STUDIES ON NEONATAL SEPSIS AND EFFECT OF PHYTOCHEMICALS FROM SELECTED MEDICINAL PLANTS ON NEONATAL SEPSIS CAUSING BACTERIA

A Thesis

Submitted in partial fulfillment of the requirements for the

Award of the degree of

DOCTOR OF PHILOSOPHY
in
MICROBIOLOGY

by

BIJAY RAJ PANDIT
(41600059)

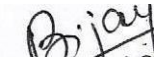
Supervised By

DR. ASHISH VYAS

LOVELY PROFESSIONAL UNIVERSITY
PUNJAB
2021

DECLARATION

I hereby declared that the work for the thesis entitled, **"Studies on Neonatal Sepsis and Effect of Phytochemicals from selected Medicinal plants on Neonatal Sepsis causing bacteria"** for the fulfilment of the requirement for the award of the degree of Doctor of Philosophy in Microbiology has been accomplished under supervision of Dr. Ashish Vyas, Professor and Head, Department of Microbiology, School of Bioengineering and Biosciences. The entirely work is my own original work and has not been submitted in part or full for any other degree/diploma at this or any other University/Institution. All the ideas and references have been duly acknowledged.



BIJAY RAJ PANDIT

Reg No.41600059

Date: 13/02/2022

CERTIFICATE

This is to certify that the thesis entitled, **"Studies on Neonatal Sepsis and Effect of Phytochemicals from selected Medicinal plants on Neonatal Sepsis causing bacteria"** submitted to School of Bioengineering and Biosciences, Lovely Professional University, Phagwara for the award of the degreeof Doctor of Philosophy in Microbiology was carried out by **Bijay Raj Pandit** under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation. No part of this thesis has ever been submitted for any other degree/diploma at this or any other University/Institution. The thesis is fit to be considered for the award of degree of Ph.D.

Dr. ASHISH VYAS

Professor and Head

(SUPERVISOR)

ABSTRACT

Neonatal sepsis is a generalized bacterial infection during the first four weeks of life associated with general systemic manifestation and positive blood culture. As per United Nation Inter-agency group for child mortality estimation report 2019, an estimated 7000 per day and 2.5 million newborns died during the first month of life during 2018. A recent study reports that about one-third of all neonatal deaths occur on the birth day and nearly three quarters succumb to death within seven days of life. It has also been estimated that if the current trends continues will lead to 52 million deaths of under-five children from 2019 to 2030 with half being will be newborns. Newborns surviving from the critical emergency condition suffer from other problems due to overdose of the antimicrobials. Some of them suffer from antimicrobial resistance in the future and also chances of re-occurrence of sepsis along with several side effects. The development of antimicrobial resistance in microorganisms is due to various reasons like the nature of the antibiotic, dose of antibiotics, host factors, and environmental cause. The manufacturing costs of synthetic antimicrobial drugs are high with severe adverse effects in comparison to plant-based drugs. The present status has compelled to look after new antimicrobial components from different plants origin which acts as novel antimicrobial chemotherapeutic agents. Focusing on these imminent issues, the present investigation was planned to formulate antibacterial syrup with the immune booster and neutraceutical properties from plant origin that collectively fight against bacterial pathogens causing neonatal sepsis.

Twelve hundred blood specimens were collected from suspected neonates. A total of eight bacterial pathogens were isolated viz. *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus* spp., *Pseudomonas aeruginosa, Escherichia coli,* Coagulase Negative *Staphylococcus, Enterobacter* spp. and *Proteus* spp.. The antibacterial activities of commonly used antibiotics were observed against isolated pathogens. The result shows the most effective antimicrobial agents against Gram-positive organisms were Linezolid showed sensitivity of 94% in comparison to Penicillin-G and Amikacin which showed 99.22% and 81.20% resistant respectively. In the case of Gram-negative organisms, Imipenem (90.19%), was highly sensitive/ effective while antibiotics as Ampicillin (95%) and 3rd generation

cephalosporin (Cefotaxime, Cefixime, Ceftriaxone, Ceftazidime) were found resistant against the isolates.

A total of seven medicinal plants viz. *Azadirachta indica*, *Moringa oleifera*, *Nyctanthes arbortristis*, *Punica granatum*, *Swertia chirata*, *Syzygium cumini* and *Tinospora cordifolia* were selected on the basis of their antibacterial properties. Each individual plants were authenticated and it's selected part were evaluated for its antibacterial activity against isolated pathogens in selected solvents (aqueous, 80% ethanol, methanol, acetone, and hexane) extract at 25, 50, 100, and 200 mg/ml concentration. The result exhibited concentration-dependent inhibition zone. The pattern of antibacterial activity of plant extracts was 80% ethanol>aqueous>methanol>acetone>hexane among solvent extract. Aqueous extract from *A. indica*, *M. oleifera* (except *P. aeruginosa*) and *S. chirata* was resistant against the bacterial isolates. All other four medicinal plants (*S. cumini*, *P. granatum*, *N. arbortristis*, and *T. cordifolia*) from aqueous extract exerted variable activity from highest (*S. cumini* against *S. aureus* 30.16±0.76 mm and *P. granatum* against *Enterobacter* spp. 22.00±0.50 mm) to lowest (*M. oleifera* against CoNS 10.66±0.57 mm and *Enterobacter* spp. 9.33±0.28 mm) to the concentration of 200 mg/ml used against gram-positive & negative bacterial cultures. Among organic solvent extracts, the highest antibacterial effect obtained from methanol extract of *P. granatum* (*S. aureus* 30.50±0.50 mm) and 80% ethanolic extract of *S. chirata* (*P. aeruginos*a 40.33±0.57 mm). Lowest activity was observed in methanol extract of *A. indica* (*Streptococcus* spp. 9.83±0.28 mm) and *T. cordifolia* (*K. pneumoniae* 12.16±0.28 mm) in gram-positive and gram-negative bacterial cultures respectively. Nine different metabolites such as alkaloids, coumarins, quinones, phenol, flavonoids, terpenoids, tannins, steroids, and saponins were assessed in each solvent extract of medicinal plants. All kinds of phytochemical classes have been detected in different solvents and shown to have significant inhibitory action against bacteria.

In the present study, phytochemicals in the aqueous extract from *Punica granatum* has been identified by HPLC/LC-MS method. Among 23 identified compounds, 22 of them were tentatively identified along with one unidentified compounds. Seventeen compounds were found in *P. granatum* and five compounds were not generally been described in literature

review related to pomegranate rather in other plant sources out of the total identified compounds. Eight out of the identified compounds were reported for antibacterial properties as also observed in this study were gallic acid, gallocatechin, catechin and epicatechin, dotriacontane, brevifolin carboxylic acid, cynaroside, gamma sitosterol, tributyl acetylcitrate.

In the present study, four trial phases were formulated (*in vitro)* in different phases along with the different composition of plant extracts (*P. granatum*, *T. cordifolia*, *N. arbortristis*, *M. oleifera*, *S. chirata*, *S. cumini*) in different solvent (aqueous and 80% ethanol) having significant antibacterial activity individually. Among 33 formulations developed, the most effective formulation found was formulation G in trial phase IV in which *S. aureus* (17.16±0.28 mm), *Streptococcus* spp. (11.16±0.57 mm), CoNS (20.16±0.76 mm), *K. pneumoniae* (18.00±1.00 mm), *E. coli* (16.00±0.00 mm), *Enterobacter* spp. (18.83±0.76 mm), *P. aeruginosa* (21.00±0.00 mm) and *Proteus* spp. (17.00±0.00 mm) were significantly sensitive. The polyherbal syrup was prepared from formulation G as per Ayurvedic Pharmacopoeia Part-II, Vol.-1 (2007) with appropriate preservatives sorbitol, sodium benzoate, sodium methyl paraben, sodium propyl paraben. The syrup was further carried out for standardization. The developed syrup exhibited brown color, sweet-bitter taste with agreeable smell having pH 6.13. Quantitative analysis of syrup for lead, arsenic, cadmium, and mercury (heavy metals) complies with the Ayurvedic pharmacopoeia of India Part-II, Vol.-2, (2018) standard. Microbial burden analysis for total aerobic count and pathogens such as *E. coli*, *Salmonella*, *P. aeruginosa*, and *S. aureus* found within the limit of API (Ayurvedic Pharmacopoeia of India Part-II, Vol.-2, 2018). Antibacterial efficacy of final syrup was determined by agar well diffusion method and was reported to show significant activity against pathogens indicates excipients and process of preparation did not affect the sensitivity of the active components present in the formulation in form of extract.

The prepared formulation was evaluated for its safety efficacy in acute dose under *in vivo* trials. Toxicity study was performed as per the procedure prescribed in OECD Guidelines 423 (2001). The test was performed on healthy laboratory animals. Young active and healthy Swiss albino mice (both sexes) of age 8-12 weeks old, weighing about 30-45 gm were

utilized for this experiment. The starting dose was 300 mg/kg body weight of polyherbal formulation dissolved in sterile distilled water administered to group 1 (male and female). No mortality was observed within four hours and 24 hours after oral prescription. Group 2 was administered with next higher dose of 2000 mg/kg body weight after 48 hours. Group 3 was fed with vehicle (sterile distilled water) as control in parallel with experiment of group 1 and 2. There was no lethal effect during the experimental period of 14 days for both male and female mice. Consumption of food, intake of water, and respective weight of all the mice are normal. The general behaviour of the extract treated animals and control group did not display any drug related changes in all kind of activities therefore, the formulation seems to be safe at a dose of 2000 mg/kg body weight and the median lethal dose (LD_{50}) of the formulation was supposed to be more than limit test dose of 2000 mg/kg body weight. The organs withdrawn from mice were observed macroscopically and found no pathological changes in the tested organs.

DEDICATION

This PhD work is dedicated to:

- **My late mother, may her soul remain in peace**
- **My Father**
- **Wife**
- **Children**

ACKNOWLEDGEMENT

In the truest sense this thesis could not exist without the support and input of more people than I can name on one written page. I would like to thank the following people who played important roles in this period of research process.

First and foremost, I offer my prayers and thanks to **"Almighty"** who gave me a role as a researcher in this life and for showing all the good people around me. I would like to thank Almighty for constant blessings and giving me ability throughout my life.

I am deeply greatful to National Medical College and Teaching Hospital, Birgunj, Parsa, Nepal for providing me research facilities to conduct and complete my reseach project.

I would like to extend profound gratitude to my supervisor Dr. Ashish Vyas, Professor and Head, Department of Microbiology and Biochemistry, Lovely Professional University for his guidance, resilience, dedication and total support with encourage from the beginning to the end of this research work. He sacrificed his valuable time (at home and in office) in guiding me throughout these years despite his busy administrative schedules.
I would also like to use this opportunity to thanks the entire faculty members of the School of Bioscience and Bioengineering and department of research division especially to Dr. Rekha mam and Dr. Akash for helping in all steps and Dr. Preeti Khurana due to her I have got opportunity to be scholar of this university.

I would also thanks to Sophisticated Analytical Instrument Facility (SAIF), Central Drug Research Institute (CDRI), Lucknow, India for carried out LC-MS of *P. granatum* peel extract.
I am greatful to Anandaban Hospital, Kathmandu, Nepal for availability of mice to carry out animal study.

I am also greatful to Gorkha Ayurved Company (P) Ltd. for providing me opportunity to prepare my formulated syrup.

I am also thanks to Nepal Ayurved Medical College and Teaching Hospital, Birgunj, Parsa, Nepal for authentication of plants.

I regard my thanks to Mrs. Bindu Malla Assistant Professor of Gandaki Medical College and Teaching Hospital, Pokhara, Nepal for providing and encouraging to carryout animal study.

The number of person to whom I owe deep thanks for the various favours received during the course of this work Mr. Anil Sah, Mr. Ravi Kumar Bhaskar, Mr. Ashish Sah, Mr. Shyambabu Rauniyar, Mr. Amrullah Shidiki, Mr. Divya Rana, Mr. Raghuraj Saran, Dr. Mukhtar Ansari, Dr. Parwez Ahmad, Mr. Santosh Sah, Mr. Satrudhan Ojha, Miss Asmita Pandit, Miss Sonali Thakur, Miss Kiran Yadav for their help inspiration and best wishes.

Finally, I wish to express my most heartily thanks and gratitude to all my friends, my family members with special mention to my wife **Kalpana,** Son **Nihal** and daughter **Nitishma** and my inlaws for their unconditional love, support and understanding which has given me strength for everyday life and work. Last but not the least I thank *the almighty* God for being with me always.

Bijay Raj Pandit

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

Chapter One

Introduction

1.1 Global Scenario for Neonatal infection

Sepsis is a critical condition of medical emergency that arises due to injury in defense mechanism of the host's own tissues and organs to response of infection and leads to mortality or significant morbidity. Globally, more than 30 million people were estimated to affect each year, leading to the death of 6 million people. It has been observed that low and middle-income countries have a probably maximum load of sepsis (Rudd *et al.*, 2020). Neonatal sepsis is a generalized bacterial infection during the first twenty-eight days of life associated with general systemic manifestation and positive blood culture (NNPD, 2005). As per United Nation Inter-agency group for child mortality (UN-IGME, 2019) estimation report, an estimated 7000/ day and 2.5 million newborns died during the first month of life in 2018. It further reports, that about one-third of all neonatal deaths are showing tendency to happen on the birth day and nearly three quarters die within seven days of life. In the year 2018, 15000/ day children died in comparison to 34,000/ day in 1990. The report further discusses that death of 54% neonates occurs during the neonatal period were from under-five children category. The report predicts that if the current trends continue can lead to about 52 million deaths of under-five children will occur between 2019 and 2030, among these halves of them will be newborns. Neonatal Mortality Rate for South Asian Countries has been depicted in Figure 1.1. Nepal has seven provinces and the Neonatal Mortality Rate (NMR) of province II was 30/ 1000 live birth, which were the second-highest among the provinces (NDHS, 2016). The leading causes of neonatal death in 2018 are Preterm birth, birth asphyxia, sepsis, pneumonia, and birth defects. Majority of the neonatal death takes place in low and lower middle income countries (UN-IGME, 2019).

1.2 Classification, Etiology and Risk factors of Neonatal Sepsis

On the basis of transmission mode and timing of infection sepsis in neonates can be divided into two subtypes as Early Onset Neonatal Sepsis (EONS) in which clinical symptoms generally appear within the first 72 hours of life. The cause of early-onset infection is frequently acquired before or through delivery by vertical mode of transmission from mother to newborn.

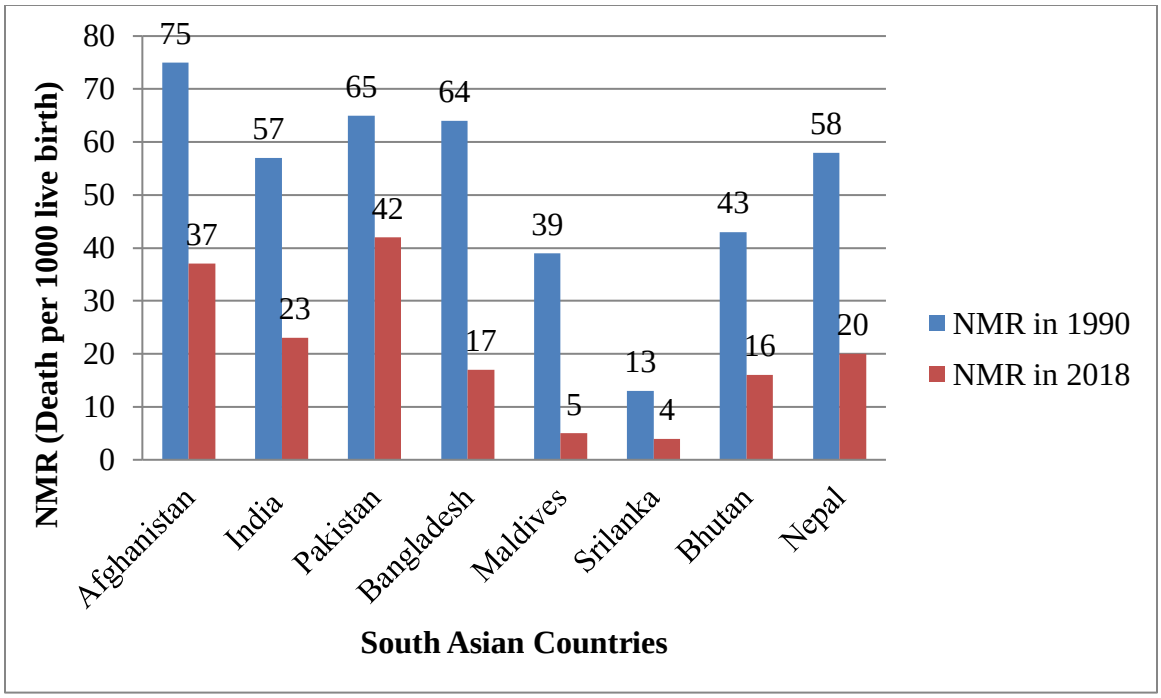


Figure 1.1: NMR of South Asian Countries (1990-2018) (UN-IGME, 2019)

Infection in ascending way or by transplacental route from the cervical region may possibly be caused by the colonized organisms in the mother's genital area during delivery. Late Onset Neonatal Sepsis (LONS) may occur after delivery or beyond 3 to 28 days of age by the colonization of the organisms present in the Neonatal Intensive Care Unit (NICU) or in the community to newborns skin, respiratory tract, conjunctiva, gastrointestinal tract, and umbilicus which lead to the possibility of infection from invasive microorganisms. The viable vector for colonization can be life assisting gadgets like vascular or urinary catheters, different indwelling lines, or via touch with caregivers (Russell, 2015).

Neonatal sepsis causing organism shows variation from place to place and even in different hospital in the different incident or in the same hospital at different time (Lakhey and Shakya, 2017). There is quite difference in bacterial spectrum causing neonatal sepsis in developing countries in comparison to developed countries. Group B Streptococcus (GBS), Coagulase Negative *Staphylococcus* (CoNS), and *Escherichia coli* (*E. coli*) are predominant pathogens in developed countries. In developing countries higher prevalence of gram-negative *Klebsiella* was the predominant pathogen causing early-onset sepsis,

liable for 25% of all septic cases, followed by *Staphylococcus aureus* (*S. aureus*) and *E. coli* (Ganatra and Zaidi, 2010).

The development of sepsis in newborn is determined by the factor like maternal, host, and environment of the surroundings. Approximately 30-40% of deaths due to the development of sepsis are transmitted during delivery and later manifestation of early-onset symptoms (Ganatra *et al.,* 2010). Newborns are at increased risk of infection due to a number of obstetric complications like pre-partum and intra-partum; the most important of which are prolonged rupture of membranes, premature onset of labour, intra-amniotic infection, and maternal fever. The poorly developed immune mechanism of the newborn is weak to defend against pathogenic organisms, because of the shortage of protective maternal immunoglobulins, immature inborn immunity, and effortlessly fragile skin (Health and Zerr, 2006). In developing countries, different risk factors associated with sepsis are listed in Table 1.1.

Table 1.1 Components of Risk factors for newborns in developingcountries

Maternal Factors	Environmental Factors	Neonatal Factors
❖ Poor antenatal care • Maternal infections • Bacterial vaginosis • Urinary tract infection • GBS colonization of genital tract ❖ Maternal malnutrition (especially micronutrient deficiency) ❖ Premature rupture of membrane ❖ Poor obstetric care and sanitation ❖ birth asphyxiation	❖ Environmental contamination ❖ Poor cord care ❖ Unsafe hand washing ❖ Artificial feeding ❖ Overcrowding ❖ Poor new born Care practices (discardingcolostrums,early bathing, prelactealfeeds)	Low birth weight and prematurity

Adapted from (Bhutta, 1999)

1.3 Transmission and Pathophysiology of Neonatal Sepsis

The shared and intricate relationship between newborns and the mothers is the main leading risk factors and cause of these infectious diseases. Normal flora that colonizes the maternal genital tract is vertically transmitted to neonates, and these acquired bacteria may lead to infection through the eyes (gonococcal conjunctivitis), umbilicus, mouth or an abrasion in the skin (Chan *et al.,* 2013). Ascending infection that may occur during the labour caused by colonized organisms that spread through the vaginal canal from the maternal perineum to the placenta and then into sterile amniotic fluid making the focal point to lungs and intestinal tract for bacterial translocation (Carroll *et al.,* 1996). The health care centers in developing countries care less about the hygienic condition during labour, delivery, postnatal care, and inappropriate basic care of the baby at the house which contributes in the development of neonatal infection leads to death of newborns in the hospital and community (Bhutta and Yusuf, 1997).

1.4 Neonatal immunity

The innate immune system which forms the early pathway for protection against infection is compromised in neonates. The function of Neutrophil and dendritic cells are also decreased. Neonatal neutrophil or polymorphonuclear (PMN) mobile cell which is essential for effective killing of bacteria is lacking in chemotaxis and killing capacity. Deterioration of the innate immune system in neonates results in an elevated vulnerability to bacterial and viral diseases in newborns (Levy, 2007). The newborn adaptive immune system gradually boosts its activity towards maturity and is intended to eliminate specific pathogens (Schelonka *et al.,* 2011). The passive immunity before birth in neonates is the placental transfer of immunoglobin-G (IgG) antibodies from mother to fetus and its level increases with gestational age. These antibodies help to protect the neonates from bacterial and viral infections (Malek, 2003). The level of antibodies is directly proportional to gestational age so that preterm infants deficient in adequate antibodies protection whereas, term infants were fully protected with maternal and most of the vaccine-protected disease in neonates through the transplacental passing from the mother's serum to the fetus. The marginal sector of the spleen is likewise not completely matured until the age of 2 years

increases the sensitivity to capsulated bacterial diseases (Zandvoort and Timens, 2002). The immunoglobulin-A (Ig-A), Immunoglobulin-E (IgE), and immunoglobulin-M (IgM) antibodies cannot cross the placenta so that there is absence of these antibodies in neonates. The paucity of Ig-A reduces the capacity of neonates to counter the environmental pathogens. All the above mentioned reasons therefore, make neonates more susceptible to gram-negative bacteria and certain viruses (Rubarth *et al.*, 2017).

1.5 Clinical symptoms and Laboratory diagnosis of neonatal sepsis

1.5.1 Clinical symptoms

Clinically, in neonatal sepsis syndrome both the EONS and LONS present common and unspecific clinical manifestations like temperature fluctuation, respiratory distress, yellowish tinge, feeding intolerance, annexation etc. that make difficult to separate the onset of sepsis (Aku *et al.*, 2018; Zakariya *et al.*, 2010).

1.5.2 Laboratory diagnosis

Neonatal sepsis is the occurrence of microorganisms in normally sterile body fluids specifically in blood, urine, cerebrospinal fluid (CSF), peritoneal, and pleural (Camacho-Gonzalez *et al.*, 2013). The laboratory finding is important along with clinical signs and symptoms because infection leads to severe danger to newborns. There is no exact test that may be hopefully used and carried out confidently by a pediatrician/neonatologist to conclude neonatal sepsis (Philip and Hewiot, 1980). Laboratory investigation like hematological, biochemical, and microbiological tests is essential for the conclusion of sepsis. Conventionally, diagnosis of neonatal sepsis is confirmed in the laboratory by isolating the infecting organism from commonly sterile body fluids viz. blood, CSF, urine, pleural, and peritoneal fluids which remains the gold standard for definitive diagnosis although, the culture report requires days and culture methods sensitivity is commonly low (Stefanovic, 2011).

1.6 Medicinal Plants

In spite of recent development in human remedies, infectious diseases caused by microorganisms are still a primary hazard to public health. The impact is even more in developing countries due to the deficiency of medicine to each individual and the emergence

of drug-resistant microorganisms. The development of antimicrobial resistance in microorganisms is due to various reasons like the nature of the antibiotic, dose of antibiotics, host factors, and environmental causes (Abiramasundari *et al*., 2011). The production costs of synthetic antimicrobial drugs are high with severe adverse effects in comparison to plant-oriented drugs. This scenario has compelled scientist to search for new antimicrobial components from different plants origin which acts as novel antimicrobial natural chemotherapeutic agents (Amenu, 2014). The uses of the medicinal plants were considered as clinically effective and safer alternatives to synthetic antimicrobial drugs (Modi *et al.,* 2012).

1.6.1Medicinal Plants selected in this study

Azadirachta indica: This plant is vernacularly known as neem. This plant belongs to the family Meliaceae. It is native to Burma, India, and Nepal (Alzohairy, 2016). Different types of the phytochemicals to be present in leaves and bark of the tree are effective as antimicrobial, antifungal, antiviral, antipyretic, antiperiodic, antimalarial, antihelminthic, insecticidal, mosquito larvicidal, antifertility, spermicidal, anti-inflammatory, hypoglycemic, gums infection in oral cavity, sores, gingivitis, and periodontitis (Lall *et al*., 2013). The components of the tree are given in different forms either as crude or as an extract for different ailments such as fever during childbirth, enlargement of spleen, malarial fever, measles, smallpox, head scald and cutaneous infections. Nimbidin from *Azadirachta indica* also has antiulcer properties (Alzohairy, 2016).

Moringa oleifera: This plant is vernacularly known as sahjan. This plant is a small to average-sized tree that belongs to the family Moringaceae and native to South Asia, India, Pakistan, Africa, Arabia, South America, the Himalayan region, the Pacific and Caribbean islands (Osman *et al*., 2015). The different parts of the plant were utilized for different purposes, roots were used in different disorder in the different ailments like relapsing fever, seizure, carminative, abdominal problem, diuretic, cardiac and circulatory tonic while leaves have been reported for its various biological properties including hypocholesterolemic, antidiabetic, anti-inflammatory, antitumor and antimicrobial whereas, seeds have reported

as antimicrobial property. Active compounds called Pteryospermin has been reported from different parts of *Moringa oleifera* plant which exhibits bacteriocidal and fungicidal properties (Dhakad *et al.,* 2019; Surendra *et al.,* 2016).

Nyctanthes arbortristis: *N. arbortristis* commonly called as night jasmine and vernacularly called as parijat or ratrani is a small tree belonging to the family Nyctanthaceae. The plant is widely distributed in Nepal, Burma, and India. The different phytochemical constituents from these plants were used as antihelminthic, antipyretic, rheumatism, skin ailments, and as a sedative (Saha *et al*., 2012). The presence of a large number of phenolic compounds in *N. arbortristis* like carotenoids and iridoids possess various biological activities like antileishmanial, anticancer, anti-inflammatory, antiallergic, immunomodulatory, and antiviral (Jain and Singh, 2013). Leaves extracts of *N. arbortristis* were also found to have antimicrobial activity (Agrawal and Pal, 2013).

Punica granatum: This plant is vernacularly called Anar. The plant posseses multifunctional and nutritional fruit and it belongs to family Lythraceae (Moga *et al*., 2021). It is a deciduous shrub or small tree, native to Asia. Plant has an abundant amount of tannins and other phytochemicals mainly phenolics which is important in reducing disease risk of like diabetic, cancer, and skin ailments (Viuda-Martos *et al.*, 2010). Pomegranate peel is a biological waste known to have possessed a broad spectrum of pharmacological activities such as anticancer, antimicrobial, antidiarrheal, anti-inflammatory, and antidiabetic (Fawole *et al*., 2012; (Moga *et al*., 2021). The phytochemicals present in *P. granatum* are gallocatechin, delphinidin, cyaniding, gallic acid, ellagic acid, pelargonidin, and sitosterol also play role in therapeutic properties (Viuda-Martos *et al.*, 2010).

Swertia chirata: This plant is vernacularly known as chirata. *S. chirata* is an erect annual or perennial herb that belongs to the family Gentianaceae. It is widely distributed in India, Bhutan, and Nepal. The whole parts of *S. chirata* are full of medicinal properties but the root is the most potential in diabetes, hepatitis, cancer, and neurological diseases (Kshirsagar *et al.,* 2019). The presence of these different type of phytochemicals like flavonoids and phenols makes the plant effective for a broad spectrum of pharmacological action like hepatoprotective, antimicrobial, anti-inflammatory, anticancer, hypoglycemic, antioxidant,

antimalarial, and CNS depressant (Roy *et al*., 2015). Recently, extract of the plant has been reported to exhibit anti-hepatitis B Virus (anti-HBV) activities (Kshirsagar *et al.,* 2019).

Syzygium cumini: This plant is vernacularly known as jamun. This evergreen tree of *S. cumini* belongs to the family Myrtaceae and is found in many regions of South Asian countries. Different parts of the plant possess various medicinal properties like antioxidant, anti-inflammatory, neuropsychological, antimicrobial, anti-HIV, antidiarrheal, antileishmanial, antifungal, antifertility, antioxidant, anorexigenic, gastroprotective, ulcerogenic, and radioprotective activities (Mohamed *et al*., 2013). Plant leaves possess broad spectrum phytochemicals like terpenoids, alkaloids, coumarins, quinones, terpenoids, tannins, steroids, saponins, and phenolics which have been reported to exhibit antimicrobial activity (Ayyanar and Subasbabu, 2012).

Tinospora cordifolia: This plant is vernacularly known as giloy. The plant is large deciduous perennial climber with glabrous heart-shaped leaves that belongs to the family Menispermaceae distributed throughout India, Nepal, and China (Chi *et al.,* 2016). Several researchers have reported various medicinal properties of *Tinospora cordifolia* and it was found effective in medication of weakness, fever, dyspepsia, dysentery, gonorrhea, urinary diseases, inflammation of the liver, anemia, antioxidant, antineoplastic, hypoglycemic, antipyretic, hepatoprotective, diuretic antistress, antihyperglycemic activity, antidiabetic, and antituberculosis activity (Chakraborty *et al*., 2014; Chi *et al.,* 2016; Sharma *et al*., 2019). The different parts of plant posseses phytochemicals like berberin, cordifolioside A and B and others are tinosporaside, tinosporin, magnoflorine, tinocordifolioside, isocolumbin (Chi *et al.,* 2016). The presence of secondary metabolites like coumarins, terpenoids, alkaloids, lignin, carbohydrates, steroids, and glycosides has been reported to play role in therapeutics (Chi *et al.,* 2016). Recently, it has been reported that the plant have very effective in immunomodulatory properties (Charles *et al*., 2021).

1.7 Structural Identification of Phytochemicals

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis: LC-MS is a chemical analysis process that mixes the physical partition potential of liquid chromatography (or HPLC) with the mass evaluation competencies of mass spectrometry (MS). LC-MS is an

effective method as it is excessively sensitive and selective having wide range of applications in the field of proteomics and metabolomics. LC-MS process involves a number of steps, after the pre-treatment sample phase, sample is added into the LC column to break molecules primarily establish on their size. LC-MS that identify the size of the phytochemicals called size exclusion chromatography while affinity to stationary phase called affinity chromatography and polarity based called ion-exchange chromatography whereas, hydrophobicity based called chromatography of reversed-phase. The retention time is the time between the sample introduction and the appearance of the highest peak of the compound after chromatographic separation.

In the case of complicated mixture analysis, many analytes elute different compound peaks simultaneously, so a single compound peak cannot be obtained with only the application of liquid chromatography. That can be resolved with Mass spectrometry which helps to separate the individual peak based on the mass per charge ratio (m/z) by analysis of compound peak. The most commonly used ionization methods among various spectrometric methods are electrospray ionization (ESI) and other is atmospheric pressure chemical ionization. The basis of mass detection depends on the instruments used for it. The most common instruments used for mass detection are quadrupole ion trap and others are triple quadrupole and next one is quadrupole time of flight mass spectrometers (Hoffmann and Stroobant, 2007).

1.8 **Herbal formulation, Standardization and *in vivo* study**

Herbal formulations are prepared from herbal extracts, herbal materials and finally finished herbal products which posseses active phytochemical ingredients plant parts and materials. These herbal medicines are specifically used for the prevention and curing of diseases. Herbal medicinal products are called botanical medicine or phytomedicine (Builders, 2018). Globally, 80% population of developing countries depends on the herbal medicinal products as a primary source of healthcare (Ekor, 2014). Herbalists favor the use of whole plants or a mixture of several herbs to enhance effectiveness for desired medicinal effect (Builders, 2018). Presently, use of herbal medicinal products is increasing because of natural herbal products, less side effect, low cost, side effect

associated with the action of synthetic drugs, easy availability, natural curing of chronic disease and management of certain diseases through consumption for longer duration of time under ineffectivity of synthetic drugs with no side effects (Ogbonna *et al*., 2012). Generally there are two main basic components of any herbal formulation first is active constituent which is responsible for therapeutic actions and another is excipients. Active constituents containing plants can be selected by different approaches like: random selection followed by chemical screening; random selection followed by one or more biological assays; biological activity reports and ethno medical use of plants. The herbal formulation possesses greater challenges compared to synthetic drugs due to the complexity, lack of complete characterization, batch to batch variation (Ogbonna *et al*., 2012). The main challenge in formulating herbal drugs is to control microbial burden in different forms that are solid form (tablets, capsules), suspension, and especially in liquid oral dosages forms (Kamil and Lupuliasa, 2011). It is considered that synthetic drugs have more side effects than the plants oriented natural drugs but few plants or those plant-derived drugs could be lethal with undesirable adverse effects. Quality control of herbal products for their safety and efficacy is essential as it directly impacts herbal medicinal products (Karimi *et al.*, 2015).

Standardization of herbal formulation is crucial with the aim of judging the quality of drugs. It is the processes regarding the evaluation of physicochemical properties of crude drug, safety for consumers, potency and reliability, assessment of finished products, specification of product facts to the customer, and product approval (Nikam *et al.*, 2012). Standardization is the study of evaluating different properties of herbal drugs like organoleptic, pharmacognostic, volatile matter, quantitative, phytochemical, xenobiotics, microbial load, toxicity, and biological activity (Modi *et al.*, 2014). Standardization test viz. organoleptic, microbial load and heavy metals has been performed as per the method of Ayurvedic pharmacopoeia of India (API-II, Vol. II, Formulations, 2018).

***In-vivo* analysis**

In Latin word *in vivo* means "within the living" that refers testing performed in animals, plants or whole cells. The herbal experimental drugs focused for humans are assessed for its safety and efficacy on animals by following one of the selected guidelines for its toxicological evaluation that should complies with the standard prior to launch in the market. More or less any substance can be harmful at a particular dose but at the same time can be safe at some lower dose. Toxicity assessment is vital for the evaluation of newly launched drugs in order to guarantee assurances of their safety to humans. Most of the toxicity testing is performed on laboratory animals. Toxicity studies are described into three types:

Acute toxicity studies: This study is a short period judgment or assessment of possible lethal of test substance or effect of single prescription of a test material. It is better described as (Lethal dose) LD_{50}, the dose which kills 50% of tested animals.

Sub-acute toxicity studies: This study evaluates the outcome of different concentration of dose that affects the organs.

Chronic toxicity studies: The knowledge is essential to observe the organs affected after a long duration of feeding the dose to ensure whether the test drug is having a carcinogenic effect or not (Agrawal and Paridhavi, 2007). In recent years newly advanced investigative methods for the performance of stability and toxicity study is performed by following the widely accepted guidelines of Organisation for Economic Co-operation and Development (OECD) for acute toxicity study (OECD 423).

Chapter Two

Review of Literature

2.1 Neonatal sepsis: history, Definition, classification, etiology and risk factor

In Greek, the word sepsis means "decomposition" or "decay," denotes the rotting of flesh. As per Hugo Schottmuller (1914), "Sepsis is the presence of pathogenic micro-organisms either constantly or periodically that invade the bloodstream presenting the relevant sign and symptoms. Recently, in the meeting between Society of Critical Care Medicine with the European Society of Intensive Care Medicine replaces the description of sepsis and septic shock. The meeting defines "Sepsis is life-threatening organ dysfunction caused by a dysregulated host response to infection" (Gul *et al.,* 2017). Neonatal sepsis is also described according to the conference of the International Pediatric Sepsis "As a clinical syndrome characterized by the presence of both infection and Systemic Inflammatory Response Syndrome (SIRS)". SIRS manifests symptoms like temperature instability, tachyarrhythmia, bradyarrhythmia, rapid breath or unexplained requirement of mechanical ventilation, and hematological parameters depicted in Table 2.1 (Gul *et al.,* 2017).

Table 2.1: Age-related critical signs and laboratory parameters

Age group	Heart Rate, Beats/Min		Respiratory rate, Breaths/min	Leukocyte count, Leukocytes $x10^3$/mm	Systolic Blood Pressure, mmHg
	Tachycardia	Bradycardia			
0 days to 1week	>180	<100	>50	>34	<65
1 week to 1 month	>180	<100	>40	>19.5 or <5	<75

(Adopted from Goldstein *et al.,* 2005)

Neonatal Sepsis is also described as clinical syndromes that represent the manifestation of infection with having or not present of bacteremia in 30 days of life (Shankar *et al.,* 2008). There are multiple definitions given by enumerable authors. Neonatal sepsis also refers to illness happening within the first four weeks in a term baby and up to the same weeks in a preterm baby behind the scheduled date of delivery (Russell, 2015). As per Goldstein *et*

al., (2005) and Barnden *et al.*, (2016), Sepsis is a life-threatening clinical syndrome with hemodynamic changes characterized by systemic invasive infection that occur due to the presence of pathogens (bacteria, virus, and fungi) in normally germ-free body fluids (blood or CSF) in the first four weeks of life. It involves bloodstream infection, meningitis, and pneumonia.

Neonatal sepsis is categorized on the representation of signs and symptoms as Early Onset and Late-Onset. Early-Onset Neonatal Sepsis (EONS) defined variably based on the age at onset: Sepsis occurring in the preterm neonates at ≤72 hr of birth in a Neonatal Intensive Care Unit (NICU) hospitalized infants, versus less than 7 days in term infants. Late-Onset Neonatal Sepsis (LONS) resulting after 72 hr in NICU neonates and one week in term infants, up to the age of less than 90 or 120 days (Simonsen *et al.*, 2014). As per American Neonatology Network report, 62% of Gram-positive microbes are responsible to cause EONS, and *Streptococcus agalactiae* (*S. agalactiae*) is having high prevalence rate of 43% out of the total cases. Gram-negative microorganism comprises 37% of the causative agents of EONS with 29% prevalence of *E. coli* strain (Procianoy and Silveira, 2019). In late-onset neonatal sepsis, 79% infection is being caused by gram-positive microorganisms of which 57% prevalence rate for CoNS in total and 12% of *S. aureus*. Gram-negative microorganisms are responsible for 19% of the total, with *E. coli* predominantly responsible for 7% of the total. Fungal strain has been reported in 6% of cases for LONS (Procianoy and Silveira, 2019). Zaidi *et al*., (2009) reported the associated pathogens with sepsis in newborns and young infants from developing countries like Africa, East Asia and Pacific, Middle East and Central Asia, South Asia. This study reports infection in the first week of life with pathogenic strains of *Klebsiella spp.* (25%), *E. coli* (15%), *Staphylococcus aureus (*18%) and Group B Streptococcus (GBS) (7%) with regional variation. As per Ganatra *et al.*, (2010) the overall ratio for gram-negative and positive organisms was 2:1. The study reports the most prevalent organism causing infection in East Asia, Pacific and South Asian countries was *Pseudomonas* and *Acinetobacter*. *S. aureus* was rarely reported in East Asia and Latin America as compared with other regions. The systematic review of Amare *et al.*, (2019)

reported that *S. aureus*, *Klebsiella* and CoNS were the common cause of neonatal infection in developing countries.

A large number of pathogenic microorganisms are linked with the disease of Neonatal sepsis. *Staphylococcus aureus* is gram positive bacteria and is distributed wide spread in nature. It has the normal habitat on the body surface along mucous sheath of mammals and aves. *S. aureus* preferred habitat is anterior nares and carried by some 40% of adults (Mackie and MacCartney, 1996; Bailey and Scott's, 2017). Most neonates become colonized immediately after birth but presence of micro-organism decreases 63.87 % at 1 month and 18.2% after 6 months. Staphylococci can be efficiently carried from one person to another or by fomites and via aerosolized droplets. The prevalence of *S. aureus* carriers is high among health care providers. Humans are frequent carriers of the colonized organism which eventually enter the germ-free site of neonates as a result of trauma to the skin or the mucosal surface. *S. aureus* causes a spectrum of toxin mediated disease such as bacteremia, endocarditis, bone, soft tissue infection, and toxic shock syndrome (Mackie and MacCartney, 1996; Bailey and Scott's, 2017). Among Staphylococci, CoNS is unable to produce coagulase. CoNS are native bacteria primitive to mammalian hosts and are a normal resident of the human skin surface. In hospital-acquired infections, the most usually established pathogen is *Staphylococcus epidermidis* (*S. epidermidis*) which colonizes the human body surface and mucosal membranes and infrequently causing infections in healthy tissues. *S. epidermidis* is able to stand by and proliferate on plastic surfaces of indwelling clinical gadgets because of its potential to form continuous multilayered biofilms that are fundamentally resistant to antibiotics with actual barrier towards immune system. *S epidermidis* usually link with preterm infants and responsible to cause neonatal sepsis (D'Angio *et al.*, 1989; Cortese *et al.*, 2016). *Streptococcus agalactiae* are Group B Streptococci (GBS) based on Lancefield classification. These are encapsulated, facultative gram-positive diplococcus capsulated bacteria with its virulence property governed by different components present in their morphological structure like capsular polysaccharide, sialic acid residues and lipo deacylated glycerol teichoic acids. Even though the utilization of Intrapartum Antibiotic Prophylaxis (IAP), *Streptococcus agalactiae* now also the pathogen chiefly accountable

(70%) for neonatal sepsis among GBS diseases and meningitis. Most of the newborns infected with *S. agalactiae* develop clinical signs and symptoms in early-stage with having respiratory distress and cardiovascular instability (Melin, 2011; Cortese *et al*., 2015). *Klebsiella pneumoniae* members of the Enterobacteriaceae family are the most common pathogen of neonatal sepsis in the developing world. The Klebsiellae are nonmotile, rod-shaped, capsulated gram-negative bacteria composed of polysaccharides. The polysaccharide layer surrounds the entire cell surface, resulting in a thick appearance that provides antiphagocytic in nature. *Klebsiella pneumoniae* is the most medically important species of this group (Mackie and MacCartney, 1996; Bailey and Scott's, 2017). *Klebsiella* spp. is the inhabitant of the nasopharynx and gastrointestinal tract. The high incidence of *Klebsiella* colonization appears to be related to the use of antibiotics, aside from medical gadgets that infected with defective hygenic protocol and blood products. The main sources of *Klebsiella* transmission in the medical institution are the unhygienic condition of patients and the hands of hospital facility employees. The dissemination capacity of *Klebsiella spp.* leads to nosocomial outbreaks, particularly in neonatal units. Large numbers of newborns are infected with *Klebsiella* spp. in Neonatal Intensive Care Units (NICUs) leads to a prominent reason for neonatal mortality due to sepsis. In NICU, Extended-Spectrum Beta-Lactamases (ESBL) producing *Klebsiella* spp. frequently causes an outbreak and these are resistant against a vast range of antibiotics (Mackie and MacCartney, 1996; Bailey and Scott's, 2017). *Escherichia coli*, a gram-negative bacterium regarded as the 2nd most principal cause of EONS in neonates, responsible for about 24% of all EONS, with 81% sufferer are preterm infants (Shane and Stoll, 2013). Coliforms which are the regular colonizers of the mother vaginal tract that can be gain access to infants at the time of delivery or just before. In EONS generally, *E. coli* leads to bacteremia at the time of delivery that may be with or without meningitis. The strain K1 and O18 are responsible for the higher rate of neonatal meningitis and septicemia which is the cause of higher mortality rates in neonates (Moulin-Schouleur *et al.,* 2006).

Pseudomonas, *Enterobacter* and *Serratia* are other gram negative bacteria responsible for nearly one-third of LONS cases with 40% to 69% of deaths result in neonatal age. Gram-negative infections generally arise by transmission from medical-related employees,

contaminated catheters, parenteral solutions and pediatric procedures. *Pseudomonas aeruginosa* is a gram-negative rod; a classic opportunistic versatile pathogen flourishes as saprophytes in the warm moist situation in the human environment and hospital setting (sinks, drains, respiratory equipment, showers, and humidifiers) and disinfectant solution. *P. aeruginosa* is the most commonly encountered gram-negative bacteria in clinical settings. Virulence factors of *P. aeruginosa* include exotoxin A, exoenzymes S and T, and production of several proteolytic enzymes (elastases) and hemolysins (phospholipase C). *P. aeruginosa* is the remarkable cause of healthcare-associated diseases of the bloodstream, urinary tracts and wounds, respiratory and even central nervous system. The highest mortality in neonates is also from infection of *Pseudomonas*. *Enterobacter* species are similar to Klebsiellae, are motile lactose fermenters that produce mucoid colonies. *Enterobacter aerogenes* and *Enterobacter cloacae* are the most common species isolated from clinical samples. The capsule possesses by bacteria provides antiphagocytic nature. The organisms are normal microbiota of the GI tract and transmit by the person to person contact, especially in hospitalized patients. The infections are typically associated with contaminated medical devices, such as respirators and other medical instruments. It causes a broad range of healthcare-associated infections of the blood, urinary, and respiratory tract. The bacteria of the genus *Proteus* are motile, non-lactose fermenters and are a normal inhabitant of the GI tract. *Proteus* species are easily identified by their swarming growth in culture media with their characteristics "fishy" smell. The organism is associated with urinary tract infections, wounds, ear, and sepsis (Mackie and MacCartney, 1996; Bailey and Scott's, 2017). Risk factors related to neonatal sepsis comprises of maternal and neonatal host factors, virulence of pathogens in different onset has been shown in Table 2.2.

The natural immune system of newborn is generally weak with proneness to various infections because of various external risk factors also like unsafe birthing practices in developing countries. As per World Health Organization (WHO) 2008 report, woman of developing countries avail only 68% of antenatal care facilities and only 35% of mothers have assessed to delivery by Skilled health personnel in the least developed countries. Ganatra *et al.,* (2010) reports that due to lack of hygiene during and after delivery, cord-cutting with contaminated

tools, poor skin and cord care, following some risky cultural practices like cow dung application to the umbilical stump, avoiding colostrum feeding in South Asian rural communities and rejection of fruitful practices such as early and exclusive breast feeding increases the chances of infection in newborns. The study also reports that there is a lack of basic sanitary facilities in hospital settings leading to the transmission of infection as the breeding ground for drug-resistant organisms in the developing countries.

Table 2.2: Risk aspect for development of neonatal sepsis

Source	Possibility Risk Factor
Early-onset neonatal sepsis	More than one pregnancies
	Prolonged (≥18 hours) rupture of sheath
	Premature rupture of sheath
	Chrorioamnionitis (maternal fever≥38^0C)
	Urinary Tract Infection in mother
	GBS colonization in vagina of mother
	Preterm Delivery
	Pre-eclampsia, cardiac disease, Diabetes mellitus
Late-onset neonatal sepsis	Necrotizing enterocolitis
	Prolonged indwelling catheter use
	H_2-receptor blocker
	Breakage of the skin and mucosa
	Long use of antibiotics
	Invasive procedures
Neonatal	Prematurity
	Maternalas well as specific Antibodies is reduced when passing to foetus
	immature function of immune system

(Camacho-Gonzalez*et al.*, 2013)

2.2 Transmission, pathophysiology and Pathogenesis of Neonatal sepsis

Pathogenesis of sepsis in newborns categorized as: early-onset infection from mother to infants and late-onset infection from a hospital setting or with health care personnel or hospital equipment, surrounding environment of the infants after birth based on epidemiological characteristics (Baltimore, 2003).

Prenatal and Early-Onset Infections: The fetus and its surrounding are generally sterile inside the amniotic membranes. The fetus is protected physically by the membranes and placenta and also the inhibitory effect of amniotic fluid against bacterial growth but

sometimes the fetus attains infection directly or via the maternal blood. Ascending infection may occur due to contagion of the amniotic fluid with mother's vaginal flora as a consequence of the frank breach of membranes before the onset of labor or invisible crack leads to intra-amniotic infection. The fetus when ingest or swallow contaminated amniotic fluid that may infect the respiratory and Gastro system before birth. The neonates get contamination of their skin and upper respiratory tract by contact with the mother's cervical or vaginal or fecal flora or during aspiration in delivery. The thinner skin of neonates cannot able to protect from exogenous organisms make susceptibility to infection that may be due to the insufficiency of physical barriers. Neonates lack gastric acid which favors to cause invasive infections by smooth colonization through environmental organisms. Pathogens like *Salmonella* spp., *Mycoplasma hominis, Haemophilus influenzae, Streptococcus pneumoniae, Listeria monocytogenes, Candida* spp., and others that colonize mother's genital parts and infection may be the cause of neonatal sepsis. Pathogens enter into the mother's lower genital tract from the outer environment immediately before or during birth. The use of defective obstetric examination, use of monitoring equipment, Scalp electrodes may be the route for entering guest pathogens. The spread of vaginal flora to newborn make them suffer from sepsis (Baltimore, 2003).

Late-Onset Infections: Neonates including all age groups acquire the hospital-oriented infection when admitted for a long duration. Newborn during the time of birth are sterile but as soon acquires flora from the hospital environment in the nursery after birth. The normal flora of our body inhibits the colonization of foreign pathogens and prepares our body for developing resistance from infection. The nature of the normal flora also depends on the long time use of antimicrobials with repeated exposure and long duration of hospital stay. Newborn when admitted to NICU they gain entrance of foreign flora that may because of invasive infection. Newborn acquires normal flora from their mother, hospital, and nursery personnel or from inanimate objects may proceed for late-onset infections. The neonate on life-supporting devices gives an opportunity for the growth of unusual organisms (Baltimore, 2003).

2.3 Immune Response to Neonatal sepsis

The improvement of the immune system includes steps of development that arise in the course of the primary years of life. Neonates, mainly preterm infants, are quite immunosuppressed due to immaturity of immune defense mechanism in addition to decline placental transfer of maternal immunoglobulins (Camacho-Gonzalez *et al.*, 2013). The inborn Immune system is regarded to be a rapid, primary line of protection at the site of microbial invasion that is based on complement system, antimicrobial peptides, phagocytes and antigen-presenting cells (Sobota *et al.*, 2005). The antimicrobial proteins and peptides found in the skin of newborns at birth, in respiratory and gastrointestinal tracts function well for microbicidal properties (Levy, 2007). Neutrophils are very vital in defensive for newborns in opposition to infections though, they are found in the low percentage of blood cells till about 32 weeks (Levy *et al.*, 1999). Blood monocytes number are normal in neonates but the mass or function are diminished of reticuloendothelial system (liver, spleen, and lungs) macrophages particularly in pre-term infants (Kumar *et al.*, 2013). Natural Killer cells (NKC) appear early in gestation and present in cord blood in equivalent numbers to those of adults. The cytokine production in neonates is directly proportional to the activation of natural killer cells. These NKC lyse infected cells (Guilmot *et al.*, 2011). The adaptive immunity is composed of mainly B and T lymphocytes. The function of these immunological cells gradually increases from infant to adult to govern for removal of specific pathogens. The growth of memory cells of the adaptive immune system occurs through mutation in nucleotide patterns in the genome encoding T-cell and B-cell receptors located in the hypervariable region. The possibility of infection is superior in infants those born between 154 to 224 days of gestational age because of maturation from early to adult-like fetal T cells (Kan *et al.*, 2016). Maternal antibodies that passages from the placenta is inversely proportional to gestational age that in the first trimester its level is less and increase in 3rd trimester to 50% (28 to 32 weeks) from nearly 10% (17 to 22 weeks) in 2nd trimester (Camacho-Gonzalez *et al.*, 2013). Therefore, preterm infants are deficient in having a sufficient amount of antibodies to defense against pathogens while term infants have plenty of antibodies gained from mother through

transplacental route to defend against vaccine-preventable infections in other hand spleen of below 2 years age are also not fully matured to counteract with capsulated bacteria leads to susceptible to infections (Zandvoort and Timens, 2002). The composition of breast milk such as IgA, lysozymes, White Blood Cells (WBCs), and lactoferrin settle the commensals like lactobacilli in the intestine and halt the growth of pathogens colonization (Levy, 2007). Finally, in premature babies, the constituents present in breast milk such as IgA, IgG, cytokines, and antibacterial peptides are not transferred in sufficient quantity so that they become susceptible to surrounding pathogens (Camacho-Gonzalez *et al.,* 2013). Infants after contact with various pathogens in several years of life become able to protect from pathogens by their developed adaptive immune mechanism. Thus newborns have to depend on the innate immune mechanism to prevent from infection as compared to adults who have developed immune systems (Kan *et al.,* 2016).

2.4Clinical manifestations and Diagnosis related to Neonatal Sepsis

Neonatal sepsis is highly severe infection therefore early diagnosis is vital by the identification of clinical features. These features depend upon gestational age and condition of severity. Both onsets of sepsis show similar types of clinical manifestations. The level of clinical features is inconsistent based on the type of bacteria, its virulence property and immune defense mechanism of neonates as it has been observed that gram-negative and fungal are more virulent than gram-positive bacterial infection (Simonsen *et al.,* 2014).

Sepsis can be diagnosed both clinically and microbiologically. The clinical prognosis of neonatal sepsis is very complex in especially preterm and Low Birth Weight (LBW) babies due to their immature immune defense mechanism which leads to the wrong diagnosis because of non-specific multiple symptoms in the early stage (Cortese *et al.,* 2016). The clinical presentation has been also observed by Simonsen *et al.,* (2014) and Stefanovic (2011) for the identification of neonatal sepsis.

a) Non-specific signs and symptoms

- ❖ Unstable body temperature, hypotension
- ❖ Respiratory distress, tachycardia or bradycardia, apnoea, grunting, cyanosis, irritability, seizures

- Lethargy, poor cry, rejection to suck, feeding intolerance, abdominal distention, jaundice, petechiae
- Poor perfusion with pallor and mottled skin
- Hypoglycemia, hyperglycemia
- Metabolic acidosis

b) The specific symptoms associated to different systems include:

- **General:** Unstable body temperature, "not doing well", refusal to feed.
- **Respiratory system:** apnea, tachypnea, dyspnea, grunting, nasal flaring, intercostal retractions
- **Central nervous system:** Excessive Cry, extreme Irritability, Coma, Seizures, and Neck Retraction, lethargy, tremors, hyporeflexia, hypotonia, irregular respirations, drowsiness, convulsions.
- **Cardiovascular system:** Hypotension, bradycardia, pallor, mottling, cold, tachycardia and Poor Perfusion, cyanosis, desaturation, reduced capillary refill.
- **Gastrointestinal:** Feed Intolerance, watery stool, Abdominal Distension, Paralytic Ileus, poor sucking, anorexia, regurgitation
- **Hepatic:** Enlargement of liver, Hyperbilirubinemia
- **Renal:** oliguria
- **Hematological:** jaundice, splenomegaly, pallor, Bleeding and Petechiae, Purpura or bleeding.
- **Skin:** Pustules, Umbilical Redness and Discharge, impetigo, cellulitis, abscesses

2.5 Diagnosis

In neonatal sepsis, the newborn presents non-specific signs and symptoms which make the diagnosis very difficult. The supportive laboratory diagnosis also does not give conformation as they are less sensitive and the normal values changes in the neonatal period (Camacho-Gonzalez *et al.,* 2013).

