identification of Bacterial Species. 24, 103-130.
Cowan, M. M. (1999). Plant Products as Antimicrobial Agents. *CLIN. MICROBIOL. REV.,* 12 564–582
CSA, A. A., Ethiopia ICF International Calverton, Maryland, USA(2012).Ethiopia Demographic and Health Survey.
Dagnew, M., Tiruneh, M., Moges, F. & Gizachew, M.(2013). Bacterial Profile and Antimicrobial Susceptibility Pattern among Food Handlers at Gondar University Cafeteria, Northwest Ethiopia. *J Infect Dis Ther,* 1(105), 2.
Daniel, N.Ohalete, U. & C., C. N. N. M. (2012).Phytochemical screening for active compounds in leaves, bark and seed extracts of *azadirachta indica* in owerri, imo state. South-Eastern nigeria.W*orld Journa of Pharmacy and Pharamceutical Sciences,* 1(4), 1181-1188.
Das, K., Tiwari, R. & Shrivastava, D.(2010).Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of medicinal plants research,* 4**,** 104-111.
DAS, K., TIWARI, R. & SHRIVASTAVA, D. (2010) Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of medicinal plants research,* 4**,** 104-111.
Das, K., Tiwari, R. K. S., & Shrivastava, D. K.(2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of medicinal plants research, 4,* 104-111.
Dejene, M. (2008). Baseline Household Survey ,Amhara National Regional State Health Bureau
Diego, S. (2005). Soap unnecessary and harmful.
Doughari, J. H., El-Mahmood, A. M. & Tyoyina,I. (2008).Antimicrobial activity of leaf extracts of *senna obtusifolia* (l). *Afri journal of pharmacy and pharmacology,* 2**,** 007-013.

Ekdahl, K., Jong, B.D. & Andersson, Y.(2005).Salmonella infections: An update on Epidemiology, management, and prevention. *Travel medicine and infectious disease,* 9**,** 263-277.

Eloff, J. (1999) It is possible to use herbarium specimens to screen for antibacterial components in some plants. *Journal of ethnopharmacology,* 67**,** 355-360.

Esser, K. B., Semagn, K. & Wolde-Yohannes, L. (2003). Medicinal use and social status of the soap berry, "Endod;,(*Phytolacca dodecandra*) in Ethiopia. *Journal of ethnopharmacology,* 85**,** 269-277.

Evans, W. C. (2009). Trease and Evans' pharmacognosy, Elsevier Health Sciences.

Farzana, K., Batool, S., Ismail, T., Asad, M., Rasool, F., Khiljee, S. & Murtaza, G.(2011). Comparative bactericidal activity of various soaps against gram-positive and gram-negative bacteria. *Scientific Research and Essays,* 6**,** 3514-3518.

Fewtrell, L., Kaufmann, R. B., Kay,D., Enanoria, W., Haller, L. & Colford JR, J. M. (2005). Water, sanitation, and hygiene interventions to reduce diarrhoea in less developed countries: a systematic review and meta-analysis. *The Lancet infectious diseases,* 5**,** 42-52.

Francis, G., Kerem, Z., Makkar, H. P. S. & Becker, K. (2002). The biological action of saponins in animal systems: a review.*British Journal of Nutrition,*88**,** 587-605.

Francis,S. P., Nagarajan, P. & Upgade, A. (2012).Prevalence of salmonella in finger swabs and nail cuts of hotel workers. *j microbiol infect dis., www.jmidonline.org* 2.

Geraldo, I. M., Gilman, A., Shintre, M. S. & Modak, S. M. (2008). Rapid antibacterial activity of 2 novel hand soaps: evaluation of the risk of development of bacterial resistance to the antibacterial agents. *Infection Control and Hospital Epidemiology,* 29**,** 736-741.

Green, E., Samie, A., Obi, C. L., Bbessong, P. O. & Ndip, R. N. (2010). Inhibitory properties of selected Mycobacterium tuberculosis. *Journal of Ethnopharmacology,* 130 ,151-157.

Guide, A. A. (2010). Guide to the Elimination of Infections in Hemodialysis.

Gupta, a., naraniwal, m. & kothari, v. (2012) modern extraction methods for preparation of bioactive plant extracts. *International journal of applied and natural sciences (ijans)*.1**,** 8-26.

Handali, S., Hosseini, H., Ameri, A. & Moghimipour, E. (2012). Formulation and evaluation of an antibacterial cream from Oxalis corniculata aqueous extract. *Jundishapur Journal of Microbiology,* 4**,** 0-0.

Harborne, J. B. (1998). Phytochemical methods A Guide to modern techniques of plant analysis, springer.