2.5.1 Culture based diagnostics

Culture of causative organism from blood, cerebrospinal fluid (CSF) or urine is the gold standard method for accurative diagnosis for neonatal sepsis. However, the result for confirmation requires 7 days with the sensitivity of being less in case of blood culture because of prior treatment with antibiotics and a small amount of blood volume which also lowers the isolation of pathogens (Chirico *et al.,* 2007).

Blood Culture: The isolation of pathogen by culturing blood is the gold standard method (Goldstein *et al.*, 2005), but the isolation rate is low (36% to 38%) and reporting takes days to confirm. The isolation rate is also depending on the volume of blood, collection procedure, stage of bacteremia, prenatal antimicrobial used, and laboratory facilities (Camacho-Gonzalez *et al.*, 2013).

In developing countries, the majority of the mortality is due to negative results of blood culture (Thaver and Zaidi, 2009). The volume of 0.5 ml blood for culture is not appropriate to isolate pathogens while 1 ml increases the isolation rate by double because of 1-log-higher number of bacteria is present in neonates than adults (Simonsen *et al.*, 2014; Connell *et al.*, 2007). About 25% of culture-positive neonates cannot be isolated from a small volume of blood due to the low burden of organisms (Schelonka *et al.*, 1996). The blood for culture is usually taken from the peripheral vein and occasionally from the umbilical artery (Pourcyrous *et al.*, 1998). The culture results with low volume (0.5) of blood in case of few numbers of bacteria report false negative in 60% cases (Schelonka *et al.*, 1996). Hence, on the basis of negative blood culture report sepsis cannot be excluded as it may be from contamination that results in bacterial growth and manifests as asymptomatic bacteremia (Kellogg *et al.*, 1997).

2.6 Medicinal plants: Alternative to Modern Antibiotics

The phytochemical potential present in plant makes it source for novel drug discovery and its wider applicability in the treatment of various diseases. The systematic screening of medicinal plants may explore for the invention of novel bioactive compounds which play role in the development of newer drugs. Firstly, being the natural source, and secondly, phytochemicals that can be utilized for human well-being. Plant-derived products were

used effectively in a broad range of diseases like in malaria (quinine, artemisinin), morphine, mevastatin, and camptothecin (Maridass and Britto, 2008). Antimicrobial drugs are one of the main therapeutic agents used for the cure of various infectious diseases which provide life to sick people. Emergence of multi-drug resistance in pathogenic organisms has created problems in the field of medical sciences. Wink (2008) has reported that synthetic antimicrobial agent is becoming less efficacious. Scientific communities are now focusing to develop alternative antimicrobial agents from medicinal plants sources. Medicinal plants have different bioactive compounds in the form of secondary metabolites which can be used for the discovery of new therapeutic agents. Secondary metabolites present in plants constitute a marvellous resource for exploring useful drugs. Secondary metabolites present in plants provide protection against different microorganisms which can be utilized in a similar way to prevent infection caused by various organisms (Wink, 2008). The current microbiological procedure displays plants regularly exhibit noteworthy potential against human pathogens. The molecular compounds of the antimicrobial agents reserved in plants belong to different classes of secondary metabolites (Maridass and Britto, 2008; Wink, 2008).

2.7 Antimicrobialpotential of medicinal Plants against human pathogens

Table 2.3 Antimicrobial activity of medicinal Plants against human Pathogenic bacteria

S.N.	Medicinal plants	Bacterial strain	References
1.	*Chassalia curviflora, Cyclea peltata, Euphorbia hirta*	*Staphylococcus aureus* ATCC 6538, *Streptococcus pyogenes, Escherichia coli* ATCC 35218, *Salmonella typhi* MTCC 733, *Proteus vulgaris, Proteus mirabilis*	Raja *et al.*, (2011)
2.	*Leonurus sibiricus, Jodina rhombifolia, Tripodanthus acutifolius, Psittacanthus cuneifolius,*	*Staphylococcus aureus,* coagulase-negative methicillin-sensitive *S. aureus, E. faecalis, E. faecium, K. pneumoniae, E. coli, E. cloacae, Pseudomonas aeruginosa, Acinetobacter freundii, Proteus mirabilis, Serratia marcescens, Morganella morganii*	Soberon *et al.*, (2007)
3.	*Crotalaria retusa, Lophira lanceolata, Crepis cameroonica, Plagiostyles africana, Ochna afzelii, Ouratea flava, Ouratea sulcata, Voacanga africana, Mammea africana, Ouratea elongata*	*S. aureus, S. epidermidis, Enterococcus hirae, Klebsiella pneumoniae, E. coli, P. aeruginosa, C. albicans*	Gangoue-Pieboji *et al.*, (2006)
4.	*Adenia lobata, Campylobacter densiflorum, Picralina nitida, Bridelia micantha, Garcinia kola, Garcinia lucida, Dorstenia picta, Barteria fistulosa, Prunus africanus, Campylobacter excavatum, Campylobacter*	*K. pneumoniae, K. oxytoca, Enterobacter cloacae, Acinetobacter baumannii, S. marcescens, Staphylococcus aureus, Staphylococcus saprophyticus, Enterococcus* spp.,	Gangoue-Pieboji *et al.*, (2009)

	zenkeri, Mallotus oppositifolius		
5.	*Thespesia populnea*	*Staphylococcus aureus, Klebsiella aerogenes, E. coli, P. aeruginosa, Bacillus subtilis, C. albicans, Aspergillus niger*	Senthil-Rajan *et al.*, (2013)
6.	*S. americana, B. pilosa, B. orellana, C. G. sepium, peltata, C. officinalis, J. mimosifolia, J. secunda, P. pulchrum, P. paniculata*	*S. aureus* ATCC 29737, *Streptococcus* β *hemolytic* ATCC 10389, *P. aeruginosa* ATCC 25619, *E. coli* ATCC 10536, *B. cereus* ATCC 14603, *C. albicans* ATCC 53324	Rojas *et al.*, (2006)
7.	*Cissus aralioides, Hileria latifolia, Piptadeniastum africana, Phyllanthus muellerianus, Gladiolus gregasius*	*S. aureus, Enterococcus faecalis, S. typhi, P. aeruginosa, Shigella flexneri, E. coli, K. pneumoniae, Proteus mirabilis, C. albicans, Candida krusei*	Assob *et al.*, (2011)
8.	*Swertia chirata*	*S. aureus, Bacillus cereus, B. megaterium, B. subtilis, Sarcina lutea, P. aeruginosa, Salmonella typhi, S. paratyphi, Shigella boydii, S. dysenteriae, Escherichia coli, Vibrio mimicus, Vibrio parahaemolyticus, Candida albicans, Aspergillus niger, Saccharomyces cerevisiae*	Alam *et al.*, (2009)
9.	*Syzygium jambos, Pityrogramma calomelanos, Tapeinochilus ananassae, Gossypium barbadense,*	*S. aureus* ATCC 6341, *S. saprophyticus* ATCC 15305, *Escherichia coli* ATCC 4157, *P. aeruginosa* ATCC 7700, *Haemophilus influenzae* ATCC 8142, *P. vulgaris* ATCC 6896, *C. albicans* ATCC 752	Luciano-Montalvo *et al.*, (2013)
10.	*Syzygium cumini*	*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 9341, *Enterococcus faecalis* ATCC 9763, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 29212, *Neisseria*	Mohamed *et al.*, (2013)

		gonorrhoeae ATCC 11778, *Bacillus subtilis* ATCC 12228.	
11.	*Lawsonia inermis*	*S. aureus*, *S. epidermidis*, *Klebsiella* spp., *E. coli*, *Salmonella typhi*, *Shigella sonnei*, *Bacillus subtilis*	Gull *et al.*, (2013)
12.	*Arbutus andrachne, Chrysanthemum coronarium, Punica granatum, Origanum syriacum, Rosmarinus officinalis, Inula viscosa*	*Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans*	Assaf *et al.*, (2016)
13.	*Bauhinia purpurea*	*S. aureus, Bacillus subtilis, E. coli, Salmonella typhi, P. aeruginosa, C. albicans*	Negi *et al.*, (2012)
14.	*Alkanna tinctoria*	*S. aureus, E. coli, Acinetobacter baumannii, P. aeruginosa*	Khan *et al.*, (2015)
15.	*Aglaia odorata, Baeckea frutescens, Cannabis sativa, Cassytha filiformis, Cinnamomum camphora, Citrus grandis, Clematis vitalba, Cratoxylum formosum ssp. Pruniflorum, Euphorbia hirta, Pedilanthus tithymaloides, Pluchea indica, Pogostemon cablin*	*Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *B. cereus* ATCC 21768	Vu *et al.*, (2016)
16.	*Rhamnus prinoides*	Reference and Clinical strain of *E. coli* ATCC 25922, *P. aeruginosa*	Molla *et al.*,

		ATCC 27853, *S. aureus* ATCC 25923, *Shigella flexneri* ATCC 12022, *S. pneumoniae* ATCC 49619, *Streptococcus pyogenes* ATCC 19615, *Salmonella typhi* ATCC 13062	(2016)
17.	*Alstonia boonei, Cassia obtusifolia, Ageratum conyzoides, Paullinia pinnata, Croton macrostachys, , Catharanthus roseus*	ATCC and clinical MDR strains of *Staphyloccocus aureus, K. pneumoniae, Enterobacter aerogenes, E. cloacae, E. coli, P. aeruginosa, Providencia stuartii*	Voukeng *et al.*, (2016)
18.	*Nyctanthes arbortristis*	*S. aureus, E. coli, P. aeruginosa, Proteus vulgaris, Salmonella typhimurium*	Jain and singh (2013)
19.	*Aloe buettneri, Alchornea floribunda, Crinumpurpurascens, Euphorbia prostrata, Markhamia tomentosa, Viscum album, Rauwolfia macrophylla*	*Staphylococcus aureus, K. pneumoniae, E. coli, Enterobacter aerogenes, E. cloacae, P. aeruginosa, Providencia stuartii*	Voukeng *et al.*, (2017)
20.	*Gymnema sylvestre*	*S. aureus* MTCC740, *S. epidermidis* MTCC 435, *Enterococcus faecalis* MTCC 439, *E. coli* MTCC 119, *K. pneumoniae* 1 MTCC 109, *K. pneumoniae* 2 MTCC 530, *Pseudomonas aeruginosa* MTCC 741, *S. typhimurium* 1 MTCC 98, *S. typhimurium* 2 MTCC 1251, *Shigella flexneri* MTCC 1457, *C. albicans* MTCC 227, *C. tropicalis* MTCC 230, Methicillin-resistant *S. aureus* (MRSA) clinical isolate	Arora and Sood (2017)
21.	*Cleome spinosa*	*S. aureus, S. epidermidis, S. mutans, Enterococcus faecalis, E. coli, Klebsiella pneumoniae, P. aeruginosa, P. mirabilis,*	Sant's Anna da Silva *et al.*,

		Salmonella enteritidis, *Bacillus subtilis*, *Micrococcus luteus*, *Myobacterium smegmatis*, Candida species	(2016)
22.	*Olea africana, Psidium guajava, Vernonia amygdalina, Lantana camara, Mangifera indica*	*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853	Cheruiyot *et al.*, (2009)
23.	*Punica granatum*	*Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* (MRSA), *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 9027, *Yersinia enterocolitica* ATCC 23715, *Candida utilis* Y-1084, *Saccharomyces cerevisae* Y- 139, *Aspergillus niger* (food isolate)	Al-Zoreky (2009)
24	*C. longa, C. molmol, P. anisum, Z. officinale*	*Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Clinical isolate)	Al-Daihan *et al.*, (2013)
25.	*Allium wallichii*	*B. cereus, B. subtilis, B. thuringiensis, E. coli, P. aeruginosa, P. mirabilis, Rhizopus, A. flavus*	Bhandari *et al.*, (2017)
26.	*Moringa oleifera*	*Staphylococcus aureus* and *Escherichia coli*	Surendra *et al.*, (2016)
27.	*Solanum nigrum and Solanum xanthocarpum*	*Staphylococcus aureus* ATCC NO. 25923, *Micrococcus varians* ATCC No. 9341, *Micrococcus luteus* ATCC No. 9342, *Klebsiella pneumoniae* ATCC NO. 700721, *E. coli* ATCC No. 25922, *S. typhi*	Abbas *et al.*, (2014)

		ATCC No.19430, *Pasteurella maltocida* ATCC No. 51687, *Vibrio cholerae* ATCC No. 39541, *Aspergillus fumigatus* ATCC No. 204305, *Aspergillus niger* ATCC No. 16404, *Aspergillus flavus* ATCC No. 204304	
28.	*Tinospora cordifolia*	*Escherichia coli, Staphylococcus aureus*	Singh *et al.*, (2015)
29.	*Rhus coriaria, Sacropoterium spinosum, Rosa damascena*	*Pseudomonas aeruginosa*	Adwan *et al.*, (2010)
30.	*Syzygium cumini*	*S. aureus* MTCC-96, *Bacillus cereus* MTCC-1305, *Enterobacter faecalis* MTCC-5112, *Klebsiella pneumoniae* MTCC-109, *E. coli* MTCC-729, *Salmonella paratyphi* MTCC-735, *P. aeruginosa* MTCC-647, *Proteus vulgaris* MTCC-426, *Serratia marcescens* MTCC-86	Reddy and Jose (2013)
31.	*Asparagus falcatus, Asteracantha longifolia, Coriandrum sativum, Epaltes divaricata, Vetiveria zizanioides*	*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 700603	De zoysa *et al.*, (2019)
32.	*Rhamnus californica, Umbellularia californica*	*S. aureus* ATCC 25923, methicillin-resistant *S. aureus* (MRSA) ATCC BAA-1683, *S. pyogenes* ATCC 10782, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 10145, *Bacillus cereus* ATCC 14579, *Mycobacterium smegmatis* ATCC 10143	Carranza *et al.*, (2015)

33.	*Azadirachta indica*	*S. aureus, E. coli, P. aeruginosa, P. vulgaris*	Al-jadidi *et al.*, (2015)
34.	*Arnica montana* (10%, w/v) and propolis	*S. aureus* ATCC 25923, *Enterococococcus faecalis* ATCC 29212, *S. sanguis* ATCC 10556, *Streptococcus mutans* OMZ-175, *Streptococcus cricetus* HS-6, *S. mutans* Ingbritt 1600, *S. sobrinus* 6715, *Candida albicans* NTCC 3736, *C. albicans* F72, *Actinomyces naeslundii* ATCC 12104, *A. naeslundii* W1053, *A. viscosus* OMZ 105, *Porphyromonas gingivalis*, *P. endodontalis*, *Prevotella denticola*.	Koo *et al.*, (2000)
35.	*Nyctanthes arbortristis*	*V. mimicus, S. typhi, S. dysenteriae, S. aureus, B. serus*.	Saha *et al.*, (2012)
36.	*Allium sativum, Baccharis trimera, Cymbopogon citratus, Mikania glomerata, Psidium guajava, Mentha piperita, Zingiber officinale, Syzygium aromaticum*	Strains of *Staphylococcus aureus*	Betoni *et al.*, (2006)
37.	*Prangos uloptera*	*Staphylococcus aureus* PTCC 1112, *Staphylococcus epidermis* PTCC 1114, *Escherichia coli* PTCC 1047, *Bacillus subtilis* ATCC 1207	Razavi *et al.*, (2010)
38.	*Nyctanthes arbortristis*	*S. aureus, E. coli, K. pneumoniae, P. aeruginosa*	Priya and

			Ganjewala (2007)
39.	*Saliva caespitosa*	S. *aureus* ATCC 6538, S. *epidermidis* ATCC 12228, *B. subtilis* ATCC 6633, *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 4352, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 27853, *P. mirabilis* ATCC 14153, *C. albicans* ATCC 10231	Ulubelen *et al.*, (2001)
40.	*Nyctanthes arbortristis*	*Staphylococcus aureus* MTCC 3160, *Escherichia coli* MTCC 1652, *P. aeruginosa* MTCC 647, *Fusarium culmorum* MTCC 349, *Rhizopus stolonifer* MTCC 2591, *Aspergillus niger* MTCC 282, *A. flavus* MTCC 2456.	Vyas and Sarin (2013)
41.	*Punica granatum*	*Staphylococcus aureus* ATCC 12600, *Klebsiella pneumoniae* ATCC 13883, *E.coli* ATCC 11775, *Bacillus subtilis* ATCC 6051	Fawole *et al.*, (2012)
42.	*Achillea millifolium, Caryophyllus aromaticus, Ocimun basilucum, Melissa offficinalis, Psidium guajava, Rosmarinus officinalis, Punica granatum, Salvia officinalis, Syzygyum joabolanum, Thymus vulgaris*	*S. aureus* ATCC 6538, *Staphylococcus aureus, K. pneumoniae, E. coli, Salmonella choleraesuis* ATCC 10708, *P. aeruginosa* ATCC 15442, *Bacillus subtilis, Candida albicans, Proteus* spp., *Shigella* spp., *P. aeruginosa, Enterobacter aerogenes*	Nascimento *et al.*, (2000)
43.	*Albizia procera, Acalypha fruticosa, Cassia auriculata, C. alata, C. fistula, Diospyros*	*S. aureus* ATCC 25923, *S. epidermidis* MTCC 3615, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 441,	Duraipandiyan *et al.*, (2006)

	ebenum, Diotacanthus albiflorus, Elephantopus scaber, Peltophorum pterocarpum, Punica granatum, Olax scandens, Pavetta indica, Pterolobium hexapetalum Syzygium cumini, Syzigium lineare, Solanum xanthocarpum, Tabernaemontana heyneana, Toddalia asiatica	*K. pneumoniae* ATCC 15380, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Ervinia* spp. MTCC 2760, *P. vulgaris* MTCC 1771, *Candida albicans* MTCC 227	
44.	*Tridax procumbens*	*Bacillus cereus, B. subtilis, Mycobacterium smegmatis, S. aureus, S. epidermidis, Escherichia coli, Klebsiella* spp., *P. vulgaris, P. aeruginosa, Salmonella* group C, *S. paratyphi, Shigella sonnei*	Taddei and Rosas-Romero (2000)
45.	*Swertia chirata*	*S. aureus* MTCC 3160, *S. pyogenes* MTCC 1927, *Bacillus subtilis* MTCC 736, *Bacillus polymyxa, E. coli* MTCC 723, *S. typhi* MTCC 3216, *Vibrio cholerae* MTCC 3906, *P. mirabilis* MTCC 1429, *P. aeruginosa* MTCC 7837, *Providentia alkalifaciens, Aspergillus niger* MTCC 1881, *A. flavus* MTCC 1883, *Cladosporium oxysporum* MTCC 1777	Ahirwal *et al.*, (2011)
46.	*Pistacia Integerrima, Swetia chirita, Polygonum Bistorta, Zingiber officinale*	*S. aureus* ATCC 6538, *Entereococcus faecalis* ATCC 14506, *Bacillus subtilis* ATCC 6633, *P. aeruginosa* ATCC 27853 and *S. typhi* ATCC 14028	Khalid *et al.*, (2011)

47.	*Swertia chirata, Terminalia bellerica and Zanthoxylum armatum*	*Acinetobacter baumanii, Aeromonas hydrophila, Staphylococcus epidermidis, Streptococcus saprophyticus, Streptococcus pyogenes, Streptococcus fecalis, Bacillus cereus, B. subtilis, B. thruingiensis, Corynebacterium diptheriae, C. hofmanii, C. xerosis, Campylobacter jejuni, Campylobacter coli, Enterobacter aerogenes, E. coli* ATCC 8739, *E. coli* multi drug resistance, *Helicobacter pylori, Hemophilus influenzae, Klebsiella pneumoniae, Salmonella typhi, S. paratyphi A, S. paratyphi B, Shigella dysenteriae, Serratia marcesens, Vibrio cholerae*	Wazir *et al.*, (2014)
48.	*Swertia cordata and Swertia chirayita*	*Staphylococcus aureus* MTCC 96, *Bacillus megaterium* MTCC 8510, *B. subtilis* MTCC 441, *B. flexus* MTCC 7024, *Clostridium perfringens* MTCC450, *Escherichia coli* MTCC 723, *K. pneumoniae* MTCC 7028, *Lactobacillus rhamnnosus* MTCC 1423, *Pseudomonas oleovorans* MTCC 617, *Salmonella enteric* MTCC 1164	Roy *et al.*, (2015)
49.	*Curcuma longa, Tinospora cordifolia, Zingiber officinale*	*S. aureus, Bacillus subtilis, E. coli, Klebsiella pneumoniae, P. aeruginosa, P. mirabilis*	Chakraborty *et al.*, (2014)
50.	*Argemone mexicana, Caesalpinia coriaria, Euphorbia tirucalli, Phyllanthus niruri, P. amarus, Tribulus terrestris, Leucas aspera, T. cordifolia, Decalepis hamiltonii*	*S. aureus* MTCC 737, *S. faecalis* MTCC 459, *E. coli* MTCC 443, *K. pneumoniae* MTCC 109, *P. mirabilis* MTCC 1429, *P. aeruginosa* MTCC 1688, *Salmonella typhi* MTCC 733, *Salmonella typhimurium* MTCC 98, *Salmonella paratyphi* A MTCC 735,	Mohana *et al.*, (2008)

		Shigella sonnei MTCC 2957, *Shigella flexneri* MTCC 1457	
51.	*Hemidesmus indicus, Ocimum sanctum, Tinospora cordifolia*	*S. aureus, Bacillus subtilis, E. coli, K. pneumoniae*	Rao *et al.,* (2013)
52.	*Tinospora cordifolia*	*Bacillus fusiformis, Bacillus cereus, Klebsiella pneumoniae, Escherichia coli*	Agnihotri *et al.,* (2012)
53.	*Moringa oleifera*	*S. aureus* ATCC 25923, *Enterococcus. Faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. enteritidis* (IH), *V. parahaemolyticus, A. caviae*	Peixoto *et al.,* (2011)
54.	*Curcuma xanthorrhiza, Ocimum sanctum, Senna alata, Kaempferia pandurata, Zingiber officinale, Moringa Oleifera, Tamarindus indica* and *Pangium edule.*	*S.. aureus* (MRSA), ESBL and carbapenemase-resistant Enterobacteriaceae	Wikaningtyas *et al.,* (2016)
55.	*Moringa oleifera*	*S. aureus, E. coli, P. aeruginosa,*	Singh (2013)
56.	*Moringa oleifera*	*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *K. pneumoniae* NCTC 13368, *P. aeruginosa* ATCC 27853	Singh and Sharma (2012)
57.	*Moringa oleifera, Passiflora edulis Xanthosoma mafaffa,*	*E. coli, K. pneumoniae* (ATCC11296, KP55, KP63, K24), *Enterobacter aerogenes* (ATCC13048, EA289, EA27, EA298, CM64), *Pseudomonas aeruginosa* (PA01, PA124), *Providencia stuartii* (ATCC29914, NEA16)	Dzotam *et al.,* (2016)
58.	*Moringa oleifera*	*S. aureus, S. albus, Enterococcus faecalis, E. coli, Klebsiella*	Osman *et al.,*

		pneumoniae, P. aeruginosa, Providencia spp., *Yersinia enterocolitica, Salmonella* spp, *Shigella* spp., *Candida albicans, Aspergillus flavus, Aspergillus niger*	(2015)
59.	*Artemisia ludoviciana, Achillea millefolium, Berberis vulgaris, Castela emoryi, Chenopodium ambrosioides, Commiphora molmol, Equisetum hyemale, Echinacea purpurea, Galium aparine, Glycyrrhiza glabra, Leucophyllum frutescens, Matricaria chamomilla, Pimenta dioica, Rosmarinus officinalis, Salvia farinacea, S. greggii, S. leucantha, S. officinalis, S. sinaloensis, Spilanthes acmella, Tagetes lucida, Uncaria tomentosa, Zea mays.*	*S. aureus, E. coli, P. aeruginosa*	Romero *et al.*, (2005)
60.	*Azadirachta indica*	*S. typhi* and *S. paratyphi*	Akeel *et al.*, (2015)
61	*Ajuga reptans, Capsella bursa-pastoris, Galium spurium, Genista Lydia, Phlomis pungens, Marrubium astracanicum,*	*Staphylococcus aureus* ATTC 25923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogenes* ATTC 19615, *Klebsiella pneumoniae* ATTC 13883, *Enterobacter cloacae* ATCC 23355,	Yildirim *et al.*, (2012)

	Mercurialis annua, Nepeta nuda, Onosma heterophyllum, Stachys annua, Nuphar lutea, Nymphaea alba, Stellaria media, Reseda luteola, Viburnum Lantana, Vinca minor	*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *Serratia marcescens* ATCC 8100, *Proteus vulgaris* ATCC 13315	
62	*Azadirachta indica, Calendula officinalis, Camellia sinensis, Psidium guajava*	*Staphylococcus aureus, Klebsiella* spp., *Escherichia coli*, *Salmonella* spp., *Pseudomonas* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*	Farjana *et al.*, (2014)
63	*Baccharis boliviensis, Chuquiraga atacamensis, Chiliotrichiopsis keidelii, Fabiana bryoides, Frankenia triandra, Fabiana densa, F. punensis, Parastrephia lucida, P. phyliciformis, P. lepidophylla, Tetraglochin cristatum*	*S. aureus, Enterococcus faecalis, E. coli, K. pneumoniae, Proteus mirabilis, Enterobacter cloacae, Morganella morganii, P. aeruginosa, Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 35218, and *K. pneumoniae* ATCC 700603	Zampini *et al.*, (2009)
64.	*Azadirachta indica, Saraca asoca* and *Curcuma longa*	*E. coli, B. subtilis, Aspegillus niger, Aspergillus fumigatus*	Lall *et al.*, (2013)
65.	*Albizia odoratissima*	*Staphylococcus aureus* (E40), *K. pneumoniae* (U98), *E. coli* (E72), *P. aeruginosa* (3023), *Proteus vulgaris* (2266)	Banothu *et al.*, (2017)
66.	*Azadirachta indica, Murraya koenigii*	*Streptococcus mutans* MTCC 497, *S. gordonii* MTCC 2695, *Pseudomonas aeruginosa* MTCC 1688	Bhuva and Dixit (2015)

67.	*Acacia nilotica, Brassica campestris, Cynodon dactylon, Emblica officinalis, Psidium guajava, Rosa indica, Terminalia chebula, Piper nigrum, Foeniculum vulgare, Lawsonia inermis, Mangifera indica, Ocimum sanctum, Myristica fragrans, Sesamum indicum, Ziziphus mauritiana, ,*	*S. aureus* NCDC 109, *S. aureus* MTCC 3160, *K. pneumoniae* MTCC 4030, *Escherichia coli* MTCC 1885, *P. aeruginosa* MTCC 7453, *P. vulgaris* MTCC 426, *Bacillus cereus* MTCC 430	Bai *et al.*, (2015)
68.	*Azadirachta indica*	*S. aureus* ATCC 6538p, *Bacillus subtilis* ATCC 6633, *E. coli* ATCC 8739, *Micrococcus luteus* ATCC 9341, *Pseudomonas aeruginosa* ATCC 9027, *S. typhiymurium* ATCC 14028, *Saccharomyces cerevisae* ATCC 2601, *Candida tropicalis* ATCC 1641, *Candida albicans* ATCC 10231	Alves *et al.*, (2009)
69.	*Andrographis paniculata, Centella asiatica, Morinda citrifolia, Piper sarmentosum, Vitex negundo*	*Staphylococcus aureus* (MRSA), *S. aureus, Klebsiella pneumoniae, E. coli, P.aeruginosa*	Zaidan *et al.*, (2005)
70.	*Anethum graveolens, Foeniculum vulgare* and *Trachyspermum ammi*	*S. aureus* MTCC 96, *Enterococcus faecalis* MTCC 439, *K. pneumoniae* 1 MTCC 109, *Klebsiella pneumoniae* 2 MTCC 530, *E. coli* MTCC 119, *P. aeruginosa* 1 MTCC 647, *P. aeruginosa* 2 MTCC 741, *S. typhimurium* 2 MTCC 1251	Kaur and Arora (2009)

2.8 Extraction Methods Use in Medicinal Plants

Plant derived medicinal products have fulfilled the basic health care needs in developing and developed countries. These products are in great demand because of their efficacy, cost-effective and natural in origin (Zhang *et al.,* 2018). Nature has provided various medicinal plants as a boon to human and just nearly 20% of the studied plants are used in the preparation of different drugs for the health care systems to treat infectious (Hepatitis, AIDS, Malaria) and non-infectious diseases (Diabetes, cancer) (Altemimi *et al.,* 2017). In recent times, plant-related medicinal products are becoming popular globally because of their easy availability in the local area, nature gift, cheap cost, easy to consume, less side effects and uses in crude or extract form. Moreover, extraction of these phytochemicals involves appropriate solvents and standard extraction procedure (Pandey and Tripathi, 2014; Azwanida, 2015; Ingle *et al.,* 2017).

2.8.1 Pre-extraction processing of plant samples: The initial steps for the processing of medicinal plants extraction is crucial as it preserves the active biomolecules present in the plant having biological activities. The parts of medicinal plants like leaves, fruits, flowers, barks, roots and stems is used for extraction either in fresh or as dried plants or parts (Azwanida, 2015).

The size of the plant materials is important as small size increases the area of contacts between the solvents and plant materials which results in the efficient extraction of plant metabolites (Azwanida, 2015). Plant materials are utilized in the fresh and dried form but generally dried sample is ideal (Azwanida, 2015). The extraction process from fresh plant materials need precaution that it should be processed within 3 hours after harvesting to prevent it from deterioration compared to dried samples (Vongsak *et al.,* 2013). The size of plant materials determines the extraction yield of medicinal plants. Coarse grinded plant particles have less contact area than the powdered sample resulting in less extraction with larger particle in comparison to smaller particles less than 0.5 mm size. These small particles ideally mix with solvent homogeneously as it has more surface area and extracts the biomolecules efficiently (Azwanida, 2015). Different drying methods are used to dried the medicinal plants/ parts for the extraction purpose like Air drying, Sun drying, Shade

Drying, Artificial heat, Microwave-drying (Azwanida, 2015; WHO, 2018), Oven-drying (Mediani *et al.,* 2013), and Freeze-drying (Azwanida, 2015).

The selection of solvent or menstrum is a pivotal step in the extraction of phytochemicals from medicinal plants. Selection of menstrum is important in which targeted metabolites from the plant materials are extracted in appropriate solvents with high solubility of bioactive molecules. Menstrum should be cost-effective and safe to use. The selection of solvent is based on the law of similarity that like dissolve likes i.e. Polar compounds of the solutes will extract more efficiently with nearly polar solvents and vice versa (Zhang *et al.,* 2018).

Table 2.4: Menstrum used for extraction of active component

Water	Ethanol	Methanol	Chloroform	Ether	Acetone
Tannins Terpenoids Lectins Anthocyanins Starches Saponins Polypeptides	Alkaloids Polyacetylenes Terpenoids Sterols Tannins Polyphenols Flavonols	Phenones Polyphenols Quassinoids Terpenoids Saponins Tannins Xanthoxyllines Totarol Lactones Flavones	Terpenoids Flavonoids	Coumarins Alkaloids Fatty acids Terpenoids	Phenol Flavonols

Adapted from Cowan, (1999)

2.8.2 Extraction:

In the process of extraction, soluble components including secondary metabolites of plant material dissolves in menstrum and separates the insoluble components and cellular matrix. Each and every extracted concentrated component has different metabolites with different functional abilities (WHO, 2018). Different extractions techniques are applied for the extraction of secondary metabolites present in plants were shown in Table 2.5.

Table 2.5: Different extraction methods used for Medicinal plants

Conventional extraction techniques		
Maceration	In this method plant materials are dissolved for certain time without application of heat in respective solvent that dissolve the soluble components of plants.	WHO (2018)
Decoction	Plant material is boiled for about 15 minutes to extract water soluble components from roots and barks that withstand at high temperature.	Pandey and Tripathi (2014)
Infusion	Selected herb or herbal components are mixed with boiled water and left for 5-20 minutes to form dilute liquid preparation.	WHO (2018)
Percolation	This method requires large amount of solvent as material is soaked in extraction solvent and then with the help of additional solvent is allowed to collect the extract through percolater dropwise. It is generally used to extract active components for tinctures and fluid extract preparation. It is applicable for small and large-scale extraction.	Salam *et al.*, (2019)
Reflux extraction	This is the heat applicable technique usually performed in round bottom flask in which condenser condenses evaporating solvents. It is not suitable for heat sensitive components.	Salam *et al.*, (2019)
Soxhlet extraction	In this method plant material is kept in thimble and extracting solvent of round flask (connected with soxhlet apparatus) is heated. The solvent vapours condense and drip into thimble upto siphon arm. After that solvent is released from bottom and cycle (fill/ siphon) is repeated.	Salam *et al*; (2019); Azwanida (2015)
Hydro distillation	This method is carried with water as solvent packed with plant materials in still compartments along with boiling. Vapour released will condense as mixtures of oil and water which can be separated in separator automatically in oil and bioactive compounds.	Silva *et al.*, (2005)

2.9 Qualitative Phytochemical Screening of Secondary Metabolites

Qualitative screening of phytochemicals is the procedure to determine the different classes of phytochemicals present in the plant. The phytochemical contents are different in individual plants and their associated parts. The identification of phytochemicals present in the plant is carried out by different chemical methods which are rapid and cost-effective. The different classes of phytochemicals have different biological properties so that it is easy to move in the right path of research (Mera *et al.,* 2019). Different methods applied for qualitative detection of secondary metabolites are shown in Appendix VII.

2.10 Antimicrobial Screening

Natural products are important resources for the discovery of novel drug molecules. Medicinal plants and other sources of nature have evolved various complex and diverse groups of compounds as potential antimicrobial agents. Different methods have been applied to assess the antimicrobial activity of extract or purified compounds by different scientific researchers. Different standard methods used for the evaluation of antimicrobial activity have been shown in Table 2.6.

2.11 Identification of Bioactive Compounds from Plant Extract

The compounds present in the extract can be identified with different procedures such as UV-visible spectroscopy, Infrared spectroscopy (IR), Mass spectroscopy, Nuclear Magnetic Resonance (NMR). The spectroscopy is based on the fundamental standard of passing electromagnetic radiation through an organic molecule that quantitatively absorbs radiation. The amount of electromagnetic radiation is measured to develop a spectrum. On the basis of developed spectra, the structure of organic molecules present in the extract can be identified (Popova *et al.,* 2009).

Table 2.6: Methods for evaluation of antimicrobial activity

S.N.	Methods	Types	Strength	Limitation	References
1.	Diffusion Methods	(Agar disc-diffusion method) Kirby Bauer disc method	This method is simple and cheap so commonly used for antimicrobial screening of plant extract, clinical samples and other drugs.	This method is not suitable for assess of minimum inhibitory concentration (MIC)	Balouiri *et al.*, (2016)
2.	Additional diffusion Methods	Agar well diffusion method	This method is extensively used to assess antimicrobial potential of plants or other microbial extracts for its antimicrobial potential.	In this method the amount and distribution of inoculum and incubation temperature can influence the inhibition zone.	Balouiri *et al.*, (2016)

Mass Spectrometry (MS): This analytical method is used to find out the mass of the compounds present in the sample. In mass spectrometry electrons or lasers are passed through organic molecules thereby changes to highly energetic charged ions which go through the collector under the activity of electric and magnetic fields. The X-axis ordinate for the mass per charge ratio (m/z) of the compounds and Y-axis symbolize for the relative abundance of fragmented ion in spectrum plot (Altemimi *et al.,* 2017; Feng *et al.,* 2019). The analysis of spectrum plot of comparative molecular mass (molecular weight) can be obtained with high accuracy along with exact molecular formula on the basis of fragmented place of the molecules (Christophoridou *et al.,* 2005). Mass spectrometry, when applied with tandem mass spectrometry, gives a lot of information on the structure of compounds. As a result, HPLC and MS provide quick and exact identification of chemical compounds present in medicinal plant extract, particularly in the case where the pure standard is unavailable. In recent times, LC/MS spectroscopic technique is broadly used for the identification of bioactive compounds in medicinal plants. Electrospray ionization (ESI) is an ideal source because of its high ionization efficiency in respect to the identification of phenolic compounds (Altemimi *et al.,* 2017). Mass spectrometry based on ion sources categorized into different types as chemical ionization mass spectrometry, electron impact mass spectrometry, fast atom bombardment mass spectrometry, field desorption mass spectrometry, others are matrix-assisted laser desorption mass spectrometry and tandem mass spectrometry, electrospray ionization mass spectrometry, and so on (Feng *et al.,* 2019).

2.12 Herbal Formulation: Doses form and Standardization

The word “herbal drug” indicates whole plant or individual parts of plants utilized for therapeutic purposes. Single medicinal plant or combination of some parts of the same plant or combination of different parts of the different plants are utilized for various biological activity in the form of Hima (cold infusion), Phanta (Hot infusion), Kwatha (Decoction), Arka (Liquid extract), Guggul (Resins and Balsams), Churna (Powders), Taila (Medicated oil), and in other various forms (Frawley and Ranade, 2000). Different approaches for drug discovery are applied for medicinal plants are: Selection of plants, chemical screening, and biological assays followed by biological activity (Ogbonna *et al*., 2012).

2.12.1 Doses forms: All medicinal plant/s used for therapeutic purposes have a certain fixed amount of form (Dose) prescribed to the diseased person to take action on the target site in the body for the cure. The different form of drugs are administered by different route generally are oral, parenteral, rectal, nasal, ophthalmic, otitic, respiratory, topical, and other routes for prescription of herbal dosage forms may include oral, rectal, topical, injectable liquids, respiratory, nasal, eye solution/ drops, and ear drops (Aulton, 2007). The new herbal products have to follow different experimental procedures like *in vivo*, stability testing, quality control, etc. to define the dose of finished products. Medicinal plants oriented finished herbal products contain active biomolecules of a single plant or in the combination of different plants in the raw state or as plant preparation (WHO, 2006).

Syrups: Syrups are liquid doses form of herbal products. These are prepared by application of different methods like decoctions, infusions, watery dissolved solution, fermented liquors, and others. Preparation of syrup needs a preservative to limit or absent the microbial growth. Honey and sugar are used to fade the unpleasant taste as well as a preservative to syrup with addition to other preservatives such as sodium methylparaben, Sodium propylparaben, Sodium benzoate, and others. Syrups are formulated by the addition of sugar to the active extract obtained by application of different methods following the standard guidelines. The extract obtained mix with solvent and other excipients in different ratios as an experimental study. Syrup can be developed in different flavors and types like simple syrup, raspberry syrup, tolu syrup, etc (Ogbonna *et al.,* 2012).

2.12.2 Standardization:

New Herbal formulations or preparations need to follow standard guidelines to standardize the formulations to evaluate the quality of drugs according to the incorporation of active ingredients, their physiochemical parameters, experimental parameters (*in vitro* and *in vivo*) before launching to market. The quality judgment of herbal preparation is vital for the acceptability in the modern medicine system (Madhav *et al.,* 2011). Herbal preparations are encouraged by World Health Organization (WHO) because of its easily available, cost-effective, safe, less side effects and faithful to people (WHA, 2003). Ministry of Ayush, India has developed General Guidelines for Drug Development of Ayurvedic

Formulations (GGDDAF) for the standardization of herbal products has been shown in Table 2.7 (GGDDAF, 2018) that has to comply before launching the drugs to consumers.

Table 2.7: Parameters for standardization

METHODS	**EVALUTION PARAMETERS**
1. Authentication	Collected parts of plants, Regional, Family, Biological source, Chemical composition
2.Morphology or Organoleptic evaluation	Taste, Odour, colour, Size, Shape, Special aspect like oral acceptability
3. Microscopy evaluation	Stomata, Leaf content, Trichome Quantitative microscopy
4. Chemical evaluation	Test for Lead, Cadmium, Mercury, Arsenic (Limits as per ASU Pharmacopoeia), Chemical assay, Phytochemical screening, Chromatographic profiles: TLC or HPTLC/HPLC/LC-MS.
5. Physical evaluation	Viscosity, Solubility, Moisture content, Ash value, Melting point, Optical rotation, Extractive value, Refractive index, Volatile oil content, Foreign matter etc.
6. Biological evaluation	A. Microbial contamination Total viable aerobic count Members of Enterobacteriaceae Total fungal count (confines as per ASU Pharmacopoeia) Test for specific pathogen *Staphyloccocus aureus, E. coli, Salmonella* spp., *P. aeruginosa* (confines as per ASU Pharmacopoeia) B. Aflatoxins (confines as per ASU Pharmacopoeia) (Bi, B2,Gi G2) C. Pesticides contamination Organo chlorine pesticides plus organophosphorus pesticides and pyrethroids should be in restrictions as per ASU Pharmacopoeia) D. Pharmacological activity of drugs

A: Ayurveda, S: Siddha, U: Unani

2.12.3 *In-vivo* Study

Toxicity is the evaluation of materials which when exposed to the living body determines the effect on a specific part (cell, tissue, or organ) or to the whole body (Denny *et al.*, 2013). Therefore, worldwide approved scientific standard methodology need to be followed for authorization of toxicological report of medicinal plants formulated as ayurvedic products. Globally accepted guidelines for toxicity studies of ayurvedic products and chemicals in laboratory animals were conducted by following the protocol of the Organization for Economic Co-operation and Development (OECD) test procedure which provides a practical approach with universal acceptance and reliability of the brand. There are different stages of guidelines to be followed for evaluation of the toxic effect of chemicals or therapeutic agents that may exhibit risk to humans and animals. These are as below:

Acute toxicity testing

This test is performed to observe the effect of chemicals in a single dose of administration on experimental animals. Acute toxicity testing can be performed in different experimental animals like rodents and mice of different species. In this testing, the chemicals which are carried out for toxic effect is administered at different concentration dose to a selected group of animals as per guidelines and the consequences are noticed for 14 days. This test is done to determine the 50% lethal dose (LD_{50}) of the chemicals that give the further selection of test. The previously large number of animals is being involved for testing the LD_{50} lethal dose and the results in high mortality. The limitation leads to the development of the following standard guidelines:

Table 2.8: Acute toxicity study guidelines

Method	Principle	Procedure
Fixed dose procedure OECD 420	The dose level is selected on the basis of a sight seen study as the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality. Further groups of animals may be dosed at higher or lower fixed doses, depending on	In this test procedure groups of single sex animals are administered with fix doses of increasing concentration in step wise manner of 5, 50, 300 and 2000 mg/kg.

	the presence or absence of signs of toxicity or mortality.	
Acute toxic class OECD 423	This test principle deals with observation of toxic effect at a particular dose generally 300 mg/kg body weight and proceeds further steps after result of mortality.	This test procedure performed with group (n=3) of animals in previously defined dose of 5, 50, 300 and 2000 mg/kg body weight.
Up and down Procedure OECD 425	The test principle deals with administered of single dose once at 48 hours gap. The first dose is based on the evaluation of LD_{50}. After dose of administration, if the animal become live then dose to next animal is increased by factor of 3.2 and if death, the dose is decreased by the same factor.	The procedure can be performed with the doses in the sequence of increasing dose factor as 1.75, 5.5, 17.5, 55, 175, 550, 1750, 2000 or 5000 mg/kg. Starting dose for the new chemicals can be initiated with 175 mg/kg body weight.

Chapter Three

Hypothesis

The antibacterial properties of medicinal plants and its effect on Neonatal sepsis causing bacteria with simultaneous formulation of oral syrup which may be used as herbal therapy for neonatal sepsis cases need to explored.

Although many synthetic antimicrobial agents have already been used yet extensive existence of natural phytochemical compounds have fewer side effects, cheaper in costs, easy availability along with immunomodulatory and nutraceutical property adding beneficial advantages to vulnerable neonates.

Chapter Four

Research Objectives

1. Screening and isolation of bacterial species from the cases of neonatal sepsis in Pediatric OPD and neonatal ward.
2. Identification of bacterial species through biochemical characterization.
3. Screening of herbal extract against the bacterial species.
4. Characterization of herbal extract for active phytochemicals.
5. Formulation of liquid oral syrup.

Chapter Five

Methods and Materials

5.1 Study plan: Descriptive cross-sectionalstudy

5.2 Study Site: The study was conducted at the National Medical College and Teaching Hospital (NMCTH), Department of Microbiology. NMCTH is situated in Birgunj of district Parsa, Province two of Nepal. It is 750 bedded teaching hospital with having Neonatal Intensive Care Unit (NICU). NMCTH is a tertiary care hospital in province two of Nepal.

5.3 Study Period: January 2017 to February 2018.

5.4 Sample Plan:

Blood were collected from neonates having probability of sepsisbased on the following sign and symptoms (Zakariya *et al*., 2010; Aku *et al*., 2018)

Table 5.1: Sign and Symptoms

Inclusion criteria	Exclusion criteria
Respiratory distress, Poor cry, Tachycardia, Cyanosis, Hypothermia, Jaundice, Lethargy, Grunting, Convulsion, Fever, Poor feeding, Vomiting, Irritability, Excessive cry	Clinically no any characteristics of probable sepsis.

The age, sex and birth weight, and symptoms were recorded of neonates and gestational age, mode of delivery and place of delivery were recorded from mother.

5.5 Sample Size Determination

Sample size was determined and calculated using proportion formula from the previous reserch in Nepal. According to the study completed at Kist Medical College and Hospital, Lalitpur, Nepal (Lakhey and Shakya, 2017) the sample size (N) was calculated with consideration of 95% confidence interval (CI) and 3% marginal error as below (Getabelew *et al.,* 2018).

Where $N= Z^2 \times P \times \frac{(1-p)}{e^2}$

N= required sample size

Z= 1.96 at 95% Confidence Interval (CI)

P= Prevalence rate of neonatal sepsis (48%) (Lakhey and Shakya, 2017)

e= margin of error that can be tolerated, 3% (0.03)

1-p=population that does not have sepsis

Therefore,

$$N= Z^2 \times P \times \frac{(1-p)}{e^2} = (1.96)^2 \, x \, (0.48) x \, \frac{(0.52)}{(0.03)^2} = 1065.40 = 1065$$

The total sample should be collected is 1065. Therefore, samples have been collected more than sample size.

5.6 Ethical Approval

As per Nepal Health Research Council (NHRC) ethical compliance from Institutional Review Committee of NMCTH, Birgunj, Nepal was taken for conducting the research on Studies on Neonatal sepsis and Effect of Phytochemicals from Selected Medicinal Plants (F-NMC/125/2016-2017). Similarly, ethical compliance from Institutional Review Committee of NMCTH, Birgunj, Nepal was taken for conducting the research on Oral Acute Toxicity of Polyherbal Syrup in Mice (F-NMC/508/2076-2077).

5.7 Collection of Sample

Blood for culture was collected from different sites of body like from marginal vein, artery or anti cubital fossa following aseptic procedure before prescription of antibiotic therapy to infected neonates. To obtain blood, the venipuncture site over the skin was moop with 70% ethanol in a circle of 5 cm diameter and was left for air dry. 1% povidone-iodine was applied centrifugally over the skin of the venipuncture site. The site was air-dried for a minute and then a needle of the sterile disposable syringe was introduced into the site and 1 or 2 ml of blood was drawn by the clinician or trained nurse and aseptically inoculated into a culture

bottle having Brain Heart Infusion (BHI) Broth (Hi Media, M210) instantly to the ratio of 1:10. Specimen containers were labeled with the patient's name, age/sex, date, and lab reference number. The needle was withdrawn from the site and again disinfect with 70% alcohol (Mackie and MacCartney, 1996; Bailey and Scott's, 2017).

5.7.1 Sample processing

The culture bottle containing 1-2 ml blood with the ratio of 1: 10 ml of BHI broth were incubated aerobically at 37^0C for one week and were routinely observed for turbidity, hemolysis of red blood cells, the appearance of coagulates and gas bubbles formed. Subculturing was done from incubated culture bottle to Sheep Blood agar (BA) (HiMedia M073), MacConkey agar (MA) (HiMedia M081), Mannitol Salt agar (MSA) (HiMedia M118), and Eosin Methylene Blue Agar (EMB) (Hi Media M). The subculture plates were incubated aerobically at 37^0C. The axenic isolates grown on subcultured plates were identified by different standard protocols used in microbiological tests which comprise studies of Gram-staining, colony morphology and various biochemical tests viz catalase, slide coagulase, cytochrome oxidase, Sulfide Indole Motility (SIM), MRVP (Methyl Red Voges Proskauer), Triple sugar iron (TSI) agar, Citrate utilization test, and urease tests. No bacterial growth after 7 days of incubation can be reported as sterile (Mackie and MacCartney, 1996; Bailey and Scott's, 2017).

5.7.2 Examination of sub cultures

The subculture plates were observed after incubation of 18-24 hrs and growth observed was recorded for colony morphology. Colony growth was followed by phenotypic and biochemical criteria (Mackie and MacCartney, 1996; Bailey and Scott's, 2017)

1. Gram Stain- to differentiate gram-positive and negative organisms
2. Preliminary tests like Catalase, Coagulase and Oxidase were performed.
3. Gram-negative bacteria were identified based on biochemical tests using SIM, TSI, MRVP, Urease, and Citrate.

Table 5.2: Colony morphology of isolated Bacteria

Isolated Bacteria	Colony Morphology
Staphylococcus aureus	Blood agar: 1-3 mm diameter, glistening, densely opaque and butyrous consistency, Creamy yellow colony with zone of beta haemolysis Mannitol Salt Agar: Golden yellow colony
Streptococcus spp.	Blood Agar: Creamy white small colony with 0.5-1 mm diameter, semi-transparent, low convex, discrete colony with matt or glossy surface
Coagulase Negative *Staphylococcus* (CoNS)	Blood agar: 1-3 mm diameter, glistening creamy white colony, densely thick and butyrous consistency
Klebsiella pneumoniae	MacConkey agar: Large mucoid pinkish red colony
Escherichia coli	MacConkey agar: Smooth, dry, flat pink red colony Eosin Methylene Blue agar: Greenish metallic colony
Pseudomonas aeruginosa	MacConkey agar: Faint yellowish flat colony, sticky consistency Blood agar: Spreading and flat. Serrated edges, confluent growth
Enterobacter spp.	MacConkey agar: Large mucoid pinkish white colony
Proteus spp.	MacConkey agar: Faint yellow colony

5.7.3 Gram stain (Mackie and MacCartney, 1996; Bailey and Scott's, 2017)

A smear was prepared from the 18-24 hrs old colonies on the glass slide with proper labeling. After air dry and heat fixation, the slide was kept on the staining rack. It was stained with 2% crystal violet solution for 1 minute. After washing Gram's Iodine was poured over the smear on the slide for 1 minute. Washing was repeated. Decolourisation was done with acetone for 2 to 3 seconds followed by washing & counter staining with 0.5% safranine for 30 seconds. The smear was then washed & blot dried. The shape and arrangement of bacteria were observed.

5.7.4 Biochemical test and test for production of enzymes

Catalase test (Mackie and MacCartney, 1996; Bailey and Scott's, 2017**)**

The enzyme Catalase converts hydrogen peroxide to water and oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Pin head colony of test bacteria was taken with a germ-free glass rod and mixed into the 3% H_2O_2 solution. Positive reactions are indicated by the production of gas bubbles/ effervescence.

Staphylococcus aureus (Positive control)

Streptococcus spp., CoNS (Negative control)

Coagulase test (Mackie and MacCartney, 1996; Bailey and Scott's, 2017)

Staphylococcus aureus has two proteins that have prothrombin-like activity. One of them secrets extracellular called the free Coagulase and the other is present on its surface called bound Coagulase. Fibrinogen is converted to fibrin by both enzymes. This test differentiates *Staphylococcus aureus* from additional species of Staphylococci.

Slide coagulase test (Mackie and MacCartney, 1996; Bailey and Scott's, 2017)

Slide coagulase test is used to detect bound Coagulase or clumping factor. The formation of fibrin strands on the surface of *Staphylococcus aureus* in presence of plasma leads to the clumping of the Staphylococcal cells. This test was performed for Catalase positive gram-positive cocci. The isolated colony is mixed with normal saline on a clear microscope slide to make a milky homogenous mixture. One drop of plasma was emulsified with a wooden stick and observed for clumping of cocci within 10 seconds which indicates a positive reaction.

Oxidase Test (Mackie and MacCartney, 1996; Bailey and Scott's, 2017)

The test is performed to detect the enzyme Cytochrome oxidase which in the presence of atmospheric oxygen oxidizes the dye tetramethyl p-phenylenediamine dihydrochloride to change its color. This test is performed by picking up the colonies with a glass rod and smeared over the commercially available Oxidase disc (Hi Media) soaked with a solution

of 1% Tetramethyl p-phenylenediamine dihydrochloride. The appearance of an intense deep purple hue within 60 seconds confirms a positive test.

Pseudomonas aeruginosa (Positive control)

Escherichia coli (Negative control)

Sulfide Indole Motility (SIM) (Mackie and MacCartney, 1996; Bailey and Scott's, 2017)

SIM medium is used to determine three characters, indole and hydrogen sulfide production and motile nature to differentiate enteric organisms. Bacteria utilize peptone, meat extract and reduced the indicator sodium thiosulphate that release H_2S which reacts with peptonized iron results in black precipitate of ferrous sulfide. Utilization of tryptophan from peptone, results in the production of indole. Indole can be detected with P-dimethyl amino benzaldehyde (Kovac's reagent). The appearance of cherry red color on the surface of inoculated SIM medium indicates the presence of indole after the addition of Kovac's reagent. The semisolid nature of the medium allows for the detection of the motile nature of bacteria. Hazzy growth along the line of inoculation indicates test organism is motile.

Citrate utilization test (Mackie and MacCartney, 1996; Bailey and Scott's, 2017**)**

The requirement of a carbon source is fulfilled by the utilization of Citrate. This can be detected by the production of alkaline by-products which alter the color of the media, devoid of protein and carbohydrates. Organisms are inoculated lightly onto the slope of Simmon's citrate media from a saline suspension. The media is incubated at 37°C for 96 hrs. The color change of the inoculated media from green to blue indicates a positive result.

Enterobacter aerogenes (Positive control)

Escherichia coli (Negative control)

Triple sugar iron (TSI) Agar test (Mackie and MacCartney, 1996; Bailey and Scott's, 2017**)**

TSI agar is a composite multi-test media which is utilized to find out whether the gram-negative bacilli can consume glucose, lactose or sucrose by fermentation with or without the production of gas. The media contains glucose 1 part lactose and sucrose 10 parts each. The indicators Phenol red and ferrous sulphate were used to find out the production

of acid and H_2S in the test respectively. In case of glucose fermentation, the color of the entire media becomes yellow within 8-12 hrs but the slant reverts to an alkaline (red) state due to oxidation of fermented by-products and production of amines in presence of oxygen within 18-24 hrs. However, if lactose or sucrose is also fermented in addition to glucose then acid production is so enormous that the whole medium remains yellow. The black color of media indicates H_2S produced. Heavy inoculums of the test organisms are streak on the slant and stabbed into the butt region. After 24 hrs incubation at 37^0C tubes are examined. Acid production by the strain results in a change of medium as of red to yellow color. If only glucose is fermented the acid production is small and the color change occurred only in the butt of the tube but if in addition lactose or sucrose or both are fermented the color change occurred both in the slant and the butt. Gas production is indicted by separation/ cracking of the media and the black color of medium or butt indicates the production of H_2S.

Table5.3: Reaction and interpretation of TSI test

Reaction	Interpretation
Alkaline slant (red)/acid butt (Yellow) (K/A)	Glucose fermented (K/A)
Acid slant (yellow)/acid butt (yellow) (A/A)	Both Lactose or sucrose are fermented
Alkaline slant/Alkaline butt (no change) (K/K)	3 sugars are not utilized
Gas bubbles or crack of medium (G)	CO_2 produced as a by-product of fermentation
Blackening of the butt and/or stab (H_2S)	Production of Hydrogen sulfide

Urease test (Mackie and MacCartney, 1996; Bailey and Scott's, 2017)

Urease is an enzyme produced by many species of bacteria which hydrolyze urea with the formation of ammonia resulting in alkalization of the medium that change the color of indicator phenol red. This test is done by inoculating the colonies of isolated organisms onto the surface of Christensen's urea agar and incubated at 37^0C. The slope is examined

for color change after 4 hrs and reported as negative after 4 days of incubation. A positive test results in change of medium color from light yellow to pink.

Klebsiella spp. (Positive control)

Escherichia coli (Negative control)

Methyl red test (Mackie and MacCartney, 1996; Bailey and Scott's, 2017**)**

Methyl red test is used to detect a sufficient amount of acid produced in process of fermentation of glucose sugar maintaining the pH below 4.5 of the culture used. The test organism was transferred to MRVP broth and incubated at 37^0C. After incubation for 48 hrs five drops of respective reagent were added and mix. The appearance of the bright red color of the whole medium indicates a positive reaction and yellow as negative.

Positive control-*Escherichia coli*

Negative control-*Enterobacter aerogenes*

Voges-Proskauer test (acetoin production test) (Mackie and MacCartney, 1996; Bailey and Scott's, 2017**)**

This test helps in the detection of acetyl methyl carbinol produced as the end product of carbohydrate fermentation. MRVP test is usually performed in conjunction. MRVP broth was inoculated and incubated with the test organism at 37^0C for 48 hrs. In incubated broth, 1 ml of 40% KOH and 3 ml of 5% solution of α-naphthol (ethanol) was added. The appearance of pink color within 2-5 minutes indicates positive and becomes crimson in 30 min.

5.8 Phenotypic tests for bacterial resistance determination including susceptibility testing

5.8.1 Anti-Microbial Sensitivity Testing

Antibiotic sensitivity testing was applied following the methods of Kirby-Bauer disc diffusion of susceptibility testing, which is taken from the Kirby-Bauer method initially described by Bauer *et al.,* (1966) and was subsequently modified by the National Committee for Clinical Laboratory Standards USA 1984 and in 1987.

5.8.2 Inoculums preparation

5 ml of autoclaved Brain heart infusion (BHI) broth was taken and 3-5 axenic colonies were inoculated in broth and incubated for 1hr at 37°C. Turbidity was adjusted to the

equivalent of around 1–2 × 10^8 colony forming units per ml. The experiment was performed as per the guidelines of Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2006).

5.8.3 Preparation of McFarland Turbidity standard:

MacFarland Turbidity Standard was prepared by the combination of 0.05 ml of 1.175% of anhydrous $BaCl_2$ to 9.95 ml of 1% H_2SO_4 in a test tube. The solution prepared was specified for its optical density (CLSI, 2006).

5.8.4 Procedure

Germ-free cotton swab was dipped into the broth inoculum within 15 minutes of adjusting the turbidity. The swab was squeezed on the wall of the test tube several times and was made carpet culture by revolving the plate around 60^0 on each occasion to make sure of homogeneous distribution of inoculum. After inoculation, the plates were left with a lid-jar for about 5 mins to allow absorption of overload surface wetness. Himedia antibiotic discs are kept over the surface of the seeded Mueller- Hinton agar and pressed gently down to make sure of complete contact. Discs are applied evenly and no closer than 24 mm from each other and 15 mm from the plate margin. A maximum of 7 discs is placed on a 90 mm plate. All antibiotic discs used in the study were of HiMedia, Mumbai, India. The antibiotic discs used in this study were levofloxacin (5 mcg), amikacin (30 mcg), cefixime (5 mcg), meropenem (10 mcg), piperacillin (100 mcg), imipenem (10 mcg), ceftriaxone (30 mcg), Cefotaxime (30 mcg), ceftazidime (30 mcg), ampicillin (10 mcg), linezolid (30 mcg), vancomycin (30 mcg), penicillin G (10 U). The plates were incubated aerobically at 37^0C for 18 to 24 hours. After the appropriate incubation period, each plate was examined for an inhibition zone around the disc. The Zone of Inhibition (ZOI) was measured with scale and interpreted according to the CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards. Results are recorded categorically as sensitive (S) or resistant (R).

Controls used with each batch:-

1. *Staphylococcus aureus* ATCC 25923
2. *Klebsiella pneumoniae* ATCC 700603

5.9.1 Selection, Collection and Identification of Medicinal Plant

Seven medicinal plants were selected for the assessment of antimicrobial activity based on ethnobotanical information, literature, references and therapeutic significance. The selected plants have been mostly reported for anti-diabetic, anti-cancer, anti-inflammatory, antimicrobial activities and for several other therapeutic values. The list of plants has been mentioned in Table 5.4.

Table 5.4: Medicinal plants used in this study

S. No.	Medicinal Plant	Family	Parts collected	Place of Collection	Common Name
1.	*Azadirachta indica*	Meliaceae	Leaves	Bara, Nepal	Neem
2.	*Moringa oleifera*	Moringaceae	Leaves	Parsa, Nepal	Shajan
3.	*Nyctanthes arbortristis*	Oleaceae	Leaves	Parsa, Nepal	Ratorani
4.	*Punica granatum*	Lythraceae	Rind	Parsa, Nepal	Anar
5.	*Swertia chirata*	Gentianaceae	Leaves, Stem, Root	Nawalprasi,Nepal	Chiraito
6.	*Syzygium cumini*	Myrtaceae	Leaves	Parsa, Nepal	Jamun
7.	*Tinospora cordifolia*	Menispermaceae	Leaves	Parsa, Nepal	Giloy

Collection and identification: Disease-free plant parts were collected from different geographical regions of Nepal mentioned in Table 5.4. The collected plants were identified by taxonomist from the Department of Drabya guna, Nepal Ayurveda Medical College, a Teaching Hospital located in Birgunj, Nepal. The plant materials collected were washed and dried under shade. Plant materials were subjected to drying at 37°C in a hot air oven for 3-4 hours with intermittent turning the material to avoid burning. The dried plant's parts were crushed by hand then crushed in a mixer grinder to a coarse powder. The crushed powder was sieved and then stored in airproof plastic bags for further extraction process (Zewdie *et al.,* 2020).

5.9.2 Extraction Methods: The extraction protocol of secondary metabolites was performed using the maceration extraction process (Zewdie *et al.,* 2020). Fifty gram of each powdered

plant materials were macerated in 300 ml (Adwan *et al.,* 2010) of different solvents with increasing polarity (Snyder, 1974) hexane, acetone, methanol, 80% Ethanol and water for a period of seven days (Abbas *et al.,* 2014) with intermittent shaking. The macerated plant materials were first filtered with muslin cloth and then by Whatman filter paper No. 1. The filtrate was made into dry residue by evaporating in hot air oven by 40°C. The obtained residues were weighed and stored at 4°C for further use in experiments (Zewdie *et al.,* 2020).

5.9.3 Determination of percentage yield

The percentage yield of different solvent extracts was calculated by means of formula (Kamarudin *et al.,* 2016).

$$\frac{W2 - W1}{W0} X\ 100$$

Here,

W2 = Combined weight (extract and container)

W1= Weight of the empty container

W0=Weight of coarsely powered plants

5.10 Agar well diffusion method for antibacterial assay of plant extract

This method was performed as per the Zewdie *et al.,* (2020) for the assessment of antibacterial potential of different concentration dissolved in distilled water (sterile) for aqueous extract as well as Dimethyl sulfoxide (DMSO) for 80% ethanol, methanol, acetone and n-hexane (Gull *et al.,* 2013) extract at concentrations of 25, 50, 100 and 200 mg/ml of residue extract (Bhandari *et al.,* 2017) of the seven plants. Mueller-Hinton agar (MHA) plates were prepared, properly labeled and inoculated the aliquot (0.5 McFarland standard bacterial suspension) with sterile swab stick under laminar airflow hood following aseptic conditions. Thirty minutes after, five equidistant wells (four wells at the sides and one well in the center) were made in the inoculated plate with a heat sterilized stainless steel cork borer of 8 mm diameter and agar plugs were removed. 100 µl of test solution (Al-Zoreky, 2009) was poured into the respective well of different concentrations and 100 µl of aqueous solvent was poured in well as a negative control for aqueous extract whereas

for other solvent extracts 100 µl DMSO was applied for negative control while vancomycin of same concentration and Levofloxacin (5 mcg/disc) was applied for positive control against gram-positive and negative isolates respectively. Each test was triplicated for each bacterial strain. The culture plates after incubation at 37°C, for 24 hrs were observed for the inhibition zone around well. The clear zone around the well represents the effectiveness of extract against bacterial isolates. The clear zone was measured in millimeters using a ruler from three plates. An agar well (8 mm) with no zone of inhibition (ZOI) was considered as no antibacterial activity.

5.11 Qualitative Screening of Phytochemical of plant extracts

The plant extracts showing substantial antimicrobial activity proceeded for detection of the phytochemical present in the plant extract. All the seven plant extracts were processed for phytochemical detection. Qualitative phytochemical screening was performed as described by Sofowora (1993; 1996), Trease and Evans (1989) and Harborne (1973). Several other publications were also referred to consolidate the actual procedures of tests which were performed for the qualitative phytochemical analysis (Al-Daihan *et al.,* 2013; Yadav *et al.,* 2014; Surendra *et al.,* 2016; Bhandari *et al.,* 2017).

Test for alkaloids (Wagner's test): This test is used for the detection of alkaloids and was conducted as per the method of Surendra *et al.,* (2016). In this test 3 ml of extract was added with Wagner's reagent (1 ml) followed by 1 ml of dilute Hydrochloric acid (HCl). The mixture was then heated for few minutes. The development of brown or reddish-brown color precipitate indicates the existence of alkaloids.

Test for Coumarins: The test for coumarins was performed as per the methods of Yadav *et al.,* (2014). 2 ml of extract amount was added with 3 ml of 10% Sodium Hydroxide (NaOH). The development of yellowish color indicates the presence of coumarins.

Test for Quinones: This analysis was carried out as per the method of Bhandari *et al.,* (2017). 1 ml of extract was added with 1 ml of conc. H_2SO_4. The positive result of red color indicates the presence of quinone.

Test for Phenol: The Ferric chloride test is used for the detection of phenol and is being performed as per the method of Surendra *et al.,* (2016). In this test, 1 ml of plant extract

was added with few drops of $FeCl_3$ solution result in the development of deep green color confirms the presence of phenol.

Test for Flavonoids (Alkaline Reagent Test): The alkaline reagent examination is used for the presence of Flavonoids. The test was performed as per the method described by Surendra *et al.,* (2016). In this test, one ml of NaOH solution was added drop by drop to 1 ml of plant extract resulting in the development of deep yellow color. This yellowish color developed changes to colorless upon addition of dil. HCl result in the presence of flavonoids.

Test for Terpenoids: This test is done for the qualitative evaluation of terpenoids in the plant extract. The test was performed as per the technique mentioned in Bhandari *et al.,* (2017). According to this method, 5 ml of extract along with 2 ml of chloroform was mixed followed by adding 3 ml of conc. H_2SO_4. The formation of reddish brown precipitate by the border indicates the presence of terpenoids in the extract.

Test for Tannins: This test was done as per the method described by Al-Daihan *et al.,* (2013) for the detection of tannins. In this technique, few drops of 1% $FeCl_3$ solution were added to the filtered sample of plant powder and distilled water. The test results in the formation of bluish-black, green or bluish-green precipitate which confirms the presence of tannins.

Test for Steroids: This test was performed as per the method of Bhandari *et al.,* (2017). According to this method, one ml of extract and 10 ml chloroform was mixed. 11 ml of concentrated H_2SO_4 was poured slowly from the inner side of the test tube. This result in the appearance of the superior layer turned into red and H_2SO_4 layer changes to yellowish with green fluorescence which indicates the presence of steroids in the plant extract.

Test for Saponins: The test for detection of saponins was done as per the method described by Al-Daihan *et al.,* (2013). In this method, one gram of crude powder of plant was boiled with 10 ml of distilled water followed by filtration. In the filtrate, 3 ml distilled water was added and shaken vigorously for about 5 min. This results in the formation of foam which confirms for the presence of saponins.

5.12 LC-MSanalysis and interpretation of *Punica granatum* Rind Extract

The analysis of the extract by HPLC/LC-MS (Waters, XEVO TQD#QCA1232) was carried out from the Department of Sophisticated Analytical Instrument Facility from Central Drug Research Institute, Lucknow, India. LC-MS system comprised of the autosampler, vacuum degasser, HPLC Pump and column compartment joined with XEVO-TQD detector with Electro Spray Ionisation (ESI) interface. Mass acquisition during the whole scan by MS/MS, was set from 20 to 2000 Da. The probe for ESI was operated in the positive mode (ES+) and negative mode (ES−) with the capillary power of 3.5 kV, cone power of 30 V, the temperature was fix to 150°C (ES+) and 120°C (ES−), desolvation 350°C, cone gas flow at the rate of 30 L/ hr and desolvation gas flow 950 (L/hr), MS and MSMS collision energy are 3 and 20 respectively.

The chromatographic separations of compounds in the sample were carried out with SUNFIRE C-18, 250X4.6, and 5 µm sample quantity. The flow rate was fixed at 1.5 ml/min, the column temperature was adjusted to 30°C, maximum pressure was also adjusted to 300 bars and volume for auto-injection was 25 µl. The mass range was selected from 150 to 1000 m/z. The each sample was run including A: water, B: acetonitrile, C: methanol and D: 0.1% formic acid. Spectra obtained from the analysis were recorded in negative and positive ionization mode ranges between m/z of 150 and 1200. The data procurement received was documented on MassLynx, MS software.

5.12.1 Interpretation of LC-MS data of isolated fractions of *P. granatum* rind extract

The Spectra obtained from negative as well as positive ion mode was used to calculate molecular masses and retention time of various components. The major peaks obtained as chromatograms were used for detection of molecular masses. LC-MS chromatogram is a useful data to identify compounds based on molecular masses and retention time.

5.13 Polyherbal Formulation

The polyherbal formulation was prepared and evaluated under *in-vitro* conditions as per the method described by Chhetri *et al*., (2010), Muguli *et al*., (2015), Mac *et al.,* (2016),. Aqueous and Ethanolic (80%) extracts of mentioned plants were used either in single solvent extract or in combination with different permutation and combination. The different

prepared combination of the solvent extract was carried for the antimicrobial test. The most effective combination on the basis of inhibition zone was further processed for the formulation of syrup. The various combinations prepared were shown in Table 5.5—5.8.

Table 5.5: Trial Formulation Phase I from aqueous extract (100 mg/ml)

Med. Plant Extract	A	B	C	D	E	F
S. cumini	20	40	30	30	30	50
P. granatum	20	30	30	40	30	50
T. cordifolia	20	10	15	10	10	NI
N. arbortristis	20	10	15	10	15	NI
M. oleifera	20	10	10	10	15	NI

NI: Not incorporated; Med.: Medicinal; A-F: Trial formulation

Table 5.6: Trial Formulation Phase II from 80% Ethanolic extracts (100 mg/ml)

Med. Plant Extract	A	B	C	D	E	F
S. cumini	20	40	30	30	30	50
P. granatum	20	30	30	40	30	50
T. cordifolia	20	10	15	10	10	NI
N. arbortristis	20	10	15	10	15	NI
M. oleifera	20	10	10	10	15	NI

NI: Not incorporated; Med.: Medicinal; A-F: Trial formulation

Table 5.7: Trial Formulation Phase III from80% Ethanolic extracts (100 mg/ml)

Med. Plant Extract	A	B	C	D	E	F	G	H	I
S. chirata	17	20	30	15	25	10	25	10	30
N. arbortristis	17	20	20	15	25	10	25	10	30
P. granatum	17	20	20	30	15	25	25	20	20
S. cumini	17	20	20	30	15	25	25	20	20
M. oleifera	16	10	5	5	10	15	NI	20	NI
T. cordifolia	16	10	5	5	10	15	NI	20	NI

NI: Not incorporated; Med.: Medicinal; A-I: Trial formulation

Table 5.8: Trial Formulation Phase IV from 80% Ethanolic and Aqueous extract (100 mg/ml)

Med. Plant Extract	A	B	C	D	E	F	G	H	I	J	K	L
S. chirata (Ethanol)	50	35	25	20	20	20	15	30	NI	NI	NI	NI
N. arbortristis(Ethanol)	50	35	25	20	20	20	15	30	NI	NI	NI	NI
P. granatum (Aqueous)	NI	30	25	20	20	30	25	20	50	25	40	30
S. cumini (Aqueous)	NI	NI	25	20	20	30	25	20	50	25	30	30
T. cordifolia (Aqueous)	NI	NI	NI	20	10	NI	10	NI	NI	25	15	20
M. oleifera (Aqueous)	NI	NI	NI	NI	10	NI	10	NI	NI	25	15	20

NI: Not incorporated; Med.: Medicinal; A-L: Trial formulation

5.14 Evaluation of Polyherbal Formulation

Selections of these plants were based on their individual effect of antibacterial activity against bacterial isolates from neonates. Antibacterial effects of these combinations were evaluated by agar well diffusion method (8 mm borer) against the isolated bacteria from neonatal sepsis cases. Zone of inhibition was measured with different combinations. The permutation which exhibited the significant inhibition zone was taken for formulation of syrup. Formulation of polyherbal syrup was prepared by incorporating active ingredients and other excipients or preservatives.

Table 5.9: Each l00ml of syrup contains

S.N.	Ingredients	Quantities
1.	*Swertia chirata* (80% Ethanolic Extract)	1500 mg
2.	*Nyctanthes arbortristis* (80% Ethanolic Extract)	1500 mg
3.	*Punica granatum* (Aqueous Extract)	2500 mg
4.	*Syzygium cumini* (Aqueous Extract)	2500 mg
5.	*Tinospora cordifolia* (Aqueous Extract)	1000 mg
6.	*Moringa oleifera* (Aqueous Extract)	1000 mg

5.15 Preparation of Polyherbal Syrup (Shakeel *et al.*, 2015)

1. 70 ml of purified water was boiled and herbal extract of the required quantity was added to the mixing vessel under stirring. 66.67 gm of sugar was dissolved under heating.

2. Adequate purified boiling water was added to make up 100 ml and boiled for approximately 10 minutes.
3. Required quantity of excipients such as sorbitol, sodium benzoate, sodium methyl and propylparaben was added after cooling at 40^0C.
4. The finally prepared syrup was filled in a 100 ml amber color airtight bottle and sealed with a proper label.

5.16 Standardization of polyherbal syrup

The herbal syrup was standardized as per the method described by Ayurvedic Pharmacopoeia Part-II, Vol.-2 (Formulations) (2018) for different criteria as mentioned below.

Colour: 5 ml final syrup was kept in watch glass against the white background and observed through naked eye.

Odor: 2 ml of final syrup was smelled sequentially in the interval of 2 minutes for aggreability of the smell.

Taste: A drop of final syrup was kept on the tongue for its taste acceptability.

pH: 10 ml of the final syrup and 90 ml of distilled water was mix and sonicated for about 10 minutes. pH was then measured by a digital pH meter.

5.16.1 Microbial Analysis

Microbial analysis of the syrup was performed as per the method described by Ayurvedic Pharmacopoeia Part-II, Vol.-2 (Formulations) (2018) as mentioned below.

5.16.1.1Total viable Aerobic Microbial Count

Pretreatment: 10 ml of the prepared syrup was dissolved in Nutrient Broth having no antimicrobial activity and adjusted the volume to 100 ml with the same medium.

Plate count of Bacteria: 1 ml of the pretreated preparation was spread on the surface of solidified medium (Soyabean Casein Digest Agar). The Petri dish was incubated at 35^0C for five days to visualize the presence of bacteria.

5.16.1.2 Test for Specified Micro-organism

Escherichia coli: A loopful of pretreated preparation was streaked on MacConkey agar medium & incubated at 37^0C for one day. Brick-red color colonies will appear having a surrounding zone of precipitated bile indicates the presence of *E. coli*.

Salmonella: 1 ml of the pretreated preparation was transferred into 100 ml of nutrient broth in sterile screw-capped jar and incubated at 37^0C for 24 hr. 1 ml of the incubated solution was mixed to 10 ml of selenite-F-broth and incubated at 37^0C for 48 hr. Subculture was performed on Xylose-lysine-deoxycholate agar. The plate was incubated for 24 hr at 37^0C and results were observed for red color colonies with or without black centres that specify the presence of *Salmonella*. Biochemical identification was done by stab and streak culture in triple sugar iron agar and incubated at 37^0C for 24 hr. The results were observed for the development of acid and gas with black or not. The lack of acidity indicated with brick red color on slant surface growth indicates the presence of *Salmonella*.

Pseudomonas aeruginosa: 1 ml of the pretreated solution was mixed with 100 ml of Soyabean-casein digest medium and incubated at 37^0C for 48 hr. The medium was examined for the presence of growth and was streaked on the slant of Cetrimide agar and incubated at 37^0C for one day. The examination was done for the growth of greenish fluorescence colonies under UV light to indicate the presence of *P. aeruginosa*.

S. aureus: 1 ml of the pretreated solution was mixed with 100 ml of Soyabean-casein digest medium and incubated at 37^0C for 48 hr. The medium was observed for the presence of growth and was streak on Mannitol Salt agar and incubated at 37^0C for one day. The examination was done for the growth of golden yellow colonies to indicate the presence of *S. aureus.*

5.17 Evaluation of Heavy metals

The present investigation was performed as per the technique of Ayurvedic Pharmacopoeia Part II Vol. II (Formulations) (2018). Heavy metal under investigation is Lead, Arsenic, Cadmium, and Mercury.

Lead (Graphite oven method)

The graphite oven method is used for Lead investigation with the preparation of test solution of 1 ml of the test syrup which was taken in a Casparian flask along with a 10 ml mixture of nitric acid (4 parts) and perchloric acid (1 part). Flask was closed with a hopper and was macerated overnight. The solution was heated till it becomes clean and transparent with the addition of the above mixture. The solution was transferred into a 50 ml volumetric flask. The container was washed with 10 ml of 2 percent nitric acid solution and the washing solution was kept in the same flask and dilutes to reach 50 ml and shake well. Simultaneously, the reagent blank solution was also prepared as mentioned in the above method. Determination of lead was performed by taking 1 ml of each test and the blank solution along with 1 ml of solution (1% ammonium dihydrogen phosphate added with 0.2% magnesium nitrate) was added and shaken well. 20 µl solutions were injected into the atomic generator of the graphite oven and absorbance was determined. The Lead content was calculated in the test solution with the calibration curve according to the calibration curve of the standard with absorbance as Y-axis and concentration as X ordinate.

Arsenic (Hydride method)

In this method standard stock solution was prepared with a single element standard with 2% nitric acid containing 1.0 µg arsenic per ml. The calibration curve was prepared by a hydride generator device. The test solution was prepared as discussed previously in the graphite oven method of Lead. Determination is done by taking10 ml of each test solution and the blank solution which was pipetted and transfer into a volumetric flask respectively. 1 ml of 25% potassium iodide solution was added with proper shaking and then mixed with 1 ml of ascorbic acid. The solution was diluted with hydrochloric acid up to the volume of 25 ml and shaken properly and closed with a stopper. Flask was then immersed in a water bath at 80^0C for 3 minutes. The flask was cooled and 10-20 µl of test solution was transferred into the hydride generator for determination of absorbance and concentration as vertical and horizontal ordinate respectively.

Cadmium (Graphite Oven Method)

Test Solution was prepared by taking 1 ml of the test syrup in a Casparian flask. The 10 ml mixture of nitric acid (4 parts) with perchloric acid (1 part) was prepared then kept in the flask. Flask was closed with a hopper and was macerated overnight. The solution was heated till it becomes clean and transparent with the addition of the above mixture. The temperature was raised and heated continuously till the mixture changes to colorless and transparent or faint yellow. The solution was transferred into a 50 ml volumetric flask. The container was washed with 10 ml of 2 % nitric acid solution and the washing solution was kept in the same flask with dilution using the same solvent to reach 50 ml with shaking. The above procedure was done for the blank solution simultaneously. Determination of cadmium is performed by taking 1 ml of each test solution and the blank solution along with 1 ml mixture of 0.2% magnesium nitrate and 1% ammonium dihydrogen phosphate and shaken properly. Pipetted out 20 µl solutions and inserted into the atomic generator of the graphite oven and absorbance was determined. The cadmium present was calculated in the test solution from the obtained calibration curve according to the calibration curve of the standard with absorbance and concentration as vertical and horizontal ordinate respectively.

Mercury (Cold absorption Method)

In this method, the standard stock solution was prepared from standard pure mercury elemental solution with two percent nitric acid containing 1.0 µg mercury per ml. 1 ml of test solution was kept in a Casparian flask. 10 ml mixture of nitric acid (4 parts) plus perchloric acid (1 part) was mixed properly with a fixing small hopper on the flask top. The flask was immersed overnight and heated on the electric hot plate at 120-140^{0}C for 4-8 hr. The flask was cooled and 4% H_2SO_4 and 0.5 ml of 5% potassium permanganate solution were added then shaken properly. Drop wise hydroxylamine hydrochloride solution (5%) was added till the violet color disappears. The solution was diluted with 4% sulfuric acid solution to make volume up to 25 ml. The supernatant was used as test solution. Blank solution was also prepared based on the same procedure simultaniously. Determination of mercury was performed by taking 1 ml of each test solution and blank solution as mentioned

previously in the mercury content in the test solution. The result was calculated with the calibration curve.

5.18 *In vitro* Efficacy of Polyherbal antibacterial syrup

Agar well diffusion method

This method was used for the efficacy of the antibacterial potential of polyherbal syrup. Muller Hinton agar (MHA) plates were prepared and seeded with help of a sterile swab stick from 3 ml each of broth culture maintained to 0.5 Mc Farland standard (1-2 x 10^8 CFU/ml) and allowed to dry. 8 mm diameter wells were made with a sterile borer on agar plates after 30 minutes followed by inoculating 100 µl of polyherbal syrup aseptically into each well. The negative control in which well was poured with sterile distilled water and DMSO. Petri plates were incubated for 24 hr at 37^0C. The susceptibility of the test organisms was recorded (mm) based on the inhibition zone from three plates.

5.19 Toxicity analysis of polyherbal syrup

Selection and maintenance of experimental animals

The prepared formulation was evaluated for its safety efficacy in acute dose under *in vivo* trials. Toxicity study was performed as per the procedure prescribed in OECD Guidelines 423 (2001). The test was performed on healthy laboratory animals. Young active and healthy Swiss albino mice (both sexes) of age 8-12 weeks old, weighing about 30-45 gm were utilized for this experiment. The animals were bought from Anandaban Hospital, Kathmandu, Nepal. Female mice are nulliparous and nonpregnant. The animals were housed in cages made up of polypropylene with wire gauzed tops and bedding of sterilized sawdust. The experimental room was maintained under a standard environment setting of temperature 22-25^{0}C, relative humidity 35-65%. The experimental room was well lighted and ventilated with 100% fresh air and artificial lighting was fitted in a sequence of 12 hr light/dark cycle. The animals were provided with enough amounts of clean tap water *ad libitum* along with food during the experimental period. Animals were allowed for acclimatization for seven days to laboratory condition prior to dosing. The bedding of the cage is prepared through sterilized sawdust and was exchanged twice a week. The intake of feed and water consumption was maintained daily by measuring the left over feed/water. Bodyweight was calculated

on weekly basis. The study procedure was presented and approved by the Institutional Review Committee, National Medical College and Teaching Hospital, Birgunj, Nepal (Approval No. F—NMC/508/076/077)

Acute toxicity Study

The Median Lethal Dose (LD_{50}) was evaluated under OECD guidelines 423 for testing of chemicals. This acute oral toxicity procedure involves oral administration of test formulation with help of gavage to mice then after daily examination for 14 days. The features/characters under observation were body weight, adverse effects (death) and ethological aspects (behavior) of the animals. 24 healthy mice of both sexes (male and female) were randomly allocated into 6 groups (3 males and 5 females) for acute toxicity. Male and female mice were marked with 2% solution of picric acid and housed separately. Mice body weight was taken prior to dosing. The polyherbal formulation was administered to the female and male mice after fasting for 4 hr (free supply of water) with help of oral gavage in a volume of 10 ml/kg body weight. The starting dose was 300 mg/kg body weight of polyherbal formulation that dissolved in sterile distilled water administered to group 1 (male and female). The mice were looked after for their behavioral response; signs and symptoms of toxic effect and mortality after ingestion of initial 4 hr, followed for a period of 48 hr. Group 2 was prescribed after 48 hr with the next higher dose of 2000 mg/kg body weight of the polyherbal formulation. The dose was dissolved in sterile distilled water after the positive result of group 1 after 48 hr of treatment. Group 3 was fed with vehicle (sterile distilled water) to establish a negative control group according to the OECD Guidelines parallel. After 2 hr of dosing, animals were fed as usual. All the experimental mice were looked after individually in the first half-hour and then periodically (1, 2, 4, 8 hr) in the first 24 hr and then daily for two weeks. Animal weights were taken on days 0, 7 and 14 days in the end. The cage side study was performed without distressing the animal concentration and animals were left individually to open area for observation of general activity, activity related coordination of motor system and muscle toning, reflexes, activities like a shiver, seizure, anesthesia, incoordination, lacrimation, cyanosis, salivation and piloerection. Furthermore, all the experimental mice were observed for mortality during

the whole study. The mice were anesthetized with diethyl ether on the 15^{th} day. Mice were then dissected and vital organs of the different system such as spleen and liver from gastrointestinal system and heart, lung and kidney from cardio, respiratory and urinary system respectively were carefully collected, weighed and gross pathological observation was recorded out for possible occurrence of macroscopic alterations like appearance, color and consistency.

Statistical analysis

Results were calculated as mean ± standard deviation. Fisher's exact test was applied for neonatal variables. The statistical data analyzed by one-way ANOVA followed by Tukey multiple comparison tests between toxicity study groups and considered significant at $P< 0.05$ (SPSS version 22)

Chapter Six

Results and Discussion

6.1 Screening of Neonatal sepsis cases

1200 blood specimens were collected in this study period from January 2017 to February 2018 at the Department of Microbiology NMCTH Birgunj, Parsa, Nepal. The sample size as per the sample size determination is 1065. Therefore, 1200 samples were collected for investigation. The samples were collected based on signs and symptoms being observed in all suspected neonates. The frequency of clinically generalized symptoms during examination of neonates suspected with sepsis in first visit were respiratory distress 245 (20.41%), fever 210 (17.50%), poor cry 177 (14.75%), jaundice 135 (11.25%), grunting 75 (6.25%), lethargy 66 (5.5%), irritability 61 (5.08%), poor feeding 60 (5%), cyanosis 50 (4.16%), vomiting 40 (3.33%), excessive cry 40 (3.33%), convulsion 19 (1.58%), hypothermia 14 (1.16%), tachycardia 8 (0.66%). The maximum frequency of culture positive growth was observed in poor cry 94 (53.10%) followed by lethargy 26 (39.39%), respiratory distress 92 (37.55%), fever 75 (35.71%), jaundice 39 (28.88%), poor feeding 14 (23.33%), hypothermia 2 (14.28%), tachycardia 1 (12.50%), vomiting 5 (12.50%), cyanosis 5 (10.00%), irritability 6 (9.83%), grunting 3 (4.00%) and excessive cry 1 (2.5%) but no bacterial growth with symptoms of convulsion in neonates was observed. The result is depicted in Figure 6.1.

Among 1200 suspected cases 843 (70.25%) were male in which 254 (30.13%) were bacterial positive and 589 (69.87%) were negative cases of neonatal sepsis whereas, 357 (29.75%) were female in which 109 (30.53%) were bacterial positive and 248 (69.47%) were bacterial negative cases of neonatal sepsis (P value 0.891). The results for male and female ratio and early and late neonatal sepsis were depicted in Figures 6.2 and 6.3. The proportion of male female neonates was found 2.3:1 in this study. In the present study, 1024 (85.33%) were EONS in which 290 (28.32%) were culture positive and 73 (41.48%) were culture positive in total of 176 (14.67%) cases of LONS (P value 0.001).

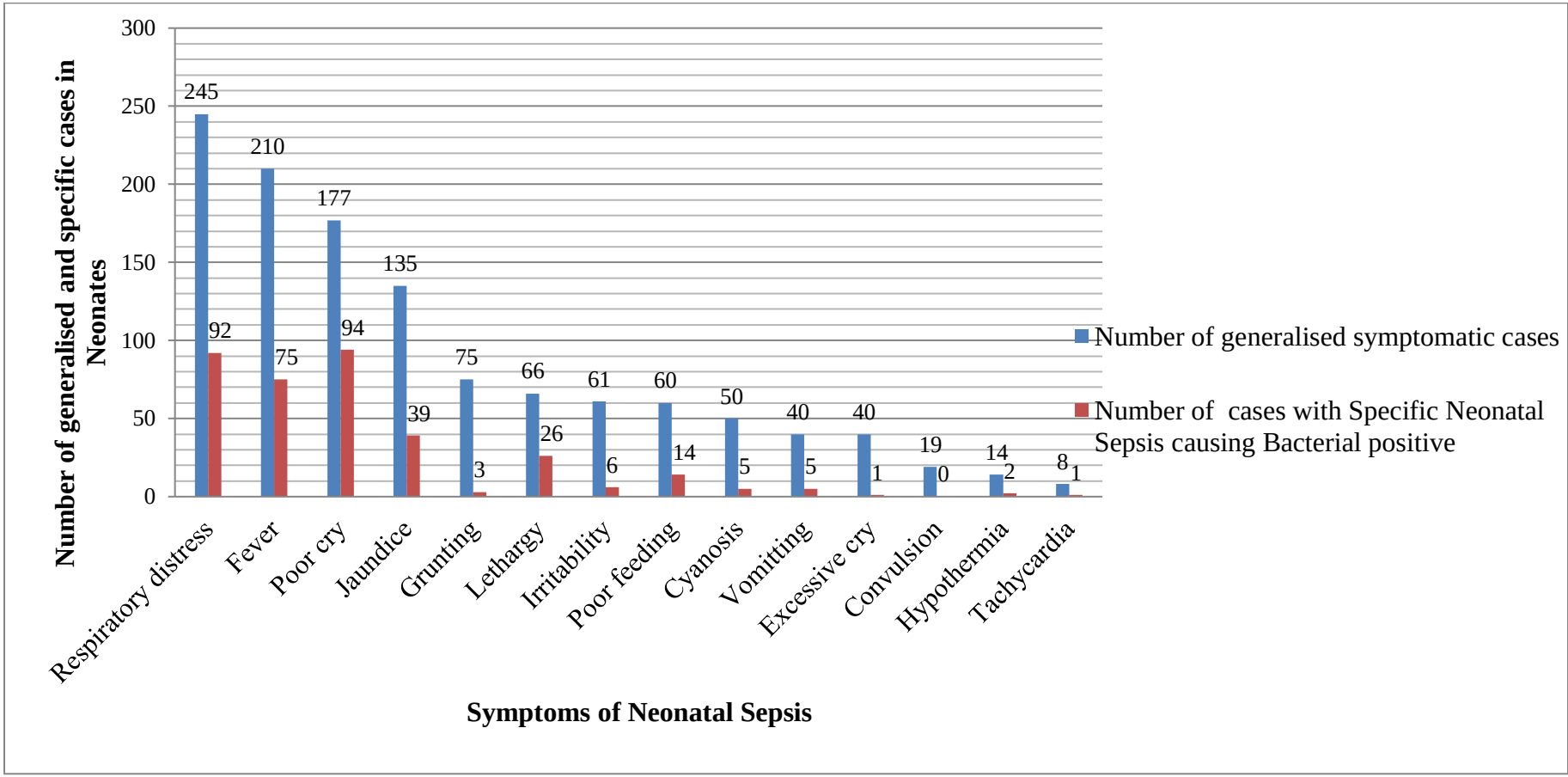


Figure 6.1: Clinical representation of Neonatal Sepsis cases amongst generalized symptomatic cases

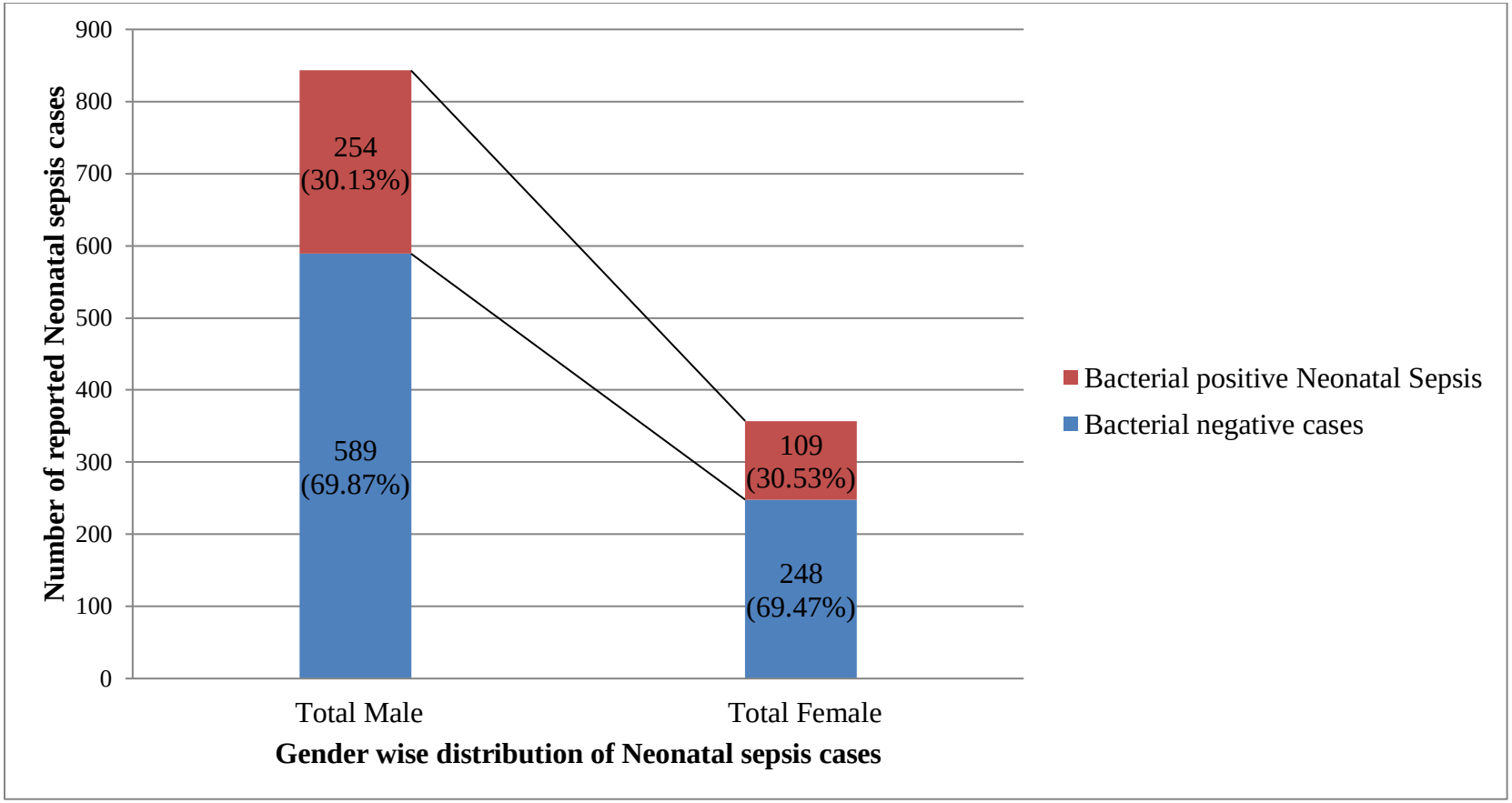
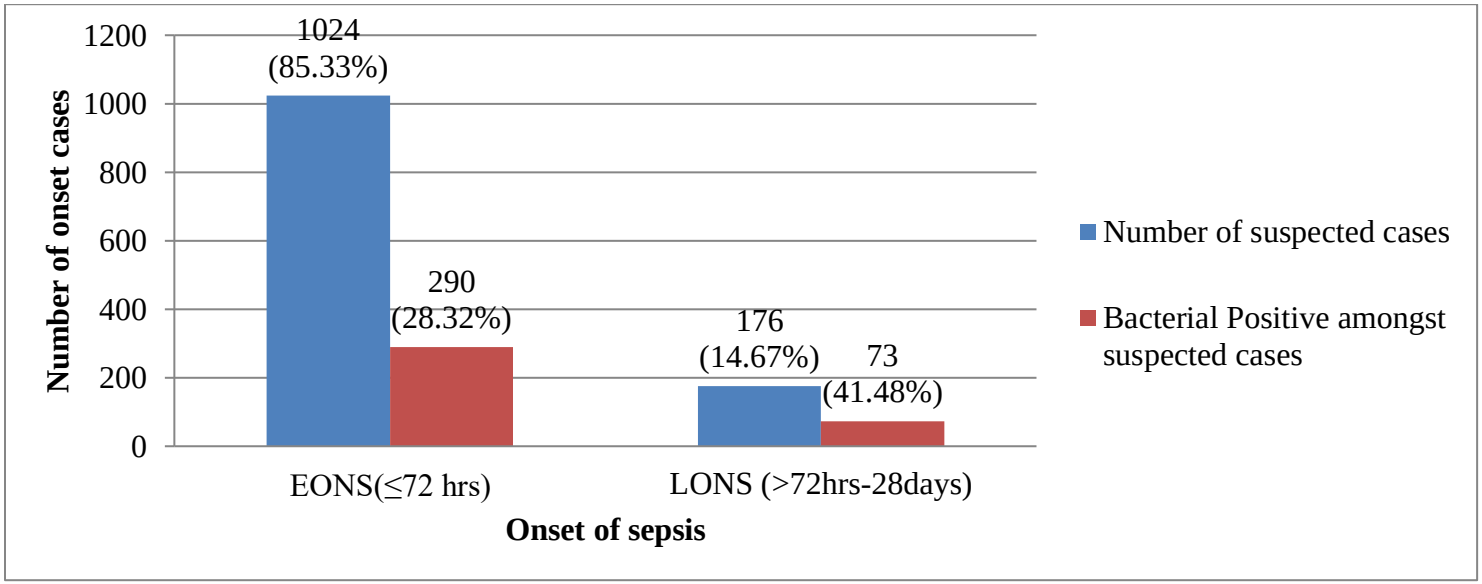


Figure 6.2: Gender wise distribution of Neonatal cases (P> 0.05)



EONS: Early Onset Neonatal Sepsis; LONS: Late Onset Neonatal Sepsis

Figure 6.3: Percentage distribution of onset in suspected cases of Neonatal sepsis (P< 0.05)

6.2 Isolation and Identification of bacteria from blood for confirmation of bacterial positive sepsis

Isolation and identification of bacterial pathogens by inoculation were performed onto different media followed by biochemical test. The result has been shown in Table 6.1. The result clearly shows that 363 cultures positive sepsis was found out of 1200 blood culture from suspected cases of neonatal sepsis. The prevalence rate of neonatal sepsis was 363 (30.25%). Among positive cases, 270 (74.38%) were Gram-positive cocci, and the rest 93 (25.62%) were gram-negative bacilli. The result has been depicted in Figure 6.4. The bacterial profile of culture isolates were depicted in Table 6.2. The percentage of total identified bacteria were *Staphylococcus aureus* 229 (63.08%), *Klebsiella pneumoniae* 48 (13.22%), *Streptococcus* spp. 28 (7.71%), *P. aeruginosa* 21 (5.79%), *Escherichia coli* 17 (4.69%), CoNS 13 (3.59%), *Enterobacter* spp. 6 (1.65%) and *Proteus* spp. 1 (0.27%) respectively.

The relation between varied neonatal risk factors and positive blood culture has been depicted in Table 6.3. The results clearly shows that maximum percentage of culture positive growth was observed in male neonates 254 (69.98%), EONS 290 (79.89%), low birth weight (<2.5kg) 284 (78.23%), preterm gestational age 265 (73%), spontaneous vaginal delivery 279 (76.86%), delivery in hospital 232 (63.91%).

1200 neonates were examined for blood cultures of suspected cases with sepsis during the interval of the study. Neonatal ages from 1 to 28 days with average of 2.69 days were selected for the study. The standard deviation (SD) was equivalent to 4.390 days, mode and median equal to 1 day. The most important clinical manifestations found among generalized symptoms in this study were respiratory distress 245 (20.41%), fever 210 (17.50%), poor cry 177 (14.75%). The present result is in accordance with earlier findings (Jain *et al.*, 2003; Thapa *et al.*, 2013; Pokhrel *et al.*, 2018; Li *et al.*, 2019). In this investigation maximum bacterial positive neonatal sepsis were found in neonates with having the symptoms of poor cry then by lethargy, respiratory distress, and the result are similar to earlier reports (Thapa *et al.*, 2013; Li *et al.*, 2019).

Table 6.1: Colony morphology and Biochemical Characterization for bacterial identification

Colony type	Gram Stain	Cell shape	Haemolysis	Cat.	Coa.	Oxi.	SIM(S=Sulphide, I=Indole, M=Motility)			Citrate test	Urease test	MR VP		TSI				Bacteria
							S	I	M			MR	VP	Slant	Butt	H_2S	Gas	
Golden yellow (MSA)	+	Cocci in cluster	β-Haemolysis	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	*S. aureus*
Cream white (BA)	+	Cocci in Chain	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	*Streptococus* spp.
Cream white (BA)	+	Cocci	-	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	CoNS
Smooth Pink (MAC)	-	Rod	-	+	ND	ND	-	+	+	-	-	+	-	Y	Y	-	+	*E. coli*
Mucoid pink (MAC)	-	Rod	-	+	ND	ND	-	-	-	+	+	-	+	Y	Y	-	+	*K. pneumoniae*
Mucoid Pink (MAC)	-	Rod	-	+	ND	ND	-	-	+	+	+	-	+	Y	Y	-	+	*Enterobacter* spp.
Pale coloured flat colony (MAC)	-	Rod	-	+	ND	+	-	-	+	+	+	-	-	R	R	-	-	*P. aeruginosa*
Pale coloured (MAC)	-	Rod	-	+	ND	ND	+	-	+	+	+	+	-	R	R	+	+	*Proteus* spp.

+: Positive; - : Negative; ND: Not done; Y: Yellow; R: Red; CoNS: Coagulase Negative *Staphylococcus*; BA: Blood Agar; MSA: Mannitol Salt Agar; MAC: MacConkey Agar; MR: Methyl Red; VP: Voges Proskaeur; TSI: Triple Sugar Iron Agar; H_2S: Hydrogen Sulphide; Cat. : Catalase test; Coa. : Coagulase test; Oxi. : Oxidase reaction

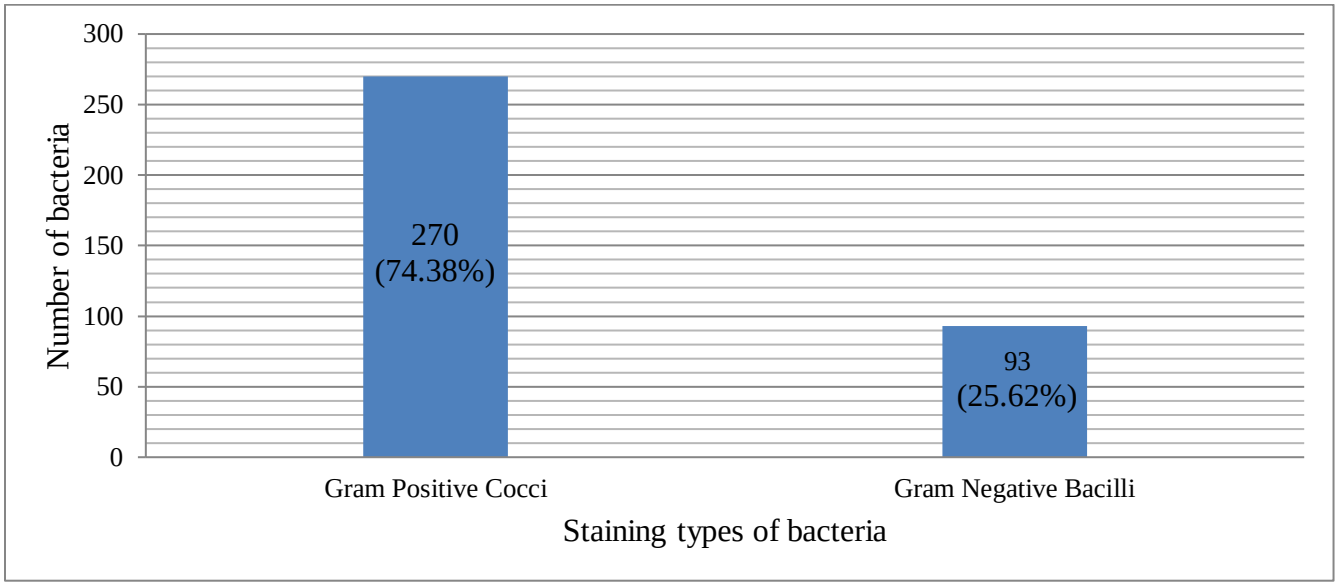


Figure 6.4: Bacterial diversification based on gram staining

Table 6.2: Distribution of bacterial cultures as percentage frequency from neonatal sepsis cases

Organisms isolated	Frequency		
	EONS (%)	LONS (%)	Total (%)
Gram-positive organisms	214 (73.80)	56 (76.71)	270 (74.39)
S. aureus	182 (62.76)	47 (64.39)	229 (63.08)
Streptococcus spp.	23 (7.93)	5 (6.84)	28 (7.71)
CoNS	9 (3.10)	4 (5.48)	13 (3.59)
Gram-negative organisms	76 (26.20)	17 (23.29)	93 (25.61)
Klebsiella pneumoniae	41 (14.13)	7 (9.58)	48 (13.22)
Pseudomonas aeruginosa	16 (5.51)	5 (6.84)	21 (5.79)
E. coli	14 (4.82)	3 (4.10)	17 (4.69)
Enterobacter spp.	4 (1.39)	2 (2.73)	6 (1.65)
Proteus spp.	1 (0.34)	0 (0)	1 (0.27)
Total	290	73	363 (100)

EONS: Early onset neonatal sepsis; LONS: Late onset neonatal sepsis; CoNS: Coagulase negative *Staphylococcus*

Table 6.3: Prevalence of positive culture in relation to different neonatal risk factors

Variables	EONS group	LONS group	Total	Percentage	Fisher's exact test p-value
Neonatal Variables					
Gender					
Male	203	51	254	69.98	1.000
Female	87	22	109	30.02	
Gestational age at birth					
Preterm(<37weeks)	212	53	265	73	1.000
Term(≥37weeks)	78	20	98	27	
Birth weight					
<2500gm	232	52	284	78.23	0.114
≥2500gm	58	21	79	21.77	
Mode of delivery					
Vaginal	225	54	279	76.86	0.536
Caesarean section	65	19	84	23.14	
Place of delivery					
Home	110	21	131	36.09	0.173
Hospital	180	52	232	63.91	

EONS: Early onset neonatal sepsis; LONS: Late onset neonatal sepsis

The present study reports that male-female proportion of 2.3:1, which is comparable to findings of earlier studies (Jain *et al.*, 2003; Shaw *et al.*, 2007; Shrestha *et al.*, 2011; Ansari *et al.*, 2015) but the result was less compare to the study by Shrestha *et al.*, (2013);

Lakhey and Shakya (2017). The present result is probably because male neonates were found to be suspected doubled the cases of neonatal sepsis than female neonates because of X-linked immune regulatory gene factor favoring susceptibility to infections in male (Chandna *et al.*, 1998). In clinically suspected onset of sepsis cases, LONS (41.47%) cases was found with higher positivity rate of bacterial growth in blood culture in comparison to EONS. This finding is in accordance with Kayange *et al.*, (2010). Kayange *et al.*, (2010) reported 51.4% which is in different from other studies as reported by Aku *et al.*, (2016); Jain *et al.*, (2003). The etiological agents associated with neonatal sepsis shows marked variation from different location and time interval from the same and different hospitals (Lakhey and Shakya, 2017). The NMR of Nepal is 21 per 1000 live birth but Province 2 has 30/ 1000 live birth (NDHS, 2016). In the present findings, the prevalence rate of neonatal sepsis was 30.25%. The present result was comparable to that of earlier reported by Jain *et al.*, (2003) (28.30%) from western and Shrestha *et al.*, (2013) (30.85%) from central region of Nepal. Li *et al.*, (2019) reported prevalence of 28.26% from Shanghai, China. In our studies, we are reporting higher prevalence rate compare to previously reported cases by Gyawali and Sanjana (2013) (15.1%), Pokhrel *et al.*, (2018) (20.7%), Chapagain *et al.*, (2015) (14%), Yadav *et al.*, (2018) (16.9%), Ansari *et al.*, (2015) (12.6%), Shrestha *et al.*, (2011) (6.1%) from the central part of Nepal. Aku *et al.*, (2016) reported 17.3% prevalence rate from Ghana, and Mehar *et al.*, (2013) reported 22% from India. The present finding reports lower prevalence rate than the report of Thapa *et al.*, (2013) (37.12%), Lakhey and Shakya (2017) (48%) from Nepal, Kayange *et al.*, (2010) (39%) from Tanzania, Zakariya *et al.*, (2010) (41.6%) from India, Ullah *et al.*, (2016) (57.1%) from Pakistan and Getabelew *et al.*, (2018) (77.9%) from Ethiopia. This inconsistent reporting of different studies regarding the positive rate of blood culture is due to difference in culture methodology, study plan, intake of antibiotics before blood culture, ineffective control over nosocomial infection (Gyawali and Sanjana, 2013; Pokhrel *et al.*, 2018). In the present investigation, male neonates (70.25%) were admitted more than female neonates. The similar type of suspected cases of neonatal admission was also found in earlier studies by Aku *et al.*, (2016) (60.7%), Getabelew *et al.*, (2018) (58.2%),

Lakhey and Shakya (2017) (56 %), Shrestha *et al.,* (2011) (63.7%), Ansari *et al.,* (2015) (61.4%). The probable reason is because of the prevalent tradition of the society regarding awareness for health concerns of male babies (Lakhey and Shakya, 2017).

In our investigation, male neonates (69.98%) were frequently caused by culture positive sepsis which is comparatively higher than the studies of Yadav *et al.,* (2018) (52.3%), Pokhrel *et al.,* (2018) (53.6%), and nearly similar with the study of Mehar *et al.,* (2013) (63.5%). The probable reason is because of the prevailing tradition of health conscious for male babies in our social culture (Lakhey and Shakya, 2017).

In the current investigation, the prevalence rate of EONS (79.89%) was found higher which is in accordance with the earlier reporting of Pokhrel *et al.,* (2018) (78.3%), Jain *et al.,* (2003) (80%) but in the study of Chapagain *et al.,* (2015) (83.3%), Yadav *et al.,* (2018) (71.2%), the prevalence of LONS was higher. This result is due to causative organisms which is acquired from mother during delivery, may chance to initiate early onset infection in neonates (Ansari *et al.,* 2015).