Hemraj, V., Diksha, S. & Gupta (2013). A review on commonly used biochemical test for bacteria. *Innovare Journal of Life Science, 1, 1-7.*

Igbinosa, O., Igbinosa, E. & Aiyegoro, O. (2009). Antimicrobial activity and phytochemical screening of stem bark extracts from Jatropha curcas (Linn). *African Journal of Pharmacy and Pharmacology,* 3**,** 058-062.

Jagessar, R. C., A.Mars & Gomes, G. (2008).Selective antimicrobial properties of phyllanthus acidus leaf extract against candida albicans, escherichia coli and staphylococcus aureus using stokes disc diffusion, well diffusion, streak plate and a dilution method. *Nature and Science,* 6**,** 24-38.

Joshi, M. G., Kamat,D. & Kamat, S. (2008). Evaluation of herbal handwash formulation.Natural product radiance. 7, 413-415.

Joy, J. M., Kumar, A. P., Mohanalakshmi, S. & Prathyusha, S. (2012).Formulation and evaluation of poly herbal hand wash.*International journal of pharmacy* 2, *39-43*.

Kamaraj, C., Rahuman, A. A., Siva, C., Iyappan, M. & Kirthi, A. V. (2012). Evaluation of antibacterial activity of selected medicinal plant extracts from south India against human pathogens. *Asian Paicfic Journal of Tropical Disease***,** S296-S301.

Kamaraj, C., Rahuman, A. A.,Siva, C., Iyappan, M., & Kirthi, A. V. (2012). Evaluation of antibacterial activity of selected medicinal plant extracts from south India against human pathogens. *Asian Pacific Journal of Tropical Disease, 2*, S296-S301.

Kiehlbauch, J. A., Hannett,G. E., Salfinger, M., Archinal, W., Monserrat, C. & Carlyn, C. (2000). Use of the National Committee for Clinical Laboratory Standards guidelines for disk diffusion susceptibility testing in New York state laboratories. *Journal of clinical microbiology*, 38**,** 3341-3348.

Kluytmans, J. Methicillin resistant Staphylococcus aureus in food products: cause for concern or case for complacency? *Clinical Microbiology and Infection*, 16**,** 11-15.

Kumar, K.,Kumar, A., Thakur, P., Patil, S., Payal,C., Kumar, A. & Sharma, P.(2012). Antibacterial activity of green tea (Camellia sinensis) extracts against various bacteria isolated from environmental sources. *Recent Research in Science and Technology*, 4,19-23.

Kumbi, E. T. (2007). Use and conservation of traditional medicinal plants by indigenous people in gimbi Woreda, Western Wellega, Ethiopia

Larson, E., Girard,R., Pessoa-Silva, C. L., Boyce, J., Donaldson, L. & Pittet, D. (2006). Skin reactions related to hand hygiene and selection of hand hygiene products. *American journal of infection control*, 34**,** 627-635.

Maatalah, M. B., Bouzidi, N. K., Bellahouel, S., Merah, B., Fortas, Z., R.Soulimani, Saidi, S. & Derdour, A. (2012). Antimicrobial activity of the alkaloids and saponin extracts of *Anabasis articulata*. *J. Biotechnol. Pharm. Res.* , 3**,** 54-57.

Mak-Mensah, E. & Firempong (2011). C. Chemical Characteristics of toilet soap prepared from neem (Azadirachta indica A. Juss) seed oil. *Asian J Plant Sci Res*, 1**,** 1-7.

Marieke Gerards (2011).International policy overview: Antibiotic resistance.

Morris, A. & Masterton, R. (2002). Antibiotic resistance surveillance: action for international studies. *Journal of Antimicrobial Chemotherapy*, 49**,** 7-10.

Natarajan, D., Gomathi, M. & Yuvarajan, R. (2012). Phytochemical and antibacterial evaluation of *barleria montana nees.* (mountain barleria).*Asian J Pharm Clin Res,Vol 5,Suppl* 3,2012,44-46.

Njume, C., Jide, A. A. & Ndip, R. N. (2011).Aqueous and organic solvent-extracts of selected south african medicinal plants possess antimicrobial activity against drug-resistant strains of helicobacter pylori: inhibitory and bactericidal potential. I*nt. J. Mol. Sci.* , 12, 5652-5665.

Paul, R., Das, N. K., Dutta, R., Bandyopadhyay, R. & Banerjee, A. K. (2011). Bacterial contamination of the hands of doctors: a study in the medicine and dermatology wards. 77, 307-313.