In the current investigation, we are reporting that higher prevalence rate of sepsis in preterm (73%) neonates which is similar with the finding of Pokhrel *et al.,* (2018) (68.01%). This study is contrary to the finding of Chapagain *et al.,* (2015) (80%) and Getabelew *et al.,* (2018) (77.89%) wherein term baby is more susceptible to sepsis has been reported. According to Petrova *et al.,* (2007) study, preterm babies have less capacity to promote the production of neutrophils in comparision to demand to fight against the factors related to bacterial sepsis. In the present study, baby delivered by normal vaginal route have higher prevalence rate (76.90%) of sepsis which is in comparable findings of Kayange *et al.,* (2010) (75.83%) and Getabelew *et al.,* (2018) (73.15%). In contrast to present study, Yadav *et al.,* (2018) (63.3%) and Li *et al.,* (2019) (51.61%) found having growth by delivery with cesarean section. The probable reason for this findings might be of unhealthy status of vagina during child birth and careless to maintain the aseptic condition by medical personnel who assisting during and after delivery (Pandit and Vyas, 2020a).

This study reports that the newborns who have been delivered in the hospital was more infected from sepsis (63.91%) and has also been reported by Getabelow *et al.,* (2018)

(75.79%) and Kayange *et al.,* (2010) (69.80%). The reason for the neonatal sepsis cases in hospital-delivered newborns is might be of contaminated environment of delivery room and hospital (Pandit and Vyas, 2020a).

In our study, it was observed that low birth weight babies (78.23%) were more vulnerable to sepsis in comparision to babies of normal weight, this finding is in similar lines with the study of 70.8% sepsis in low birth weight neonates by Lakhey and Shakya (2017), 66.6% by Yadav *et al.,* in 2018, 63.8% by Pokhrel *et al.,* in 2018. In contrary to this, Chapagain *et al.,* in 2015 and Getabelew *et al.,* in 2018 reported 67% and 72.10% sepsis in the baby weighing normal birth weight respectively. According to Benitz (1999), fetus of around 32–34 weeks of gestation age starts maturation of immunological barriers. The maturity is accelerated after birth leading to lowering the level of mucosal antibody in malnourished newborns to fight against bacterial pathogens.

The bacterial spectrum revealed that the higher prevalence of *Staphylococcus aureus*, followed by *K. pneumoniae*, *Streptococcus* spp., *Pseudomonas aeruginosa*, *E. coli*, CoNS, *Enterobacter* spp., and *Proteus* spp. The findings of these prominent bacteria in this study were also reported from earlier studies (Ansari *et al.,* 2015; Aku *et al.,* 2016*;* Lakhey and Shakya, 2017; Pokhrel *et al.,* 2018; Getabelew *et al.,* 2018; Yadav *et al.,* 2018; Li *et al.,* 2019).

The result shown in Table 6.2 revealed the higher prevalence of gram-positive bacteria 270/363 (74.39%) causing neonatal sepsis than that of gram-negative bacteria 93/363 (25.61%). This findings was similar with previous findings of Ansari *et al.,* (2015) (63.8%), Khanal *et al.,* (2004) (73%) Lakhey and Shakya (2017) (72.3%) from Nepal likewise Aku *et al.,* (2016) reported 69% from Ghana, Geyesus *et al.,* (2017) reported 67.5% from Ethiopia. The result indicates that large numbers of bacteria were transferred from contaminated hands of medical personnel and family members of newborns because *S. aureus* is omnipresent in environment and major cause of hospital-acquired infection. *Staphylococcus aureus* is also present frequently as normal flora of body surface and nose (Bailey and Scott's, 2017). Human and medical personnel careless about hygiene and are involved in caring for neonates and the management of peripheral intravenous lines set up on neonates

create higher chances of acquiring these infections (Aku *et al.,* 2016). In contrary to our findings Zakariya *et al.,* (2010) (82%), Kayange *et al.,* (2010) (61.1%), Mehar *et al.,* (2013) (56.7%), Gyawali and Sanjana (2013) (55.9%), Ullah *et al.,* (2016) (78.6%), Pokhrel *et al.,* (2018) (77%), Yadav *et al.,* (2018) (54%) reported preponderance of gram-negative bacteria. We are reporting that most frequent causative agents of neonatal sepsis was *S. aureus* responsible for 63.08% and *K. pneumoniae* for 25.61% among total bacterial isolates. This present result was similar to the reports of Gyawali and Sanjana (2013); Mehar *et al.,* (2013); Shrestha *et al.,* (2013); Geyesus *et al.,* (2017) and in contrast to our studies of Ansari *et al.,* (2015); Pokhrel *et al.,* (2018); Zakariya *et al.,* (2010). The variation of causative bacteria may be due to the hygienic condition of the hospital personnel, sanitation of the hospital and surroundings and geographical region (Pandit and Vyas, 2020a). *S. aureus* was common finding in EONS and LONS both which was similar to the study of Khanal *et al.,* (2004); Geyesus *et al.,* (2017). In contrast to this study, Aku *et al.,* (2016); Ansari *et al.,* (2015); Li *et al.,* (2019) reported different organism in the different onset of sepsis.

6.3 Antibacterial profile for bacterial cultures

In the present investigation antibacterial profiling has been performed in case of isolated bacterial cultures. Selected antibiotics viz. levofloxacin (5 mcg), amikacin (30 mcg), cefixime (5 mcg), meropenem (10 mcg), piperacillin (100 mcg), imipenem (10 mcg), ceftriaxone (30mcg), cefotaxime (30 mcg), ceftazidime (30 mcg), ampicillin (10 mcg), linezolid (30 mcg), vancomycin (30 mcg), penicillin G (10 U) have been used in the present investigation. The result has been shown in Tables 6.4 and 6.5. The result clearly shows that the most efficient antibiotic against Gram-positive organisms were Linezolid with 94% sensitivity. The result also showed that Penicillin-G (99.22%) and Amikacin (81.20%) were found resistant. Gram-negative bacteria were highly sensitive against Imipenem (90.19%) while antimicrobial agents such as Ampicillin (95%) and 3rd generation cephalosporin (Cefotaxime, Cefixime, Ceftriaxone, Ceftazidime) were found resistant against the isolates. In this study, the most efficient antimicrobial agents against gram positive isolate were Linezolid followed by vancomycin which is similar to the earlier studies (Mehar *et al.,* 2013;

Li *et al.,* 2019; Pokhrel *et al.,* 2018). It has been observed in the present investigation, for Gram-negative bacterial isolates antibiotics such as meropenem, imipenem and levofloxacin are choice drugs for the management of sepsis in neonates. Gram-negative bacterial isolates showed resistance to normally used antimicrobials like ampicillin, Piperacillin and 3rd generation Cephalosporins. This study was in accordance with studies of Khanal *et al.,* (2004); Gyawali and Sanjana (2013); Shrestha *et al.,* (2013); Ansari *et al.,* (2015). Almuneef *et al.,* (2001) reported that the haphazardly use of broad-spectrum antibiotics increases the chances of gaining bacterial pathogens by inhibiting the growth of normal flora which are resistant to multiple antibiotics.

6.4 Extract yield and Evaluation of Antibacterial Effect of medicinal Plants

6.4.1 Extraction:

Extraction is the initial step for the study of medicinal plants and the determination of phytochemical constituent of the herbs. In our study, maceration process was applied for the extraction of dried powder of medicinal plants. The result was obtained by maceration of dry powder of selected parts of medicinal plants in a different solvent extract with an increasing polarity viz. hexane, acetone, methanol, 80% ethanol, and water. The percentage weight of the residue obtained after dry of solvent was presented in Figure 6.5. The result clearly shows that aqueous and hydroalcoholic solvent extract more amount of residue compared to methanol, acetone, and the least from hexane in all used medicinal plants. Romero *et al.,* (2005) reported that dried powdered plants retain more concentrated active phytochemical compounds than present in the fresh plant while Kamarudin *et al.,* (2016) reported that exposure of plant materials to high temperature for a long time will evaporate the volatile compounds as well as solvents used with loss of energy. Therefore, by considering the above problem cold maceration of extraction is still a better method.

Table 6.4: Antibiotic sensitivity/ resistance profile of Gram-positive bacteria

Antibiotics	*Staphylococcus aureus* N= 229		CoNS N=13		*Streptococcus* spp. N=28		Total Gram Positive N= 270	
	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)
Vancomycin (30 mcg)	193/226 (85.40)	33/226 (14.60)	12/12 (100)	0/12 (0)	25/28 (89.29)	3/28 (10.71)	230/266 (86.47)	36/266 (13.53)
Linezolid (30 mcg)	210/226 (92.92)	16/226 (7.08)	12/12 (100)	0/12 (0)	28/28 (100)	0/28 (0)	250/266 (94)	16/266 (6)
Penicillin-G (10 U)	0/225 (0)	225/225 (100)	0/10 (0)	10/10 (100)	2/21 (9.52)	19/21 (90.48)	2/256 (0.78)	254/256 (99.22)
Amikacin (30 mcg)	29/197 (14.72)	168/197 (85.28)	11/12 (91.67)	1/12 (8.33)	4/25 (16)	21/25 (84)	44/234 (18.80)	190/234 (81.20)
Meropenem (10 mcg)	132/169 (78.10)	37/169 (21.90)	6/11 (54.55)	5/11 (45.45)	3/18 (16.67)	15/18 (83.33)	141/198 (71.21)	57/198 (28.79)
Cefotaxime (30 mcg)	10/164 (6.10)	154/164 (93.90)	3/9 (33.33)	6/9 (66.67)	2/19 (10.53)	17/19 (89.47)	15/192 (7.81)	177/192 (92.19)
Levofloxacin (5 mcg)	20/173 (11.56)	153/173 (88.44)	4/12 (33.33)	8/12 (66.67)	5/19 (26.32)	14/19 (73.68)	29/204 (14.22)	175/204 (85.78)

N: Number of isolates; CoNS: Coagulase Negative *Staphylococcus*

Table 6.5: Antibiotic sensitivity/ resistance profile of gram-negative bacteria

Antibiotics	*K. pneumoniae* N=48		*P. aeruginosa* N=21		*E. coli* N=17		*Enterobacter spp.* N=6		*Proteus spp.* N=1		Total Gram Negative (N=93)	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
Amikacin (30 mcg)	23/47 (48.94)	24/47 (51.06)	16/19 (84.21)	3/19 (15.79)	5/8 (62.50)	3/8 (37.50)	2/5 (40)	3/5 (60)	0/1 (0)	1/1 (100)	46/80 (57.50)	34/80 (42.50)
Meropenem (10 mcg)	19/39 (48.72)	20/39 (51.28)	16/17 (94.12)	1/17 (5.88)	5/9 (55.55)	4/9 (44.44)	4/6 (66.67)	2/6 (33.33)	1/1 (100)	0/1 (0)	45/72 (62.50)	27/72 (37.50)
Cefotaxime (30 mcg)	0/33 (0)	33/33 (100)	4/13 (30.77)	9/13 (69.23)	0/5 (0)	5/5 (100)	0/6 (0)	6/6 (100)	0/1 (0)	1/1 (100)	4/58 (6.90)	54/58 (93.10)
Levofloxacin (5 mcg)	33/43 (76.74)	10/43 (23.26)	14/18 (77.78)	4/18 (22.22)	12/17 (70.59)	5/17 (29.41)	5/6 (83.33)	1/6 (16.67)	1/1 (100)	0/1 (0)	65/85 (76.47)	20/85 (23.53)
Cefixime (5 mcg)	2/33 (6.06)	31/33 (93.94)	1/7 (14.29)	6/7 (85.71)	1/5 (20)	4/5 (80)	0/6 (0)	6/6 (100)	0/1 (0)	1/1 (100)	4/52 (7.69)	48/52 (92.31)
Piperacillin (100 mcg)	8/48 (16.67)	40/48 (83.33)	10/21 (47.62)	11/21 (52.38)	8/17 (47.06)	9/17 (52.94)	0/6 (0)	6/6 (100)	0/1 (0)	1/1 (100)	26/93 (27.96)	67/93 (72.04)
Imipenem (10 mcg)	30/32 (93.75)	2/32 (6.25)	3/4 (75)	1/4 (25)	8/9 (88.89)	1/9 (11.11)	4/5 (80)	1/5 (20)	1/1 (100)	0/1 (0)	46/51 (90.20)	5/51 (9.80)
Ceftriazone (30 mcg)	4/36 (11.11)	32/36 (88.89)	3/10 (30)	7/10 (70)	0/5 (0)	5/5 (100)	0/6 (0)	6/6 (100)	0/1 (0)	1/1 (100)	7/58 (12.07)	51/58 (87.93)
Ceftazidime (30 mcg)	4/13 (30.77)	9/13 (69.23)	4/16 (25)	12/16 (75)	0/17 (0)	17/17 (100)	0/3 (0)	3/3 (100)	0/1 (0)	1/1 (100)	18/50 (36)	42/50 (84)
Ampicillin (10 mcg)	1/21 (4.76)	20/21 (95.24)	NT	NT	1/16 (6.25)	15/16 (93.75)	0/2 (0)	2/2 (100)	0/1 (0)	1/1 (100)	2/40 (5)	38/40 (95)

NT: Not tested; S: Sensitive; R: Resistant; N: Number of isolates

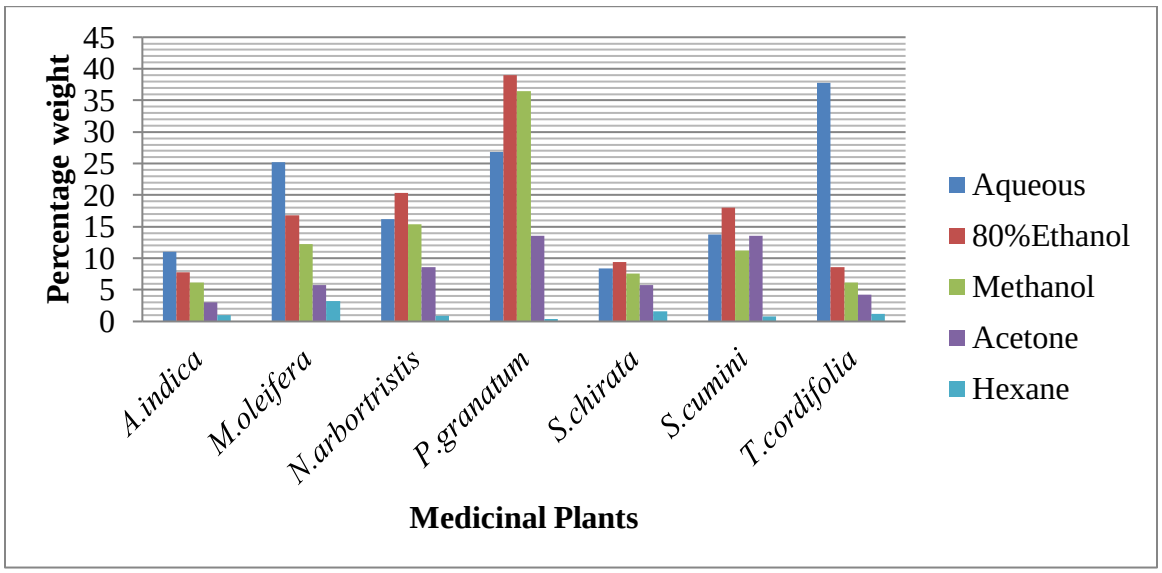


Figure 6.5: Percentage yield of medicinal plants

The extract obtained by using different types of solvents of increasing polarity viz. hexane, acetone, methanol, 80% ethanol and water were made a comparison for the effectiveness of solvents by yield amount of residue obtained. The results in Figure 6.5 clearly indicate broad extraction yield for different solvents used (0.42 -39%). It can be seen that the extraction yield of aqueous and 80% ethanolic is higher than pure methanol and acetone. This result is somewhat consistent with the extraction yield of De Zoysa *et al.*, (2019) and Abbas *et al.*, (2014). This is because of the solvent capacity to extract the components based on the polarity and compounds that will be extracted with respected solvents. 80% ethanolic solvents act as polar and nonpolar and it extracts both polar and non-polar components together in comparison to absolute ethanol (Kamarudin *et al.*, 2016) similarity with *P. granatum*, *S. cumini*, and *N. arbortristis* yields. Water is the most polar solvent and extracted highly polar components from powdered plants. Among these solvents used, water was found to have the most polar potential, results in the extraction of highly polar components from powders. The highest extraction yield in the aqueous solvent is because of the highest polarity of water which also plays its role as universal solvents.

Furthermore, hexane is a non-polar solvent and it extracts the non-polar materials present in lesser amounts in plants leading to yield in the less quantity (De Zoysa *et al.*, 2019).

Methanol, acetone possesses intermediate polarities, but methanol extracted more components from peel than those of water and acetone. This type of report has also been reported by Negi and Jayaprakasha (2003); Molla *et al.,* (2016). The study from Hassine *et al.,* (2013) discusses that variation in the extraction yield may be associated with the laboratory procedure and wide varieties of polar and non-polar compounds. The amount of yield does not always influence the antibacterial activity rather active ingredients found in the extract play a major role in biological activity (Uthayarasa *et al.,* 2010).

6.4.2 Antibacterial efficacy and Qualitative analysis of phytoconstituents

The antibacterial efficacy was determined of different concentration ranging from 25 to 200 mg/ml from selected medicinal plants. Residues obtained by maceration extraction method were tested against different isolated bacteria to study the concentration-dependent inhibition with agar well diffusion method (Perez *et al.,* 1990; Bhandari *et al.,* 2017). The result has been shown in Table 6.6 to 6.12 clearly indicate the inhibition zones (in mm) obtained at different concentration from selected medicinal plants against eight bacterial isolates.

6.4.2.1 *Azadirachta indica*:

The antibacterial activities of *Azadirachta indica* were studied and the result has been shown in Table 6.6. The aqueous fraction of neem leaves extracts exhibited no activity against gram-positive isolates even at the highest concentration of 200 mg/ml and gram-negative bacteria other than *P. aeruginosa* (14.33±0.57 mm) and *Proteus* spp.(9.83±0.76 mm) which exhibited least activity. This finding is in accordance with studies of Khan *et al.,* (2014); Al-Jadidi and Hossain (2015). Among the organic solvent extract, ethanol and methanol showed least sensitivity against *S. aureus* (13.66±0.28 mm, 15.00±1.00 mm), CoNS (11.33±0.57 mm, 14.50±0.50 mm), *Streptococcus* spp. (11.33±0.28 mm, 9.83±0.28 mm) respectively whereas, acetone extract exhibited remarkable sensitivity against *S. aureus* (19.00±0.00 mm) and CoNS (20.33±0.57 mm). Similarly, hexane extract also exhibited moderate activity against *S. aureus* (17.16±0.28 mm) and *Streptococcus* spp. (15.66±1.15 mm) at the concentration of 200 mg/ml. All the gram-negative isolates were resistant against organic solvent extracts at all the concentration used. The result is similar

to the study of Vasantharaj *et al.*, (2013) wherein, *S. aureus* and *Streptococcus pyogenes* showed sensitivity in methanolic extract. In contrary to our study *E. coli*, *K. pneumoniae* and *P. aeruginosa* were sensitive in the same solvent. Khan *et al.,* (2014) reported resistant activity in case of *E.coli* and *K. pneumoniae* in hexane extract but sensitive for *E. coli* in case of ethanolic extract. Lakshmi *et al.,* (2015) reported the effectiveness of the antibacterial nature of *A. indica* is due to the presence of active compounds like azadirachtin which inhibit the formation of the cellular membrane.

The results of phytochemical screening for all plants have been depicted in Table 6.13. The result of *A. indica* in different solvent extract shows marked variation in presence of phytochemical content. The aqueous extract contains all the phytochemical except phenol but it does not exhibited antibacterial activity it may be due to the absence of a sufficient quantity of antimicrobial compounds. The phytochemicals viz. alkaloids, coumarins, quinones, phenols, flavonoids, terpenoids, tannins, steroids and saponins reported from *A. indica* in the present study have also been reported in previous studies (Vasantharaj *et al.,* 2013; Jafari *et al.,* 2013; Al-Jadidi and Hossain, 2015). The presence of steroids in all solvent extract except acetone may be responsible for antibacterial activity because it has been associated with lipid membrane and cause leakage from liposomes (Raquel *et al.*, 2007). Overall, the presence of active biomolecules in the extract exhibit in terms of effectiveness against gram-positive bacteria in the present investigation.

6.4.2.2 *Moringa oleifera*:

In the present investigation, inhibitory effects of *M. oleifera* leaf extract against eight isolates were determined. The result has been shown in Table 6.7. The result clearly depicts that aqueous extract showed highly significant activity against *P. aeruginosa* (20.00±1.00 mm) while others gram-negative exhibited less activity except *Proteus* spp.. *S. aureus* being resistant, whereas *Streptococcus* spp. (14.33±0.57 mm) being moderately sensitive and CoNS (10.66±0.57 mm) as least sensitive at concentration of 200 mg/ml. The present results were in accordance with earlier studies by Oluduro (2012) in which *S. aureus* being resistant and opposite to our study related to gram-negative bacteria. High sensitivity was observed in case of *S. aureus* (22.50±0.50 mm) and *P. aeruginosa* (30.50±0.50 mm) from

80% ethanol. Similar to our study, highest sensitivity was also observed from ethanol extract with *S. aureus* (Elgamily *et al.*, 2016) and *P. aeruginosa* in methanol extract (Pandit and Vyas, 2020b) respectively. In the present study, *S. aureus* (18.33±0.57 mm) in methanol extract, CoNS (13.16±0.28 mm, 16.33±0.57 mm) in 80% ethanol and methanol extract, *Streptooccus* spp. (13.33±0.28 mm, 17.33±0.57 mm) in 80% ethanol and hexane *P. aeruginosa* (19.00±0.50 mm) in 80% ethanol were moderately sensistive. Whereas, *S. aureus* (10.16±0.28 mm), CoNS (12.83±0.28 mm), *K. pneumoniae* (12.33±0.28 mm), *Enterobacter* spp. (12.50±0.86 mm) are least sensitive in hexane, acetone, hexane and 80% ethanol solvent extract respectively. This finding of moderate sensitivity was similar to the reports of Oluduro (2012); Singh (2013); Elgamily *et al.*, (2016). Singh (2013) also reported least sensitive with hexane extract. *E. coli* and *Proteus* spp. are resistant to organic solvent extract resemble to the finding of Oluduro (2012).

Aqueous extract of *M. oleifera* contains all the phytochemical tested (Alkaloids, Coumarins, Quinones, Phenol, Flavonoids, Terpenoids, Tannins, Steroids and Saponins) and has been represented in Table 6.13. The extract did not exhibited antibacterial activity except for *P. aeruginosa*. It indicates that aqueous extract is not able to extract sufficient amount of antimicrobial components from *M. oleifera* leaves. All other solvent extract composed of diversified secondary metabolites although being less in number than aqueous extract. These solvents exhibited remarkable sensitivity towards isolates. Various authors also found a similar type of results (Doughari *et al.*, 2007; Patel *et al.*, 2014; Dzotam *et al.*, 2016).

6.4.2.3 *Nyctanthes arbortristis*

The antibacterial activities of *Nyctanthes arbortristis* were studied and the result has been shown in Table 6.8. The result clearly shows that maximum inhibition was observed against *P. aeruginosa* (20.50±0.76 mm) followed by *S. aureus* (14.83±0.76 mm), CoNS (14.00±0.50 mm), and *Enterobacter* spp. (14.33±0.57 mm) with moderate activity. *Streptococcus* spp., *K. pneumoniae*, *E. coli*, and *Proteus* spp. was found to show resistant activity from aqueous extract of *N. arbortristis*. The present result was similar to the studies of Jain and Singh (2013) wherein, *S. aureus, P. aeruginosa, E. coli* and *Proteus vulgaris* were significantly

sensitive. In the present study, 80% ethanolic fraction proved most effective among in all concentration and against all the isolates, showing maximum sensitivity against *P. aeruginosa* (30.50±0.50 mm), *S. aureus* (29.00±0.00 mm), and CoNS (22.33±0.57 mm) while moderate activity against *Streptococcus* spp. (18.33±0.76 mm), *Proteus* spp. (18.33±0.57 mm), *E. coli* (18.16.±0.28 mm), *K. pneumoniae* (17.33±0.28 mm), and *Enterobacter* spp. (14.83±0.28 mm). Sathiya *et al.,* (2008) reported similar pattern of sensitivity in consistent to our study against *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *S. aureus* and *Streptococcus* spp.. Methanol, acetone and hexane fraction showed moderate activity against gram-positive isolates, whereas resistant against gram-negative tested bacterial strains at concentration of 200 mg/ml. *S. aureus* (16.33±0.57 mm, 13.33±0.28 mm) and CoNS (14.33±0.28 mm, 15.33±0.28 mm) were moderately active in methanol and acetone extract while *Streptococcus* spp. (17.33±0.57 mm) showed moderate inhibition in hexane extract. In contrast to present investigation, Jain and Singh (2013) found significant inhibition against *E. coli*, *P. aerugonosa* and *Proteus vulgaris* in methanolic fraction.

The result related to the phytochemical analysis of *N. arbortristis* has been represented in Table 6.13. In the present study, aqueous extract of *N. arbortristis* contain only alkaloids, tannins, and saponins whereas, 80% ethanol and methanol extract revealed the existence of all the phytochemical tested except terpenoids and saponin respectively. The result also showed that acetone extract contains more number of phytochemical tested in comparison to hexane, as it contains only phenol. The result has been also reported by various authors (Thangavelu and Thomas, 2010; Chouhan *et al.,* 2014; Chidi *et al.,* 2015; Patel and Gokhle, 2016).

6.4.2.4 *Punica granatum*

Antibacterial activity of *P. granatum* has been depicted in Table 6.9. The study shows that aqueous extract of rind gave significant antibacterial activity against *Enterobacter* spp. (22.00±0.50 mm), *S. aureus* (20.83±0.76 mm), CoNS (20.50±0.50 mm) and *Streptococcus* spp. (20.16±0.76 mm) whereas moderate activity was observed in *E. coli* (16.33±0.33 mm) and *P. aeruginosa* (17.00±1.00 mm). *Klebsiella pneumoniae* and *Proteus* spp. were found to be resistant in comparison to control. This result shows a similar pattern of finding in

accordance with Malviya *et al.,* (2014) in which, *S. aureus* and *Enterobacter aerogenes* were significantly sensitive. In contrast, Al-Zoreky *et al.*, (2009) reported *S. aureus, E. coli, P. aeruginosa* were resistant. The present investigation clearly shows that 80% ethanolic extract showed highly significant antibacterial potential against *S. aureus* (22.83±0.76 mm), *P. aeruginosa* (21.83±.28 mm), and moderate activity against CoNS (17.00±1.00 mm), *Streptococcus* spp. (15.66±0.57 mm), *K. pneumoniae* (15.50±0.50 mm), *E. coli* (15.66±0.28 mm), *Enterobacter* spp. (16.83±0.28 mm) and *Proteus* spp. (15.50±0.50 mm). Malviya *et al.,* (2014) and Eddebbagh *et al.,* (2018) also reported significant activity of *S. aureus, Enterobacter aerogenes, K. pneumoniae* and *P. aeruginosa, E. coli* in similar lines with our findings. In this study, high significant antibacterial activity was found against *S. aureus* (30.50±0.50 mm, 20.00±1.00 mm), CoNS (24.16±0.28 mm, 20.66±0.57 mm) and *P. aeruginosa* (21.83±0.28 mm, 25.16±0.28 mm) while moderate activity against *Proteus* spp. (14.50±0.50 mm, 14.16±0.28 mm) and least activity with *Streptococcus* spp. (10.16±0.28 mm) in methanol and acetone extracts respectively. In addition moderate activity was observed against *Enterbacter* spp. (15.66±0.28 mm) in acetone extract. Our result are in consistent with previously published research of Malviya *et al.,* (2014) and Eddebbagh *et al.,* (2018) wherein, *S. aureus*, *Enterobacter aerogenes*, and *P. aeruginosa*, *E. coli* were exhibiting significant antibacterial activity from methanol and acetone extract respectively. In this report, hexane extract was only sensitive against *Streptococcus* spp. (16.33±0.57 mm) and *Proteus* spp. (13.00±0.00 mm) whereas resistant against other isolates.

Phytochemical analysis of *P. granatum* has been depicted in Table 6.13. The existence of all the phytochemical tested except alkaloids, coumarins, and quinones in aqueous extract, beside this methanol extract contain all the tested phytochemicals whereas, 80% ethanolic, acetone and hexane extract devoid of phenol. Saponin was absent in 80% ethanolic extract. Similar reports have been reported by various authors (Al-Zoreky, 2009; Eddebbagh *et al.,* 2018).

6.4.2.5 *Swertia chirata*

The antibacterial activity of *Swertia chirata* has been depicted in Table 6.10. In the present finding, aqueous extract exhibited no activity against all the bacteria studied at

the highest concentration of 200 mg/ml tested. This type of similar finding was reported by Ahirwal *et al.,* (2011). Ahirwal *et al.,* (2011) also reported the moderate activity of *E. coli*, and Khalid *et al.,* (2011) found moderate activity against *P. aeruginosa*. The present results revealed 80% ethanol extract from the whole part exhibited the highest inhibitory action against *S. aureus* (30.33±0.57 mm), CoNS (30.33±0.57 mm), *Streptococcus* spp. (22.83±0.28 mm), *P. aeruginosa* (40.33±0.57 mm) and *Enterobacter* spp. (20.33±0.57 mm) nearly equal to or more effective than control used. *K. pneumoniae* (18.33±0.28 mm), *E. coli* (17.16±0.28 mm), and *Proteus* spp. (18.66±0.28 mm) showed moderate activity. Naqvi *et al.,* (2013) reported *Streptococcus* spp. and *E. coli* showed strong antibacterial potential with increased concentration similarly as per our investigation. Methanolic solvent extracts exhibited the highest activity towards *S. aureus* (22.33±0.28 mm), and CoNS (20.83±0.28 mm) while moderate activity against *Streptococcus* spp., *E.coli* (15.16±0.28 mm), *P. aeruginosa* (13.16±0.28 mm). The present finding was in agreement with Naqvi *et al.,* (2013) and Kweera *et al.,* (2011) wherein, *E. coli* and *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans* were significantly active with methanol extract respectively. Acetone extract exhibited moderate activity against *S. aureus* (17.00±0.50 mm), CoNS (19.50±0.50 mm), and *P. aeruginosa* (15.16±0.28 mm). The finding is in agreement with Kweera *et al.,* (2011) wherein, *S. epidermidis* is significantly sensitive. Hexane extract exhibited moderate activity against *Streptococcus* spp., (15.50±0.50 mm) only.

Phytochemical analysis of *Swertia chirata* has been depicted in Table 6.13. The most active ethanolic extract contains all phytochemicals excluding phenol. Although methanol extract possesses all phytochemical tested but was not as sensitive as ethanolic extract. Acetone and hexane extract also have shown the same activity as methanol. Aqueous extract only devoid of coumarins, quinones, phenol, and the presence of others phytochemicals, are not able to exhibit antibacterial activity against bacterial isolates. The result was similar to the study of Ahirwal *et al.,* (2011); Kweera *et al.,* (2011); Khanal *et al.,* (2015).

6.4.2.6 *Syzygium cumini*

The antibacterial activities of *S. cumini* were performed against isolated bacteria and results were depicted in Table 6.11. The result clearly showed that *in vitro* antibacterial properties of aqueous extract possessed the most significant antibacterial potential against gram positive bacteria *S. aureus* (30.16±0.76 mm), CoNS (20.33±0.57 mm), *Streptococcus* spp. (20.00±1.00 mm) and moderate activity against *P. aeruginosa* (13.83±0.76 mm). All other gram-negative bacteria did not exhibited antibacterial potential towards the extract. The present study was in accordance with the study of Gowri and Vasantha (2010) in which aqueous leaf extract was studied and found that *S. aureus* and *P. aeruginosa* exhibited significant activity in contrary to our study. Researcher observed that *E.coli* have moderate activity. 80% ethanolic extract of *S. cumini* leaf exhibited moderate antibacterial activity against CoNS (17.66±0.28 mm), *Streptococcus* spp. (13.16±0.28 mm), *K. pneumoniae* (16.33±0.28 mm), *E. coli* (14.83±.28 mm), *Enterobacter* spp. (13.33±0.28 mm), *Proteus* spp. (15.33±0.28 mm) except *S. aureus* (20.50±0.50 mm) and *P. aeruginosa* (20.33±0.28 mm) which showed highest activity. Similar to our study significant activity was also found by Nascimento *et al.,* (2000) against *S. aureus*, *Proteus* spp., *K. pneumoniae*, *P. aeruginosa*, *Enterobacter aerogenes*. The study also resembles with the study of Prabhahar *et al.*, (2012) reporting high significant activity from ethanolic extract against *S. aureus, Pseudomonas aeruginosa, E. coli* and *K. pneumoniae*. Methanol and Acetone extract exhibited significant activity against *S. aureus* (20.33±.57 mm, 16.33±0.57 mm), CoNS (20.50±0.50 mm, 20.00±1.00 mm), *P. aeruginosa* (15.16±0.28 mm, 24.50±0.86 mm), and moderate activity against *Streptococcus* spp. (12.50±0.50 mm, 13.16±0.28 mm) and *Enterobacter* spp. (14.66±0.73 mm) respectively. The significant antibacterial results were obtained from the methanolic extract in our study and the results were similar to the study of Reddy and Jose (2013) wherein, *Enterobacter* spp., *S. aureus, P. aeruginosa* were significantly active. *K. Pneumoniae*, *E. coli,* and *Proteus* spp. are resistant to all the solvent extract except 80% ethanol while hexane extract was moderately sensitive to *Streptococcus* spp. (14.33±0.57 mm) only. In contrary to our result, Kumar *et al.,* (2014) found significant activity against *K. pneumoniae* and *E. coli* in methanolic extract.

Phytochemical analysis of *S. cumini* has been depicted in Table 6.13. The aqueous extract contains alkaloids, flavonoids, terpenoids, tannins and steroids. 80% ethanolic extract have quinones and saponins. Methanol and acetone extract does not have the presence of coumarins, flavonoids, and saponin respectively. Hexane possesses coumarins, steroids and exhibiting the least activity among solvents. 80% ethanolic extract was highly active for antibacterial activity. The current findings are in consistent with studies of Gowri and Vasantha (2010), Ugbabe *et al.,* (2010), Reddy and Jose (2013) in leaf extract of *S. cumini* in different solvent.

6.4.2.7 *Tinospora cordifolia*

The antibacterial activities were performed under *in vitro* and results have been depicted in Table 6.12. The antibacterial activity of aqueous fraction of *T. cordifolia* leaf extracts exhibited high sensitivity against *P. aeruginosa* (20.00±1.00 mm) and less sensitivity against *K. pneumoniae* (12.16±0.76 mm), *E. coli* (12.33±0.57 mm), and *Enterobacter* spp. (10.16±0.76 mm). All other bacterial isolates (*S. aureus*, CoNS, *Streptococcus* spp., *Proteus* spp.) are resistant at concentration of 200 mg/ml tested. The present study was in similar lines with that of earlier report by Mohana *et al.,* (2008) in reporting resistance by *S. aureus* and *Streptococcus* spp.. In contrary to our result the author also reported no activity against *Pseudomonas aeruginosa*, *K. pneumoniae* and *Escherichia coli*. 80% ethanolic solvent extract exhibited moderate activity against *P. aeruginosa* (15.66±0.28 mm) and least with *S. aureus* (11.83±0.76 mm) in contrary to other isolates which showed resistant activity. Inacio *et al.,* (2013) reported resistant activity in *S. aureus* which is in similar lines with present investigation. Methanol extracts showed high sensitivity against *P. aeruginosa* (26.16±0.28 mm) while moderate activity against CoNS (14.16±0.28 mm) and least with *K. pneumoniae* (12.6±0.28 mm) whereas, other isolates showed resistance activity. Hossain *et al.,* (2013) reported resistant activity by *E. coli* in methanol extract which is in similar lines with our present investigation. Acetone and hexane extract exhibited moderate activity against *S. aureus* (13.16±0.28 mm), CoNS (13.16±0.28 mm, 18.33±0.57 mm) *Streptococcus* spp., (16.33±0.57 mm) respectively at concentration of 200 mg/ml tested.

Phytochemical analysis of *T. cordifolia* has been shown in Table 6.13. The preliminary qualitative phytochemical screening showed the presence of alkaloids, coumarins, quinones, terpenoids, steroids, and saponins in aqueous extract. Tannin was present in 80% ethanolic extract in addition to phytochemical from aqueous extract. Methanol extract contain alkaloids, coumarins and flavonoids whereas, acetone extract consists of alkaloids, coumarins, quinones and terpenoids. Hexane possesses only terpenoids. The variable result was also observed in the study from different authors (Kavitha *et al.,* 2011; Desai *et al.,* 2012; Rao *et al.,* 2013)

Table 6.6: Antibacterial activity of *Azadirachta indica* (Leaves) in different solvent extract

Solvent	Extract Concentration	*Staphylococcus aureus*	CoNS	*Streptococcus* spp.	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enterobacter* spp.	*Proteus* spp.
Aqueous	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	-	-	-	-	-	-	-	-
	100 mg/ml	-	-	-	-	-	12.00±1.00	-	-
	200 mg/ml	-	-	-	-	-	14.33±0.57	-	9.83±0.76
80% Ethanol	25 mg/ml	11.33±0.28	-	9.00±0.00	-				
	50 mg/ml	12.83±0.28	11.16±0.28	9.16±.28	-	-	-	-	-
	100 mg/ml	13.16±0.28	11.16±0.28	9.33±0.28	-	-	-	-	-
	200 mg/ml	13.66±0.28	11.33±0.57	11.33±0.28	-	-	-	-	-
Methanol	25 mg/ml	10.33±0.57	-	-	-	-	-	-	-
	50 mg/ml	12.33±0.57	12.33±0.57	-	-	-	-	-	-
	100 mg/ml	14.50±0.50	13.16±0.28	-	-	-	-	-	-
	200 mg/ml	15.00±1.00	14.50±0.50	9.83±0.28	-	-	-	-	-
Acetone	25 mg/ml	15.33±0.57	13.33±0.28	-	-	-	-	-	-
	50 mg/ml	17.16±0.28	18.00±1.00	-	-	-	-	-	-
	100 mg/ml	18.16±0.28	18.33±0.57	-	-	-	-	-	-
	200 mg/ml	19.00±0.00	20.33±0.57	-	-	-	-	-	-
Hexane	25 mg/ml	-	-	14.16±0.28	-	-	-	-	-
	50 mg/ml	-	-	14.66±0.28	-	-	-	-	-
	100mg/ml	10.16±0.28	-	15.50±0.50	-	-	-	-	-
	200mg/ml	17.16±0.28	-	15.66±1.15	-	-	-	-	-
NC	DW/DMSO	-	-	-	-	-	-	-	-
PC(VA)	25 mg/ml	31.16±1.04	18.16±1.04	33.33±1.52	NT	NT	NT	NT	NT
	50 mg/ml	32.83±0.76	21.00±1.00	34.16±0.28	NT	NT	NT	NT	NT
	100 mg/ml	33.50±0.86	23.16±1.25	36.33±0.28	NT	NT	NT	NT	NT
	200 mg/ml	35.00±1.00	23.83±0.76	38.50±0.50	NT	NT	NT	NT	NT
PC(LE)	5 mcg/disc	NT	NT	NT	20.33±0.57	17.83±0.28	32.66±0.57	17.33±0.57	24.33±0.57

Values are the mean ± SD of inhibition zone in mm; Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well;CoNS: Coagulase negative *Staphylococcus*; NC: Negative control as solvent; PC: Positive control; DW: Distilled water; DMSO: Dimethylsulfoxide; VA: Vancomycin; LE: Levofloxacin; NT: Not tested;

Table 6.7: Antibacterial activity of *Moringa oleifera* (Leaves) in different solvent extract

Solvent	Extract Concentration	*Staphylococcus aureus*	CoNS	*Streptococcus* spp.	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enterobacter* spp.	*Proteus* spp.
Aqueous	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	-	-	-	-	-	-	-	-
	100 mg/ml	-	-	-	-	-	16.00±1.00	-	-
	200 mg/ml	-	10.66±0.57	14.33±0.57	10.00±1.00	10.83±0.76	20.00±1.00	9.33±0.28	-
80% Ethanol	25 mg/ml	12.33±0.28	10.50±0.50	10.66±0.57	-	-	14.50±0.50	-	-
	50 mg/ml	12.83±0.28	11.83±0.28	11.33±0.28	-	-	14.33±0.28	10.00±0.00	-
	100 mg/ml	14.83±0.28	12.66±0.28	12.16±0.28	-	-	14.83±0.28	11.00±1.00	-
	200 mg/ml	22.50±0.50	13.16±0.28	13.33±0.28	-	-	19.00±0.50	12.50±0.86	-
Methanol	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	-	-	-	-	-	22.50±0.50	-	-
	100 mg/ml	16.66±0.57	-	-	-	-	25.16±0.28	-	-
	200 mg/ml	18.33±0.57	16.33±0.57	-	-	-	30.50±0.50	-	-
Acetone	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	-	10.33±0.57	-	-	-	-	-	-
	100 mg/ml	-	12.50±0.50	-	-	-	-	-	-
	200 mg/ml	-	12.83±0.28	-	-	-	-	-	-
Hexane	25 mg/ml	-	-	13.16±0.28	-	-	-	-	-
	50 mg/ml	-	-	14.33±0.28	-	-	-	-	-
	100 mg/ml	-	-	15.33±0.28	11.16±0.28	-	-	-	-
	200 mg/ml	10.16±0.28	-	17.33±0.57	12.33±0.28	-	-	-	-
NC	DW/DMSO	-	-	-	-	-	-	-	-
PC(VA	25 mg/ml	31.16±1.04	18.16±1.04	33.33±1.52	NT	NT	NT	NT	NT
	50 mg/ml	32.83±0.76	21.00±1.00	34.16±0.28	NT	NT	NT	NT	NT
	100 mg/ml	33.50±0.86	23.16±1.25	36.33±0.28	NT	NT	NT	NT	NT
	200 mg/ml	35.00±1.00	23.83±0.76	38.50±0.50	NT	NT	NT	NT	NT
PC(LE)	5 mcg/disc	NT	NT	NT	20.33±0.57	17.83±0.28	32.66±0.57	17.33±0.57	24.33±0.57

Values are the mean ± SD of inhibition zone in mm; Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well;CoNS: Coagulase negative *Staphylococcus*; NC: Negative control; PC: Positive control; DW: Distilled water; DMSO: Dimethylsulfoxide; VA: Vancomycin; LE: Levofloxacin; NT: Not tested;

Table 6.8: Antibacterial activity of *Nyctanthes arbortristis* (Leaves) in different solvent extract

Solvent	Extract Concentration	*Staphylococcus aureus*	CoNS	*Streptococcus* spp.	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enterobacter* spp.	*Proteus* spp.
Aqueous	25 mg/ml	-	9.83±0.28	-	-	-	12.00±1.00	-	-
	50 mg/ml	-	12.50±0.50	-	-	-	14.33±0.57	10.83±0.76	-
	100 mg/ml	12.00±1.00	13.50±0.50	-	-	-	17.83±0.76	12.16±0.76	-
	200 mg/ml	14.83±0.76	14.00±0.50	-	-	-	20.50±1.32	14.33±0.57	-
80% Ethanol	25 mg/ml	18.33±0.28	15.16±0.28	9.16±0.28	12.83±0.28	12.16±0.28	14.33±0.28	11.16±0.28	12.16±0.28
	50 mg/ml	20.50±0.50	16.16±0.28	9.16±0.28	12.83±0.28	13.66±0.28	16.16±0.28	13.16±0.28	13.50±0.50
	100 mg/ml	22.33±0.28	19.33±0.57	12.50±0.50	16.16±0.28	15.50±0.50	22.16±0.28	13.50±0.50	17.66±0.28
	200 mg/ml	29.00±0.00	22.33±0.57	18.33±0.76	17.33±0.28	18.16±0.28	30.50±0.50	14.83±0.28	18.33±0.57
Methanol	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	12.50±0.50	-	-	-	-	-	-	-
	100 mg/ml	14.16±0.28	12.33±0.28	-	-	-	-	-	-
	200 mg/ml	16.33±0.57	14.33±0.28	-	-	-	-	-	-
Acetone	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	10.16±0.28	10.16±0.28	-	-	-	-	-	-
	100 mg/ml	12.16±0.28	13.33±0.28	-	-	-	-	-	-
	200 mg/ml	13.33±0.28	15.33±0.28	-	-	-	-	-	-
Hexane	25 mg/ml	-	-	15.33±0.28	-	-	-	-	-
	50 mg/ml	-	-	16.16±0.28	-	-	-	-	-
	100 mg/ml	-	-	17.00±1.00	-	-	-	-	-
	200 mg/ml	-	-	17.33±0.57	-	-	-	-	-
NC	DW/DMSO	-	-	-	-	-	-	-	-
PC(VA)	25 mg/ml	31.16±1.04	18.16±1.04	33.33±1.52	NT	NT	NT	NT	NT
	50 mg/ml	32.83±0.76	21.00±1.00	34.16±0.28	NT	NT	NT	NT	NT
	100 mg/ml	33.50±0.86	23.16±1.25	36.33±0.28	NT	NT	NT	NT	NT
	200 mg/ml	35.00±1.00	23.83±0.76	38.50±0.50	NT	NT	NT	NT	NT
PC(LE)	5 mcg/disc	NT	NT	NT	20.33±0.57	17.83±0.28	32.66±0.57	17.33±0.57	24.33±0.57

Values are the mean ± SD of inhibition zone in mm; Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well;CoNS: Coagulase negative *Staphylococcus*; NC: Negative control; PC: Positive control; DW: Distilled water; DMSO: Dimethylsulfoxide; VA: Vancomycin; LE: Levofloxacin; NT: Not tested;

Table 6.9: Antibacterial activity of *Punica granatum* (Rind) in different solvent extract

Solvent	Extract Concentration	*Staphylococcus aureus*	CoNS	*Streptococcus* spp.	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enterobacter* spp.	*Proteus* spp.
Aqueous	25 mg/ml	11.83±0.28	13.66±0.28	-	-	-	10.00±1.00	12.83±0.28	-
	50 mg/ml	13.66±0.57	14.83±0.28	11.16±0.76	-	-	12.66±0.28	16.33±0.28	-
	100 mg/ml	17.83±0.76	16.00±0.50	13.00±0.00	-	10.16±0.44	15.16±0.76	17.83±0.28	-
	200 mg/ml	20.83±0.76	20.50±0.50	20.16±0.76	-	16.33±0.33	17.00±1.00	22.00±0.50	-
80% Ethanol	25 mg/ml	16.16±0.76	9.16±0.28	11.00±0.50	12.66±0.57	-	14.83±0.76	13.16±0.28	9.16±0.28
	50 mg/ml	17.00±1.00	13.16±0.28	12.00±0.50	13.16±0.28	13.50±0.50	16.50±0.50	14.50±0.50	11.33±0.28
	100 mg/ml	20. 50±0.50	13.00±1.00	13.16±0.28	14.33±0.57	13.33±0.28	20.50±0.50	15.50±0.50	12.33±0.28
	200 mg/ml	22.83±0. 76	17.00±1.00	15.66±0.57	15.50±0.50	15.66±0.28	21.83±0.28	16.83±0.28	15.50±0.50
Methanol	25 mg/ml	18.16±0.28	15.50±0.50	-	-	-	12.50±0.50	-	-
	50 mg/ml	20. 50±0.50	20.50±0.50	-	-	-	15.33±0.57	-	-
	100 mg/ml	24.16±0.28	21.50±0.86	-	-	-	17.83±0.76	-	-
	200 mg/ml	30. 50±0.50	24.16±0.28	10.16±0.28	-	-	21.83±0.28	-	14.50±0.50
Acetone	25 mg/ml	13. 50±0.50	18.50±0.50	-	-	-	15.50±0.50	13.16±0.28	-
	50 mg/ml	14.16±0.28	19.50±0.50	-	-	-	17.66±0.28	13.33±0.28	-
	100 mg/ml	16.83±0.76	20.16±0.28	-	-	-	20.33±0.57	14.33±0.28	12.16±0.28
	200 mg/ml	20.00±1.00	20.66±0.57	-	-	-	25.16±0.28	15.66±0.28	14.16±0.28
Hexane	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	-	-	12.83±0.28	-	-	-	-	11.16±0.28
	100 mg/ml	-	-	14.16±0.28	-	-	-	-	12.16±0.28
	200 mg/ml	-	-	16.33±0.57	-	-	-	-	13.00±0.00
NC	DW/DMSO	-	-	-	-	-	-	-	-
PC(VA)	25 mg/ml	31.16±1.04	18.16±1.04	33.33±1.52	NT	NT	NT	NT	NT
	50 mg/ml	32.83±0.76	21.00±1.00	34.16±0.28	NT	NT	NT	NT	NT
	100 mg/ml	33.50±0.86	23.16±1.25	36.33±0.28	NT	NT	NT	NT	NT
	200 mg/ml	35.00±1.00	23.83±0.76	38.50±0.50	NT	NT	NT	NT	NT
PC(LE)	5 mcg/disc	NT	NT	NT	20.33±0.57	17.83±0.28	32.66±0.57	17.33±0.57	24.33±0.57

Values are the mean ± SD of inhibition zone in mm; Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well;CoNS: Coagulase negative *Staphylococcus*; NC: Negative control; PC: Positive control; DW: Distilled water; DMSO: Dimethylsulfoxide; VA: Vancomycin; LE: Levofloxacin; NT: Not tested;

Table 6.10: Antibacterial activity of *Swertia chirata* (Whole plant) in different solvent extract

Solvent	Extract Concentration	*Staphylococcus aureus*	CoNS	*Streptococcus* spp.	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enterobacter* spp.	*Proteus* spp.
Aqueous	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	-	-	-	-	-	-	-	-
	100 mg/ml	-	-	-	-	-	-	-	-
	200 mg/ml	-	-	-	-	-	-	-	-
80% Ethanol	25 mg/ml	20.33±0.57	22.33±0.57	11.16±0.28	14.83±0.28	15.16±0.28	25.16±0.28	12.66±0.76	-
	50 mg/ml	25.16±0.28	25.16±0.28	12.83±0.28	16.33±0.57	15.33±0.28	30.33±0.57	15.50±0.50	15.50±0.50
	100 mg/ml	28.33±0.57	27.50±0.50	20.33±0.57	17.33±0.57	16.00±0.00	37.83±0.28	17.33±0.57	17.50±0.50
	200 mg/ml	30.33±0.57	30.33±0.57	22.83±0.28	18.33±0.28	17.16±0.28	40.33±0.57	20.33±0.57	18.66±0.28
Methanol	25 mg/ml	18.33±0.57	15.16±0.28	-	-	-	-	-	-
	50 mg/ml	19.33±0.57	16.00±0.00	-	-	-	-	-	-
	100 mg/ml	20.33±0.28	18.33±0.57	13.00±0.00	-	12.83±0.28	12.16±0.28	-	-
	200 mg/ml	22.33±0.28	20.83±0.28	15.16±0.28	-	15.16±0.28	13.16±0.28	-	-
Acetone	25 mg/ml	14.16±0.28	16.83±0.28	-	-	-	10.16±0.28	-	-
	50 mg/ml	15.16±0.28	18.33±0.57	-	-	-	12.33±0.28	-	-
	100 mg/ml	16.33±0.28	19.33±0.57	-	-	-	14.66±0.28	-	-
	200 mg/ml	17.00±0.50	19.50±0.50	-	-	-	15.16±0.28	-	-
Hexane	25 mg/ml	-	-	12.66±0.57	-	-	-	-	-
	50 mg/ml	-	-	13.50±0.50	-	-	-	-	-
	100 mg/ml	-	-	14.33±0.28	-	-	-	-	-
	200 mg/ml	-	-	15.50±0.50	-	-	-	-	-
NC	DW/DMSO	-	-	-	-	-	-	-	-
PC(VA)	25 mg/ml	31.16±1.04	18.16±1.04	33.33±1.52	NT	NT	NT	NT	NT
	50 mg/ml	32.83±0.76	21.00±1.00	34.16±0.28	NT	NT	NT	NT	NT
	100 mg/ml	33.50±0.86	23.16±1.25	36.33±0.28	NT	NT	NT	NT	NT
	200 mg/ml	35.00±1.00	23.83±0.76	38.50±0.50	NT	NT	NT	NT	NT
PC(LE)	5 mcg/disc	NT	NT	NT	20.33±0.57	17.83±0.28	32.66±0.57	17.33±0.57	24.33±0.57

Values are the mean ± SD of inhibition zone in mm; Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well;CoNS: Coagulase negative *Staphylococcus*; NC: Negative control; PC: Positive control; DW: Distilled water; DMSO: Dimethylsulfoxide; VA: Vancomycin; LE: Levofloxacin; NT: Not tested;

Table 6.11: Antibacterial activity of *Syzygium cumini* (Leaves) in different solvent extract

Solvent	Extract Concentration	*Staphylococcus aureus*	CoNS	*Streptococcus* spp.	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enterobacter* spp.	*Proteus* spp.
Aqueous	25 mg/ml	12.83±0.28	14.00±1.00	-	-	-	-	-	-
	50 mg/ml	25.16±0.76	15.00±1.00	-	-	-	-	-	-
	100 mg/ml	26.83±0.28	17.16±1.04	18.00±0.50	-	-	12.00±0.50	-	-
	200 mg/ml	30.16±0.76	20.33±0.57	20.00±1.00	-	-	13.83±0.76	-	-
80% Ethanol	25 mg/ml	14.16±0.28	13.33±0.28	11.16±0.28	12.83±0.28	10.16±0.28	13.33±0.28		12.50±0.50
	50 mg/ml	16.50±0.50	14.16±0.28	10.83±0.76	12.83±0.28	10.16±0.28	17.83±0.28	10.16±0.28	13.33±0.28
	100 mg/ml	19.33±0.28	16.50±0.50	12.16±0.28	14.33±0.28	13.00±0.50	20.16±0.28	12.16±0.28	14.33±0.28
	200 mg/ml	20.50±0.50	17.66±0.28	13.16±0.28	16.33±0.28	14.83±0.28	20.33±0.28	13.33±0.28	15.33±0.28
Methanol	25 mg/ml	16.16±0.28	16.16±0.28	-	-	-	-	-	-
	50 mg/ml	18.00±0.00	18.33±0.57	-	-	-	12.16±0.28	-	-
	100 mg/ml	20.33±0.57	18.50±0.50	11.16±0.28	-	-	13.16±0.28	12.50±0.50	-
	200 mg/ml	20.33±0.57	20.50±0.50	12.50±0.50	-	-	15.16±0.28	14.66±0.73	-
Acetone	25 mg/ml	12.50±0.50	14.16±0.28	-	-	-	17.50±0.50	-	-
	50 mg/ml	14.16±0.28	15.33±0.28	12.16±0.28	-	-	20.66±0.76	-	-
	100 mg/ml	15.16±0.28	16.33±0.28	12.33±0.28	-	-	22.83±0.28	-	-
	200 mg/ml	16.33±0.57	20.00±1.00	13.16±0.28	-	-	24.50±0.86	-	-
Hexane	25 mg/ml	-	-	12.33±0.28	-	-	-	-	-
	50 mg/ml	-	-	13.33±0.28	-	-	-	-	-
	100 mg/ml	-	-	13.50±0.00	-	-	-	-	-
	200 mg/ml	-	-	14.33±0.57	-	-	-	-	-
NC	DW/DMSO	-	-	-	-	-	-	-	-
PC(VA)	25 mg/ml	31.16±1.04	18.16±1.04	33.33±1.52	NT	NT	NT	NT	NT
	50 mg/ml	32.83±0.76	21.00±1.00	34.16±0.28	NT	NT	NT	NT	NT
	100 mg/ml	33.50±0.86	23.16±1.25	36.33±0.28	NT	NT	NT	NT	NT
	200 mg/ml	35.00±1.00	23.83±0.76	38.50±0.50	NT	NT	NT	NT	NT
PC(LE)	5 mcg/disc	NT	NT	NT	20.33±0.57	17.83±0.28	32.66±0.57	17.33±0.57	24.33±0.57

Values are the mean ± SD of inhibition zone in mm; Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well;CoNS: Coagulase negative *Staphylococcus*; NC: Negative control; PC: Positive control; DW: Distilled water; DMSO: Dimethylsulfoxide; VA: Vancomycin; LE: Levofloxacin; NT: Not tested;

Table 6.12: Antibacterial activity of *Tinospora cordifolia* (Leaves) in different solvent extract

Solvent	Extract Concentration	*Staphylococcus aureus*	CoNS	*Streptococcus* spp.	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Enterobacter* spp.	*Proteus* spp.
Aqueous	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	-	-	-	-	-	15.83±0.28	-	-
	100 mg/ml	-	-	-	10.16±0.28	-	18.16±0.28	-	-
	200 mg/ml	-	-	-	12.16±0.76	12.33±0.57	20.00±1.00	10.16±0.76	-
80% Ethanol	25 mg/ml	-	-	-	-	-	11.66±0.28	-	-
	50 mg/ml	10.33±0.28	-	-	-	-	13.33±0.28	-	-
	100 mg/ml	11.00±1.00	-	-	-	-	14.16±0.28	-	-
	200 mg/ml	11.83±0.76	-	-	-	-	15.66±0.28	-	-
Methanol	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	-	-	-	-	-	22.50±0.50	-	-
	100 mg/ml	-	-	-	-	-	24.00±0.00	-	-
	200 mg/ml	-	14.16±0.28	-	12.16±0.28	-	26.16±0.28	-	-
Acetone	25 mg/ml	-	-	-	-	-	-	-	-
	50 mg/ml	11.83±0.76	10.50±0.50	-	-	-	-	-	-
	100 mg/ml	12.16±0.28	12.16±0.28	-	-	-	-	-	-
	200 mg/ml	13.16±0.28	13.16±0.28	-	-	-	-	-	-
Hexane	25 mg/ml	-	12.16±0.28	-	-	-	-	-	-
	50 mg/ml	-	14.33±0.28	-	-	-	-	-	-
	100 mg/ml	-	17.16±0.28	14.33±0.57	-	-	-	-	-
	200 mg/ml	-	18.33±0.57	16.33±0.57	-	-	-	-	-
NC	DW/DMSO	-	-	-	-	-	-	-	-
PC(VA)	25 mg/ml	31.16±1.04	18.16±1.04	33.33±1.52	NT	NT	NT	NT	NT
	50 mg/ml	32.83±0.76	21.00±1.00	34.16±0.28	NT	NT	NT	NT	NT
	100 mg/ml	33.50±0.86	23.16±1.25	36.33±0.28	NT	NT	NT	NT	NT
	200 mg/ml	35.00±1.00	23.83±0.76	38.50±0.50	NT	NT	NT	NT	NT
PC(LE)	5 mcg/disc	NT	NT	NT	20.33±0.57	17.83±0.28	32.66±0.57	17.33±0.57	24.33±0.57

Values are the mean ± SD of inhibition zone in mm; Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well;CoNS: Coagulase negative *Staphylococcus*; NC: Negative control; PC: Positive control; DW: Distilled water; DMSO: Dimethylsulfoxide; VA: Vancomycin; LE: Levofloxacin; NT: Not tested;

Table 6.13: Phytochemical analysis of medicinal plants under study

S.N.	Phytochemicals	Solvents	Selected plant samples						
			A. indica	*M. oleifera*	*N. arbortristis*	*P. granatum*	*S. chirata*	*S. cumini*	*T. cordifolia*
1.	Alkaloids	Aqueous	+	+	+	-	+	+	+
		80%ethanol	+	+	+	+	+	+	+
		Methanol	-	+	+	+	+	+	+
		Acetone	+	+	-	+	+	+	+
		Hexane	-	-	-	+	+	-	-
2.	Coumarins	Aqueous	+	+	-	-	-	-	+
		80%ethanol	+	+	+	+	+	-	+
		Methanol	-	+	+	+	+	-	+
		Acetone	-	-	+	+	+	+	+
		Hexane	-	-	-	+	-	+	-
3.	Quinones	Aqueous	+	+	-	-	-	-	+
		80%ethanol	-	+	+	+	+	+	+
		Methanol	-	-	+	+	+	+	-
		Acetone	+	+	+	+	+	+	+
		Hexane	+	-	-	+	+	-	-
4.	Phenol	Aqueous	-	+	-	+	-	-	-

		80%ethanol	-	-	+	-	-	-	-
		Methanol	+	+	+	+	+	+	-
		Acetone	+	+	+	-	+	+	-
		Hexane	-	-	+	-	-	-	-
5.	Flavonoids	Aqueous	+	+	-	+	+	+	-
		80%ethanol	+	+	+	+	+	+	-
		Methanol	-	+	+	+	+	-	+
		Acetone	-	-	+	+	+	+	-
		Hexane	-	-	-	+	+	-	-
6.	Terpenoids	Aqueous	+	+	-	+	+	+	+
		80%ethanol	-	+	-	+	+	+	+
		Methanol	-	-	+	+	+	+	-
		Acetone	+	+	+	+	+	+	+
		Hexane	+	-	-	+	+	-	+
7.	Tannins	Aqueous	+	+	+	+	+	+	-
		80%ethanol	-	+	+	+	+	+	+
		Methanol	+	-	+	+	+	+	-
		Acetone	-	-	-	+	-	+	-
		Hexane	+	-	-	+	-	-	-
8.	Steroids	Aqueous	+	+	-	+	+	+	+

		80%ethanol	+	+	+	+	+	+	+
		Methanol	+	-	+	+	+	+	-
		Acetone	-	-	-	+	+	+	-
		Hexane	+	+	-	+	+	+	-
9.	Saponins	Aqueous	+	+	+	+	+	-	+
		80%ethanol	-	-	+	-	+	+	+
		Methanol	-	-	-	+	+	+	-
		Acetone	-	-	-	+	+	-	-
		Hexane	-	-	-	+	-	-	-

+: Present; -: Absent

The study of Bhandari *et al.*, (2017) showed concentration-dependent antimicrobial activity with the zone of inhibition increasing in a dose-dependent manner against the isolated bacteria. The similar results were depicted in Table 6.6 to 6.12 observed in our study. The highest antibacterial activity was found at concentration of 200 mg/ml and lowest at 25 mg/ml, concluded that the step-by-step decrease in concentration showed a similar decrease in ZOI by Bhandari *et al.*, (2017). Water extracted phytochemicals from *A. indica*, *M. oleifera* (except *P. aeruginosa*) and *S. chirata* did not exhibit any antibacterial activity against the bacterial isolates. All other four medicinal plants (*S. cumini*, *P. granatum*, *N. arbortristis*, and *T. cordifolia*) from aqueous extract exerted variable activity from highest (*S. cumini* against *S. aureus* 30.16±0.76 mm and *P. granatum* against *Enterobacter* spp. 22.00±0.50 mm) to lowest (*M. oleifera* against CoNS 10.66±0.57 mm and *Enterobacter* spp. 9.33±0.28 mm) to the concentration of 200 mg/ml used against gram-positive & negative bacteria. Among all organic solvent extract, the maximum antibacterial effect was from methanol extract of *P. granatum* (*S. aureus* 30.50±0.50 mm) and 80% ethanolic extract from *S. chirata* (*P. aeruginos*a 40.33±0.57 mm). Lowest activity was observed in methanol extract of *A. indica* (*Streptococcus* spp. 9.83±0.28 mm) and *T. cordifolia* (*K. pneumoniae* 12.16±0.28 mm) which is gram-positive and gram-negative bacteria respectively. Among all solvent extract tested *A. indica* (80% ethanol, methanol, acetone, and hexane), *M. oleifera* (acetone), *N. arbortristis* (methanol, acetone, and hexane), *S. chirata* (aqueous and hexane), *S. cumini* (hexane), and *T. cordifolia* (acetone and hexane) extract showed resistant activity in respective solvents against gram-negative bacteria from lowest (25 mg/ml) to the highest concentration of 200 mg/ml. This present findings leads to conclusion that bioactive phytochemicals are present in the medicinal plants used but they don't have antibacterial activity rather due to the inadequate existence of antibacterial bioactive compounds in an appropriate amount for the activity of antibacterial function (De Zoysa *et al.*, 2019). The strong antimicrobial action of the aqueous extracts of medicinal plants found in this study may be because of the presence of naturally water soluble components and presence of thiocyanate, nitrate, chlorides, and sulfates as anionic components (Darout *et al.*, 2000). Among the extract evaluated, 80%

ethanol exhibited the highest antibacterial activity followed by methanol and aqueous. This type of result may be due to the polarity of the compounds extracted with different solvents and the capacity of the extract to dissolve and diffuse in the culture media used for antibacterial activity (De Zoysa *et al.*, 2019). In the present study, 80% ethanolic extract exhibited maximum sensitivity compared to aqueous and other solvent extracts which is in similar lines with that of the earlier studies reported by De Zoysa *et al.*, (2019). Among the plants evaluated for antibacterial efficacy *N. arbortristis* (80%ethanol), *P. granatum* (80% ethanol and aqueous), *S. chirata* (80% ethanol) and *S. cumini* (80% ethanol and aqueous) exhibited high activity against the isolates. This may be due to the presence of a low polar compound that is not extracted with highly polar solvent in the case of aqueous extract (De Zoysa *et al.*, 2019). This also gives information that ethanol is the better solvent to extract biologically active compounds from the plant parts than other solvent extracts (Khan *et al.*, 2014). Most of the medicinal plants in the study exhibited highly effective antibacterial potential against gram-positive bacteria as compared to gram-negative bacteria resulting in the broad spectrum activity of medicinal plants (De Zoysa *et al.*, 2019). This phenomenon may be due to the diversity in morphological symphony between Gram-positive and Gram-negative bacteria. Gram-negative bacteria contain lipopolysaccharide in their cell wall causing the impermeability to chemical substances having antimicrobial properties. The Gram-positive bacteria composed peptidoglycan in the cell wall, which makes it more permeable to substances that have antibacterial potential than lipopolysaccharide layer of the cell wall of gram-negative bacteria. Gram-negative bacteria have complex cell wall composition than Gram-positive bacteria and this probably is the reason for more vulnerability to chemical substances with antibacterial potential than Gram-negative bacteria (De Zoysa *et al.*, 2019; Pandit and Vyas, 2020b). The variable result of present findings are unexpected because phytochemicals may vary with ecological factors, different seasonal variation, environmental condition of growth, presence of the compounds, and solvent used for extraction and age of plants (Khan *et al.*, 2014).

The evaluation of the phytochemical report of each plant extract in different solvents showed marked variation in the phytochemical content used in this study as tabulated in Table 6.13. Nine different metabolites such as alkaloids, coumarins, quinones, phenol, flavonoids, terpenoids, tannins, steroids, and saponins were assessed from each solvent extract of medicinal plants. In fact, all kinds of phytochemical classes are detected into the different solvents and are well known to have significant inhibitory action against bacteria (Hayek *et al.,* 2013). This shows the reason for maximum extract exhibited antibacterial effect. These bioactive metabolites possess one or more pharmacological activity and play role in therapeutic value hence it is important to study phytochemical screening while studying medicinal plants. Detection of phytochemical revealed the presence of alkaloids in maximum solvent extract followed by terpenoids, steroids, quinones, flavonoids, tannins, coumarins, saponins, and phenols. In studies of De Zoysa *et al.*, (2019) and Nwonuma *et al.*, (2020) documented the detection of the variability of phytochemicals in different solvent extracts. These bioactive compounds follow different physiological mechanisms and exert antibacterial activity by acting on the cytoplasmic membrane, the most common site for secondary metabolites. These metabolites cause cell lysis by triggering the leakage of cellular components and ultimately cell death (Sant's Anna da Silva *et al.*, 2013). The therapeutic action is also due to the possibility of interaction with metabolites and genetic material that able to change the function of genetic machinery and results in ineffective transcription and disturb the vital function of the cell and stop protein synthesis leads to lysis of cell (Hayek *et al.,* 2013). The preliminary studies showed that the presence of alkaloids seems to affect bacteria by disturbing cell division process, respiratory as well as enzyme inhibition for a different process, membrane disruption and suppress or affect virulence genes in organisms (Othman *et al.,* 2019). Alkaloids are large and have structurally different groups of different origin of natural products from either microbial or plant and animal (Cushnie *et al.,* 2014). Bioactive compounds of tannins and flavonoids provide a source of stable free radicals and react with nucleophilic amino acids of protein prominently inactivate the protein result in loss of function. The compounds possibly aim for microbial cell surface exposed adhesins, cell wall polypeptides and membrane bound enzymes. These mechanisms

play role in having potential of antimicrobial activity (Stern *et al.*, 1996). Terpenoids make membranous tissue fragile and dissolve the cell wall of microorganisms leading to the death of microbes by the flow out of cytoplasmic content (Hernandez *et al.*, 2000). Saponin also causes seepage of proteins and definite enzymes from the cell resulting in the block of the functional activity of the cell and death (Zablotowicz *et al.*, 1996). Structural composition of saponins having lipophilic portion into its structure and a hydrophilic core made able to exhibit the antibacterial property (Costa *et al.*, 2010). Steroidal compounds are the reason for leakages of cellular components from liposomes and cause the death of cells which makes the activity of antimicrobial potential of metabolites (Raquel *et al.*, 2007). Quinones are aromatic ring compounds ability to donate free radicals. It inactivates microorganisms by forming irreversible complexes with amino acids in proteins make it favourable for attack surface adhesions, polypeptide chain of the cell wall, and membrane enzymes (Cowan, 1999). Simple phenols and phenolic acids have evidence that increased hydroxylation of those phenols likes cinnaic and caffeic acid substitution results in better toxicity or antibacterial activities. Simple phenols interact with sulfhydryl groups present in microbial enzymes, leading to the inactivation of enzymes because of nonspecific protein interactions (Cowan, 1999). Coumarins are present in many plants and microorganisms. It is able to suppress the quorum sensing network of pathogenic bacteria and affects their ability in the development of biofilm and showing of virulence factors (Cowan, 1999).

6.5 Interpretation of LC-MS analysis for *P. granatum*

In the present study, the bioactive molecules in the aqueous extract can be well characterized by HPLC/LC-MS method. The experiment was carried out in two ionization modes negative and positive. The spectra obtained were illustrated in Figures 6.6 and 6.7. The analysis was carried out by the method of full scan and MS2 data-dependent, operative mode. The analysis results from the most intense peak come by ESI-MS1 spectrum correspond to the deprotonated molecular ion $[M–H]^-$ which was proceeded further to MS2 fragmentation. The tentative categorization of the compounds was based on the mass spectral database and by comparing with the data published in the previous literature. In this study, identification

of bioactive compounds was characterized by comparing with the reported molecular weight of mass spectrometry data obtained from literature survey. *P. granatum* rind has been reported with the identification of 23 bioactive molecules which are depicted in Table 6.14. These compounds belong to different classes with tentative identification of 22 compounds and one unidentified. Among identified compounds (Citric acid, Gallic acid, Gallocatechin, Granatin A, Catechin and Epicatechin, Corilagin, Kaempferol-3-*O*-rhamnoglucoside, Dotriacontane, Brevifolin carboxylic acid, Estriol, Quercetin, Cynaroside, 3,3'-Di-O-methylellagic acid, Gamma sitosterol, Gadoleic acid, 1,2-Benzenedicarboxylic acid,bis(2-ethylpropyl)ester; Tributyl acetylcitrate) were found from *P. granatum* and five compounds (Sebiferine; 9-12-octadecadienoic acid, ethyl ester; Limonen -6-ol, pivalate; Hazaleamide monoeptixide; Dihydromyricetin 3-O-rhamnoside) were not been previously described in literature review related to pomegranate to our knowledge rather in other plant sources (*Litsea cubeba*, *Bougainvillea buttiana*, *Nigella sativa*, *Stylosanthes fruticosa* and Italian millet) respectively. The compounds identified were mainly phenolic, flavonoids, Organic acids, Catechin and Procyanidins, Ellagitannins and Gallotannins, Sterols and Terpenoids. The phytochemicals being reported from *P. granatum* in present investigation is in consistant with the previous studies and is depicted in Table 6.14. Eight out of the total identified compounds were reported for antibacterial properties were Gallic acid (Borges *et al.*, 2013), Gallocatechin (Cosarca *et al.*, 2019), Catechin and Epicatechin (Cosarca *et al.*, 2019), Dotriacontane (Soosairaj and Dons, 2016), Brevifolin carboxylic acid (N'Guessan *et al.*, 2007), Cynaroside (Mogana *et al.*, 2020), Gamma sitosterol (Abu-Lafi *et al.*, 2019), Tributyl acetylcitrate (Hussein *et al.*, 2016).

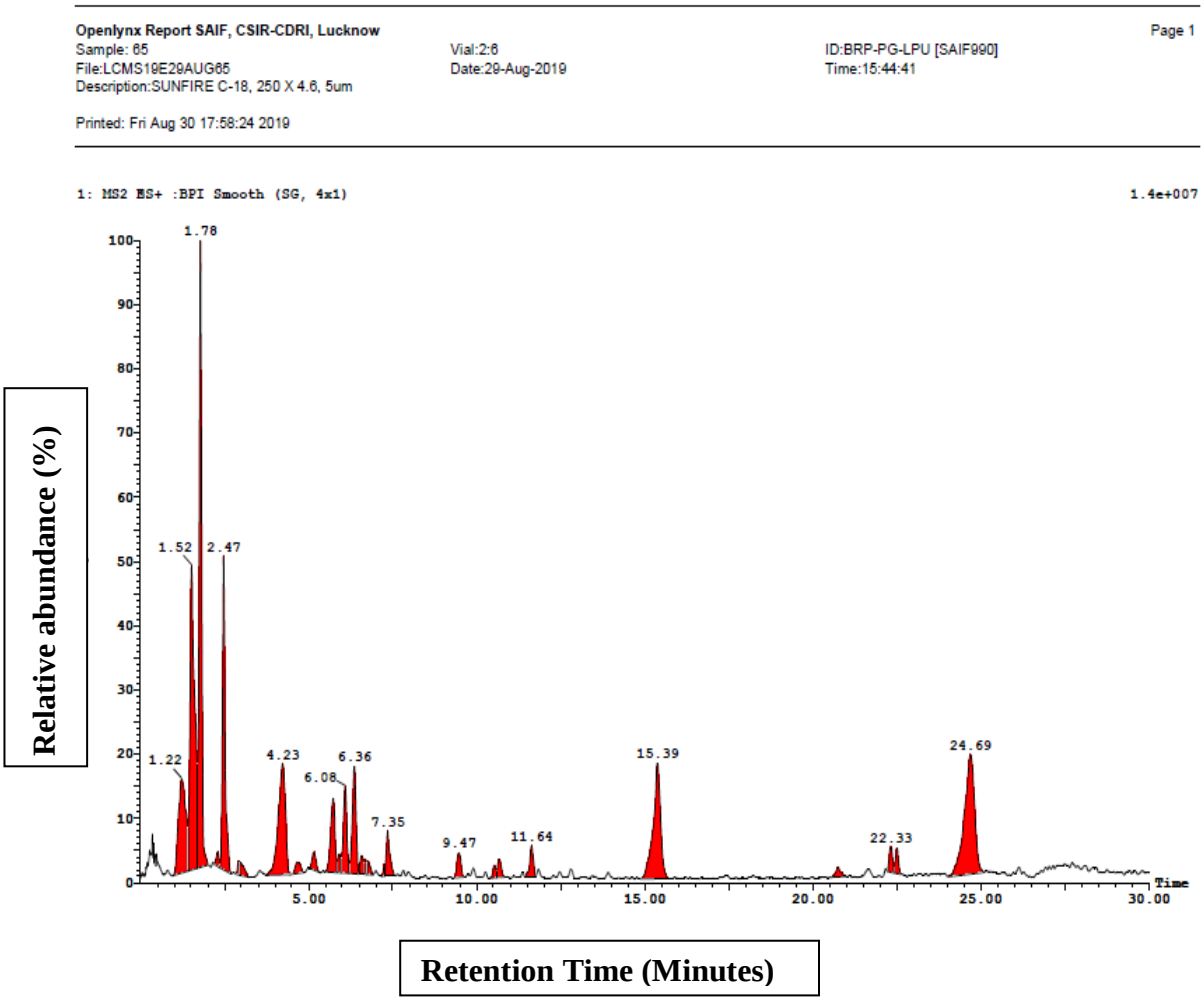


Figure 6.6: Different peaks of compounds obtained from LC-MS analysis (ES+)

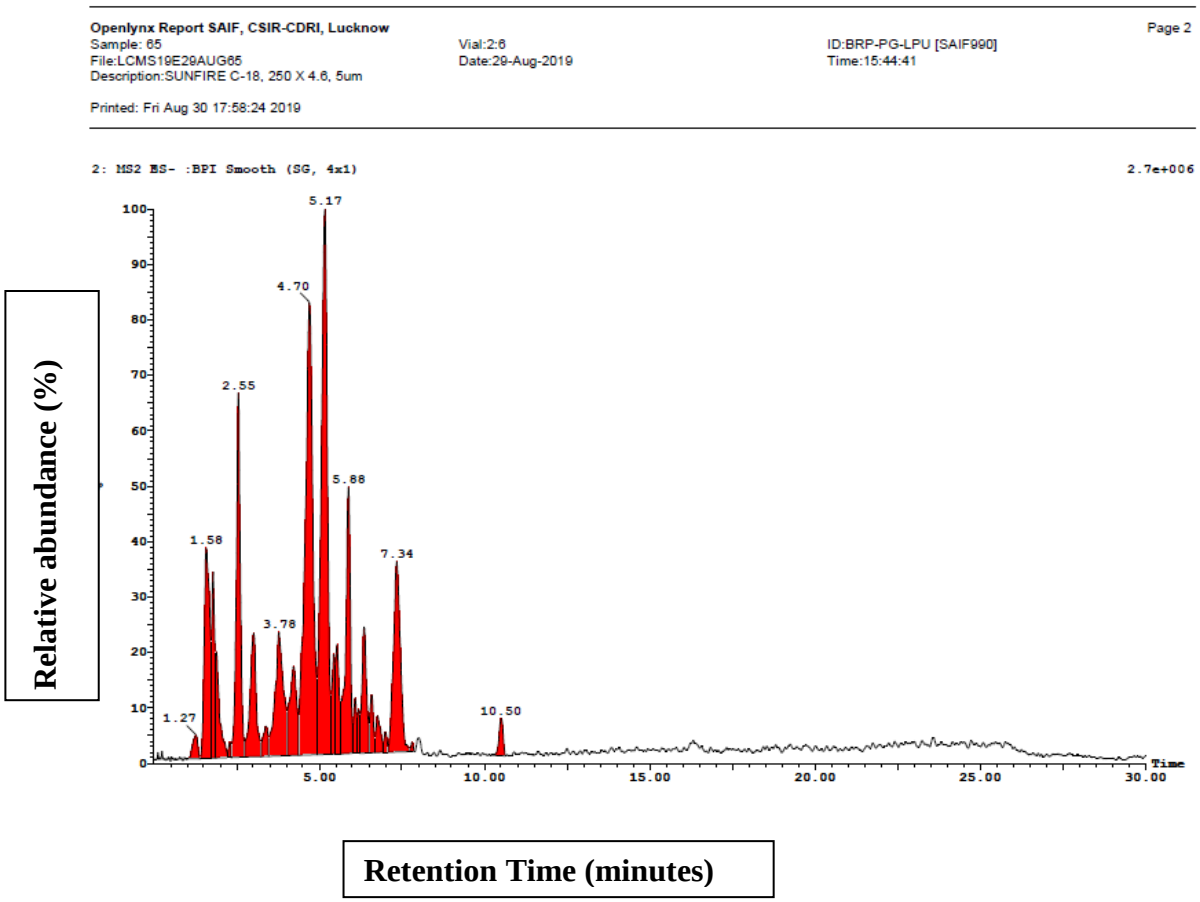
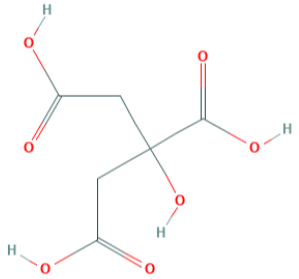
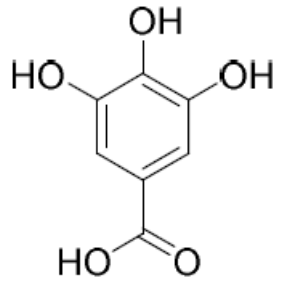
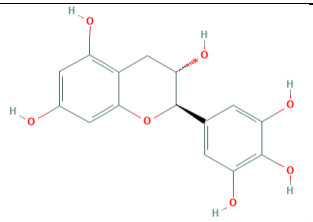
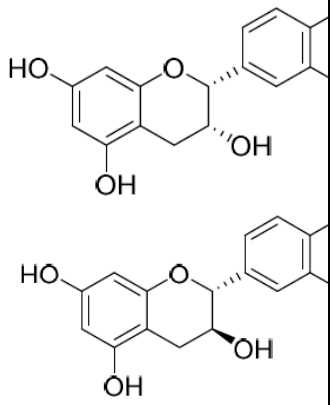
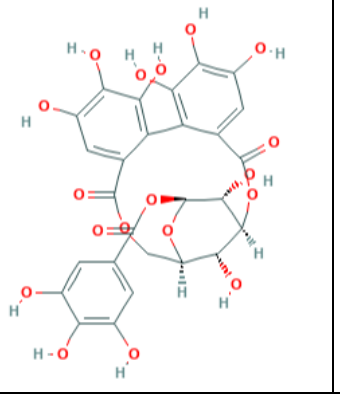
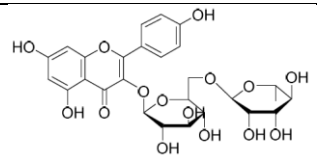
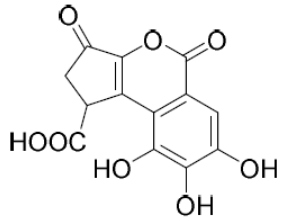
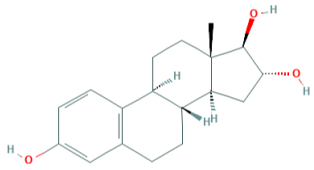
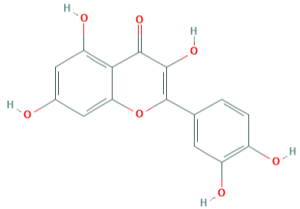
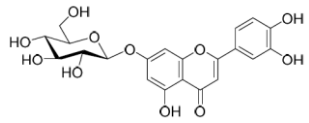


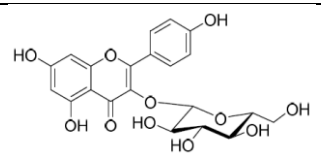
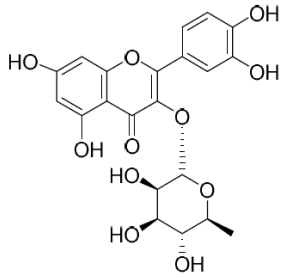
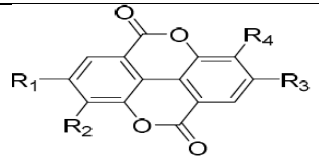
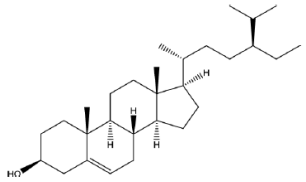
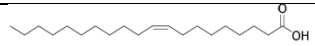
Figure 6.7: Different peaks of compounds obtained from LC-MS analysis (ES-)

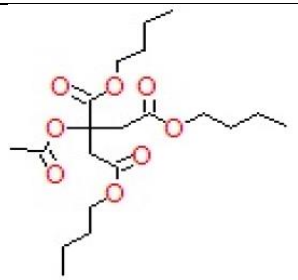
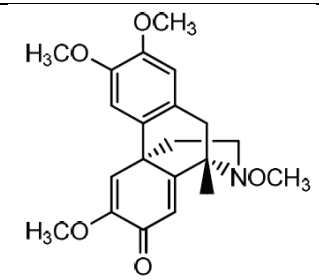
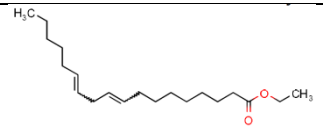
Table 6.14 Compounds identified in aqueous extracts of *P. granatum* rind during mass spectrometry analysis

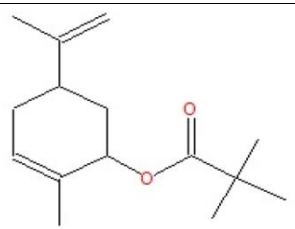
S.N.	Experimental				Tentative identification				
	Mol. Wt.	$[M+H]^+$	$[M-H]^-$	RT	Compounds	Chemical class (Chemical Formula)M. Wt.	Structure	Plant parts	References
1.	192	193	191	2.55	Citric acid	Organic acids ($C_6H_8O_7$) 192.12		*P. granatum* Juice	Seeram (2006)
2.	170	171	169	2.92	Gallic acid	Organic acids ($C_7H_6O_5$) 170.1200		*P. granatum* Peel	Sheng and Tian (2017)
3.	306	307	305	4.23	Gallocatechin	Catechin and Procyanidins ($C_{15}H_{14}O_7$) 306.27		*P. granatum* Peel	Seeram (2006)

4.	800	801	799	5.43	Granatin A	Ellagitannins and Gallotannins ($C_{34}H_{24}O_{23}$) 800.54		*P. granatum* Pericarp	Seeram (2006)
5.	290	291	289	5.74	Catechin and Epicatechin	Catechin and Procyanidins ($C_{15}H_{14}O_6$) 290.27		*P. granatum* Peel, juice, leaf	Sheng and Tian (2017)
6.	634		633	5.91	Corilagin	Ellagitannins and Gallotannins ($C_{27}H_{22}O_{18}$) 634.45		*P. granatum* Pericarp, peel	Seeram (2006)

7.	432		431	6.08	Kaempferol-3-*O*-rhamnoglucoside	Flavonoids ($C_{21}H_{20}O_{10}$) 432.3810		*P. granatum* Juice	Sheng and Tian (2017)
8.	450	451	449	6.36	Dotriacontane (CAS)	($C_{32}H_{66}$) 450		*P. granatum* Peel	Attia (2019)
9.	292	293	291	6.59	Brevifolin carboxylic acid	Simple Gallyol Derivatives ($C_{13}H_8O_8$) 292.1990		*P. granatum* Leaf, flower,	Seeram (2006)
10.	288	289	287	6.99	Estriol	Sterols and Terpenoids ($C_{18}H_{24}O_3$) 288.38		*P. granatum* Seed oil	Seeram (2006)
11.	302	303	301	7.35	Quercetin	Flavonols ($C_{15}H_{10}O_7$) 302.04		*P. granatum* Peel	Seeram (2006)
12.	448	449	447	7.81	Cynaroside (Luteolin 7-*O*-glycoside)	Flavonols ($C_{21}H_{20}O_{11}$) 448.3800		*P. granatum* Peel	Sheng and Tian (2017)

					Astragalin (Kaempferol 3-*O*-glucoside)	Flavonoids ($C_{21}H_{20}O_{11}$) 448.3800		*P. granatum* Peel	Sheng and Tian (2017)
					Quercetin 3-*O*-rhamnoside	Flavonoids ($C_{21}H_{20}O_{11}$) 448.3800		*P. granatum* Peel	Sheng and Tian (2017)
13.	330	331	329	10.53	3,3'-Di-O-methylellagic acid	Ellagitannins, gallotannins ($C_{16}H_{10}O_8$) 330.2480	 R1 = R3 = OH, R2 = R4 = OCH3	*P. granatum* seed	Wang *et al.*, (2004)
14.	414	415		15.39	Gamma sitosterol	(C29H50O) 414.286		*P. granatum* rind	Sangeeta and Vijayalaxmi (2011)
15.	310	311		20.75	Gadoleic acid (9Z-icosenoic acid)	Fatty acids and lipids ($C_{20}H_{38}O_2$) 310.5220		*P. granatum* seed	Sheng and Tian (2017)

16.	278	279		22.50	1,2-Benzenedicarboxylic acid,bis(2-ethylpropyl)est	(C_{16} H $_{22}O_4$) 278.34		*P. granatum* rind	Sangeeta and Vijayalaxmi (2011)
17.	402	403		24.69	Tributyl acetylcitrate	($C_{20}H34O_8$) 402.225		Pomegranate husks	Al—Hashimia *et al.,* (2018)
18.	628		627	6.20					unknown
19.	341	342	340	1.22	Sebiferine	Alkaloids ($C_{20}H_{23}O_4$ N) 341.1700			Kamle *et al.,* (2019) [not reported from *P. granatum*]
20.	308	309	307	1.52	9-12-octadecadienoic acid,ethyl ester	Polyenoic fatty acid ($C_{20}H_{36}O_2$) 308.49			Guerrero *et al.,* (2017) [Not reported from *P. granatum*]

21.	236	237		2.30	Limonen -6-ol ,pivalate	($C_{15}H_{24}O_2$) 236.17			Hadi *et al.*, (2016) [Not reported from *P. granatum* peel]
22.	291	292	290	2.47	Hazaleamide monoeptixide	($C_{18}H_{29}NO_2$) 291			Peter *et al.*, (2012) [Not reported from *P. granatum*]
23.	466	467	465	6.78	Dihydromyricetin 3-O-rhamnoside	Phenolic compounds ($C_{21}H_{22}O_{12}$) 466.1113			Ofosu *et al.*, (2020) [Not reported from *P. granatum*

MW: exact mass; m/z: mass to charge ratio; RT: Retention time

6.6 Formulation of Polyherbal syrup

6.6.1 *In-vitro* Formulation and Evaluation

The polyherbal syrup is the mixture of extract of more than one medicinal plant in different compositions in sweet taste. Polyherbal syrup contains various bioactive compounds in large amounts to exert its effect in controling a particular disease. Polyherbal syrup cures by activating the associated system compare to monoherbal syrup which is lacking sufficient amount of bioactive compounds for action towards the disease efficiently. Different trial phases (four) formulated and tested under *in vitro* conditions in different phases along with the different composition of plant extract (*P. granatum*, *T. cordifolia*, *N. arbortristis*, *M. oleifera*, *S. chirata*, *S. cumini*) in different solvent (aqueous and 80% ethanol) having significant antibacterial activity performed individually. The extracts were mixed in different ratios in different permutations and combinations. The experimental trial has been conducted in four phases. In trial formulation phase I (six formulations), phase II (Six formulations), Phase III (Nine formulations), Phase IV (Twelve formulations) were prepared and has been represented in Table 5.5—5.8. The formulation with all the combinations was tested against the bacterial isolates (*S. aureus*, CoNS, *Streptococcus* spp., *K. pneumoniae*, *E. coli*, *P. aeruginosa*, *Enterobacter* spp., *Proteus* spp.) for its effectiveness and the results have been depicted in Table 6.15—6.18. The result for the formulation phase I have been shown in Table 6.15. The result showed that among the six formulations tested for antibacterial activity, the significant activity was shown by formulation D against *S. aureus* (24.50±0.50 mm), CoNS (23.50±0.50 mm), *Streptococcus* spp. (26.66±0.28 mm) and formulation B against *P. aeruginosa* (35.00±1.00 mm). *K. pneumoniae*, *Escherichia coli*, *Enterobacter* spp., and *Proteus* spp. were found resistant against all the formulation tested. The result for the formulation phase II has been shown in Table 6.16. The result showed that all the formulation was significantly effective against all the tested bacteria except *Streptococcus* spp. which showed resistant activity. The most effective formulation was F against *S. aureus* (23.66±0.57 mm), CoNS (24.00±0.00 mm), *Enterobacter* spp. (20.00±0.00 mm) and *Proteus* spp. (20.66±0.57 mm) whereas, formulation D was most effective against *K. pneumoniae* (20.50±0.50 mm), and formulation A was effective against *E. coli*

(20.00±1.00 mm) and C against *P. aeruginosa* (35.66±1.15 mm). In the formulation phase II, the most effective combination was F which showed the most sensitive against four tested bacterial strains with ZOI in *S. aureus* (23.66±0.57 mm), CoNS (24.00±0.00 mm), *Enterobacter* spp. (20.00±0.00 mm) and *Proteus* spp. (20.66±0.57 mm) and moderate activity against *K. pneumoniae* (19.33±0.28 mm), *E. coli* (19.33±1.52 mm), and *P. aeruginosa* (30.33±0.57 mm). The result for the formulation phase III has been shown in Table 6.17. The result showed that *S.aureus* (20.50±0.50 mm), CoNS (16.66±0.57 mm), *E. coli* (17.33±1.52 mm), *P. aeruginosa* (13.00±1.00 mm) and *Proteus* spp. (17.66±1.52 mm) were sensitive towards all the formulation developed with having highest ZOI respectively whereas, *Streptococcus* spp. was found resistant. The tested bacteria, *K. pneumoniae* (12.33±0.57 mm, 11.16±0.28 mm, 11.66±1.15 mm) was found least sensitive in formulation B, E, and I while, *Enterobacter* spp. was less sensitive (11.83±0.28 mm, 12.00±0.00 mm, 12.00±0.00 mm) with A, B, and I formulation respectively. Among the nine formulations developed in phase III, the most effective formulation was I against *S. aureus* (20.33±0.57 mm), CoNS (16.66±0.57 mm), *K. pneumoniae* (11.66±1.15 mm), *E. coli* (17.00±1.00 mm), *Enterobacter* spp. (12.00±0.00 mm), *P. aeruginosa* (11.83±0.76 mm), *Proteus* spp. (17.66±1.52 mm). The result of the formulation phase IV has been shown in Table 6.18. The result showed that all the formulation was effective against all the tested bacteria. Among the 12 formulations developed, the most effective formulation was G in which *S. aureus* (17.16±0.28 mm), *Streptococcus* spp. (11.16±0.57 mm), CoNS (20.16±0.76 mm), *K. pneumoniae* (18.00±1.00 mm), *E. coli* (16.00±0.00 mm), *Enterobacter* spp. (18.83±0.76 mm), *P. aeruginosa* (21.00±0.00 mm), and *Proteus* spp. (17.00±0.00 mm) were significantly sensitive. Moreover, among all the formulation developed in different phases (I—IV), the formulation developed in phase IV was most significant. The formulation F, G, H shows nearly equal ZOI against all the tested bacteria. In the present investigation, further study was carried out with formulation G. The formulation G contain *T. cordifolia* and *M. oleifera* leaf extract in comparison to formulation F and H. *T. cordifolia* is better known as immune modulator/ immune enhancer and *M. oleifera* as malnutrition supplement which

contains the essential nutrient source to overcome the deficiency of micronutrient as well as macronutrient to neonates.

6.6.2 Preparation of Polyherbal syrup

Polyherbal syrup constitutes the mixture of different plant parts extract in different ratios obtained from different solvents by extraction methods to ensure better effectivity compare to individual plant extract syrup. The polyherbal syrup was formulated by a combination of extracts with appropriate preservatives to preserve from different bacterial and fungal growth along with sugar combination to make the syrup soothing to taste buds and also to minimize the bitter taste of the extract. Polyherbal syrup is more effective than the single plant extract syrup due to the presence of variety of components of different bioactive compounds in polyherbal syrup which combinatorially work to cure the disease as compared to few bioactive compounds present in single extract syrup. The composition of the final syrup was prepared according to Ayurvedic Pharmacopoeia (API-II, Vol-1, 2007; Shakeel *et al.,* 2015). The composition of Polyherbal syrup was shown in Table 6.19. In the present investigation, high amount of extract residue was obtained from ethanol compared to aqueous extract. Polyherbal Ayurvedic Formulation (PHAF) is prescribed in hot water extract form to patients but due to some difficulties like the unpleasant taste, lowers stability, and require freshly prepared on each dose as per traditional Ayurvedic Practice. The scientific and industrial community has therefore researched to develop syrups and tonics to avoid the above mentioned drawback. The syrup was prepared according to the procedural methodology described in materials and methods as described in Ayurvedic Pharmacopoeia with little modification (API-II, Vol-1, 2007; Shakeel *et al.,* 2015; Muguli *et al*., 2015). The contents of the prepared syrup were shown in Table 6.19.

The plant extract can be obtained by use of different solvents like hexane, methanol, Acetone, Chloroform, Petrolium ether, etc. This extract contains bioactive secondary metabolites of medicinal plants. In our study, ethanolic and aqueous extract are safe to formulate a modern drug as PHAF on similar lines with Wakkumbura *et al.,* (2020). PHAF developed by Kumar *et al.,* (2016) (Indian Pharmacopoeia), Kumar and Prasan, (2013) (United State Pharmacopoeia), Shakeel *et al.,* (2015) (Ayurvedic Pharmacopoeia) by used of different

pharmacopoeia methods to prepare syrup for antimicrobial and antidiabetic, energy booster and gastrointestinal tract problems. Likewise in our study, Ayurvedic Pharmacopoeia was used for the preparation of PHAF having antibacterial properties. Experimental polyherbal syrup contained bioactive components and mandatory appropriate excipients including preservative, increased viscosity, and sweetening agents. The PHAF was evaluated immediately after preparation.

6.6.3 Standardization

The developed syrup exhibited brown color, sweet-bitter taste with agreeable smell tabulated in Table 6.20. The developed syrup by other researchers also found the same organoleptic character (Kumar *et al.,* 2016; Shakeel *et al.,* 2015) as observed in this study. The taste of the drug is an important character to prevent it from undesirable taste to remove unfavorable taste (Avbunudiogba *et al.,* 2013). The syrup was also aimed to develop pleasant and suitable. The developed syrup was found pH 6.13 which plays a vital role in quality assessment. Variation in pH and moisture favor microbial growth and cause worsening followed by hydrolysis (Modi *et al.,* 2014).

The analysis for heavy metal was carried out at Nepal Research Foundation Pvt. Ltd., Birgunj, Nepal (AR No.: NRF-049/P7). The analyses were performed for Lead, Arsenic, Cadmium, and Mercury. The concentration of the heavy metals in the syrup was found to be within the limit developed by Ayurvedic Pharmacopoeia of India (API-II, Vol-2, 2018) with prescribed standard. The result was depicted in Table 6.21**.** The concentration of heavy metal obtained were Lead **(**<0.1ppm**),** Arsenic **(**<0.001ppm**),** Cadmium **(**<0.005ppm**),** and Mercury **(**<0.0005ppm**)** respectively. The results were in similar lines with Thangarathinam *et al.,* (2013) wherein, concentration of heavy metals within the limits as per standard in polyherbal syrup.

Table 6.15: Zone of inhibition in formulation phase I

Bacterial isolates	A	B	C	D	E	F
S. aureus	20.83±0.76	21.33±0.57	21.00±1.00	24.50±0.50	23.66±0.57	23.00±1.00
Streptococcus spp.	23.83±0.76	-	24.00±0.00	26.66±0.28	-	24.50±0.50
CoNS	19.66±0.57	22.16±0.28	22.50±0.50	23.50±0.50	21.33±0.57	23.33±0.57
K. pneumoniae	-	-	-	-	-	-
E. coli	-	-	-	-	-	-
Enterobacter spp.	-	-	-	-	-	-
P. aeruginosa	30.33±0.57	35.00±1.00	29.66±0.57	30.33±0.57	28.00±0.00	30.00±1.00
Proteus spp.	-	-	-	-	-	-

Values are the mean ± SD of inhibition zone in mm (triplicates); Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well; CoNS: Coagulase negative *Staphylococcus*; -: ZOI absent

Table 6.16: Zone of inhibition in formulation phase II

Bacterial isolates	A	B	C	D	E	F
S. aureus	22.00±1.00	22.33±0.57	21.00±1.00	22.50±0.50	22.83±0.76	23.66±0.57
Streptococcus spp.	-	-	-	-	-	-
CoNS	20.66±1.15	23.33±0.57	22.00±0.50	23.16±1.25	23.00±1.00	24.00±0.00
K. pneumoniae	16.50±0.50	18.33±0.57	19.00±1.00	20.50±0.50	19.33±0.57	19.33±0.28
E. coli	20.00±1.00	19.66±0.57	18.16±0.28	18.83±0.76	19.00±1.00	19.33±1.52
Enterobacter spp.	16.00±1.00	17.33±0.57	16.50±0.50	18.83±0.76	20.00±1.00	20.00±0.00
P. aeruginosa	32.33±0.57	30.66±0.57	35.66±1.15	33.33±1.52	32.83±0.76	30.33±0.57
Proteus spp.	17.83±0.76	19.00±0.00	20.33±0.57	20.33±0.57	19.16±0.28	20.66±0.57

Values are the mean ± SD of inhibition zone in mm (triplicates); Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well; CoNS: Coagulase negative *Staphylococcus*; -: ZOI absent

Table 6.17: Zone of inhibition in Formulation phase III

Bacterial isolates	A	B	C	D	E	F	G	H	I
S. aureus	20.33 ±0.57	19.16 ±0.28	19.16 ±0.28	20.50 ±0.50	20.00 ±1.00	20.33 ±0.57	20.00 ±0.00	19.00 ±0.00	20.33 ±0.57
Streptococcus spp.	-	-	-	-	-	-	-	-	-
CoNS	16.33 ±0.57	15.33 ±0.28	14.33 ±0.28	15.50 ±0.50	15.83 ±0.76	15.00 ±1.00	14.83 ±0.28	15.33 ±0.57	16.66 ±0.57
K. pneumoniae	-	12.33 ±0.57	-	-	11.16 ±0.28	-	-	-	11.66 ±1.15
E. coli	14.66 ±0.57	14.00 ±0.86	14.50 ±0.50	14.50 ±0.50	14.00 ±0.00	14.00 ±0.00	11.50 ±0.50	17.33 ±1.52	17.00 ±1.00
Enterobacter spp.	11.83 ±0.28	12.00 ±0.00	-	-	-	-	-	-	12.00 ±0.00
P. aeruginosa	11.00 ±0.00	11.50 ±0.50	12.00 ±0.50	12.33 ±0.28	13.00 ±1.00	10.33 ±0.57	12.16 ±0.28	9.83 ±0.28	11.83 ±0.76
Proteus spp.	16.33 ±0.57	15.33 ±0.57	17.33 ±0.57	16.66 ±0.57	14.00 ±0.00	14.00 ±0.00	16.00 ±0.00	14.33 ±0.57	17.66 ±1.52

Values are the mean ± SD of inhibition zone in mm (Triplicates); Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well; CoNS: Coagulase negative *Staphylococcus*; -: ZOI absent

Table 6.18: Zone of inhibition in Formulation phase IV

Bacterial isolates	A	B	C	D	E	F	**G**	H	I	J	K	L
S. aureus	13.16 ±0.28	19.33 ±0.57	18.33 ±0.57	16.50 ±0.50	14.50 ±0.50	18.50 ±0.50	**17.16 ±0.28**	17.83 ±0.76	20.00 ±1.00	12.50 ±0.50	15.83 ±0.76	16.33 ±0.57
Streptococcus spp.	13.83 ±0.76	12.83 ±0.28	10.16 ±0.28	10.00 ±0.00	10.33 ±0.57	11.83 ±0.28	**11.16 ±0.57**	11.83 ±0.28	10.50 ±0.50	10.00 ±0.00	9.83 ±0.28	9.83 ±0.28
CoNS	12.50 ±0.50	20.33 ±0.57	20.83 ±0.28	18.00 ±0.00	19.16 ±1.25	21.16 ±0.76	**20.16 ±0.76**	19.83 ±0.76	19.50 ±0.50	20.00 ±0.00	21.00 ±0.00	17.66 ±0.57
K. pneumoniae	9.66 ±0.57	14.83 ±0.76	16.00 ±1.00	15.33 ±0.57	13.33 ±0.57	17.66 ±0.57	**18.00 ±1.00**	16.83 ±1.04	16.50 ±0.50	14.16 ±0.76	16.00 ±1.00	14.00 ±0.00
E. coli	11.83 ±0.28	19.00 ±1.00	16.66 ±0.28	18.66 ±0.28	20.00 ±1.00	16.16 ±0.28	**16.00 ±0.00**	18.00 ±0.00	11.00 ±0.00	12.50 ±0.50	13.33 ±0.57	12.83 ±0.28
Enterobacter spp.	16.83 ±0.28	21.00 ±1.00	20.33 ±0.57	18.33 ±0.28	20.50 ±0.00	20.66 ±0.57	**18.83 ±0.76**	20.33 ±0.57	17.83 ±0.28	16.16 ±0.28	17.00 ±0.00	15.50 ±0.50
P. aeruginosa	15.00 ±0.00	20.66 ±0.57	21.66 ±0.57	21.33 ±0.28	20.16 ±0.28	23.00 ±0.00	**21.00 ±0.00**	20.00 ±0.00	22.83 ±0.28	21.66 ±0.57	24.16 ±0.28	23.16 ±0.28
Proteus spp.	12.33 ±0.57	14.66 ±1.15	16.33 ±0.57	17.16 ±0.28	13.33 ±0.57	18.00 ±0.00	**17.00 ±0.00**	17.16 ±0.28	16.00 ±0.00	13.33 ±0.57	15.00 ±1.00	13.00 ±0.00

Values are the mean ± SD of inhibition zone in mm (Triplicates); Well size: 8 mm (Diameter), Sample quantity: 100 µl for each well;
CoNS: Coagulase negative *Staphylococcus*

Table 6.19: Composition of Polyherbal Syrup (l00 ml)

S.N.	Ingredients	Quantities
1.	*Swertia chirata* (80% Ethanolic Extract)	1500 mg
2.	*Nyctanthes arbortristis* (80% Ethanolic Extract)	1500 mg
3.	*Punica granatum* (Aqueous Extract)	2500 mg
4.	*Syzygium cumini* (Aqueous Extract)	2500 mg
5.	*Tinospora cordifolia* (Aqueous Extract)	1000 mg
6.	*Moringa oleifera* (Aqueous Extract)	1000 mg
7.	Sorbitol (70% IP)	7 ml
8.	Sodium benzoate (IP)	1 gm
9	Sodium Methyl paraben (IP)	0.5 ml
10.	Sodium Propyl Paraben (IP)	0.025 ml
11.	Sugar syrup (66.67%)	77 ml

IP: Indian Pharmacopoeia

Table 6.20: Organoleptic Description of Polyherbal syrup

Colour	Brown
Appearance	Syrup
Texture	Liquid
Taste	Sweet Bitter
Smell	Agreeable
pH	6.13

Table 6.21: Quantitative analysis of syrup

Heavy metals	Results	Limit as per API
Lead	<0.1ppm	Not more than 10ppm
Arsenic	<0.001ppm	Not more than 3ppm
Cadmium	<0.005ppm	Not more than 0.3ppm
Mercury	<0.0005ppm	Not more than 1ppm

API: Ayurvedic Pharmacopoeia of India; ppm: Part per million

The microbial load analysis in polyherbal syrup was carried out and results have been depicted in Table 6.22. The analysis was performed for total aerobic count and pathogens such as gram-positive *S. aureus* & negative *E. coli*, *Salmonella*, and *P. aeruginosa* as per the methods

of API-II, Vol.-2 (formulations) (2018). Total aerobic viable count was 10^3 CFU/ml, total yeast and molds was 10 CFU/ml whereas; specific micro-organism *E. coli*, *Salmonella*, *P. aeruginosa*, and *S. aureus* were absent. This is in accordance within the limit of API developed for standard evaluation of microbial load in syrup. The syrup developed by Shakeel *et al.,* (2015) and Thangarathinam *et al.,* (2013) reported industrial compliance with the permissible microbial limits which is in similar lines with present investigation.

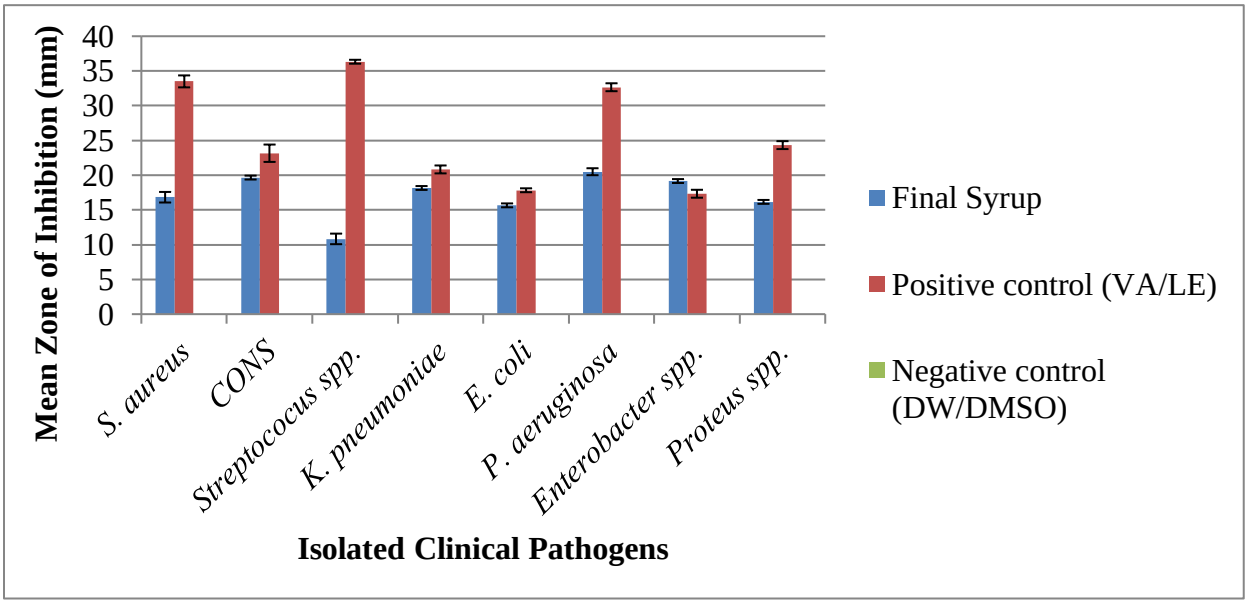
Table 6.22: Bio-Burden analysis of polyherbal Syrup

Parameters	Results	Limit as per API
Total aerobic Viable count	Comply (10^3CFU/ml)	10^7/g
Total yeast and Molds	Comply (10 CFU/ ml)	10^3/g
Test for specific Micro-organism		
Escherichia coli	Absent	Absent
Salmonella	Absent	Absent
Pseudomonas aeruginosa	Absent	Absent
Staphylococcus aureus	Absent	Absent

API: Ayurvedic Pharmacopoeia of India; CFU: Colony Forming Units

6.7 Efficacy of Polyherbal syrup *In Vitro*: Post Preparation

Polyherbal Ayurvedic syrup was assessed for its *in vitro* antibacterial potential by the agar well diffusion method and results were shown in Table 6.23. It is the combination of plants of medicinal values that have been used for antibacterial activity since prehistoric times. In our study, these plants had also shown the antibacterial effect against neonatal sepsis causing bacteria. A sweetening agent sugar has been incorporated to fade the bitter taste of the extract. The final prepared syrup halted the growth of tested organisms which was depicted in Figure 6.8. Therefore, it concludes that the excipients and process of preparation did not affect the sensitivity of the active components present in the formulation in the form of extract. The inhibition zone of the developed formulation was compared with the positive control.