Chandrashekar, R., Kumar, A. K., Reddy, Y. R., Chaitanya, P. J., Bhavani, N. L. & Pochampalli,J. (2013). Journal of Pharmacognosy and Phytochemistry Isolation of Gossypol and Analysis of Phytochemicals in Seed Extract of Bt and Non-Bt Varieties of Cotton. *Journal of Pharmacognosy and Phytochemistry*, 2**,** 180-186.

Revelas, A. (2012). Acute gastroenteritis among children in the developing world:review. *South Afr J Epidemiol Infect* 27(4),156-162.

Sahu, M. C. & Padhy, R. N. (2013).In vitro antibacterial potency of butea monosperma lam. Against 12 clinically isolated multidrug resistant bacteria.*Asian pac j trop dis,* 3, 217-226.

Saskatchewan, C. O. P. A. S. O. (2010). Procedures/guidelines for the microbiology laboratory.

SDA, Soap and detergent association, (1994). Soaps and detergents association, 2nd edition.

Sharma, A. & Sharma, K. (2011). Should Solubility and Zone of Inhibition Be the Only Criteria for Selection of Solvent in Antimicrobial Assay. *Advan. Biol. Res.,* 5**,** 241-247.

Sharma, B. & Kumar, P. (2008). Extraction and pharmacological evaluation of some extracts of Tridax procumbens and Capparis decidua. *International journal of applied research in natural products,* 1**,** 5-12.

Siendou, C., Djakalia, O., Konkon N, D. G., Kagohire, K. & Hilaire, K. T. (2012). Spontaneous Plants Used in the Traditional Soap Making in CoteD'Ivoire. *International Journal of Plant & Soil Science, 1, 16-29.*

Simonne, A. (2011). Hand hygiene and hand sanitizers.

Snyder, O. P. (1997). A Safe Hands; hand wash program for retail food operations. *St. Paul, MN: Hospitality Institute of Technology and Management*.

Soothill, G., HU, Y. & Coates, A. (2013). Can We Prevent Antimicrobial Resistance by Using Antimicrobials Better? *Pathogens,* 2**,** 422-435.

Spitz, L. (2004). SODEOPEC*:* Soaps, Detergents, Oleochemicals, and Personal Care Products, AOCS Press Skokie, IL.

Sule, I. & Agbabiaka, T. (2008). Antibacterial effect of some plant extracts on selected enterobacteriaceae. *Ethnobotanical Leaflets,* 2008**,** 137.

Suleman, B. & Alemu, T. (2012).A survey on utilization of ethnomedicinal plants in Nekemte Town,East Wellega (Oromia), Ethiopia. *Journal of herbs, spices & medicinal plants,* 18 34-57.

Tambekar, D. & Shirsat, S. (2009). Hand washing: a cornerstone to prevent the transmission of diarrhoeal infection. *Asian Journal of Medical Sciences,* 1**,** 100-103.

Tambekar, D., Shirsat, S., Suradkar, S., Rajankar, P. & Banginwar, Y. (2007). Prevention of transmission of infectious disease: Studies on hand hygiene in health-care among students. *Continental Journal of Biomedical Sciences,* 1**,** 6-10.

Taye, B., Giday, M., Animut, A. & Seid, J. (2011). Antibacterial activities of selected medicinal plants in traditional treatment of human wounds in Ethiopia. *Asian Pacific journal of tropical biomedicine,* 1**,** 370-375.

Tiwari, H. K., Das, A. K., Sapkota, D., Sivrajan, K. & Pahwa, V. K. (2009). Methicillin resistant Staphylococcus aureus: prevalence and antibiogram in a tertiary care hospital in western Nepal. *The Journal of Infection in Developing Countries,* 3**,** 681-684.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G. & Kaur, H. (2011). Phytochemical screening and extraction: a review. *Internationale pharmaceutica sciencia,* 1**,** 98-106.

Ubani, C. S., Oje, O. A., Ihekogwo, F. N. P., Eze, E. A. & Okafor, C. L. (2012). Effect Of Varying Soil Minerals And Phytochemical Parameters On Antibacterial Susceptibility Of Mitracarpus Villosus Ethanol Extracts; Using Samples From South East And South-Southern Regions Of Nigeria. *Global Advanced Research Journal of Microbiology* 1,120-125.

Ulger, F., Esen, S., Dilek, A., Yanik, K., Gunaydin, M. & Leblebicioglu,H. (2009). Are we aware how contaminated our mobile phones with nosocomial pathogens? *Annals of Clinical Microbiology and Antimicrobials,* 8**,** 7.