VA: Vancomycin (100 mg/ml); LE: Levofloxacin (5mcg/disc); DW: Distilled Water; DMSO: Dimethyl Sulfoxide

Figure 6.8: Efficacy of prepared syrup for antibacterial potential

6.8 Acute Toxicity Study

Acute toxicity of any plant or chemicals indicates the effect of that material within the short time period after inoculation of the substances. It is performed to find out the immediate effect of the substance that may be plant extract or chemicals so that further testing can proceed. In the present investigation, acute toxicity testing of the polyherbal formulation was conducted. After the treatment, no mortality was observed within four hours and thereafter for 24 hours after oral prescription of the tested plant extract at a dose of 300 mg/kg and 2000 mg/kg body weight. There was negative lethal effect during the experimental period of 14 days for both male and female mice. Consumption of food, intake of water, and respective weight of all the mice are normal. The general behavior of the extract prescribed mice and control group did not showed any drug-related changes in all kind of activities therefore, the formulation seems to be safe at a dose of 2000 mg/kg body weight and the median lethal dose (LD_{50}) of the formulation was supposed to be higher than limit test dose of 2000 mg/kg body weight. The parameters observed for the acute toxicity study after the

administration of the test formulation compared with the control group are presented in Table 6.25. The organs withdrawn from mice were observed macroscopically and found no pathological changes in the tested organs which were illustrated in Figure 6.9 - 6.11.

Acute toxicity is regarded as the primitive study which provides initial information about the toxic nature of the new substance so that we can determine the dose (Ping *et al.*, 2013). The most useful method for normally followed for toxicity testing is oral administration, although the absorption is slow the method is cheap and painless to animals (Kumar and Lalitha, 2013). In this experimental study, *in vivo* testing was performed with mice. Selection of mice will generate reliable data of lethal dose and then assumption related to toxic effect in human beings can be studied (Walum *et al.*, 1995). In this present investigation, single-dose prescription of polyherbal formulation with raising doses did not show any serious or lethal effect. General behavioral and body weight change is an important index for assessment of lethal effect due to various chemicals and drugs (Ezeja *et al.*, 2014). In this study administration of polyherbal formulation did not decrease the bodyweight of mice suggesting that the formulation did not influence any harmful result on the growth of mice. In addition, the increase in body weight during the time interval showed that formulation did not alter feed uptake of mice. Likewise, the formulation also did not exhibit variation in the organ weight of mice so, therefore, considered nontoxic because decreases in body weight are important markers of toxicity (Waller and Sampson, 2018). The study data is in similar lines with Illuri *et al.*, (2019).

Table 6.23: Effect of polyherbal formulation on body weight, food intake and water consumption in mice

Parameters	Day	Control	300 mg/kg	2000 mg/kg
Body weight (gm)	1(Female)	201.68±0.58	173.68±0.39	176.89±0.61
	7	201.87±0.97	174.74±0.43	177.62±0.74
	14	203.08±0.62	175.08±0.33	179.48±0.18
Food intake (gm/24hr)	1	32.90±0.01	31.83±0.02	29.31±0.01
	7	28.75±0.01	28.77±0.01**	30.31±0.01
	14	35.67±0.00	36.01±0.02	39.69±0.01
Water intake (ml/24hr)	1	16.06±0.05	15.53±0.05	16.60±0.10
	7	17.13±0.05	17.16±0.11**	17.13±0.11**
	14	19.13±0.11	17.13±0.11	20.10±0.10
Body weight (gm)	1(Male)	115.86±0.17	116.77±0.28	110.35±0.31
	7	116.80±1.08	116.72±0.26**	110.70±0.44
	14	118.94±0.48	117.26±0.70	111.12±0.10
Food intake (gm/24hr)	1	20.91±0.01	21.27±0.02	20.22±0.02
	7	20.13±0.02	17.37±0.02	21.69±0.01
	14	21.13±0.02	27.81±0.05	22.45±0.01
Water intake (ml/24hr)	1	12.06±0.05	15.13±0.11	12.10±0.10**
	7	13.10±0.10	14.10±0.10	14.03±0.05
	14	13.06±0.05	13.06±0.05**	16.03±0.05

Values are demonstrated as mean±SD. Sex (Male n=3, Female n=5). (**P>0.05) was found between test & control group as interpretated by ANOVA *Post hoc* tukey test

Table 6.24: Absolute and relative mice organ weight in the acute toxicity study of polyherbal formulation (Control and Treated)

Organs	Absolute Organ Weight			Relative Organ Weight		
	Control	300 mg/kg	2000 mg/kg	Control	300 mg/kg	2000 mg/kg
Kidney (F)	0.47±0.04	0.45±0.07	0.41±0.04	1.16±0.08	1.27±0.11	1.18±0.24
Liver (F)	2.25±0.38	2.22±0.34	1.80±0.18	5.54±0.92	6.38±1.08	5.20±1.18
Lung (F)	0.26±0.04	0.27±0.06	0.27±0.04	0.64±0.08	0.78±0.17	0.76±0.18
Heart (F)	0.24±0.01	0.23±0.02	0.23±0.04	0.58±0.03	0.67±0.10	0.65±0.13
Spleen (F)	0.23±0.05	0.20±0.04	0.18±0.02	0.56±0.10	0.57±0.11	0.52±0.09
Kidney (M)	0.83±0.31	0.77±0.08	0.73±0.07	2.05±0.42	1.97±0.09	1.97±0.05
Liver (M)	2.12±0.23	2.58±0.19	2.45±0.12	5.44±0.76	6.60±0.51	6.65±0.60
Lung (M)	0.27±0.11	0.23±0.03	0.25±0.03	0.68±0.20	0.59±0.06	0.69±0.10
Heart (M)	0.22±0.06	0.27±0.03	0.27±0.03	0.54±0.66	0.68±0.05	0.72±0.06*
Spleen (M)	0.31±0.00	0.21±0.02*	0.30±0.03	0.82±0.21	0.53±0.08	0.82±0.10

F: Female; M: Male; Values are demonstrated as mean±SD; *P<0.05 indicate significant by ANOVA *Post hoc* tukey test

Table 6.25: Acute study on female mice for 14 days

Mice group	Parameters										
	Autonomic effect			CNS effect			Skin				
	Lacrimation	Salivation	Piloerection	Drowsiness	Tremors	Convulsions	Fur	Respiration	Food intake	Water con.	Mortality
F1 (Control)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 2 (Control)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 3 (Control)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 4 (Control)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 5 (Control)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 1 (300mg/kg)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 2 (300mg/kg)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 3 (300mg/kg)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 4 (300mg/kg)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 5 (300g/kg)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 1(2000mg/kg)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 2(2000mg/kg)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 3(2000mg/kg)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 4 (2000mg/kg)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF
F 5 (2000mg/kg)	NF	NF	NF	NF	NF	NF	N	N	N	N	NF

NF: Not Found; N: Normal; CNS: Central Nervous System; con.: consumption

Figure 6.9: Vital organ study of female mice control (FC: Female Control mice)

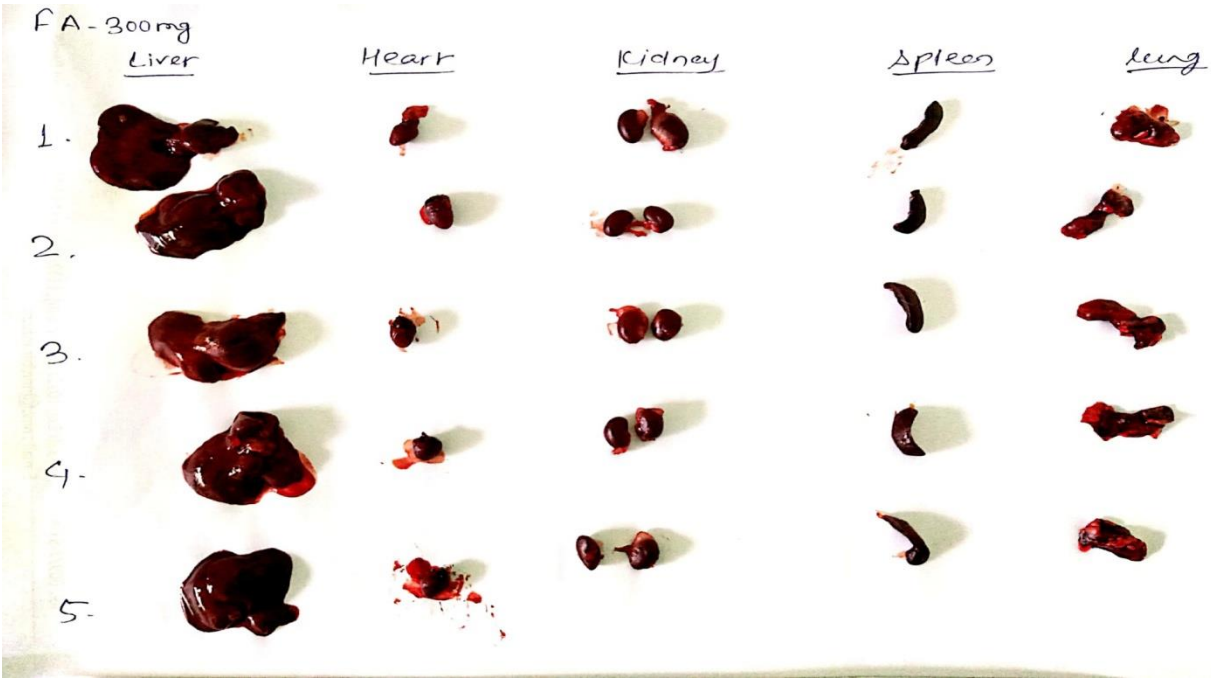


Figure 6.10: Vital organ study of female mice prescribed with dose of 300 mg/kg body weight

Figure 6.11: Vital organ study of female mice prescribed with dose of 2000 mg/kg body weight

Chapter Seven

Summaryand Conclusions

7.1 Summary

Medicinal plants are the gift from God to nature and possessed enormous potential rendering various products to human mankind. Plants are used all over the world as drugs and applied in various remedies in different diseases since ages. Many medicinal plants occupy very significant place in modern medicine. Most drugs of plant origin are utilized in the form of extract from whole plant material or parts which shows efficient biological activity. In present times many herbal medicines are effectively used in the treatment of several diseases such as malaria, dysentery, diabetes, gonorrhea, cancers, HIV, Hepatitis and in many infectious diseases. Medicinal plants have the powerful potential effect that can be identified by research in diverse areas. Over the past few decades, the emergence of MDR bacteria has created a threat in combating many microbial infections. The decreasing effectiveness of synthetic chemotherapeutics and their side effect has forced the selection of various plants for their latent of antimicrobial capacity. Neonatal sepsis is one of the infectious diseases caused by different microbes within 28 days of life. Newborns open their eyes in the lap of this beautiful nature but without enjoying this nature approximately 7000 newborns per day leave the earth and keep the heart-rending condition of their parents by killing hope and happiness. Neonates getting cured of sepsis have a different side effect in the future due to the overuse of synthetic antimicrobial drugs. Therefore, efforts were made to combat neonatal sepsis with objectives of screening and evaluating medicinal plants against causative organisms, evaluation of formulation and preparation, *in vitro* standardization and efficacy, and *in vivo* determination of oral toxicity.

7.1.1 Screening, Isolation, Identification and antibiogram of Neonatal sepsis causing Bacteria

The blood sample was collected from suspected neonates. Twelve hundred blood samples were processed for culture. The frequent clinical symptoms observed at the time of admission

were respiratory distress 245 (20.41%), fever 210 (17.50%), poor cry 177 (14.75%), jaundice 135 (11.25%), grunting 75 (6.25%), lethargy 66 (5.5%), irritability 61 (5.08%), poor feeding 60 (5%), cyanosis 50 (4.16%), vomitting 40 (3.33%), excessive cry 40 (3.33%), convulsion 19 (1.58%), hypothermia 14 (1.16%), tachycardia 8 (0.66%). Eight bacterial species were isolated viz. *S. aureus*, CoNS, *Streptococcus* spp., *K. pneumoniae*, *Escherichia coli*, *P. aeruginosa*, *Enterobacter* spp., *Proteus* spp. through the procedure of blood culture. *S. aureus* (63.08%) and *K. pneumoniae* (13.22%) was prominent organism to cause neonatal sepsis. The maximum percentage of bacterial growth was observed in poor cry 94 (53.10%) followed by lethargy 26 (39.39%), respiratory distress 92 (37.55%), fever 75 (35.71%), jaundice 39 (28.88%), poor feeding 14 (23.33%), hypothermia 2 (14.28%), tachycardia 1 (12.50%), vomiting 5 (12.50%), cyanosis 5 (10.00%), irritability 6 (9.83%), grunting 3 (4.00%) and excessive cry 1 (2.5%) but no bacterial growth with symptoms of convulsion in neonates. The occurrence of neonatal sepsis was 363 (30.25%). Among positive cases, 270 were Gram-positive cocci, and remaining 93 were gram-negative bacilli. The highest frequency of bacterial growth observed in male neonates (69.98%); EONS (79.89%); low birth weight (78.23%); preterm gestational age (73%); spontaneous vaginal delivery (76.86%); delivery in hospital (63.91%). The antibacterial profile of isolated bacteria, gram-positive were found most effective against Linezolid (94%) and resistance to Penicillin-G (99.22%) and Amikacin (81.20%) while gram-negative were highly sensitive to Imipenem (90.19%) and resistance to Ampicillin (95%) and 3rd generation cephalosporin (Cefotaxime, Cefixime, Ceftriaxone, Ceftazidime).

7.1.2 Screening of medicinal plants along with phytochemical and antibacterial profile

Among the seventy medicinal plants, seven plants were selected based on ethnobotanical information, literature, references, and therapeutic significance. The plants and plants parts were collected from different places of Nepal and were authenticated from Nepal Ayurved Medical College and Teaching Hospital, Birgunj, Nepal. The plants/ parts were shade dried and experimented using the extraction procedure of maceration with different solvents (aqueous, 80% ethanol, methanol, acetone, and hexane). The highest extract residues were

obtained with 80% ethanol, aqueous, methanol, acetone, and least with hexane. The dried extracts were dissolved in respective solvents (aqueous in distilled water and organic extract in DMSO) in different concentration of 25, 50, 100, and 200 mg/ml. The aqueous fraction of *A. indica* leaves extracts exhibited no activity against gram-positive isolates even at the highest concentration of 200 mg/ml and gram-negative bacteria other than *P. aeruginosa* (14.33±0.57 mm) and *Proteus* spp.(9.83±0.76 mm) which exhibited least activity. Among the organic solvent extract, ethanol and methanol showed least sensitivity against *S. aureus* (13.66±0.28 mm, 15.00±1.00 mm), CoNS (11.33±0.57 mm, 14.50±0.50 mm), *Streptococcus* spp. (11.33±0.28 mm, 9.83±0.28 mm) respectively whereas, acetone extract exhibited remarkable sensitivity against *S. aureus* (19.00±0.00 mm) and CoNS (20.33±0.57 mm). Similarly, hexane extract also exhibited moderate activity against *S. aureus* (17.16±0.28 mm) and *Streptococcus* spp. (15.66±1.15 mm) at the concentration of 200 mg/ml. All the gram-negative isolates were resistant against organic solvent extracts at all the concentration used. Aqueous leaf extract of *M. oleifera* showed highly significant activity against *P. aeruginosa* (20.00±1.00 mm) while others gram-negative exhibited less activity except *Proteus* spp.. *S. aureus* being resistant, whereas *Streptococcus* spp. (14.33±0.57 mm) being moderately sensitive and CoNS (10.66±0.57 mm) as least sensitive at concentration of 200 mg/ml. High sensitivity was observed in case *S. aureus* (22.50±0.50 mm) and *P. aeruginosa* (30.50±0.50 mm) from 80% ethanol. In the present study, *S. aureus* (18.33±0.57 mm) in methanol extract, CoNS (13.16±0.28 mm, 16.33±0.57 mm) in 80% ethanol and methanol extract, *Streptooccus* spp. (13.33±0.28 mm, 17.33±0.57 mm) in 80% ethanol and hexane. *P. aeruginosa* (19.00±0.50 mm) in 80% ethanol were moderately sensistive. *S. aureus* (10.16±0.28 mm), CoNS (12.83±0.28 mm), *K. pneumoniae* (12.33±0.28 mm), *Enterobacter* spp. (12.50±0.86 mm) are least sensitive in hexane, acetone, hexane and 80% ethanol solvent extract respectively. Aqueous extract of *N. arbortristis* leaf exhibited maximum inhibition against *P. aeruginosa* (20.50±0.76 mm) followed by *S. aureus* (14.83±0.76 mm), CoNS (14.00±0.50 mm), and *Enterobacter* spp. (14.33±0.57 mm) with moderate activity. *Streptococcus* spp., *K. pneumoniae*, *E. coli*, and *Proteus* spp. was found to show resistant activity from aqueous extract of *N. arbortristis*. In the present study, 80% ethanolic fraction

proved most effective among all concentration and against all the isolates, showing maximum sensitivity against *P. aeruginosa* (30.50±0.50 mm), *S. aureus* (29.00±0.00 mm), and CoNS (22.33±0.57 mm), while moderate activity against *Streptococcus* spp. (18.33±0.76 mm), *Proteus* spp. (18.33±0.57 mm), *E. coli* (18.16.±0.28 mm), *K. pneumoniae* (17.33±0.28 mm), and *Enterobacter* spp. (14.83±0.28 mm). Methanol, acetone and hexane fraction showed moderate activity against gram-positive isolates, whereas, resistant against gram-negative tested bacterial strains at concentration of 200 mg/ml. *S. aureus* (16.33±0.57 mm, 13.33±0.28 mm) and CoNS (14.33±0.28 mm, 15.33±0.28 mm) were moderately active in methanol and acetone extract while *Streptococcus* spp. (17.33±0.57 mm) showed moderate inhibition in hexane extract. Aqueous extract of *P. granatum* rind exhibited significant antibacterial activity against *Enterobacter* spp. (22.00±0.50 mm), *S. aureus* (20.83±0.76 mm), CoNS (20.50±0.50 mm) and *Streptococcus* spp. (20.16±0.76 mm), whereas moderate activity was recorded in *E. coli* (16.33±0.33 mm) and *P. aeruginosa* (17.00±1.00 mm). *Klebsiella pneumoniae* and *Proteus* spp. were found to be resistant in comparison to control. The present investigation clearly shows that 80% ethanolic extract showed highly significant antibacterial potential against *S. aureus* (22.83±0.76 mm), *P. aeruginosa* (21.83±.28 mm), and moderate activity against CoNS (17.00±1.00 mm), *Streptococcus* spp. (15.66±0.57 mm), *K. pneumoniae* (15.50±0.50 mm), *E. coli* (15.66±0.28 mm), *Enterobacter* spp. (16.83±0.28 mm) and *Proteus* spp. (15.50±0.50 mm). In this study, high significant antibacterial activity was found against *S. aureus* (30.50±0.50 mm, 20.00±1.00 mm), CoNS (24.16±0.28 mm, 20.66±0.57 mm) and *P. aeruginosa* (21.83±0.28 mm, 25.16±0.28 mm), while moderate activity against *Proteus* spp. (14.50±0.50 mm, 14.16±0.28 mm) and least activity with *Streptococcus* spp. (10.16±0.28 mm) in methanol and acetone extracts respectively. Additionally moderate activity was observed against *Enterbacter* spp. (15.66±0.28 mm) in acetone extract. In the present report, hexane extract was only sensitive against *Streptococcus* spp. (16.33±0.57 mm) and *Proteus* spp. (13.00±0.00 mm) whereas resistant against other isolates. The aqueous extract of *S. chirata* did not exhibited antibacterial activity against all the isolates but surprisingly 80% ethanolic extract showed highly significant activity against *S. aureus* (30.33±0.57 mm), CoNS (30.33±0.57 mm), *Streptococcus*

spp. (22.83±0.28 mm), *P. aeruginosa* (40.33±0.57 mm) and *Enterobacter* spp. (20.33±0.57 mm) nearly equal to or more effective than control used. *K. pneumoniae* (18.33±0.28 mm), *E. coli* (17.16±0.28 mm), and *Proteus* spp. (18.66±0.28 mm) showed moderate activity. Methanolic solvent extracts exhibited the highest activity towards *S. aureus* (22.33±0.28 mm), and CoNS (20.83±0.28 mm) while moderate activity against *Streptococcus* spp., *E. coli* (15.16±0.28 mm), *P. aeruginosa* (13.16±0.28 mm). Acetone extract exhibited moderate activity against *S. aureus* (17.00±0.50 mm), CoNS (19.50±0.50 mm), and *P. aeruginosa* (15.16±0.28 mm). Hexane extract exhibited moderate activity against *Streptococcus* spp., (15.50±0.50 mm) only. Aqueous leaf extract of *S. cumini* revealed most considerable antibacterial potential against gram-positive bacteria *S. aureus* (30.16±0.76 mm), CoNS (20.33±0.57 mm), *Streptococcus* spp. (20.00±1.00 mm) and moderate activity against *P. aeruginosa* (13.83±0.76 mm). All other gram-negative bacteria are resistant. 80% ethanolic extract of *S. cumini* leaf exhibited moderate antibacterial activity against CoNS (17.66±0.28 mm), *Streptococcus* spp. (13.16±0.28 mm), *K. pneumoniae* (16.33±0.28 mm), *E. coli* (14.83±.28 mm), *Enterobacter* spp. (13.33±0.28 mm), *Proteus* spp. (15.33±0.28 mm) except *S. aureus* (20.50±0.50 mm) and *P. aeruginosa* (20.33±0.28 mm) which showed highest activity. Methanol and acetone extract exhibited significant activity against *S. aureus* (20.33±.57 mm, 16.33±0.57 mm), CoNS (20.50±0.50 mm, 20.00±1.00 mm), *P. aeruginosa* (15.16±0.28 mm, 24.50±0.86 mm), and moderate activity against *Streptococcus* spp. (12.50±0.50 mm, 13.16±0.28 mm) and *Enterobacter* spp. (14.66±0.73 mm) respectively. *K. pneumoniae*, *E. coli*, and *Proteus* spp. are resistant to all the solvent extract except 80% ethanol while hexane extract was moderately sensitive to *Streptococcus* spp. (14.33±0.57 mm) only. Aqueous leaf extract of *T. cordifolia* exhibited high sensitivity against *P. aeruginosa* (20.00±1.00 mm) and less sensitivity against *K. pneumoniae* (12.16±0.76 mm), *E. coli* (12.33±0.57 mm), and *Enterobacter* spp. (10.16±0.76 mm). All other bacterial isolates (*S. aureus*, CoNS, *Streptococcus* spp., *Proteus* spp.) are resistant at concentration of 200 mg/ml tested. 80% ethanolic solvent extract exhibited moderate activity against *P. aeruginosa* (15.66±0.28 mm) and least with *S. aureus* (11.83±0.76 mm). Methanol extracts showed high sensitivity against *P. aeruginosa* (26.16±0.28 mm) while moderate activity

against CoNS (14.16±0.28 mm) and least with *K. pneumoniae* (12.6±0.28 mm) whereas, other isolates showed resistance activity. Acetone and hexane extract exhibited moderate activity against *S. aureus* (13.16±0.28 mm), CoNS (13.16±0.28 mm, 18.33±0.57 mm) *Streptococcus* spp., (16.33±0.57 mm) respectively at concentration of 200 mg/ml tested.

In the present investigation, phytochemical screening was performed for the detection of alkaloids, Coumarins, Phenol, Flavonoids, Terpenoids, Tannins, Steroids, and Saponins. These phytochemicals were screened for the different solvent extract of seven medicinal plants used. The result showed the presence of alkaloids in 28 different solvents extract out of 35 solvent extract followed by terpenoids (26), Steroids (26), Quinones (23), Flavonoids (23), Tannins (21), Coumarins (21), Saponins (16), and Phenol (15). The presence of phytochemicals was abundant in aqueous, 80% ethanol, methanol and acetone extract, and least in hexane. The detection of phytochemicals showed variable results.

7.1.3 Identification of Bioactive compounds from *P. granatum* rind by LC-MS analysis

The study for LCMS analysis and interpretation of chromatogram was carried out from SAIF, CDRI located in Lucknow. As per the suggestion of the CDRI, with further search for the different literature for the identification of compounds that has been previously reported on the basis of molecular weight and retention time. *P. granatum* rind led to the identification of 23 bioactive molecules belonging to different chemical classes and 22 of them were identified along with one unidentified compound. Among identified compounds (Citric acid, Gallic acid, Gallocatechin, Granatin A, Catechin and Epicatechin, Corilagin, Kaempferol-3-*O*-rhamnoglucoside, Dotriacontane, Brevifolin carboxylic acid, Estriol, Quercetin, Cynaroside, 3,3'-Di-O-methylellagic acid, Gamma sitosterol, Gadoleic acid, 1,2-Benzenedicarboxylic acid,bis (2-ethylpropyl) ester; Tributyl acetylcitrate) were found in different parts of *P. granatum* and five compounds (Sebiferine; 9-12 octadecadienoic acid, ethyl ester; Limonen-6-ol, pivalate; Hazaleamide monoeptixide; Dihydromyricetin 3-O-rhamnoside) were not generally been described in the pomegranate literature to our knowledge rather in other plant sources (*Litsea cubeba*, *Bougainvillea buttiana*, *Nigella sativa*, *Stylosanthes fruticosa* and Italian millet) respectively. The compounds identified

were mainly phenolic, flavonoids, Organic acids, Catechin and Procyanidins, Ellagitannins and Gallotannins, Sterols and Terpenoids.

7.1.4 Polyherbal syrup: Formulation, Evaluation, Preparation and Standardization

Formulation of polyherbal syrup was performed by permutation and combination of different solvent extract of six plants which have exhibited significant antibacterial activity individually against all the isolated bacteria. Four phases of the trial formulation were developed and evaluated for antibacterial activity. Among these trial phases, four was found most significant with the ratio of formulation of G in which *S. aureus* (17.16±0.28 mm), *Streptococcus* spp. (11.16±0.57 mm), CoNS (20.16±0.76 mm), *K. pneumoniae* (18.00±1.00 mm), *E. coli* (16.00±0.00 mm), *Enterobacter* spp. (18.83±0.76 mm), *P. aeruginosa* (21.00±0.00 mm), and *Proteus* spp. (17.00±0.00 mm) were significantly sensitive. The ratio of combination G was used to formulate the syrup with the help of Gorkha Ayurved Company (P) Ltd., Gorkha, Nepal following the guidelines of Ayurvedic Pharmacopoeia. The prepared syrup was standardized according to the criteria of ayurvedic pharmacopoeia. The prepared syrup was observed for the organoleptic character, Quantitative analysis of heavy metals, and microbial burden. The developed syrup exhibited brown color, sweet-bitter taste with agreeable smell having pH 6.13.The concentration of heavy metal obtained were Lead **(**<0.1ppm**),** Arsenic **(**<0.001ppm**),** Cadmium **(**<0.005ppm**),** and Mercury **(**<0.0005ppm**)** respectively. The microbial load analysis results total aerobic viable count was 10^3 CFU/ml, total yeast and molds was 10 CFU/ml whereas; specific micro-organism *E. coli*, *Salmonella*, *P. aeruginosa*, and *S. aureus* were absent**.** All these criteria are in accordance with Ayurvedic Pharmacopoeia of India Part-II, Vol.-2 formulations. The final prepared syrup halted the growth of tested organisms which concludes that the excipients and process of preparation did not affect the sensitivity of the active components present in the formulation in the form of extract.

7.1.5 *In vivo* study

The developed formulation was carried out for acute oral toxicity by following the guidelines of OECD 423. After the treatment, no mortality was observed within four hours and thereafter for 24 hours after oral prescription of the tested plant extract at a dose of 300 mg,

2000 mg/kg BW. There was no lethal effect during the experimental period of 14 days for both male and female mice. Food, water intake, body weight, and respiration were normal. The general behaviour of the extract administered mice and control group did not show any drug related change in behaviour, breathing, water consumption, unable to take food and temperature therefore, the formulation seems to be safe at a dose of 2000 mg/kg body weight and the median lethal dose (LD_{50}) of the formulation was assumed to be more than limit test dose of 2000 mg/kg body weight.

7.2 Conclusions

Neonatal sepsis is a life intimidating disease which compels to understand the complete knowledge of its major symptoms, care about associated risk factors, prevalent etiological organism and current effective antibiotics. Respiratory distress is the major symptom and common risk factors were vaginal delivery in hospital, birth weight less than 2.5 kg, and a gestational period of less than 37 weeks. We report that *S. aureus* is the most common isolate in both onsets (EONS and LONS) of sepsis. We strongly recommend medical personnel working in NICU should be trained and maintain hand hygiene with proper disinfection during the procedure and use of Linezolid and Imipenem against gram-positive & negative bacteria respectively as compared to the broad-spectrum antibiotics which are more rampantly used now a day. This vital information helps Neonatologist to manage and treat neonates with sepsis. Thus, reduction in mortality will reduce the NMR in the southern part along with overall in Nepal to meet the Sustainable Development Goals -3.
The study demonstrated the extract of the selected plants has antibacterial potential against *S. aureus*, CoNS, *Streptococcus* spp., *K. pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter* spp. and *Proteus* spp. which may be attributed to the polar and nonpolar phytochemicals present in plants. Furthermore, the polyherbal syrup developed by combination of six medicinal plants comprise of 80% ethanolic (*S. chirata*, *N. arbortristis*) and aqueous (*P. granatum*, *S. cumini*, *T. cordifolia*, *M. oleifera*) extract. Based on the physicochemical and analytical procedures, it can be concluded that the syrup is well standardized product at the baseline parameters. The finding of acute study concluded that this polyherbal formulation is non-toxic with a single oral dose of 2000 mg/kg/body weight.

The emergence of antibiotic resistance and severe side effect of synthetic antibiotics in the present time has made mankind to shift towards herbal products. This antibacterial syrup against neonatal sepsis may play a vital role in controlling the development of resistance in bacteria, treatment and reoccurrence of sepsis in neonates with fewer side effects. It also helps the neonates to enhance their immunity and micronutrients availability. It is recommended that in future the study related to efficacy in induced septic mice model along with subacute and chronic toxicity *in vivo* need to be performed of this polyherbal syrup for more effective product in ayurvedic medicine, as antibacterial syrup for the cure of sepsis in neonates and to reduce the death of millions of newborn happening per year in world.

Bibliography

Abbas K, Niaz U, Hussain T, Saeed MA, Javaid Z, Idrees A, Rasool S. 2014. Antimicrobial Activity of Fruits of *Solanum nigrum* and *Solanum xanthocarpum.* Acta Poloniae Pharmaceutica -Drug Research 71:415-421. https://www.ptfarm.pl/pub/File/Acta_Poloniae/2014/3/415.pdf

Abiramasundari P, Priya V, Jeyanthi GP, Devi GS. 2011. Evaluation of the Antibacterial activity of *Cocculushirsutus*. *Hygeia*. Journal for Drugs andMedicines3: 26-31. http://www.hygeiajournal.com/downloads/8818372494.gayathri.pdf

Abu-Lafi S, Rayan M, Masalha M, Abu-Farich B, Al-Jaas H, Abu-Lafi M, Rayan A. 2019. Phytochemical Composition and Biological Activities of Wild *Scolymus maculatus* L. Medicines 6:53.https://doi.org/10.3390/medicines6020053.

Adwan G, Abu-Shanab B, Adwan K. 2010.Antibacterial activities of some plant extracts alone and in combination with different antimicrobials against multidrug-resistant *Pseudomonas aeruginosa* strains. Asian Pacific Journal of Tropical Medicine 3:266-269. https://doi.org/10.1016/S1995-7645(10)60064-8.

Agnihotri A, Kumar K, Srivastava A, Bhadauria S. 2012. Antibacterial Potential of *Tinospora cordifolia* against resistant Human Pathogenic Bacteria**.**Int J Pharm Pharm Sci 4:146-147.

Agrawal J, Pal A. 2013. *Nyctanthes arbor-tristis* Linn-A critical ethnopharmacological review. Journal of Ethnopharmacology 146:645-658 https://doi.org/10.1016/j.jep.2013.01.024.

Agrawal SS, Paridhavi M. Herbal drug technology. Universities press, India. 2007:607-614.

Ahirwal L, Singh S, Mehta A. 2011. Antimicrobial Screening of Methanol and Aqueous Extracts of *Swertia chirata.*Int J Pharm Pharm Sci 3:142-146. https://innovareacademics.in/journal/ijpps/Vol3Suppl4/2375.pdf.

Akeel RA, Mateen A, Janardhan K, Gupta VC. 2015. Analysis of anti-bacterial and anti oxidative activity of *Azadirachta indica* bark using various solvents extracts.Saudi Journal of Biological Sciences 24:11–14. http://dx.doi.org/10.1016/j.sjbs.2015.08.006.

Aku FY, Akweongo P, Nyarko K, Sackey S, Wurapa F, Afari EA, Ameme DK, Kenu E. 2018. Bacteriological profile and antibiotic susceptibility pattern of common isolates of neonatal

sepsis, Ho Municipality, Ghana-2016. Maternal Health, Neonatology, and Perinatology 4:1-8.https://doi.org/10.1186/s40748-017-0071-z.

Alam KD, Ali MS, Parvin S, Mahjabeen S, Akbar MA, Ahamed R. 2009. *In Vitro* Antimicrobial activities of different fractions of *Swertia chirata* ethanolic extract. Pak J Biol Sci 12:1334-1337. https://doi.org/10.3923/pjbs.2009.1334.1337.

Al-Daihan S, Al-Faham M, Al-shawi N, Almayman R, Brnawi A, Zargar S, Bhat RS. 2013. Antibacterial activity and phytochemical screening of some medicinal plants commonly used in Saudi Arabia against selected pathogenic microorganisms.Journal of King Saud University-Science 25:115–120. https://doi.org/10.1016/j.jksus.2012.11.003.

AL-Hashimia AG, AL-Salmanb HNK, AL-Jadaanb SAN. 2018. GC-Mass Analysis and Estimation of Pomegranate Husks Extracts and the Biological Efficacy of Compound Tri-butyl Acetyl Citrate as one of the Extract Against Food Fungi.Oriental Journal of Chemistry 34:2089-2097. http://dx.doi.org/10.13005/ojc/3404049.

Al-Jadidi HSK, Hossain MA. 2015. Studies on total phenolics, total flavonoids and antimicrobial activity from the leaves crude extracts of neem traditionally used for the treatment of cough and nausea.Beni- Suef university journal of basic and applied sciences 4:93-9 8. http://dx.doi.org/10.1016/j.bjbas.2015.05.001.

Almuneef MA, Baltomore RS, Farrel PA, Reagan-Cirincione P, Dembry LM. 2001. Molecular typing demonstrating transmission of gram-negative rods in neonatal intensive care unit in the absence of a recognized epidemic. Clin Infect Dis 32:221-227. https://doi.org/10.1086/318477.

Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA. 2017. Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. Plants 6:1-23. https://doi.org/10.3390/plants6040042.

Alves PD, Brandao MGL, Nunan EA, Vianna-Soares CD. 2009. Chromatographic evaluation and antimicrobial activity of Neem (*Azadirachta indica* A. Juss., Meliaceae) leaves hydroalcoholic extracts. Rev Bras Farmacogn Brazilian Journal of Pharmacognosy 19:510-515. http://dx.doi.org/10.1590/S0102-695X2009000400001.

Alzohairy MA. 2016. Therapeutics Role of *Azadirachta indica* (Neem) and Their Active Constituents in Diseases Prevention and Treatment. Evidence-Based Complementary and Alternative Medicine 2016:1-11. http://dx.doi.org/10.1155/2016/7382506.

Al-Zoreky NS. 2009. Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. Int J Food Microbiol 134:244–248. https://doi.org/10.1016/j.ijfoodmicro.2009.07.002.

Amare D, Mela M, Dessie G. 2019. Unfinished agenda of the neonates in developing countries: magnitude of neonatal sepsis: systematic review and meta-analysis. Heliyon 5:e02519. https://doi.org/10.1016/j.heliyon.2019.e02519.

Amenu D. 2014. Antimicrobial activity of medicinal plant extracts and their synergistic effect on some selected pathogens. American Journal of Ethnomedicine l:018-029.

Ansari S, Nepal HP, Gautam R, Shrestha S, Neopane P, Chapagain ML. 2015. Neonatal Septicemia in Nepal: Early-Onset versus Late-Onset.International Journal of Pediatrics 2015:1-6. http://dx.doi.org/10.1155/2015/379806.

Arora DS, Sood H. 2017. In vitro antimicrobial potential of extracts and phytoconstituents from *Gymnema sylvestre* R.Br. leaves and their biosafety evaluation.AMB Expr 7:1-13. https://doi.org/10.1186/s13568-017-0416-z.

Assaf AM, Amro BI, Mashallah S, Haddadin RN. 2016. Antimicrobial and anti-inflammatory potential therapy for opportunistic microorganisms.J Infect Dev Ctries 10:494-505. https://doi.org/10.3855/jidc.7610.

Assob JCN, Kamga HLF, Nsagha DS, Njunda AL, Nde PF, Asongalem EA, Njouendou AJ, Sandjon B, Penlap VB. 2011. Antimicrobial and toxicological activities of five medicinal plant species from CameroonTraditional Medicine. BMC Complement Altern Med 11:1-11. http://doi.org/10.1186/1472-6882-11-70

Attia EA. 2019. Antimicrobial activity and bio-active compounds analysis in ethanolic plant extract of *Punica granatum* (Pomegranate) using GC-MS. Egypt J Exp Biol (Bot) 15:325–331. https://doi.org/10.5455/egyjebb.20190707112923.

Aulton ME. 2007. Aulton's pharmaceutics: the design and manufacture of medicines. 3rd ed. Edinburgh; New York : Churchill Livingstone

Avbunudiogba JA, Alalor CA, Builders PF, Odozie S. 2013. Development and evaluation of liquid oral phytoformulation of *Phyllanthus amarus*. Journal of Pharmacy Research 6:908-912. https://doi.org/10.1016/j.jopr.2013.08.029.

Ayurvedic Pharmacopoeia of India (API) (2018) (1st edition) Part II, Volume II (Formulations). Government of India, Ministry of Health and Family Welfare, Department of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homeopathy (AYUSH), New Delhi

Ayurvedic Pharmacopoeia of India (API) (2007) (1st edition) Part II, Volume I (Formulations). Government of India, Ministry of Health and Family Welfare, Department of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homeopathy (AYUSH), New Delhi

Ayyanar M, Subash-Babu P. 2012.*Syzygium cumini* (L.) Skeels: A review of its phytochemical constituents and traditional uses. Asian Pac J Trop Biomed 2:240-246. https://doi.org/10.1016/S2221-1691(12)60050-1.

Azwanida NN. 2015. A review on the extraction methods use in medicinal plants, principle, strength, and limitation. Med Aromat Plants 4:1-6. http://dx.doi.org/10.4172/2167-0412.1000196.

Bai S, Bharti P, Seasotiya L, Malik A, Dalal S. 2015. In vitro screening and evaluation of some Indian medicinal plants for their potential to inhibit Jack bean and bacterial ureases causing urinary infections. Pharm Biol 53:326-333. https://doi.org/10.3109/13880209.2014.918158.

Balouiri M, Sadiki M, Ibnsouda SK. 2016. Methods for in vitro evaluating antimicrobial activity: Areview. Journal of Pharmaceutical Analysis 6:71–79. http://dx.doi.org/10.1016/j.jpha.2015.11.005.

Baltimore RS. 2003. Neonatal Sepsis Epidemiology and Management**.** Pediatr Drugs 5:723-740. http://doi.org/1174-5878/03/0011-0723/$30.00/0.

Banothu V, Neelagiri C, Adepally U, Lingam J, Bommareddy K. 2017. Phytochemical screening and evaluation of in vitro antioxidant and antimicrobial activities of the indigenous medicinal plant *Albizia odoratissima*. Pharmaceutical Biology 55:1155–1161. http://dx.doi.org/10.1080/13880209.2017.1291694.

Barnden J, Diamond V, Heaton PA, Paul SP. 2016. Recognition and management of sepsis in early infancy. Nurs Child Young People 28:36–45. http://doi.org/10.7748/ncyp.2016.e775.

Bauer AW, Kirby WM, Sherris JC, Turck M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45: 493–6.

Benitz WE, Gould JB, Druzin ML. 1999. Risk Factors for Early-onset Group B Streptococcal Sepsis: Estimation of Odds Ratios by Critical Literature Review. PEDIATRICS 103:1-14. https://doi/org/10.1542/peds.103.6.e77.

Betoni JEC, Mantovani RP, Barbosa LN, Stasi LCD, Junior AF. 2006. Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. Mem Inst Oswaldo Cruz 101:387-390. https://doi.org/10.1590/s0074-02762006000400007.

Bhandari J, Muhammad BT, Pratiksha Thapa P, Shrestha BG. 2017. Study of phytochemical, anti-microbial, anti-oxidant, and anti-cancer properties of *Allium wallichii*. BMC Complement Altern Med 17:1-9. https://doi.org/10.1186/s12906-017-1622-6.

Bhutta ZA, Yusuf K. 1997. Early-onset neonatal sepsis in Pakistan: a case control study of risk factors in a birth cohort. Am J Perinatol 14: 577-581. https://doi.org/10.1055/s-2007-994338.

Bhutta ZA. 1999. Neonatal bacterial infections in developing countries: strategies for prevention. Seminars in Neonatology 4: 159-71. https://doi.org/10.1016/S1084-2756(99)90040-4.

Bhuva RM, Dixit YM. 2015. Comparative Antimicrobial Activities of Neem and curry leaf extracts and their synergistic effect against selected pathogenic bacteria and fungus. Int Res J Pharm 6:755-759. https://doi.org/10.7897/2230-8407.0611147.

Borges A, Ferreira C, Saavedra MJ, Simoes M. 2013. Antibacterial Activity and Mode of Action of Ferulic and Gallic Acids against Pathogenic Bacteria.Microbial Drug Resistance. 0:1-10. https://doi.org/10.1089/mdr.2012.0244.

Builders PF. 2018. Introductory Chapter: Introduction to Herbal Medicine. pp 1-11. http://dx.doi.org/10.5772/intechopen.78661.

Cai LY, Shi FX, Gao X. 2011. Preliminary phytochemical analysis of *Acanthopanan trifoliatus* (L.) Merr. J Med Plant Res 5:4059-4064. https://doi.org/10.5897/JMPR.9001037.

Camacho-Gonzalez A, Spearman PW, Stoll BJ. 2013. Neonatal infectious diseases: evaluation of neonatal sepsis. Pediatr Clin North Am 60:367–389. https://doi.org/10.1016/j.pcl.2012.12.003.

Carranza MG, Sevigny MB, Banerjee D, Fox-Cubley L. 2015. Antibacterial activity of native California medicinal plant extracts isolated from *Rhamnus californica* and *Umbellularia californica*. Ann Clin Microbiol Antimicrob 14:1-6. https://doi.org/10.1186/s12941-015-0086-0.

Carroll SG, Papaioannou S, Ntumazah IL, Philpott-Howard J, Nicolaides KH. 1996. Lower genital tract swabs in the prediction of intrauterine infection in preterm prelabour rupture of the membranes. Br J Obstet Gynaecol 103: 54-59. https://doi.org/10.1111/j.1471-0528.1996.tb09515.x.

Chakraborty B, Nath A, Saikia H, Sengupta M. 2014. Bactericidal activity of selected medicinal plants against multidrug resistant bacterial strains from clinical isolates. Asian Pac J Trop Med 7:S435-S441. https://doi.org/10.1016/S1995-7645(14)60271-6.

Chan GJ, Modak JK, Mahmud AA, Baqui AH, Black RE and Saha SK. 2013. Maternal and neonatal colonization in Bangladesh: prevalences, etiologies and risk factors. J Perinatol 33: 971-976.https://doi.org/10.1038/jp.2013.99.

Chandna A, Rao MN, Srinivas M, Shyamala S. 1998. Rapid diagnostic tests in neonatal septicemia. Indian Journal of Pediatrics 55:947-953. https://doi.org/10.1007/BF02727835.

Chapagain RH, Acharya R, Shrestha N, Giri BR, Bagale BB, Kayastha M. 2015. Bacteriological Profile of Neonatal Sepsis in Neonatal Intermediate Care Unit of Central Paediatric Referral Hospital in Nepal. J Nepal Health Res Counc 13:205-208. https://doi.org/10.33314/jnhrc.v0i0.

Charles RY, Bruno EJ, Mary EDY. 2021. *Tinospora Cordifolia*: A review of its immunomodulatory properties. Journal of Dietary Supplements 22:1-15 https://doi.org/10.1080/19390211.2021.1873214

Cheruiyot KR, Olila D, Kateregga J. 2009. In-vitroantibacterial activity of selected medicinal plants from Longisa region of Bomet district, Kenya. Afr Health Sci 9:S42-46. https://pubmed.ncbi.nlm.nih.gov/20589160/.

Chhetri HP, Yogol NS, Sherchan J, Anupa KC, Mansoor S, Thapa P. 2010. Formulation and Evaluation of Antimicrobial herbal ointment. Kathmandu University journal of science, engineering and technology 6: 102-107

Chi S, She G, Han D, Wang W, Liu Z, Liu B. 2016. Genus *Tinospora*: Ethnopharmacology, Phytochemistry, and Pharmacology. Evidence-Based Complementary and Alternative Medicine 2016:1-32. http://doi.org/10.1155/2016/9232593.

Chidi BB, Pandeya S, Gharti KP, Bharati L. 2015. Phytochemical Screening and Cytotoxic activity of *Nyctanthes arbor-tristis.*Ind Res J Pharm & Sci. 2:205-217.

Chirico G, Cortinovis S, Fonte C, Giudici G. 2007. Bacterial Sepsis. Journal of Chemotherapy 19:28-30. https://doi.org/10.1080/1120009X.2007.11782440.

Chouhan A, Gokhale M, Bans S. 2014. Antimicrobial potential of *Nyctanthes arbor-tristis* and isolation of *Colletotrichum gleosporioides* –an endophyte. Journal of Pharmacy Research 8:1082-1090. https://jprsolutions.info/files/final-file-58031f6ccdd497.55908389.pdf.

Christophoridou S, Dais P, Tseng LH, Spraul M. 2005. Separation and identification of phenolic compounds in olive oil by coupling high-performance liquid chromatography with postcolumn solid-phase extraction to nuclear magnetic resonance spectroscopy (LC-SPE-NMR). J Agric Food Chem 53:4667–4679. https://doi.org/10.1021/jf040466r.

Clinical Laboratory Standards Institute (CLSI) 2006. Performance standards for antimicrobial susceptibility testing. In: 24th informational supplement (M100-S24) CLSI, Wayne PA, USA.

Collee JG, Fraser AG, Marmion BP, Simmons A, editors. Mackie and McCartney Practical Medical Microbiology. 14th edi. New York: Churchill Livingstone; 1996.

Connell TG, Rele M, Cowley D, Buttery JP, Curtis **N.** 2007. How reliable is a negative blood culture result? Volume of blood submitted for culture in routine practice in a children's hospital. Pediatrics 119:891– 896. http://dx.doi.org/10.1542/peds.2006-0440.

Cortese F, Scicchitano P, Gesualdo M, Filaninno A, Giorgi ED, Schettini F, Laforgia N, Ciccone MM. 2016. Early and Late Infections in Newborns: Where Do We Stand? A Review. Pediatrics and Neonatology 57:265-273. http://dx.doi.org/10.1016/j.pedneo.2015.09.007.

Cosarca S, Tanase C, Muntean DL. 2019. Therapeutic Aspects of Catechin and Its Derivatives – An Update. ABMJ 2:21-29. https://doi.org/10.2478/abmj-2019-0003.

Costa DA, Chaves MH, Silva WCS, Costa CLS. 2010. Constituintes químicos, fenois totais e atividade antioxidante de *Sterculiastriata* St. Hil.et Naudin. Acta Amaz 40:207–212. https://doi.org/10.1590/S0044-59672010000100026.

Cowan MM. 1999. Plant products as antimicrobial agents. Clin Microbiol Rev 12:564–582. https://doi.org/10.1128/CMR.12.4.564.

Cushnie TPT, Cushnie B, Lamb AJ. 2014. Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. Int J Antimicrob Agents 44:377–386. http://doi.org/10.1016/j.ijantimicag.2014.06.001.

D'Angio CT, McGowan KL, Baumgart S, St Geme J, Harris MC. 1989. Surface colonization with coagulase-negative staphylococci in premature neonates. J. Pediatr. 114:1029–1034. http://dx.doi.org/10.1016/S0022-3476(89)80457-3.

Darout IA, Christy AA, Skaug N, Egeberg PK. 2000. Identification and quantification of some potentially antimicrobial anionic components in Miswak extract. Ind J Pharmacol.32:11–14.

De Zoysa MHN, Rathnayake H, Hewawasam RP, Wijayaratne WMDGB. 2019. Determination of *In Vitro* Antimicrobial Activity of Five Sri Lankan Medicinal Plants against Selected Human Pathogenic Bacteria. International Journal of Microbiology 2019:1—8 pages. https://doi.org/10.1155/2019/7431439.

Deepak TK, Hegde K, Hassainar A, Swapna D. 2014. Phytochemical screening and Haemolytic activities of hydroalcoholic extract of *Santalum album* .L leaves. International Journal of Pharma Sciences and Research 5:514-517. https://www.ijpsr.info/docs/IJPSR14-05-08-013.pdf.

Denny KH, Stewart CW. 2017. Acute, sub-acute, sub-chronic and chronic general toxicity testing for preclinical drug development. In: A Comprehensive Guide to Toxicology in Preclinical Drug Development. Chapter 5109-127. Elsevier Inc.: London https://doi.org/10.1016/B978-0-12-803620-4.00005-0.

Desai S, Metrani R, Vantamuri S, Ginigeri V, Phadke K, Hungund B. 2012. Phytochemical analysis, Antimicrobial and Antitumor screening of endophytes of *Tinospora cordifolia*. Int J Pharm Bio Sci 3:(B)533–540.

Dhakad AK, Ikram M, Sharma S, Khan S, Pandey VV, Singh A. 2019. Biological, nutritional, and therapeutic significance of *Moringaoleifera* Lam. Phytotherapy Research 33:2870–2903. https://doi.org/10.1002/ptr.6475.

Doughari JH, Pukuma MS, De N. 2007. Antibacterial effects of *Balanites aegyptiaca* L. Drel. and *Moringa oleifera* Lam. on *Salmonella typhi.* Afr J Biotechnol 6:2212-2215. https://doi.org/10.5897/AJB2007.000-2346.

Duraipandiyan V, Ayyanar M, Ignacimuthu S. 2006. Antimicrobial activity of some ethnomedicinal plants used byPaliyar tribe from Tamil Nadu, India. BMC Complement Altern Med, 6:1-7. https://doi.org/10.1186/1472-6882-6-35.

Dzotam JK, Touani FK, Kuete V. 2016. Antibacterial and antibiotic modifying activities of three food plants (Xanthosoma mafaffa Lam., *Moringa oleifera* (L.) Schott and Passiflora edulis Sims) against multidrug-resistant (MDR) Gram-negative bacteria. BMC Complement Altern Med 16:1-8. https://doi.org/10.1186/s12906-016-0990-7.

Eddebbagh M, Ziyate N, Moughaoui F, Abourriche A, Berrada M, Darkaoui S, Bennamara A. 2018. Antimicrobial activity of pomegranate (*Punica granatum*) waste. The second international conference on smart application and data analysis for smart cities 27th and 28th February 2018.

Ekor M. 2014. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front. Pharmacol.4:1-10. doi: 10.3389/fphar.2013.00177.

Elgamily H, Moussa A, Elboraey A, EL-Sayed H, Al-Moghazy M, Abdalla A. 2016. Microbiological Assessment of *Moringa Oleifera* Extracts and Its Incorporation in Novel

Dental Remedies against Some Oral Pathogens. Open Access Maced J Med Sci.4:585-590. http://dx.doi.org/10.3889/oamjms.2016.132.

Ezeja MI, Anaga AO, Asuzu IU. 2014. Acute and sub-chronic toxicity profile of methanol leaf extract of Gouania longipetala in rats. J Ethnopharmacol 151:1155–1164. https://doi.org/10.1016/j.jep.2013.12.034.

Farjana A, Zerin N, Kabir MS. 2014. Antimicrobial activity of medicinal plant leaf extracts against pathogenic Bacteria. Asian Pac J Trop Dis 4:S920-S923. https://doi.org/10.1016/S2222-1808(14)60758-1.

Fawole OA, Makunga NP, Opara UL. 2012. Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. BMC Complement and Altern Med12:1-11. https://doi.org/10.1186/1472-6882-12-200.

Feng W, Li M, Zhiyou H, Zhang J. 2019. Analytical Methods of Isolation and Identification.In Phytochemicals in Human Health. http://dx.doi.org/10.5772/intechopen.88122.

Frawley D, Ranade S. Ayurveda, Nature's Medicine. Wisconsin: Lotus Press; 2000. https://books.google.com.np/books?id=aTIVnugZofsC&printsec=copyright#v=onepage&q&f=false.

Geyesus T, Moges F, Eshetie S, Yeshitela B, Abate E. 2017. Bacterial etiologic agents causing neonatal sepsis and associated risk factors in Gondar, Northwest Ethiopia. BMC Pediatrics 17:1-10. https://doi.org/10.1186/s12887-017-0892-y.

Ganatra HA, Stoll BJ and Zaidi AKM. 2010. International perspective on early-onset neonatal sepsis. Clin Perinatol 37: 501-523. https://doi.org/10.1016/j.clp.2010.02.004.

Ganatra HA, Zaidi AKM. 2010. Neonatal Infections in the Developing World. Semin Perinatol 34:416-425. https://doi.org/10.1053/j.semperi.2010.09.004.

Gangoue-Pieboji J, Eze N, Djintchui AN, Ngameni B, Tsabang N, Pegnyemb DE, Biyiti L, Ngassam P, Koulla-Shiro S, Galleni M. 2009. The *in-vitro* antimicrobial activity of some traditionally used medicinal plants against beta-lactam-resistant bacteria. J Infect Dev Ctries3:671-680. https://doi.org/10.3855/jidc.77.

Gangoue-Pieboji J, Pegnyemb DE, Niyitegeka D, Nsangou A, Eze N, Minyem C, Mbing JN, Ngassam P, Tih RG, Sodengam BL, Bodo B. 2006. The in-vitro antimicrobial activities of some medicinal plants from Cameroon. Ann Trop Med Parasitol 100:237–243. https://doi.org/10.1179/136485906X86365.

General Guidelines for Drug Development of Ayurvedic Formulations (GGDDAF). 2018. 1st Edition, Vol—1. Central Council for Research in Ayurvedic Sciences Ministry of Ayush, Govt. of India, New Delhi

Getabelew A, Aman M, Fantaye E, Yeheyis T. 2018. Prevalence of Neonatal Sepsis andAssociated Factors among Neonates in Neonatal Intensive Care Unit at Selected Governmental Hospitals in Shashemene Town, Oromia Regional State, Ethiopia, 2017. International Journal of Pediatrics 2018:7801272, 1-7. https://doi.org/10.1155/2018/7801272.

Goldstein B, Giroir B, Randolph A, and the members of the International Consensus Conference on Pediatric Sepsis. 2005. International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics Pediatr Crit Care Med 6:2–8. http://doi.org/10.1097/01.PCC.0000149131.72248.E6.

Gowri SS, Vasantha K. 2010. Phytochemical Screening and Antibacterial Activity of *Syzygium cumini* (L.) (Myrtaceae) Leaves Extracts. Int J PharmTech Res 2:1569-1573. http://sphinxsai.com/s_v2_n2/PT_V.2No.2/phamtech_vol2no.2_pdf/PT=95%20_1569-1573_.pdf.

Guerrero RV, Abarca-Vargas R, Petricevich VL. 2017. Chemical Compounds and Biological Activity of an Extract From *Bougainvillea X buttiana* (Var. Rose) Holttum and Standl. International Journal of Pharmacy and Pharmaceutical Sciences 9:42-46. http://dx.doi.org/10.22159/ijpps.2017v9i3.16190.

Guilmot A, Hermann E, Braud VM, Carlier Y, Truyens C. 2011. Natural killer cell responses to infections in early life. J Innate Immun 3:280–288. http://doi.org/10.1159/000323934.

Gul F, Arslantas MK, Cinel I, Kumar A. 2017. Changing definitions of sepsis. Turk J Anaesthesiol Reanim 45: 129-38. http://doi.org/10.5152/TJAR.2017.93753.

Gull I, Sohail M, Aslam MS, Athar MA. 2013. Phytochemical, toxicological and antimicrobial evaluation of *lawsonia inermis* extracts against clinical isolates of pathogenic bacteria. Annals of Clinical Microbiology and Antimicrobials 12:1-6. https://doi.org/10.1186/1476-0711-12-36.

Gyawali N, Sanjana RK. 2013. Bacteriological Profile and Antibiogram of Neonatal Septicemia. Indian J Pediatr 80:371–374. https://doi.org/10.1007/s12098-012-0911-9.

Hadi MY, Mohammed GJ, Hameed IH. 2016. Analysis of bioactive chemical compounds of *Nigella sativa* using gas chromatography-mass spectrometry. Journal of Pharmacognosy and Phytotherapy 8:8-24. https://doi.org/10.5897/JPP2015.0364.

Hassine BD, Abderrabba M, Cazaux S, Bouajila J. 2013. Contribution to the Chemical and Biological Study of Eucalyptus Leaves Extracts: Effect on Frying Oil. Journal of Medical and Bioengineering 2:242–247. https://doi.org/10.12720/jomb.2.4.242-247.

Hayek SA, Gyawali R, Ibrahim SA. 2013. Antimicrobial natural products in Microbial Pathogens and Strategies for combating them: Science, Technology and Education, (A. Mendez-Vilas Ed.) 910–921.

Heath JA, Zerr DM. 2006. Infections Acquired in the Nursery: Epidemiology and Control.Infectious Diseases of the Fetus and Newborn Infant 1179–1205. https://doi.org 10.1016/B0-72-160537-0/50037-2.

Hernandez NE, Tereschuk ML, Abdala LR. 2000. Antimicrobial activity of flavonoids in medicinal plants from Tafi del Valle (Tucuman, Argentina). J Ethnopharmacol 73:317–322. https://doi.org/10.1016/s0378-8741(00)00295-6.

Hoffmann ED, Stroobant V. 2007. Mass spectrometry: Principles and applications. 3rd edition. England, John Wiley & Sons. http://www.usp.br/massa/2014/qfl2144/pdf/MassSpectrometry.pdf.

Hossain MS, khatun MT, Hassan MM. 2013. In vitro antibacterial effect of *Tinospora cordifolia* extracts against some selective bacterial pathogens. Int J Biosci 3:156-161. http://dx.doi.org/10.12692/ijb/3.7.156-161.

Hugo Schottmuller BG. 1969. The problem of sepsis (1867-1936). Internist (Berl) 10:92–101

Hussein HM, Hameed IH, Ibraheem OA. 2016. Antimicrobial Activity and Spectral Chemical Analysis of Methanolic Leaves Extract of Adiantum Capillus-Veneris Using GC-MS andFT-IR Spectroscopy. International Journal of Pharmacognosy and Phytochemical Research 8:369-385.

Illuri R, Venkataramana SH, Daguet D, Kodimule S. 2019. Sub-acute and acute toxicity of *Ferula asafoetida* and *Silybum marianum* formulation and effect of the formulation on delaying gastric emptying. BMC Complement Altern Med 19:1-11. https://doi.org/10.1186/s12906-019-2576-7.

Inacio MC, Carmona F, Paz TA, Furlan M, Silva FAD, Bertoni BW, Franca SDC, Pereira AMS. 2013. Screening Test for Antibiotics in Medicinal Plants (STAMP): Using Powdered Plant Materials Instead of Extracts.American Journal of Plant Sciences 4:2340-2350. http://dx.doi.org/10.4236/ajps.2013.412290.

Ingle KP, Deshmukh AG, Padole DA, Dudhare MS, Moharil MP, Khelurkar VC. 2017. Phytochemicals: Extraction methods, identification, and detection of bioactive compounds from plant extracts. J Pharmacogn Phytochem 6: 32-36. https://www.phytojournal.com/archives/2017/vol6issue1/PartA/6-1-23-924.pdf.

Jafari S, Saeidnia S, Ardekani MRS, Hadjiakhoondi A, Khanavi M. 2013. Micromorphological and preliminary phytochemical studies of *Azadirachta indica* and *Melia azedarach*. Turk J Bot37: 690-697. https://doi.org/10.3906/bot-1205-14.

Jagessar RC. 2017. Phytochemical screening and chromatographic profile of the ethanolic and aqueous extract of *Passiflora edulis* and *Vicia faba* L. (Fabaceae). Journal of Pharmacognosy and Phytochemistry 6:1714-1721.

Jain AK, Singh KP. 2013. Evaluation of antibacterial studies in Harsingaar. Int Res J Pharm 4:151-153. https://doi.org/10.1186/s13104-017-2665-y.

Jain NK, Jain VM, Maheshwari S. 2003. Clinical profile of neonatal sepsis. Kathmandu University Medical Journal 1:117-120. http://www.kumj.com.np/issue/2/117-120.pdf.

Kamarudin NA, Markom M, Latip J. 2016. Effects of Solvents and Extraction Methods on Herbal Plants *Phyllanthus niruri*, *Orthosiphon stamineus* and *Labisia pumila*. Indian Journal of Science and Technology 9:1-5.

https://doi.org/ 10.17485/ijst/2016/v9i21/95235.

Kamil OH, Lupuliasa D. 2011. Modern aspects regarding the microbial spoilage of pharmaceutical products. Farmacia 59: 133- 146. http://www.revistafarmacia.ro/20112/art01%20-%20oday%20133-146.pdf.

Kamle M, Mahato DK, Lee KE, Bajpai VK, Gajurel PR, Gu KS, Kumar P. 2019. Ethnopharmacological Properties and Medicinal Uses of *Litsea cubeba*. Plants 8:1-13. https://doi.org/10.3390/plants8060150.

Kan B, Razzaghian HR, Lavoie PM. 2016. An Immunological Perspective on Neonatal Sepsis. Trends Mol Med 22:290-302. http://dx.doi.org/10.1016/j.molmed.2016.02.001.

Karimi A, Majlesi M, Rafieian-Kopaei M. 2015. Herbal versus synthetic drugs; beliefs and facts. J Nephropharmacol 4:27-30. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5297475/pdf/npj-4-27.pdf.

Kaur GJ, Arora DS. 2009. Antibacterial and phytochemical screening of *Anethum graveolens*, *Foeniculum vulgare* and *Trachyspermum ammi*. BMC Complement Altern Med 9:1-10. https://doi.org/10.1186/1472-6882-9-30.

Kavitha BT, Shruthi SD, Rai SP, Ramachandra YL. 2011. Phytochemical analysis and hepatoprotective properties of *Tinospora cordifolia* against carbon tetrachloride-induced hepatic damage in rats. J Basic Clin Pharm 2:139-142.

Kayange N, Kamugisha E, Mwizamholya DL, Jeremiah S, Mshana SE. 2010. Predictors of positive blood culture and deaths among neonates with suspected neonatal sepsis in a tertiary hospital, Mwanza- Tanzania. BMC Pediatrics 10:1-9. https://doi.org/10.1186/1471-2431-10-39.

Kellogg JA, Ferrentino FL, Goodstein MH, Liss J, Shapiro SL, Bankert DA. 1997. Frequency of low level bacteremia in infants from birth to two months of age. Pediatr Infect Dis J 16:381–385. http://doi.org/10.1097/00006454-199704000-00009.

Khalid A, Waseem A, Saadullah M, Uzair-Ur-Rehman, Khiljee S, Sethi A, Asad MHHB, Rasool F, Khurram M, Waqas, Murtaza G. 2011. Antibacterial activity analysis of extracts of various plants against gram -positive and -negative bacteria. African Journal of Pharmacy and Pharmacology 5:887-893.https://doi.org/10.5897/AJPP11.215.

Khan N, Abbasi AM, Dastagir G, Nazir A, Shah GM, Shah MM, Shah MH. 2014. Ethnobotanical and antimicrobial study of some selected medicinal plants used in Khyber Pakhtunkhwa (KPK) as a potential source to cure infectious diseases. BMC Complement Altern Med 14:1-10. https://doi.org/10.1186/1472-6882-14-122.

Khan UA, Rahman H, Qasim M, Hussain A, Azizllah A, Murad W, Khan Z, Anees M, Adnan M. 2015. *Alkanna tinctoria* leaves extracts: a prospective remedy against multidrug resistant human pathogenic bacteria. BMC Complement Altern Med 15:1-6. https://doi.org/10.1186/s12906-015-0646-z.

Khanal B, Shariff M, Deb M. 2004. Neonatal Septicaemia: A Hospital Based Study in Eastern Nepal. Journal of Nepal Medical Association 43:231-234. https://doi.org/10.31729/jnma.488.

Khanal S, Shakya N, Thapa K, Pant DR. 2015. Phytochemical investigation of crude methanol extracts of different species of Swertia from Nepal. BMC Res Notes 8:1-9. https://doi.org/10.1186/s13104-015-1753-0.

Koo H, Gomes BP, Rosalen PL, Ambrosano GM, Park YK, Cury JA. 2000. In vitro antimicrobial activity of propolis and *Arnica montana* against oral pathogens. Arch Oral Biol 45:141-148. https://doi.org/10.1016/s0003-9969(99)00117-x.

Kshirsagar PR, Jagtap UB, Gaikwad NB, Bapat VA. 2019. Ethanopharmacology, phytochemistry and pharmacology of medicinally potent genus Swertia: An update. South African Journal of Botany 124:444–483. https://doi.org/10.1016/j.sajb.2019.05.030.

Kumar D, Arora S, Alam M. 2014. Pharmacognostical Standard and antimicrobial activity of leaves of *Syzygium cumini* (Linn.) from various region of north India. Int Res J Pharm 5:62-65. https://doi.org/10.7897/2230-8407.050212.

Kumar RSA, Rajeswari R, Rajeswari S, Romola CVJ, Priya RS, Rajeshwari K, Sukasani S. 2016. Formulation of simple syrup from *Acorus calamus* and *Ocimum kilimandscharicum* based on their antioxidant and antimicrobial activity. Der Pharmacia Lettre 8:75-78. http://doi.org/ 10.3109/1040841X.2012.706249.

Kumar SP, Prasan ND. 2013. Design, Development & Evaluation of a Poly herbal Syrup from some herbs used as Energy booster.International Journal of Ayurvedic Medicine 4:374-378. https://doi.org/10.47552/ijam.v4i4.354.

Kumar VK, Lalitha K. 2013. Acute oral toxicity studies of Anacyclus pyrethrum dc root in albino rats. Int J Pharm Pharm Sci 5:675–678. https://innovareacademics.in/journal/ijpps/Vol5Issue4/7847.pdf.

Kweera B, Sharma N, Jadon V, Negi Y, Parcha V. 2011. Phytochemical analysis and *in-vitro* antibacterial activity of *Swertia chirata* whole plant in different solvents. Journal of Pharmacy Research 4:4448-4449.

Lakhey A, Shakya H. 2017. Role of sepsis screening in early diagnosis of neonatal sepsis. Journal of Pathology of Nepal **7**:1103 -1110. https://doi.org/10.3126/jpn.v7i1.16944.

Lakshmi T, Krishnan V, Rajendram R, Madhusudhanan N. 2015. *Azadirachta indica*: a herbal panacea in dentistry-an update. Pharmacogn Rev 9:41–44. https://doi.org/10.4103/0973-7847.156337.

Lall WS, Charan AA, Akhilesh B. 2013. Antimicrobial Activity of Methanolic and Acetonic Extracts Of*Azadirachta Indica*, *Saraca Asoca* and *Curcuma Longa*. International Journal of Medicine and Pharmaceutical Sciences 3:79-86.

Levy O, Martin S, Eichenwald E, Ganz T, Valore E, Carroll SF, Lee K, Goldman D, Thorne GM. 1999. Impaired innate immunity in the newborn: newborn neutrophils are deficient in bactericidal/permeability-increasing protein. Pediatrics 104: 1327–1333. http://doi.org/10.1542/peds.104.6.1327.

Levy O. 2007. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol 7:379–90. https://doi.org/10.1038/nri2075.

Li X, Ding X, Shi P, Zhu Y, Huang Y, Li Q, Lu J, Li Z, Zhu L. 2019. Clinical features and antimicrobial susceptibility profiles of culture-proven neonatal sepsis in a tertiary children's hospital, 2013 to 2017. Medicine 98:1-8. http://dx.doi.org/10.1097/MD.0000000000014686.

Luciano-Montalvo C, Boulogne I, Gavillan-Suarez J. 2013. A screening for antimicrobial activities of Caribbean herbal remedies. BMC Complement Altern Med 13:1-9. https://doi.org/10.1186/1472-6882-13-126.

Mac DR, Camargo SS, Meyre-Silva C, Quintao NLM, Filho VC, Bresolin TMB, Lucinda-Silva RM. 2016. Development of an oral suspension containing dry extract of *Aleurites moluccanus* with anti-inflammatory activity. Revista Brasileira de Farmacognosia 26:68–76. http://dx.doi.org/10.1016/j.bjp.2015.06.011.

Madhav NVS, Upadhya K, Bishti A. 2011. Phytochemical screening and standardization of poly herbal formulation for Dyslipidemia. Int J Pharm pharm Sci 3:235-238. https://innovareacademics.in/journal/ijpps/Vol3Suppl3/2149.pdf.

Malek A. 2003. Ex vivo human placenta models: transport of immunoglobulin G and its subclasses. Vaccine 21:3362—3364. https://doi.org/10.1016/s0264-410x(03)00333-5.

Malviya S, Arvind, Jha A. Hettiarachchy N. 2014. Antioxidant and antibacterial potential of pomegranate peel extracts. J Food Sci Technol 51:4132–4137. https://doi.org/ 10.1007/s13197-013-0956-4.

Maridass M, Britto AJD. 2008. Origins of plant derived medicines. *EthnobotanicalLeaflets* 12:373-387.

Mediani A, Abas F, Khatib A, Tan CP. 2013. Cosmos Caudatus as a potential source of polyphenolic compounds: Optimisation of oven drying conditions and characterisation of its functional properties. Molecules 18:10452-10464. https://doi.org/10.3390/molecules180910452.

Mehar V, Yadav D, Somani P, Bhatambare G, Mulye S, Singh K. 2013. Neonatal sepsis in a tertiary care center in central India: Microbiological profile, antimicrobial sensitivity pattern and outcome. Journal of Neonatal-Perinatal Medicine 6:165–172. https://doi.org/10.3233/NPM-1367312.

Melin P. 2011. Neonatal group B streptococcal disease: from pathogenesis to preventive strategies. Clin Microbiol Infect 17: 1294–1303. http://doi.org/10.1111/j.1469-0691.2011.03576.x.

Mera IFG, Falconi DEG, Cordova VM. 2019. Secondary metabolites in plants: main classes, phytochemical analysis and pharmacological activities. 4:1000-1009. https://doi.org/10.21931/RB/2019.04.04.11.

Ministry of Health. 2016. Nepal demographic and health survey (NDHS). Kathmandu, Nepal. https://www.dhsprogram.com/pubs/pdf/fr336/fr336.pdf.

Modi C, Mody S, Patel H, Dudhatra G, Kumar A, Awale M. 2012. Herbal antibacterials, a review. Journal of Ethnopharmacology 1:52-61. https://doi.org/ 10.5455/jice.20120207113423

Modi J, Soni H, Pandya K, Patel G, Patel N. 2014. A detail phyto-chemical evaluation of herbo mineral formulation used in respiratory diseases. Journal of Pharmacognosy and Phytochemistry 2:36-42. https://www.phytojournal.com/archives/2014/vol2issue5/PartA/14.1.pdf

Moga MA, Dimienescu OG, Balan A, Dima L, Toma SI, Bigiu NF, Blidaru A. 2021. Pharmacological and Therapeutic Properties of *Punica granatum* Phytochemicals: Possible Roles inBreast Cancer. Molecules 26:1-20. https://doi.org/10.3390/molecules26041054

Mogana R, Adhikari A, Tzar MN, Ramliza R, Wiart C. 2020. Antibacterial activities of the extracts, fractions and isolated compounds from *Canarium patentinervium* Miq. Against bacterial clinical isolates. BMC Complementary Medicine and Therapies 20:55. https://doi.org/10.1186/s12906-020-2837-5.

Mohamed AA, Ali SI, El-Baz FK. 2013. Antioxidant and Antibacterial Activities of Crude Extracts and Essential Oils of *Syzygium cumini* Leaves. PLoS ONE 8: e60269. https://doi.org/10.1371/journal.pone.0060269.

Mohana DC, Satish S, Raveesha KA .2008. Antibacterial Evaluation of Some Plant Extracts against Some Human Pathogenic Bacteria. Advan Biol Res 2:49-55. https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.547.2948&rep=rep1&type=pdf.

Molla Y, Nedi T, Tadesse G, Alemayehu H, Shibeshi W. 2016. Evaluation of the in vitro antibacterial activity of the solvent fractions of the leaves of *Rhamnus prinoides* L'Herit (Rhamnaceae) against pathogenic bacteria. BMC Complement Altern Med 16:1-9.

https://doi.org/10.1186/s12906-016-1279-6.

Moulin-Schouleur M, Schouler C, Tailliez P, Kao MR, Bree A, Germon P, Oswald E, Mainil J, Blanco M, Blanco J. 2006. Common virulence factors and genetic relationships between O18:K1:H7 *Escherichia coli* isolates of human and avian origin. J Clin Microbiol 44:3484-3492. http://doi.org/10.1128/JCM.00548-06.

Muguli G, Gowda VD, Dutta V, Jadhav AN, Mendhe BB, Paramesh R, Babu UV. 2015. A contemporary approach on design, development, and evaluation of Ayurvedic formulation - *Triphala Guggulu*. Ayu 36:318-322.

N'Guessan JD, Bidie AP, Lenta BN,Weniger B, Andre P,Guede-Guina F. 2007. *In vitro* assays for bioactivity-guided isolation of antisalmonella and antioxidant compounds in *Thonningia sanguinea* flowers. African Journal of Biotechnology 6:1685-1689.

Naqvi SAR, Qurat-Ul-Ain, Khan ZA, Hussain Z, Shahzad SA, Yar M, Ghaffar A, Mahmood N, Kousar S. 2013. Antioxidant, Antibacterial and Antiproliferative Activities of Areal Parts of *Swertia chirata* (Bush Ham) Plant Extracts Using *In Vitro* Models. Asian Journal of Chemistry 25:5448-5452. http://dx.doi.org/10.14233/ajchem.2013.14700.

Nascimento GGF, Locatelli L, Freitas PC, Silva GL. 2000. Antibacterial activity of Plant Extracts and Phytochemicals on antibiotic resistant Bacteria. Braz J Microbiol 31:247-256. https://doi.org/10.1590/S1517-83822000000400003.

National Neonatology Forum. 2002-2003. National Neonatal Perinatal Database report. NNPD Nodal Center at Department of Pediatrics, WHO Collaborating Centre Newborn Training & Research, All India Institute of Medical Sciences, New Delhi, India 2005 Jan 70 pages. http://www.unicefrosa progressreport.org/savenewborns.html cited on 11/12/2019 https://www.newbornwhocc.org/pdf/nnpd_report_2002-03.PDF.

Negi BS, Dave B, Agarwal YK. 2012. Evaluation of Antimicrobial Activity of *Bauhinia purpurea* Leaves under in Vitro Conditions. Indian J Microbiol 52:360–365. https://doi.org/10.1007/s12088-012-0264-0.

Negi PS, Jayaprakasha GK. 2003. Antioxidant and Antibacterial Activities of *Punica granatum* Peel Extracts. Journal of Food Science 68:1473- 1477. https://doi.org/10.1111/j.1365-2621.2003.tb09669.x.

Nikam PH, Kareparamban J, Jadhav A, Kadam V. 2012. Future Trends in Standardization of Herbal Drugs. Journal of Applied Pharmaceutical Science 02: 38-44.

Nwonuma CO, Adelani-Akande TA, Osemwegie OO, Olaniran AF, Adeyemo TA. 2020. Preliminary *in vitro* antimicrobial potential and phytochemicals study of some medical plants. F1000Research 8:1-19.https://doi.org/10.12688/f1000research.17094.3.

OECD (2001). Test Guideline 420. Acute oraltoxicity – fixed dose procedure (FDP). In: OECD Guidelines for the Testing of Chemicals.Organisation for Economic Cooperation and Development, Paris

OECD (2001). Test Guideline 423. Acute OralToxicity – Acute Toxic Class Method. In: OECD Guidelines for the Testing of Chemicals.Organisation for Economic Cooperation and Development, Paris.

OECD (2006). Test Guideline 425. Acute Oral Toxicity – Up and Down Procedure (UDP. In: OECD Guidelines for the Testing of Chemicals.Organisation for Economic Cooperation and Development, Paris.

Ofosu FK, Elahi F, Banan-Mwine Daliri E, Chelliah R, Ham HJ, Kim JH, Han S, Hur JH, Oh D. 2020. Phenolic Profile, Antioxidant, and Antidiabetic Potential Exerted by Millet Grain Varieties. Antioxidants 9:1-14. https://doi.org/10.3390/antiox9030254.

Ogbonna JDN, Kenechukwu FC, Attama AA, Salome Amarachi Chime SA. 2012. Different Approaches to Formulation of Herbal Extracts/ Phytopharmaceuticals/ Bioactive Phytoconstituents- A Review. Int J Pharm Sci Rev Res 16: 1-8.

Oluduro AO. 2012. Evaluation of Antimicrobial properties and nutritional potentials of *Moringa oleifera* Lam. leaf in South-Western Nigeria. Mal J Microbiol 8: 59-67. https://doi.org/10.21161/mjm.02912.

Osman NA, Ali ZKM, Elden NYS, Elrahman SAA. 2015. Antibacterial and Antifungal Activity of Different Extract of *Moringa oleifera* Leaves – An In-Vitro Study. International journal of microbiology 1:110.

Othman L, Sleiman A, Abdel-Massih RM. 2019. Antimicrobial Activity of Polyphenols and Alkaloids in Middle Eastern Plants. Front Microbiol 10:1-28. https://doi.org/10.3389/fmicb.2019.00911.

Pandey A, Tripathi S. 2014. Concept of standardization, extraction and pre-phytochemical screening strategies for herbal drug. J Pharmacogn Phytochem 2:115-119. https://www.phytojournal.com/vol2Issue5/Issue_jan_2014/11.pdf.

Pandit BR,Vyas A. 2020a. Clinical Symptoms, Pathogen Spectrum, Risk factors and Antibiogram of Suspected Neonatal Sepsis cases in Tertiary Care Hospital of Southern Part of Nepal. JNMA 58:932-938. https://doi/org/10.31729/jnma.5498.

Pandit BR, Vyas A. 2020b. Efficacy of Anti-Bacterial action on Seven Medicinal Plants Extract against Neonatal Sepsis causing Bacteria-An In Vitro Study. Plant Archives 20: 2487-2494. http://www.plantarchives.org/SPL%20ISSUE%2020-2/412__2487-2494_.pdf.

Patel P, Patel N, Patel D, Desai S, Meshram D. 2014. Phytochemical analysis and Antifungal activity of *Moringa oleifera*. Int J Pharm Pharm Sci6:144-147. https://innovareacademics.in/journal/ijpps/Vol6Issue5/9066.pdf.

Patel S, Gokhale M. 2016. Comparative Study of Antioxidant Activity of Ethanol and Aqueous Extracts of Different Parts of *Nyctanthes arbor-tristis* Linn. Pharmacognosy Journal 8:113-116. https://doi.org/10.5530/pj.2016.2.3.

Peixoto JRO, Silva GC, Costa RA, Fontenelle JLS, Vieira GHF, Filho AAF, Vieira RHSF. 2011. In vitro antibacterial effect of aqueous and ethanolic Moringa leaf extracts. Asian Pacific Journal of Tropical Medicine 4:201-204. https://doi.org/10.1016/S1995-7645(11)60069-2.

Perez C, Pauli M, Bazerque P. 1990. An Antibacterial Assay by Agar Well Diffusion Method. Acta Biologiae et Medicinae Experimentalis 15:113–115.

Periyanayagam K, Mubeen M. 2018. Physiochemical and preliminary phytochemical analysis on the leaves and volatile oil of *Piper betle* (L.) var. Sirugamani 1. Drug Invention Today 10:169-173. https://jprsolutions.info/files/ms-file-5aebe83f19d5b3.97209820.pdf.

Peter MPJ, Venkatesan M, Raj JY. 2012. The Antibacterial Activity and Phytochemicals of the Leaves of *Stylosanthes fruticosa.* International Journal of Phytopharmacy 2:98-106. https://doi.org/10.7439/ijpp.v2i4.689.

Petrova A, Mehta R. 2007. Dysfunction of innate immunity and associated pathology in neonates. Indian J Pediatr 74:185–191. https://doi.org/10.1007/s12098-007-0013-2.

Philip AGS, Hewiot JR. 1980. Early diagnosis of neonatal sepsis. Paediatrics 65:1036-1041. https://pediatrics.aappublications.org/content/65/5/1036.long.

Ping KY, Darah I, Chen Y, Sreeramanan S, Sasidharan S. 2013. Acute and subchronic toxicity study of Euphorbia hirta L. methanol extract in rats. Biomed Res Int 2013:182064. https://doi.org/10.1155/2013/182064.

Pokhrel B, Koirala T, Shah G, Joshi S, Baral P. 2018. Bacteriological profile and antibiotic susceptibility of neonatal sepsis in neonatal intensive care unit of a tertiary hospital in Nepal. BMC Pediatrics 18:1-8. https://doi.org/10.1186/s12887-018-1176-x.

Popova IE, Hall C, Kubatova A. 2009. Determination of lignans in flaxseed using liquid chromatography with time-of-flight mass spectrometry. J Chromatogr A 1216:217–229. https://doi.org/10.1016/j.chroma.2008.11.063.

Pourcyrous M, Korones SB, Bada HS, Patterson T, Baselski V. 1988. Indwelling umbilical arterial catheter: a preferred sampling site for blood culture. Pediatrics 81:821–825. https://pediatrics.aappublications.org/content/pediatrics/81/6/821.full.pdf.

Prabhahar C, Saleshrani K, Saranraj P, Tharmaraj K. 2012. Antibacterial Effect of Ethanol and Ethyl Acetate Extracts of *Syzygium cumini* against Bacterial Pathogens.International Journal of Recent Scientific Research 3:155 -158. https://recentscientific.com/sites/default/files/Download_172.pdf.

Priya K, Ganjewala D. 2007. Antibacterial Activities and Phytochemical Analysis of Different Plant Parts of *Nyctanthes arbortristis* (Linn.). Res J Phytochem 1:61-67. https://doi.org/10.3923/rjphyto.2007.61.67

Procianoy RS, Silveira RC. 2019. The challenges of neonatal sepsis management. J Pediatr (Rio J) 842:1—7. https://doi.org/10.1016/j.jped.2019.10.004.

Rahman MA, Rahman MA, Ahmed NU. 2013. Phytochemical and biological activities of ethanolic extract of *C. hirsute* leaves. Bangladesh J Sci Ind Res 48:43-50. https://doi.org/10.3329/bjsir.v48i1.15413.

Raja RDA, Jeeva S, Prakash JW, Antonisamy JM, Irudayaraj V. 2011. Asian Pacific Journal of Tropical Medicine 375-378. https://oaji.net/articles/2016/3555-1470303761.pdf.

Rao PV, Sujana P, Vijaykanth T, Sankar NS, Kumar BV, Naidu D, Gan SH. 2013. Analysis of the Phytochemical Content and the Antibacterial, Antifungal and antioxidant Activities of the roots, Stems and Leaves of *Hemidesmus indicus*, *Ocimum sanctum* and *Tinospora cordifolia*. Int J Pharmacol 9:277-287. https://doi.org/10.3923/ijp.2013.277.287.

Raquel FE, Paul BS, Richard ME. 2007. Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds (Ceragenins). Biochimica et Biophysica Acta (BBA)-Bioembranes 1768:2500-2509. https://doi.org/10.1016/j.bbamem.2007.05.023.

Razavi SM, Zarrini G, Zahri S, Mohammadi S. 2010. Biological activity of *Prangos uloptera* DC. roots, a medicinal plant from Iran. Natural Product Research 24:797-803. https://doi.org/10.1080/14786410802588667.

Reddy LJ, Jose B. 2013. Evaluation of Antibacterial and DPPH Radical Scavenging activities of the Leaf Extracts and Leaf Essential Oil of *Syzygium cumini* Linn. from South India. Int J Pharm Pharm Sci 5:358-361. https://innovareacademics.in/journal/ijpps/Vol5Suppl3/7330.pdf.

Rojas JJ, Ochoa VJ, Ocampo SA, Munoz JF. 2006. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: A possible alternative in the treatment of non-nosocomial infections. BMC Complement Altern Med 6:1-6. https://doi.org/10.1186/1472-6882-6-2.

Romero CD, Chopin SF, Buck G, Martinez E, Garcia M, Bixby L. 2005. Antibacterial properties of common herbal remedies of the southwest. Journal of Ethnopharmacology 99:253–257. https://doi.org/10.1016/j.jep.2005.02.028.

Roy P, Abdulsalam FI, Pandey DK, Bhattacharjee A, Eruvaram NR, Malik T. 2015. Evaluation of antioxidant, antibacterial, and antidiabetic potential of two traditional medicinal plants of India: *Swertia cordata* and *Swertia chirayita*. Pharmacognosy Res 7:S57-62. https://doi.org/10.4103/0974-8490.157997.

Rubarth LB, Christensen CM, Riley C. 2017. Bacterial sepsis in the neonate. Nurse Pract 42:25–32. https://doi.org/10.1097/01.NPR.0000521994.61181.6a.

Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, Colombara DV, Ikuta KS, Kissoon N, Finfer S, Fleischmann-Struzek C, Machado FR, Reinhart KK, Rowan K, Seymour CW, Watson RS, West TE, Marinho F, Hay SI, Lozano R, Lopez AD, Angus DC Murrey CJL, Naghvi M. 2020. Global, regional, and national sepsis incidence and mortality, 1990-2017: analysis for the Global Burden of Disease Study. Lancet 395:200-11. https://doi.org/10.1016/S0140-6736(19)32989-7.

Russell ARB. 2015. Neonatal sepsis Symposium: Neonatology. Paediatrics and Child Health 25:271-275. https://doi.org/10.1016/j.paed.2015.02.005.

Saha RK, Acharya S, Shovon SSH, Apu AS, Roy P. 2012. Biochemical investigation and biological evaluation of the methanolic extract of the leaves of *Nyctanthes arbortristis* in vitro. Asian Pacific Journal of Tropical Biomedicine 2:S1534-S1541. https://doi.org/10.1016/S2221-1691(12)60449-3.

Salam AM, Lyles JT, Quave CL. 2019. Methods in the Extraction and Chemical Analysis of Medicinal Plants. In: Albuquerque U., de Lucena R., Cruz da Cunha L., Alves R. (eds) Methods and Techniques in Ethnobiology and Ethnoecology. Springer Protocols Handbooks. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-8919-5_17.

Sangeetha J, Vijayalakshmi K. 2011. Determination of Bioactive Components of Ethyl Acetate Fraction of *Punica granatum* Rind Extract. International Journal of Pharmaceutical Sciences and Drug Research 3:116-122. https://ijpsdr.com/index.php/ijpsdr/article/view/183.

Shankar MJ, Agarwal R, Deorari AK, Paul VK. 2008. Sepsis in newborn. Indian J Paediatr 75:261-266. http://doi.org/10.1007/s12098-008-0056-z.

Sant's Anna da Silva AP, Nascimento da Silva LC, Martins da Fonseca CS, M de Araujo J, Correia MTDS, Cavalcanti MS, Lima VLM. 2016. Antimicrobial Activity and Phytochemical Analysis of Organic Extracts from *Cleome spinosa* Jaqc. Front Microbiol 7:1-10. https://doi.org/10.3389/fmicb.2016.00963.

Sathiya M, Parimala P, Muthuchelian K. 2008. Preliminary Phytochemical Screening and Antibacterial Studies on the Ethanolic Leaf Extract of *Nyctanthes arbortristis* Linn.. Ethnobotanical Leaflets 12:337-342. https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.972.9600&rep=rep1&type=pdf.

Schelonka RL, Chai MK, Yoder BA, Hensley D, Brockett RM, Ascher DP. 1996. Volume of blood required to detect common neonatal pathogens. J Pediatr 129:275–278. http://dx.doi.org/10.1016 /S0022-3476(96)70254-8.

Schelonka RL, Maheshwari A, Carlo WA, Taylor S. Hansen NI, Schendel DE, Thorsen P, Skogstrand K, Hougaard DM, Higgins RD. 2011. T cell cytokines and the risk of blood stream infection in extremely low birth weight infants. Cytokine 53: 249–255. https://doi.org/10.1016/j.cyto.2010.11.003.

Seeram NP, Schulman RN, David Heber D (Editors) (2006) Pomegranates Ancient Roots to Modern Medicine. 1st Edition .6000 Broken Sound Parkway NW, Suite 300. Taylor and Francis

Senthil-Rajan D, Rajkumar M, Srinivasan R, Kumarappan C, Arunkumar K, Senthilkumar KL, Srikanth MV. 2013. Investigation on antimicrobial activity of root extracts of *Thespesia populnea* Linn. Trop Biomed30:570–578. https://pubmed.ncbi.nlm.nih.gov/24522124/.

Shakeel S, Shaikh ZA, Zaidi SF, Khan U. 2015. Development and evaluation of polyherbal Entoban syrup.Spatula DD 5:97-102. https://doi.org/10.5455/spatula.20151001032308.

Shane AL, Stoll BJ**.** 2013. Recent developments and current issues in the epidemiology, diagnosis, and management of bacterial and fungal neonatal sepsis. Am. J. Perinatol. 30:131–142. http://dx.doi.org/10.1055/s-0032-1333413.

Sharma P, Dwivedee BP, Bisht D, Dash AK, Kumar D. 2019. The chemical constituents and diverse pharmacological importance of *Tinospora cordifolia*. Heliyon 5: e02437 https://doi.org/10.1016/j.heliyon.2019.e02437

Shaw CK, Shaw P, Thapalial A. 2007. Neonatal sepsis bacterial isolates and antibiotic susceptibility patterns at a NICU in a tertiary care hospital in western Nepal: A retrospective analysis. Kathmandu University Medical Journal 5:153-160.

http://www.kumj.com.np/issue/18/153-160.pdf.

ShengWu, Li Tian. 2017. Diverse Phytochemicals and Bioactivities in the Ancient Fruit and Modern Functional Food Pomegranate (*Punica granatum*). Molecules 22:1-17. https://doi.org/10.3390/molecules22101606.

Shrestha NJ, Subedi KU, Rai GK. 2011. Bacteriological profile of neonatal sepsis: a hospital based study. Journal of Nepal Paediatric Society 31:1–5. https://doi.org/10.3126/jnps.v31i1.4158.

Shrestha RK, Rai SK, Khanal LK, Manda PK. 2013. Bacteriological study of neonatal sepsis and antibiotic susceptibility pattern in Kathmandu, Nepal. Nepal Med Coll J 15:71–73. https://pubmed.ncbi.nlm.nih.gov/24592800/.

Silva LV, Nelson DL, Drummond MFB, Dufosse L, Gloria MBA. 2005. Comparison of hydrodistillation methods for the deodorization of turmeric. Food Research International 38:1087–1096. https://doi.org/10.1016/j.foodres.2005.02.025.

Simonsen KA, Anderson-Berry AL, Delair SF, Davies HD. 2014. Early-onset neonatal sepsis.Clin Microbiol Rev 2014; 27:21-47. http://doi.org/10.1128/CMR.00031-13.

Singh GP, Sharma SK. 2012. Antimicrobial evaluation of Leaf extract of *Moringa oleifera* Lam. IRJP 3:212-214. https://www.irjponline.com/admin/php/uploads/1008_pdf.pdf.

Singh P, Katiyar D, Singh B, Srivastava A. 2015. Antimicrobial activity of *Tinospora cordifolia* Extracts against Urinary Tract Infections causing Bacteria. Int J Pharm Bio Sci 6:571 – 577.

Singh SK. 2013. Antibacterial activity of different extract of *Moringa oleifera* leaf against some pathogenic bacteria. JPSI 2:13-15. https://doi.org/10.7897/2277-4572.02206.

Singh V, Kumar R. 2017. Study of Phytochemical Analysis and Antioxidant Activity of *Allium sativum* of Bundelkhand Region. Int J Life Sci Scienti Res 3:1451-1458. https://doi.org/10.21276/ijlssr.2017.3.6.4.

Snyder LR. 1974. Classification of the Solvent Properties of Common Liquids. Journal of Chromatography, 92:223-230.

Soberon JR, Sgariglia MA, Sampietro DA, Quiroga EN, Vattuone MA. 2007. Antibacterial activity of plant extracts from northwestern Argentina. Journal of Applied Microbiology 102:1450–1461. https://doi.org/10.1111/j.1365-2672.2006.03229.x.

Sobota A, Strzelecka-Kiliszek A, Gladkowska E, Yoshida K, Mrozinska K, Kwaitkowska K. 2005. Binding of IgG-opsonized particles to FcγR is an active stage of phagocytosis that involves receptor clustering and phosphorylation. J Immunol 175:4450–4457. http://doi.org/10.4049/jimmunol.175.7.4450.

Soosairaj S, Dons T. 2016. Bio-active compounds analysis and characterization in Ethanolic plant extracts of Justicia tranquebariensis L. (Acanthaceae) – using GC-MS. International Journal of ChemTech Research 9:260-265. http://sphinxsai.com/2016/ch_vol9_no7/1/(260-265)V9N7CT.pdf.

Stefanovic IM. 2011. Neonatal sepsis. Biochemia Medica 21:276–281. https://doi.org/10.11613/bm.2011.037.

Stern JL, Hagerman AE, Steinberg PD, Mason PK. 1996. Phlorotannin–protein interactions. J Chem Ecol 22:1877–1899. https://doi.org/10.1007/BF02028510.

Surendra TV, Roopan SM, Arasu MV, Al-Dhabi NA, Sridharan M. 2016. Phenolic compounds in drumstick peel for the evaluation of antibacterial, hemolytic and photocatalytic activities. Journal of Photochemistry & Photobiology, B: Biology 161:463–471. http://dx.doi.org/10.1016/j.jphotobiol.2016.06.013.

Taddei A, Rosas-Romero AJ. 2000. Bioactivity studies of extracts from *Tridax procumbens.* Phytomedicine 7:235-238. https://doi.org/10.1016/S0944-7113(00)80009-4.

Thangarathinam N, Jayshree N, Metha AV, Ramanathan L. 2013. Development, Standardization and Evaluation of a Polyherbal Syrup. Int J Pharm Sci Rev Res 20:149-154. https://globalresearchonline.net/journalcontents/v20-2/25.pdf.

Thangavelu NR, Thomas S. 2010. In vitro anti-oxidant studies on ethanolic extracts of leaves and stems of *Nyctanthes arbortristis*. L (Night-flowering Jasmine). Int J Biol Med Res 1:188-192.

Thapa B, Thapa A, Aryal DR, Thapa K, Pun A, Khanal S, Mahat K. 2013. Neonatal Sepsis as a major cause of Morbidity in a tertiary center in Kathmandu. J Nepal Med Assoc 52:549-56.

https://doi.org/10.31729/jnma.2424.

Thaver D, Zaidi AKM. 2009. Burden of neonatal infections in developing countries: a review of evidence from community based studies. Pediatr Infect Dis J 28:S3–9. http://doi.org/10.1097/INF.0b013e3181958755.

Tille PM. 2017. Bailey and Scott's Diagnostic Microbiology. (14th Edition). 3251 Riverport Lane, St. Louis, Missouri 63043: Elsevier

Ugbabe GE, Ezeunala MN, Edmond IN, Apev J, Salawu OA. 2010. Preliminary Phytochemical, Antimicrobial and AcuteToxicity Studies of the Stem, bark and the Leaves of a cultivated *Syzygium cumini* Linn. (Family: Myrtaceae) in Nigeria. African Journal of Biotechnology 9:6943-6747. https://doi.org/10.5897/AJB09.1205.

Ullah O, Khan A, Ambreen A,Ahmad I, Akhtar T, Gandapor AJ, Khan AM. 2016. Antibiotic Sensitivity pattern of Bacterial Isolates of Neonatal Septicemia in Peshawar, Pakistan. Archives of Iranian Medicine 19:866–869. http://www.ams.ac.ir/AIM/NEWPUB/16/19/12/009.pdf.

Ulubelen A, Oksuz S, Topcu G, Goren AC, Bozok-johansson C, Celik C, Gamze Kokdil G, Voelter W. 2001. A New Antibacterial Diterpene from the Roots of *Salvia caespitosa*. Natural Product Letters 15:307-314. http://dx.doi.org/10.1080/10575630108041297.

United Nations Children's Fund, World Bank Group, World Health Organization, United Nations Population Division. 2019. United Nations Inter-agency Group for Child Mortality Estimation (UN-IGME), Levels and trends in Child Mortality-2019. Switzerland& USA.

Uthayarasa K, Pathmanathan K, Jeyadevan JP, Jeyaseelan EC. 2010. Antibacterial activity and qualitative phytochemical analysis of medicinal plant extracts obtained by sequential extraction method. International Journal of Integrative Biology 10:76-81.

Vasantharaj S, Sathiyavimal S, Hemashenpagam N. 2013. Phytochemical Analysis and Antibacterial Activity of *Azadirachta indica*. Int J Pharm Sci Rev Res 22:59-61. https://globalresearchonline.net/journalcontents/v22-1/12.pdf.

Viuda-Martos M, Fernandez-Lopez J, Perez-Alvarez JA. 2010. Pomegranate and its Many Functional Components as Related to Human Health: A Review. Comprehensive Reviews in Food Science and Food Safety 9:635-654.

https://doi.org/10.1111/j.1541-4337.2010.00131.x.

Vongsak B, Sithisarn P, Mangmool S, Thongpraditchote S, Wongkrajang Y, Gritsanapan W. 2013. Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method Ind. Crops Prod 44: 566-571. https://doi.org/10.1016/j.indcrop.2012.09.021.

Voukeng IK, Beng VP, Kuete V. 2016. Antibacterial activity of six medicinal Cameroonian plants against Gram-positive and Gram-negative multidrug resistant phenotypes. BMC Complement Altern Med 16:1-9. https://doi.org/10.1186/s12906-016-1371-y.

Voukeng IK, Beng VP, Kuete V. 2017. Multidrug resistant bacteria are sensitive to *Euphorbia prostrata* and six others Cameroonian medicinal plants extracts. BMC Res Notes 10:1-8. https://doi.org/10.1186/s13104-017-2665-y.

Vu TT, Kim H, Tran VK, Dang QL, Nguyen HT, Kim H, Kim IS, Choi GJ, Kim JC. 2016. In vitro antibacterial activity of selected medicinal plants traditionally used in Vietnam against human pathogenic Bacteria. BMC Complement Altern Medicine 16:1-6. https://doi.org/10.1186/s12906-016-1007-2.

Vyas A, Sarin R. 2013. Analysis of the phytochemical content and antimicrobial activity of *Nyctanthes arbor- tristis.* Int J Pharm Bio Sci 4:B201 – B206.

Wakkumbura HP, Wickramaarachchi WMD, Arawwawala L DAM, Liyanage JA, Rajapakse RPVJ. 2020. Assessment of the Quality and Evaluation of the Antioxidant Potential of a Novel Sri Lankan Ayurvedic Polyherbal Formulation. Evidence-Based Complementary and Alternative Medicine 2020:1-7. https://doi.org/10.1155/2020/2319315.

Waller DG, Sampson AP. 2018. Medical pharmacology and therapeutics. 5th ed. Elsevier, China

Walum E. 1998. Acute Oral Toxicity. Environment Health Perspectives 106:497-503. https://ehp.niehs.nih.gov/doi/pdf/10.1289/ehp.98106497.

Wang RF, Xie WD, Zhang Z, Xing DM, Ding Y, Wei Wang W, Ma C, Du LJ. 2004. Bioactive Compounds from the Seeds of *Punica granatum* (Pomegranate). J Nat Prod 67:2096-2098. https://doi.org/10.1021/np0498051.

Wazir A, Mehjabeen, Noor-Jahan, Sherwani SK, Ahmad M. 2014. Antibacterial, Antifungal and antioxidant activities of some medicinal plants. Pak J Pharm Sci 27:2145-2152. https://applications.emro.who.int/imemrf/Pak_J_Pharm_Sci/Pak_J_Pharm_Sci_2014_27_6_2145_2152.pdf.

Wikaningtyas P, Sukandar EY. 2016. The antibacterial activity of selected plants towards resistant bacteria isolated from clinical specimens. Asian Pac J Trop Biomed 6:16–19. http://dx.doi.org/10.1016/j.apjtb.2015.08.003.

Wink M. 2008. Plant secondary metabolism: diversity, function and its evolution. Natural Product Communications 3:1205–1216. https://doi.org/10.1177/1934578X0800300801.

World Health Assembly (WHA) (2003). Traditional medicine: report by the Secretariat. World Health Organization. https://apps.who.int/iris/handle/10665/78244.

World Health Organization (WHO). 2006. Stability Testing of Active Substances and Pharmaceutical Products. Restricted Working document QAS/06.179; p. 10-32.

World Health Organization Department of Reproductive Health and Research: Proportion of Births Attended by a Skilled Health Worker. Geneva, World Health Organization, 2008. https://apps.who.int/iris/bitstream/handle/10665/69950/WHO_RHR_08.22_eng.pdf?sequence=1.

World Health Organization & WHO Expert Committee on Specifications for Pharmaceutical Preparations. 2018. Fifty-second report of the WHO Expert Committee on Specifications for Pharmaceutical Preparations. World Health Organization. https://apps.who.int/iris/handle/10665/272452.

Yadav M, Chatterji S, Gupta SK, Watal G. 2014. Preliminary Phytochemical Screening of Six Medicinal Plants Used in Traditional Medicine. Int J Pharm Pharm Sci 6:539-542. https://innovareacademics.in/journal/ijpps/Vol6Issue5/9439.pdf.

Yadav NS, Sharma S, Chaudhary DK, Panthi P, Pokhrel P, Shrestha A, Mandal PK. 2018. Bacteriological profile of neonatal sepsis and antibiotic susceptibility pattern of isolates admitted at Kanti Children's Hospital, Kathmandu, Nepal. BMC Res Notes 11:1-6. https://doi.org/10.1186/s13104-018-3394-6.

Yildirim AB, Karakas FP, Turker AU. 2012. In vitro antibacterial and antitumor activities of some medicinal plant extracts, growing in Turkey. Asian Pac J Trop Med 6:616-624. https://doi.org/10.1016/S1995-7645(13)60106-6.

Zablotowicz RM, Hoagland RE, Wagner SC. 1996. Effect of saponins on the growth and activity of rhizosphere bacteria. In: Waller G.R., Yamasaki K. (eds) Saponins Used in Food and Agriculture. Advances in Experimental Medicine and Biology 405:83–95. Springer, Boston, MA.https://doi.org/10.1007/978-1-4613-0413-5_8.

Zaidan MRS, Rain AN, Badrul AR, Adlin A, Norazah A, Zakiah I. 2005. *In vitro* screening of five local medicinal plants for antibacterial activity using disc diffusion method. Trop Biomed22:165–170. http://www.msptm.org/files/165_-_170_In_vitro_screening.pdf.

Zaidi AKM, Thaver D, Ali SA, Khan TA. 2009. Pathogens associated with sepsis in newborns and young infants in developing countries. Pediatr Infect Dis J 28:S10–S18. http://doi.org/10.1097/INF.0b013e3181958769.

Zakariya BP, Bhat V, Harish BN, Arun Babu T, Joseph NM. 2010. Neonatal sepsis in a tertiary care hospital in South India: bacteriological profile and antibiotic sensitivity pattern. Indian J Pediatr 78:413-7. https://doi.org/10.1007/s12098-010-0314-8.

Zampini IC, Cuello S, Alberto MR, Ordonez RM, Almeida RD, Solorzano E, Isla MI. 2009. Antimicrobial activity of selected plant species from "the Argentine Puna" against sensitive and multi-resistant bacteria. Journal of Ethnopharmacology 124:499–505. https://doi.org/10.1016/j.jep.2009.05.011.

Zandvoort A, Timens W. 2002. The dual function of the splenic marginal zone: essential for initiation of anti-TI-2 responses but also vital in the general first-line defense against blood-borne antigens. Clin Exp Immunol 130:4–11. https://doi.org/10.1046/j.1365-2249.2002.01953.x.

Zewdie KA, Bhoumik D, Wondafrash DZ, Tuem KB. 2020. Evaluation of in-vivo antidiarrhoeal and invitro antibacterial activities of the root extract of *Brucea antidysenterica* J. F. Mill (Simaroubaceae). BMC Complementary Medicine and Therapies 20:1-11. https://doi.org/10.1186/s12906-020-03001-7.

Zhang QW, Lin LG, Ye WC. 2018. Techniques for extraction and isolation of natural products: a comprehensive review. Chin Med 13:1-26. https://doi.org/10.1186/s13020-018-0177-x.