Vashist, H., Sharma, D. & Gupta,A. (2013).A review on commonly used biochemical test for bacteria. *Innovare Journal of Life Science,* 1**, *1-7.***

Vila, J. & Pal, T.(2010). Update on antibacterial resistance in low-income countries: factors favoring the emergence of resistance. *Open Infectious Diseases Journal*, 4**,** 38-54.

Vinoth, B., Manivasagaperumal, R. & Balamurugan., S. (2012). Phytochemical analysis and antibacterial activity of *moringa oleifera* lam. *International journal of research in biological sciences* 2, 98-102.

Vyas, P., Ggalib, Patgiri, B. J. & Prajapati, P.K.(2011).Antimicrobial Activity of Ayurvedic Hand Sanitizers. *International Journal of Pharmaceutical & Biological Archives,* 2**,** 762-766.

Wallace, R. (2004). Antimicrobial properties of plant secondary metabolites. *Proc Nutr Soc***,** 621-629.

Wani, N. S., Bhalerao, A. K., Ranaware, V. P. & Zanje, R. (2013). Formulation and Evaluation Of Herbal Sanitizer.*International Journal of PharmTech Research*.5 40-43.

WHO (2012). The evolving threat of antimicrobial resistance. Options for action.Geneva: Switzerland.

Wolf, R., & Wolf, D. (2001). Soaps, shampoos, and detergents. *Clinics in dermatology*, *19*(4), 393-397.

Zimudzi, C., Msiteli, S., Jere, J., Kativu, S. & Kunonga, N. (2013). Phytochemical, antibacterial and cytotoxic evaluation of *Euphorbia memoralis* R.A Dyer stem extracts. *Topclass Journal of Herbal Medicine,* 2,36-42.

ANNEX

PROCEDURES FOR PREPARATION OF CULTURE MEDIA BIOCHEMICAL TEST.

I. CULTURE MEDIA PREPARATION

1. **Xylose lysine desoxycholate (XLD) agar.**
   - ✓ The media was measured and mixed in to distilled water according to manufacturer's instructions
   - ✓ Heated with agitation just until the medium boiled.
   - ✓ The flask was cooled to about 50^0c.
   - ✓ Poured on sterile Petridish and waited until solidified.
2. **Preparation of Mac Conkey agar (MAC)**
   - ✓ 49.53 grams of medium was suspended in 1000 ml distilled water.
   - ✓ Heated to boil to dissolve the medium completely.
   - ✓ Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
   - ✓ Finally, cooled to 45-50°C and poured to sterile Petridish and waited until solidified.
3. **Manitol salt agar**
   - ✓ 111g was suspended in 1000ml distilled water
   - ✓ heated to boil until the medium mixed completely
   - ✓ Autoclaved at 121^0C for 15 minutes
   - ✓ Cooled to around 50^0c
   - ✓ Dispensed in sterile Petridish
4. **Nutrient Agar Slants:**
   - ✓ Nutrient agar medium was Prepared and boiled with stirring until the entire agar is melted.
   - ✓ 5 ml of molten agar using a pipette was transferred to test tubes.
   - ✓ The caps were loosely placed on the tubes while the media is hot.
   - ✓ The tubes were sterilized at 121° C for 15 minutes with caps.
   - ✓ While the medium is still hot, the tubes with the media were tilted so that the medium in the tubes is slanted.
   - ✓ The caps of the cool media were tightened.
5. **Preparation of Selenite broth**
   - ✓ 23 grams the medium was mixed in 1 liter of distilled water.
   - ✓ 10 ml was distributed in sterile test tubes.

- ✓ The tubes were sterilized by boiling in a water bath.
    - ➢ 1ml of swab sample was transferred in to the 10 ml selenite broth then Incubate at 37°C for 24 hours. Finally, planting by streaking a loopful sample on SS agar and XLD was performed under aseptic condition.

II. GRAM STAINING AND BIOCHEMICAL TEST MEDIA PREPARATION

6. **Gram staining**

- ✓ Bacteria cell were spreaded on clean glass slide with a drop of distilled water then dried to form smear.
- ✓ The smear heated by passing over the flame to fix on the slide and purple **dye, crystal violet** was added.
- ✓ Then the smear was treated with iodine which a mordant solution
- ✓ The smear was decolorized using alcohol solution.
- ✓ The pink color safranin, counter stain was added to make it more visible under microscope.
    - ❖ Bacteria that are not decolorized by alcohol are gram positive bacteria where as those lost the crystal violet are gram negative bacteria.

7. **KIA (Kliger iron agar)**

- ✓ known weight of KIA was measured and added in distilled water based on the manufacture standard
- ✓ Boiled to dissolve the medium completely.
- ✓ Cooled to 50–55^{0}C, mixed well and 7ml was dispensed in test tubes.
- ✓ The test tubes the medium was sterilized by autoclaving (with caps loosened) at 121^{0}C for 15 minutes.
- ✓ The medium was allowed to solidify in a sloped position.
    - ❖ Glucose fermenters - cause the butt of the tube to become acid (yellow) and some also produce gas. Lactose-fermenting organisms produce an acid (yellow) slant and Lactose-non-fermenting organisms will have an alkaline (red) slant.

8. **Preparation of LIA**

- ✓ known weight of LIA was measured in distilled water based on the manufacture standard
- ✓ Boiled to dissolve the medium completely.
- ✓ Cooled to 50–55^{0}C, mixed well and 7ml was dispensed in test tubes.

- ✓ The test tubes the medium was sterilized by autoclaving (with caps loosened) at 121^0C for 15 minutes.
- ✓ The medium was allowed to solidify in a sloped position to get enough butt and slope.
    - ➢ Organisms that produce **lysine decarboxylase** in lysine iron agar (LIA), Cause an alkaline reaction (purple color) in the butt of the medium and also on the slant and H_2S production is indicated by a blackening of the medium.

9. **Simmons Citrate Test**
    - ✓ 24.28 grams was suspended in 1000 ml distilled water
    - ✓ Heated to boiling point to dissolve the medium completely.
    - ✓ Mixed well and distributed in tubes and Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes and tilted/slanted until it solidified.
        - ➢ Metabolism of these salts causes the medium to become alkaline, indicated by a change in colour of the pH indicator from green to blue

10. **Urease test**
    - ✓ 18.71 grams of urea agar base was suspended in 950 ml distilled water
    - ✓ Heated to dissolve the medium completely followed by Sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
    - ✓ **Cooled to 55°C then 50ml of 40% sterile Urea solution was aseptically added and mixed well.**
    - ✓ Finally, 10ml of the solution was distributed in each test tube to make slat.
        - ➢ Urea is hydrolyzed to liberate ammonia and Phenol red indicator detects the alkalinity generated by visible colour change from orange to pink.

11. **Motility media**
    - ✓ 20 grams was suspended in 1000 ml distilled water.
    - ✓ Heated to boiling to dissolve the medium completely.
    - ✓ The medium was dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
    - ✓ The tubes were allowed to cool in upright position.
        - ❖ The test organism was inoculated in tubes of motility medium by stabbing the medium to a depth of about 5cm and incubated at 37^0c for 24 hours.Motile organisms migrated through the medium changed to turbid where as non-motile organisms is confined to the stab.

12. **Indole test**

- ✓ Peptone water was prepared based on manufacturer direction.
- ✓ 3ml of peptone solution was added in test tubes then sterilized using autoclave.
- ✓ Test organisms was inoculated in sterile peptone water then incubated at 37^{o}c for 24 up to 48 hours.
- ✓ Test for indole was done by adding 0.5 ml or 3-5 drops of Kovac's Reagent and shaked gently.
- ✓ Formation a red colour in the surface layer was examoned within 10 minutes.
    - ❖ Red ring = positive Indole and Colourless ring = negative Indole

13. **Catalase activity**

- ✓ A drop of 3% hydrogen peroxide was placed on sterile/clean glass slide.
- ✓ An isolated colony was picked up using sterile loop
- ✓ Placed on the drop of hydrogen peroxide and mixed well slowly.
    - ❖ Production of Oxygen **Gas bubbles** is positive test.

14. Coagulase test

- ✓ A drop of physiological saline was placed on each end of a slide.
- ✓ Two thick suspensions were made by emulsifying a pure colony of the test organism on each of the drops.
- ✓ A drop of fresh **plasma** was added to one of the suspensions, and mixed gently.
- ✓ The suspension was clumped within **10 seconds** which is indication of *S.aurues*.
    - ❖ Colonies from a nutrient agar was used and plasma was not added to the second suspension for comparison of appearance true coagulase clumping.

15. **Oxidase test**

- ✓ One gram of oxidase powder was added to 100ml of distilled water.
- ✓ Heated in a water bath until dissolved completely.
- ✓ Cooled at room temperature and the fresh solution was immediately used for identification of bacteria.
    - ❖ A piece of filters paper was placed on a clean Petridish and 2 or 3 drops oxidase reagent was added on it. A **sterile** glass rod was used to remove a colony of the test organism and smeared on the filter paper. Development of blue-purple color within 10 seconds was taken as positive for oxidase test.

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