APPENDIX- I

ABBREVIATIONS

S.No.	Abbreviation	Full meaning
1	()	Open and Close Bracket
2	%	Percentage
3	NMR	Neonatal Mortality Rate
4	EONS	Early Onset Neonatal sepsis
5	LONS	Late Onset Neonatal Sepsis
6	CSF	Cerebrospinal Fluid
7	CRP	C-reactive protein
8	PCT	Procalcitonin
9	PCR	Polymerase Chain Reaction
10	DNA	Deoxyribose Nucleic Acid
11	HIV	Human Immunodeficiency virus
12	LC-MS	Liquid Chromatography Mass Spectrometry
13	HPLC	High Performance Liquid Chromatography
14	m/z	Mass to Charge ratio
15	ESI	Electrospray Ionization
16	WHO	World Health Organisation
17	LD	Lethal Dose
18	OECD	Organisation for Economic Co-operation and Development
19	>	More than
20	<	Less than
21	min	minute
22	qSOFA	Quick Sequential Organ Failure Assessment
23	LODS	Logistic Organ Dysfunction System
24	SIRS	Systemic Inflammatory Response Syndrome
25	mmHg	Millimeters of Mercury
26	≤	Less than equal to
27	IAP	Intrapartum Antibiotic Prophylaxis
28	NICU	Neonatal Intensive Care Unit
29	ESBL	Extended Spectrum Beta -Lactamase
30	≥	More than equal to
31	^{0} C	Degree Centigrade
32	PAMPs	Pathogen-Associated Molecular Patterns
33	NKC	Natural Killer Cells
34	2nd	Second
35	LBW	Low Birth Weight Babies
36	CBC	Complete Blood Count
37	cell/mm^3	Cell per cubic millimeter
38	ANC	Absolute Neutrophils Count

39	WBC	White Blood Cells
40	PCT	Procalcitonin
41	NPV	Negative Predictive Value
42	B.C.	Before christ
43	ATCC	American Type Culture Collection
44	MDR	Multidrug Resistance
45	MTCC	Microbial Type Culture Collection
46	MRSA	Methicillin Resistance *Staphylococcus aureus*
47	PTCC	Persian Type Culture Collection
48	NCDC	National Collection of Dairy Cultures
49	AIDS	Acquired Immunodeficiency Syndrome
50	KHz	Kilo Hertz
51	PSI	Per Square Inch
52	ml	Milli litre
53	NMR	Nuclear Magnetic Resonance
54	UV	Ultra Voilet
55	IR	Infra Red
56	WHA	World Health Assembly
57	GGDDAF	General guidelines forDrug Development of Ayurvedic Formulations
58	TLC	Thin Layer Chromatography
59	HPTLC	High Performance Thin Layer Chromatography
59	ASU	Ayuvedic, Siddha and Unani
60	NMCTH	National Medical College and Teaching Hospital
61	NICU	Neonatal Intensive Care Unit
62	mcg/disc	Microgram per disc
63	mm	Millimetre
64	ZOI	Zone of Inhibition
65	KV	Kilo volt
66	ES^+	Electrospray Ionization in Positive Mode
67	ES^-	Electrospray Ionization in Negative Mode
68	L/Hr	Litre per Hour
69	µl	Micro litre
70	mg/ml	Milligram per Millilitre
71	±	Plus or Minus
72	&	and
73	µg	Microgram
74	hr	Hour
75	CFU	Colony Forming Unit
76	ANOVA	Analysis of Variance
77	β	Beta
78	NDHS	Nepal Demography and Health Survey

APPENDIX II

LIST OF CHEMICALS USED IN THIS RESEARCH

S.No.	Name of Chemicals
1	70% ethanol
2	1% Povidine Iodine solution
3	2% Crystal violet
4	Gram's Iodine
5	Acetone
6	0.5% Safranine
7	3% Hydrogen Perooxide
8	1% Tetramethyl P-Phenylene diamine dihydrochloride
9	P-dimethyl aminobenzaldehyde
10	40% Potassium hydrooxide
11	5% solution of α-naphthol in ethanol
12	Barium Chloride
13	Sulphuric acid
14	Hexane
15	Methanol
16	Dimethyl sulfoxide
17	Hydrochloric acid
18	Sodium Hydrooxide
19	Ferric Chloride
20	chloroform
21	Formic acid
22	Sorbitol
23	Sodium benzoate
24	Sodium methyl Paraben
25	Sodium Propyl Paraben
26	Nitric acid
27	Perchloric acid
28	Ammonium dihydrogen phosphate
29	Magnesium Nitrate
30	Potassium iodide
31	Potassium Permaganate
32	Hydroxylamine hydrochloride
33	Picric acid

INSTRUMENTS USED IN THIS STUDY

S.No.	Instruments
1	Weight machine
2	Incubator
3	Laminar Air flow
4	Hot air Oven
5	Autoclave
6	Pipetts
7	Stainless Steel Borer
8	Pipette Tips
9	Refrigerator
10	Gavage syringe
11	Forcep
12	Inoculating Loop
13	Straight wire
14	Measuring scale

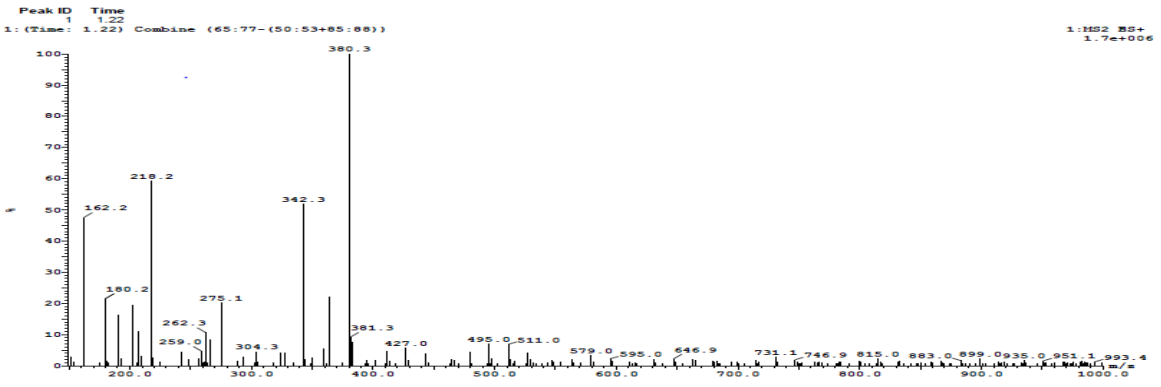
NAME OF MICROORGANISM AND THEIR ABBREVIATIONS

S. No.	Abbreviation	Full meaning
1	CoNS	Coagulase Negative *Staphylococcus*
2	GBS	Group B Streptococcus
3	*E. coli*	*Escherichia coli*
4	*S. aureus*	*Staphylococcus aureus*
5	*S. agalactiae*	*Streptococcus agalactiae*
6	*S. epidermidis*	*Staphylococcus epidermidis*
7	*K. pneumoniae*	*Klebsiella pneumoniae*
8	*P. aeruginosa*	*Pseudomonas aeruginosa*
9	*E. aerogenes*	*Enterobacter aerogenes*
10	*E. cloacae*	*Enterobacter cloacae*
11	*M. hominis*	*Mycoplasma hominis*
12	*H. influenzae*	*Haemophilus influenzae*
13	*S. pneumoniae*	*Streptococcus pneumoniae*
14	*L. monocytogenes*	*Listeria monocytogenes*
15	*E. faecalis*	*Enterococcus faecalis*
16	*E. faecium*	*Enterococcus faecium*
17	*C. albicans*	*Candida albicans*
18	*K. oxytoca*	*Klebsiella oxytoca*
19	*S. typhi*	*Salmonella typhi*
20	*B. megaterium*	*Bacillus megaterium*
21	*B. subtilis*	*Bacillus subtilis*

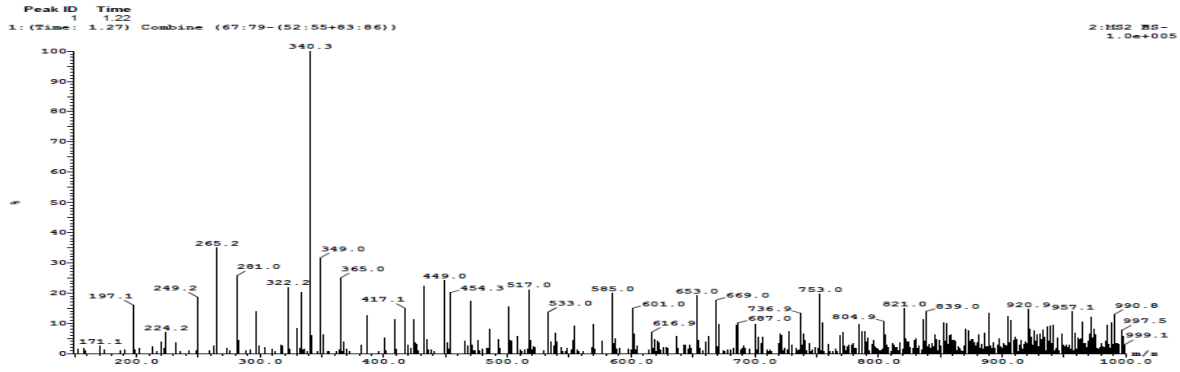
22	*S. saprophyticus*	*Staphylococcus saprophyticus*
23	*P. vulgaris*	*Proteus vulgaris*
24	*S. mutans*	*Streptococcus mutans*
25	*S. sanguis*	*Streptococcus sanguis*
26	*S. cricetus*	*Streptococcus cricetus*
27	*P. endodontalis*	*Porphyromonas endodontalis*
28	*S. dysenteriae*	*Shigella dysenteriae*
29	*P. mirabilis*	*Proteus mirabilis*
30	*A. flavus*	*Aspergillus flavus*
31	*B. thruingiensis*	*Bacillus thruingiensis*
32	*C. hofmani*	*Corynebacterium hofmani*
33	*C. xerosis*	*Corynebacterium xerosis*
34	*S. paratyphi A*	*Salmonella paratyphi A*
35	*S. paratyphi B*	*Salmonella paratyphi B*
36	*B.flexus*	*Bacillus flexus*
37	*V. parahaemolyticus*	*Vibrio parahaemolyticus*
38	*S. gordonii*	*Streptococcus gordonii*
39	*S. typhimurium*	*Salmonella typhimurium*
40	*Streptococcus spp.*	*Streptococcus species*
41	*Enterobacter spp.*	*Enterobacter species*
42	*Proteus spp.*	*Proteus species*
43	*Klebsiella spp.*	*Klebsiella species*

APPENDIX III

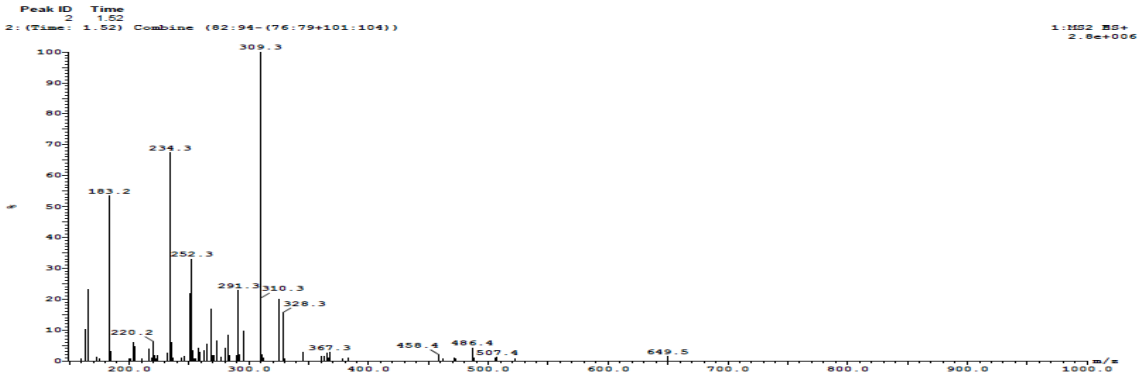
PROFILING OF MASS SPECTRA OF DIFFERENT COMPOUNDS AT DIFFERENT RETENTION TIME



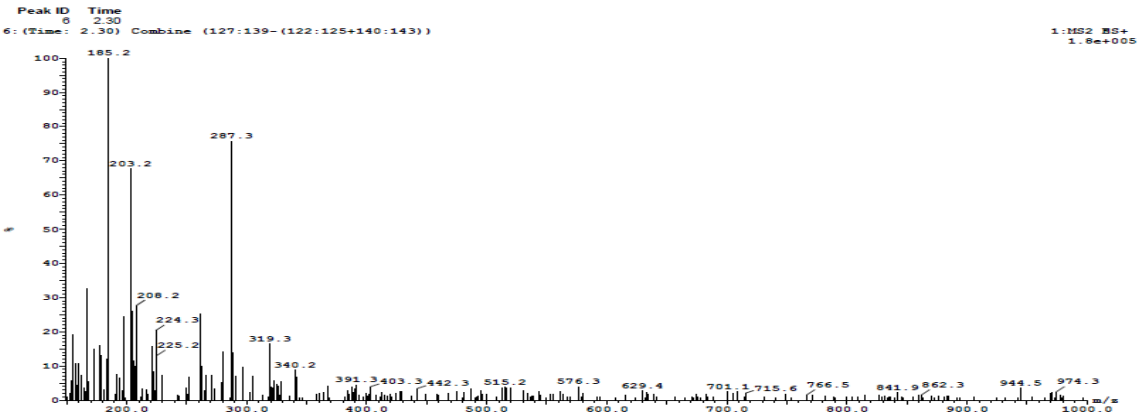
Mass Spectrum at Retention time 1.22 (ESI+)



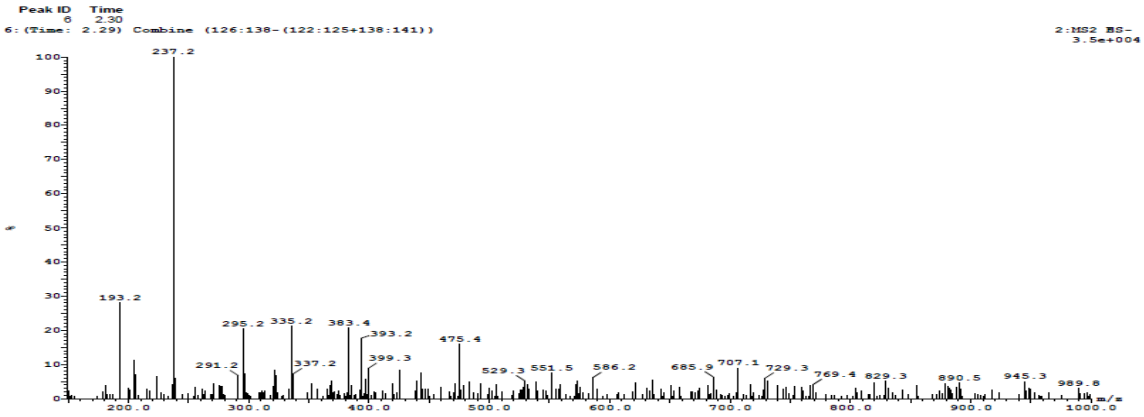
Mass Spectrum at Retention time 1.22 (ESI-)



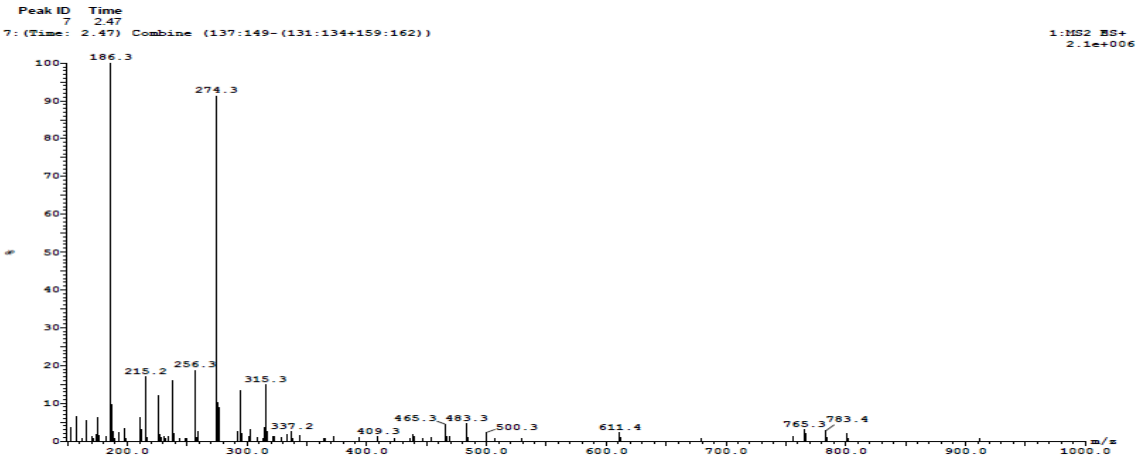
Mass Spectrum at Retention time 1.52 (ESI+)



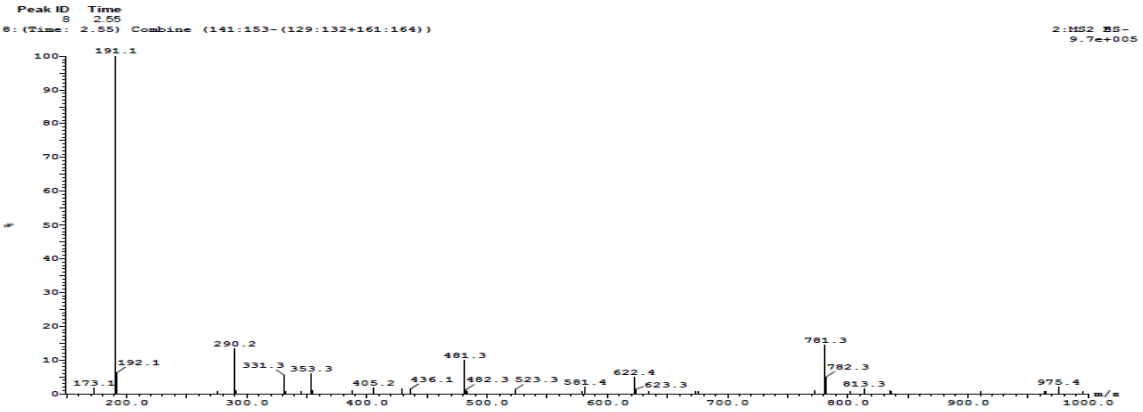
Mass Spectrum at Retention time 2.30 (ESI+)



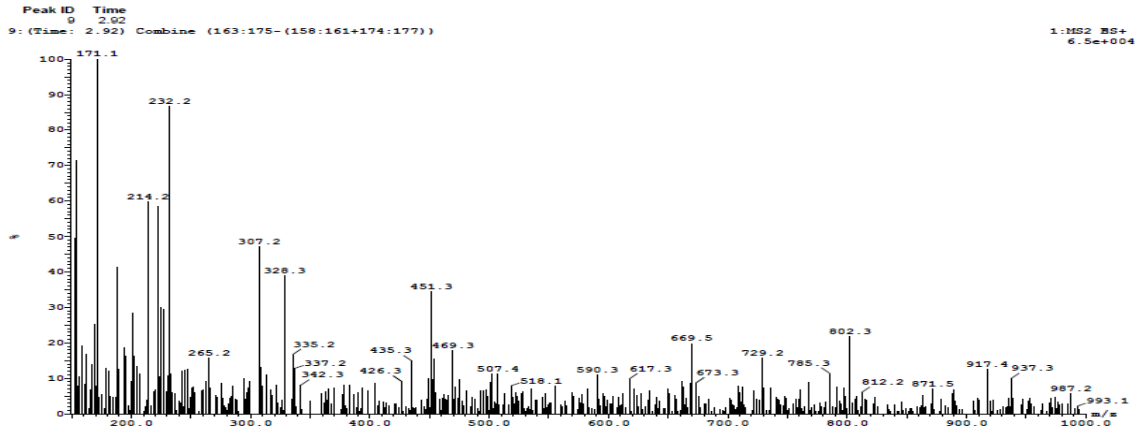
Mass Spectrum at Retention time 2.30 (ESI-)



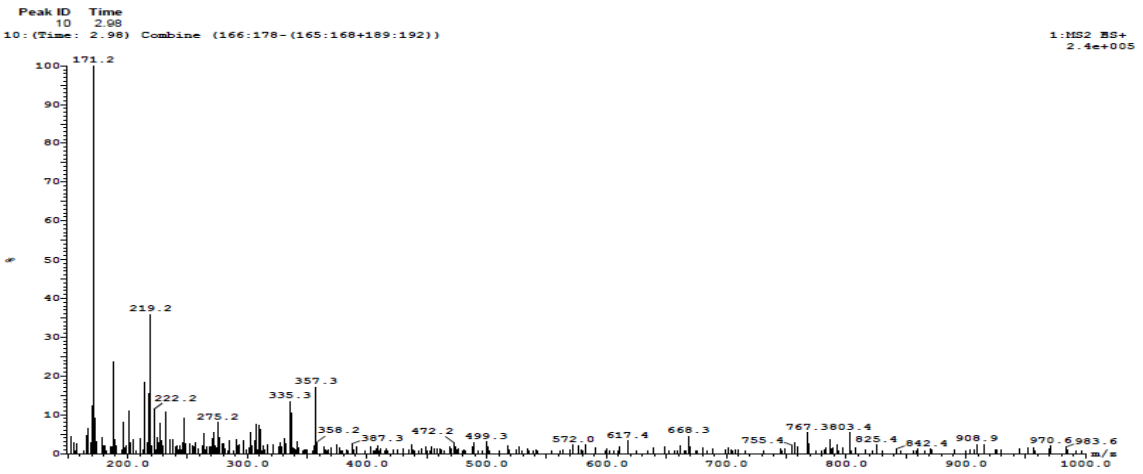
Mass Spectrum at Retention time 2.47 (ESI+)



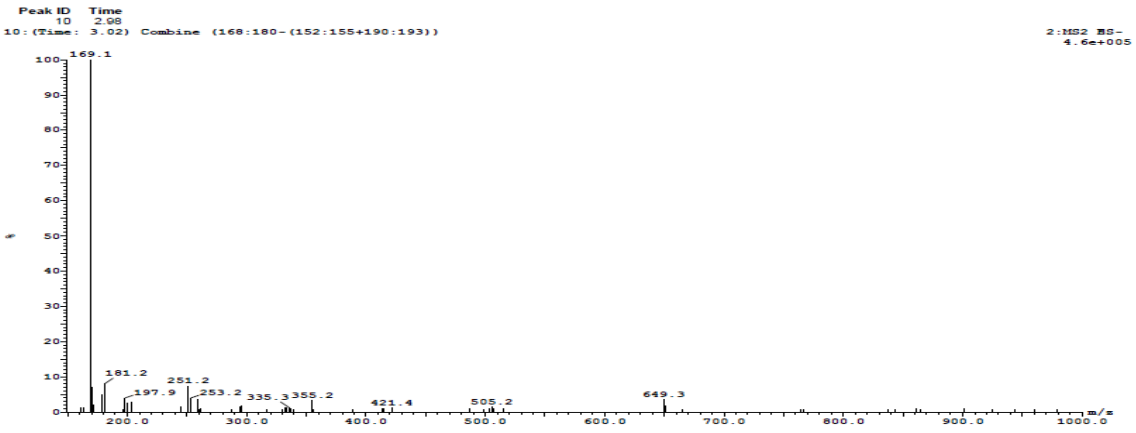
Mass Spectrum at Retention time 2.55 (ESI-)



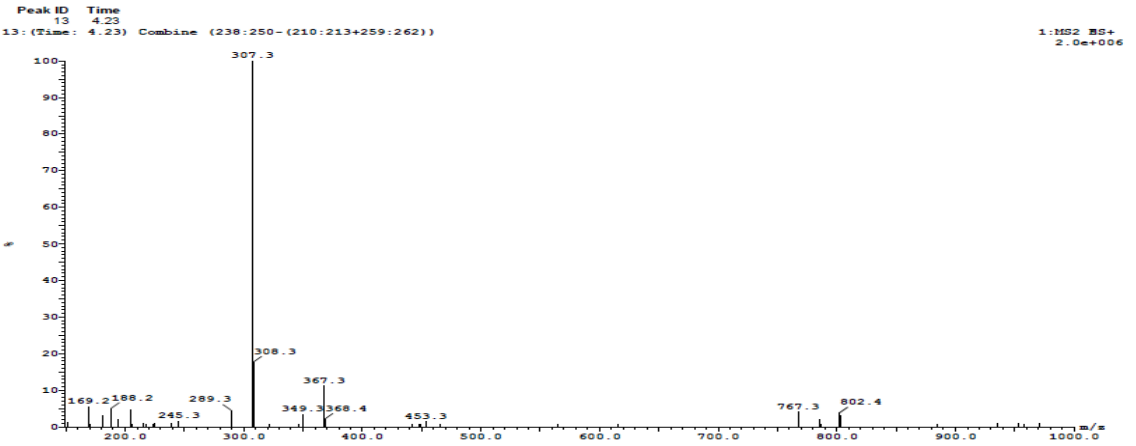
Mass Spectrum at Retention time 2.92 (ESI+)



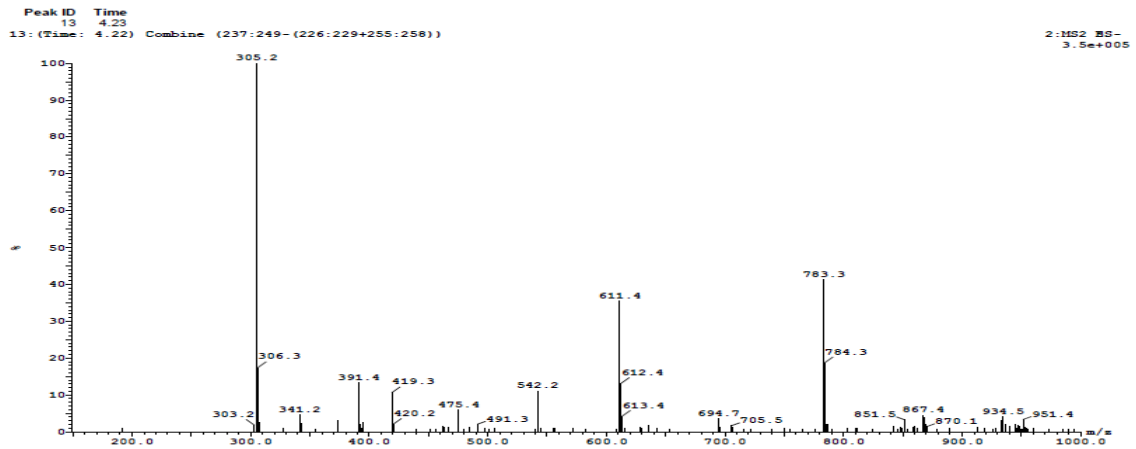
Mass Spectrum at Retention time 2.98 (ESI+)



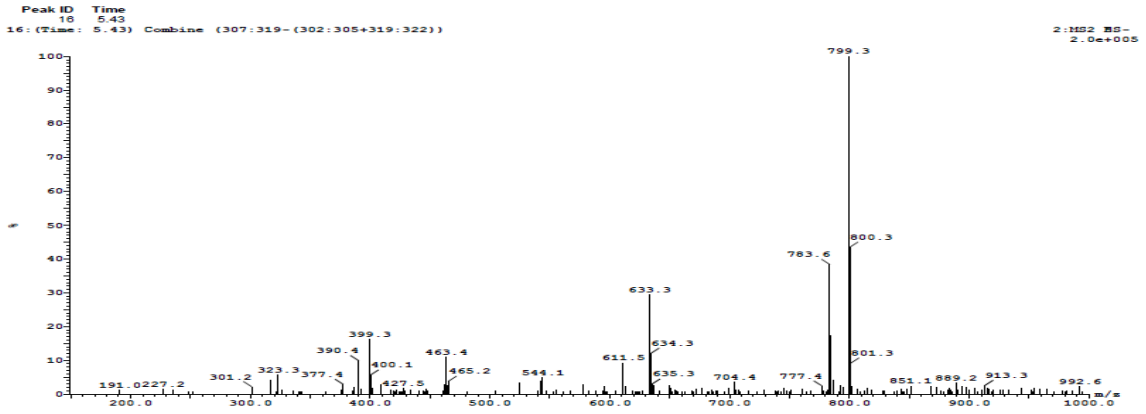
Mass Spectrum at Retention time 2.98 (ESI-)



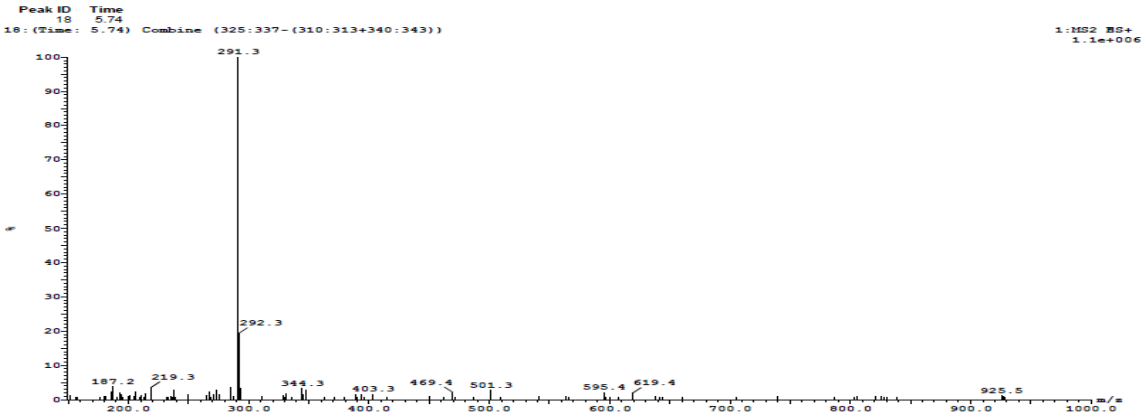
Mass Spectrum at Retention time 4.23 (ESI+)



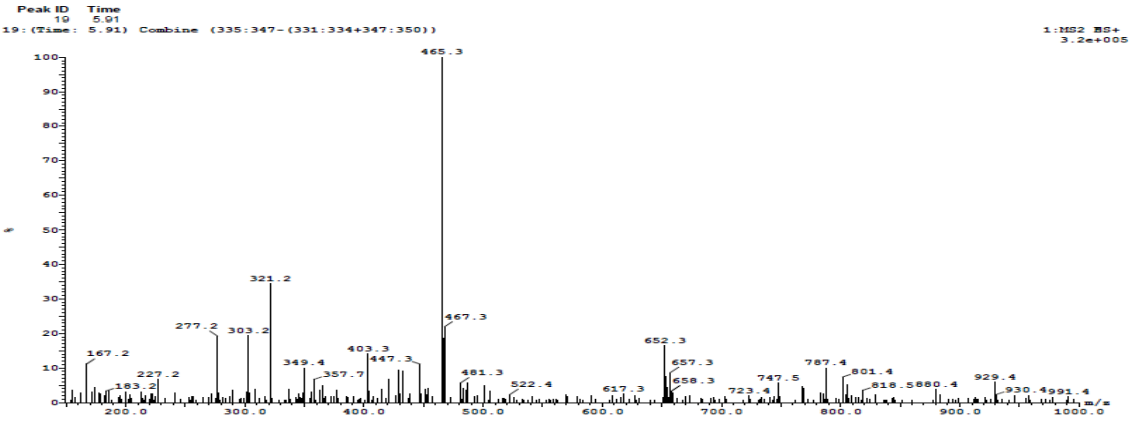
Mass Spectrum at Retention time 4.23 (ESI-)



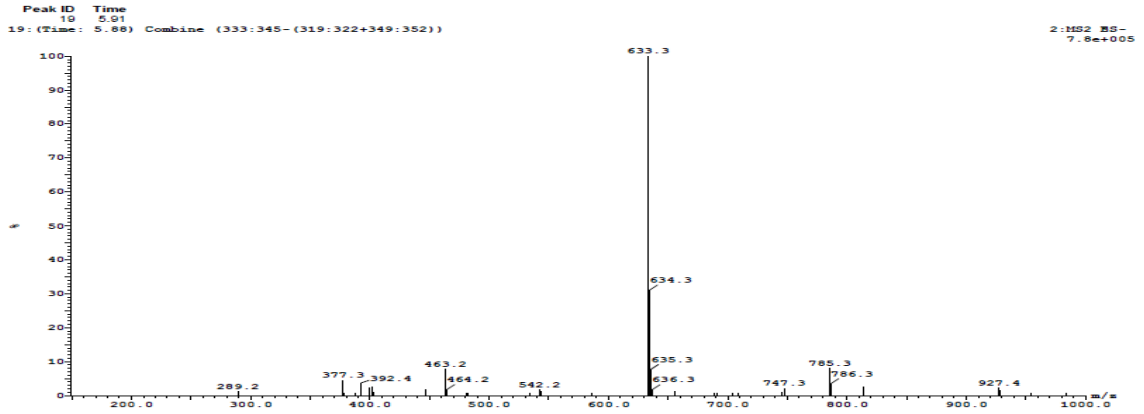
Mass Spectrum at Retention time 5.43 (ESI-)



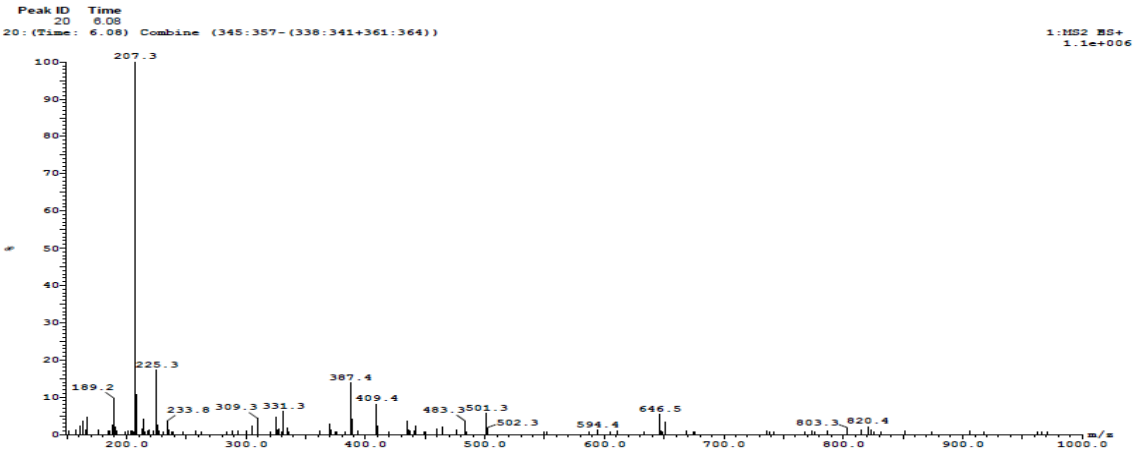
Mass Spectrum at Retention time 5.74 (ESI+)



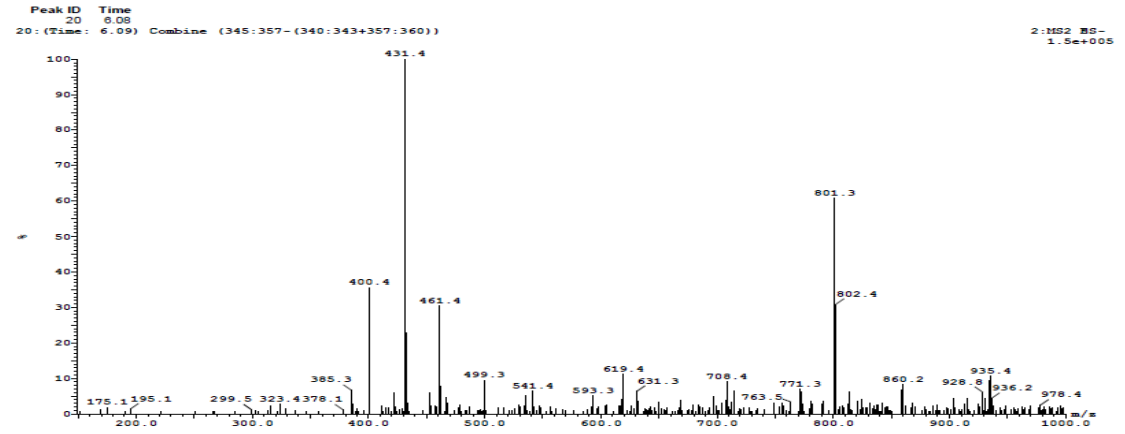
Mass Spectrum at Retention time 5.91 (ESI+)



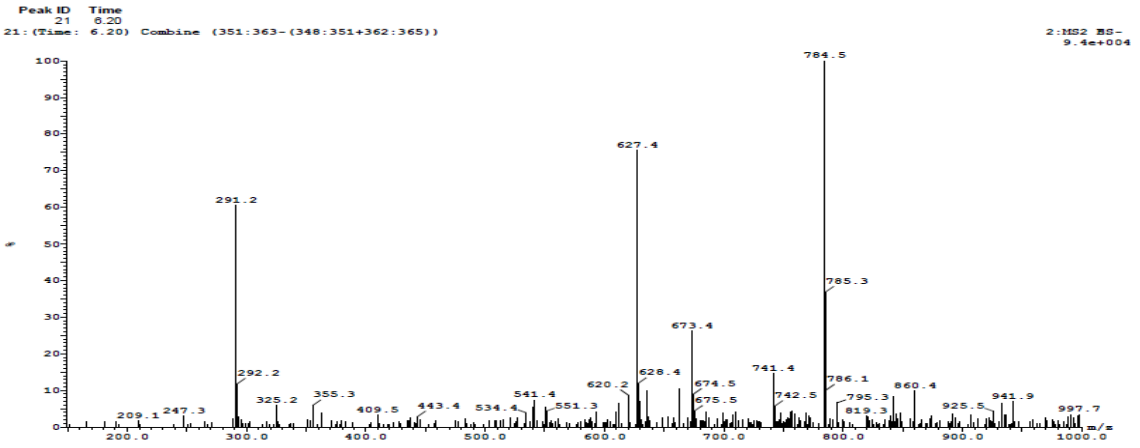
Mass Spectrum at Retention time 5.91 (ESI-)



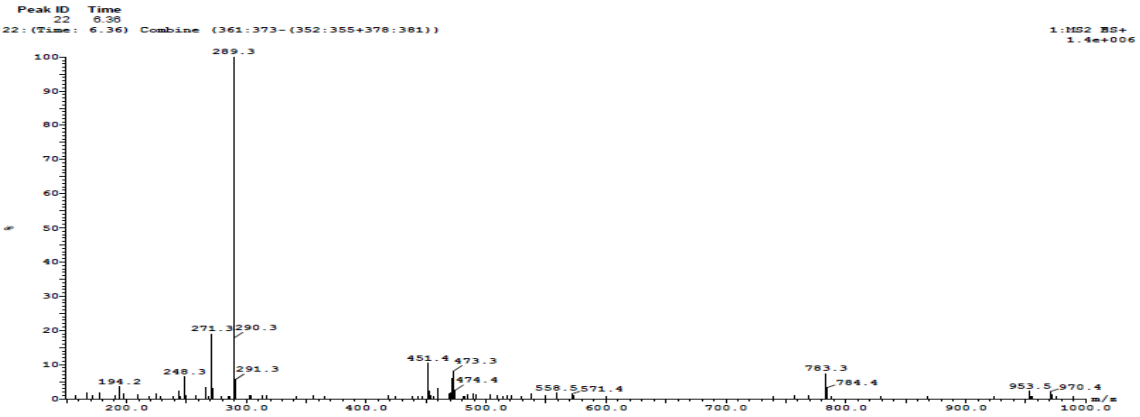
Mass Spectrum at Retention time 6.08 (ESI+)



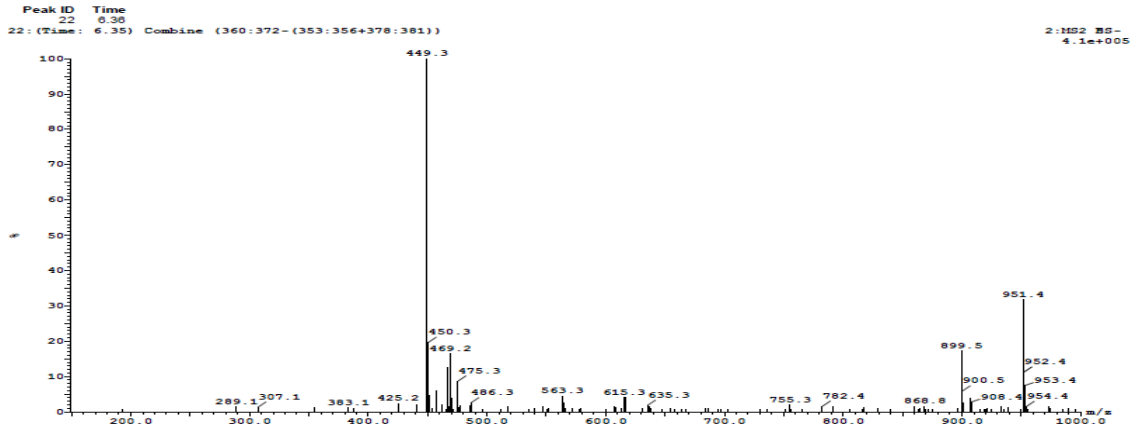
Mass Spectrum at Retention time 6.08 (ESI-)



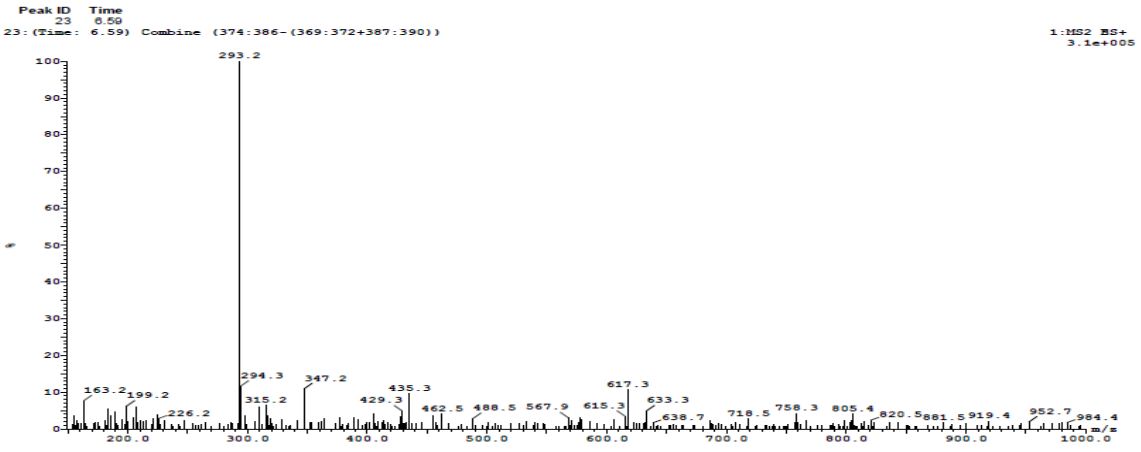
Mass Spectrum at Retention time 6.20 (ESI-)



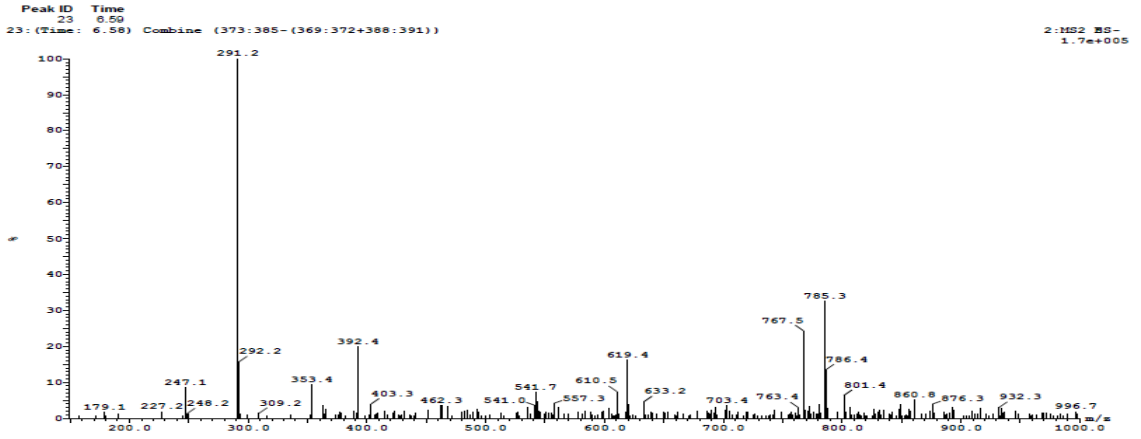
Mass Spectrum at Retention time 6.36 (ESI+)



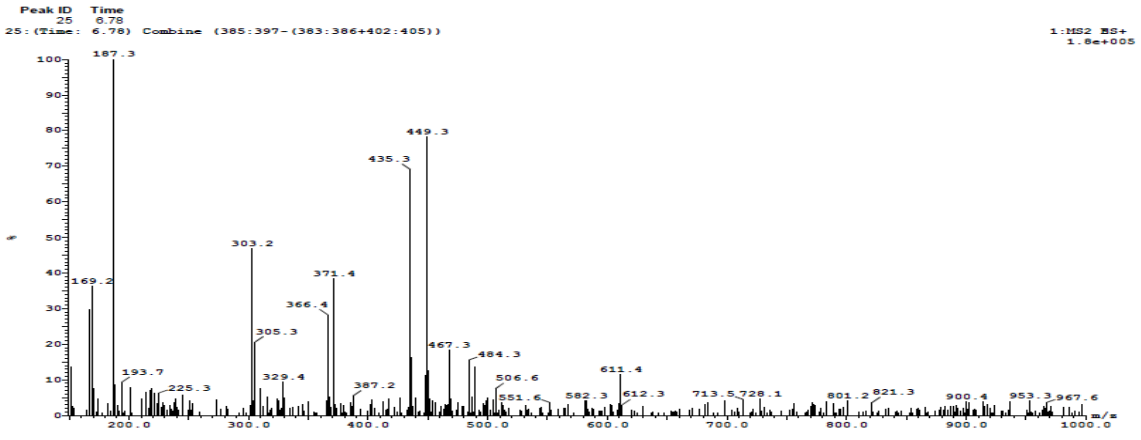
Mass Spectrum at Retention time 6.36 (ESI-)



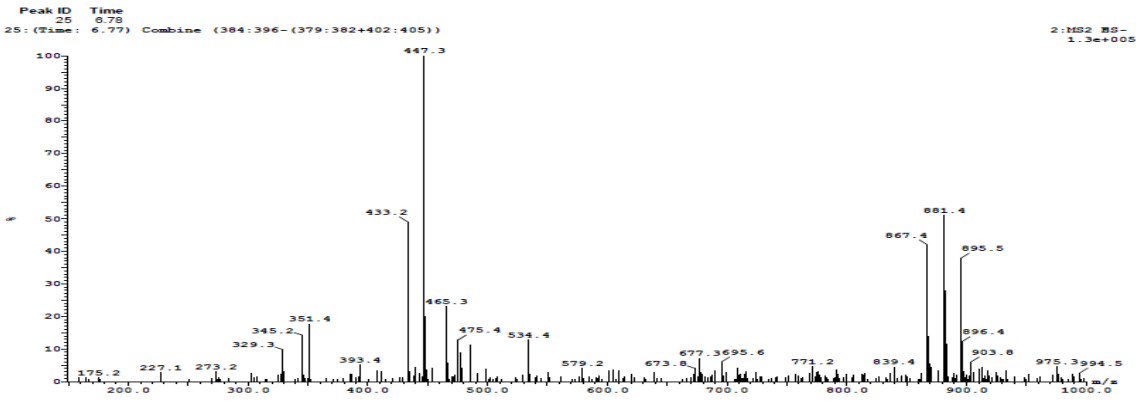
Mass Spectrum at Retention time 6.59 (ESI+)



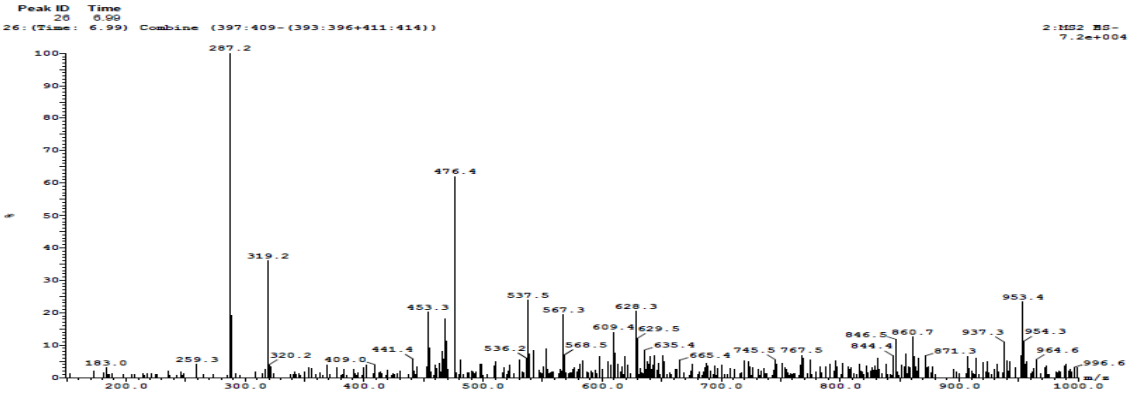
Mass Spectrum at Retention time 6.59 (ESI-)



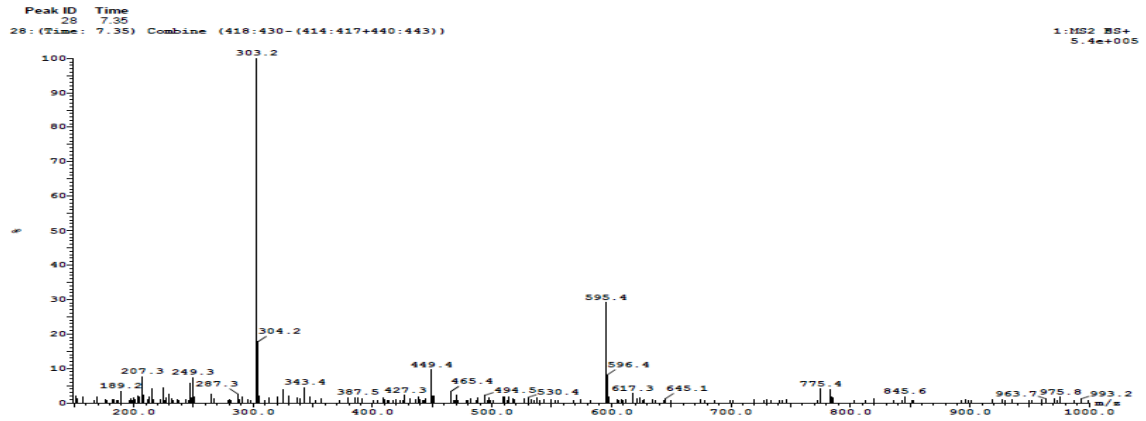
Mass Spectrum at Retention time 6.78 (ESI+)



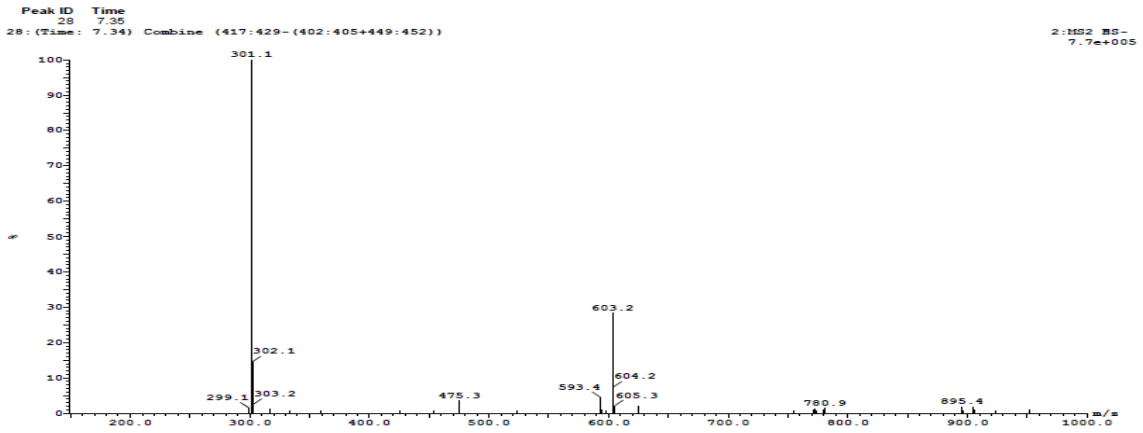
Mass Spectrum at Retention time 6.78 (ESI-)



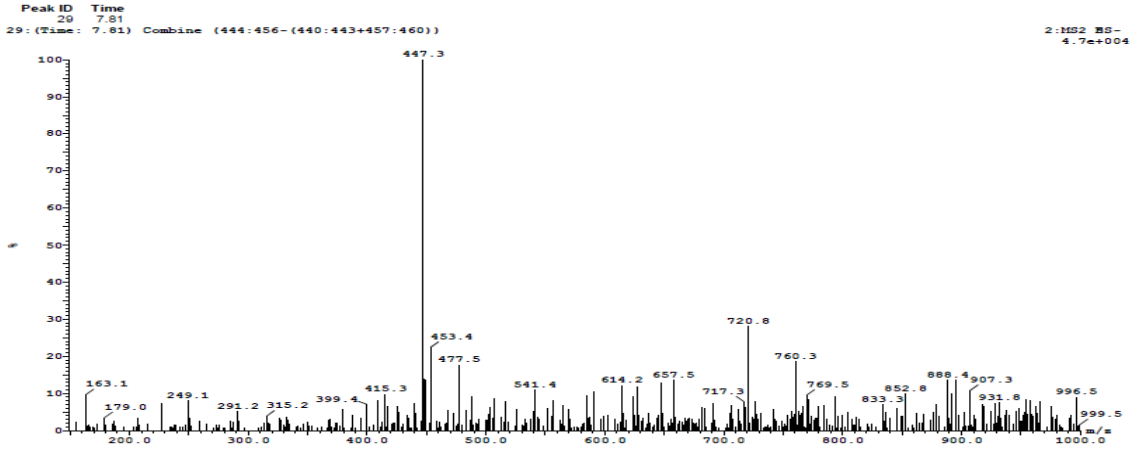
Mass Spectrum at Retention time 6.99 (ESI-)



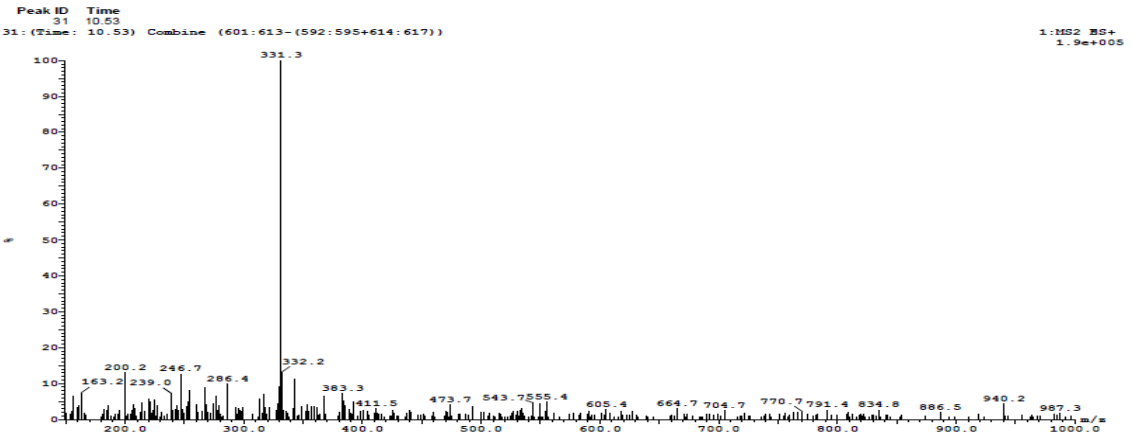
Mass Spectrum at Retention time 7.35 (ESI+)



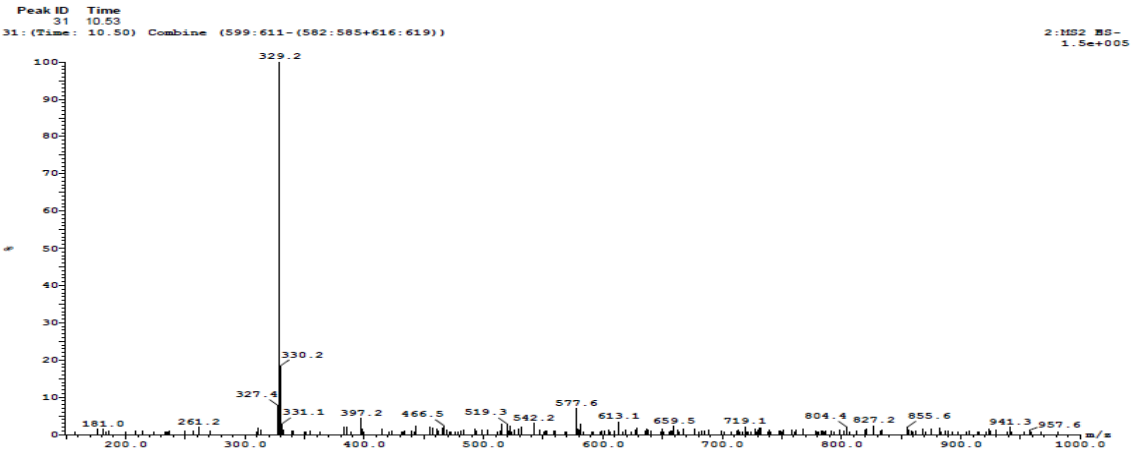
Mass Spectrum at Retention time 7.35 (ESI-)



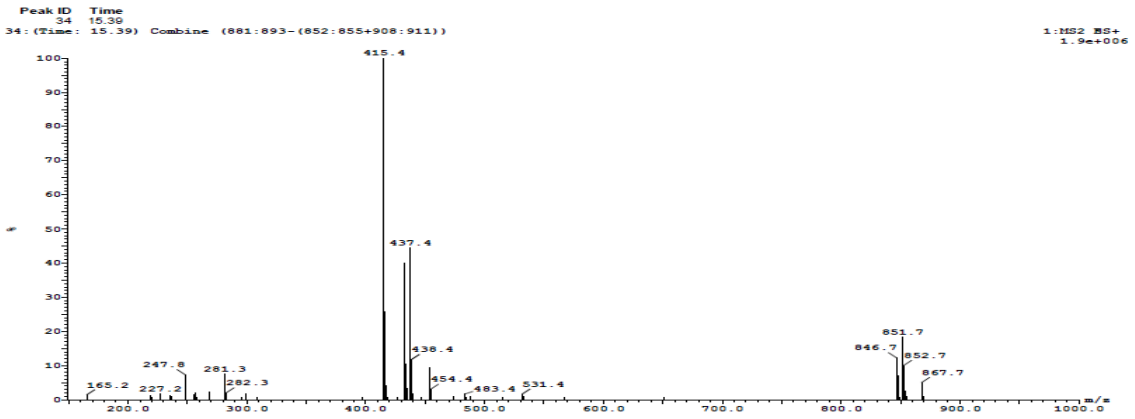
Mass Spectrum at Retention time 7.81 (ESI-)



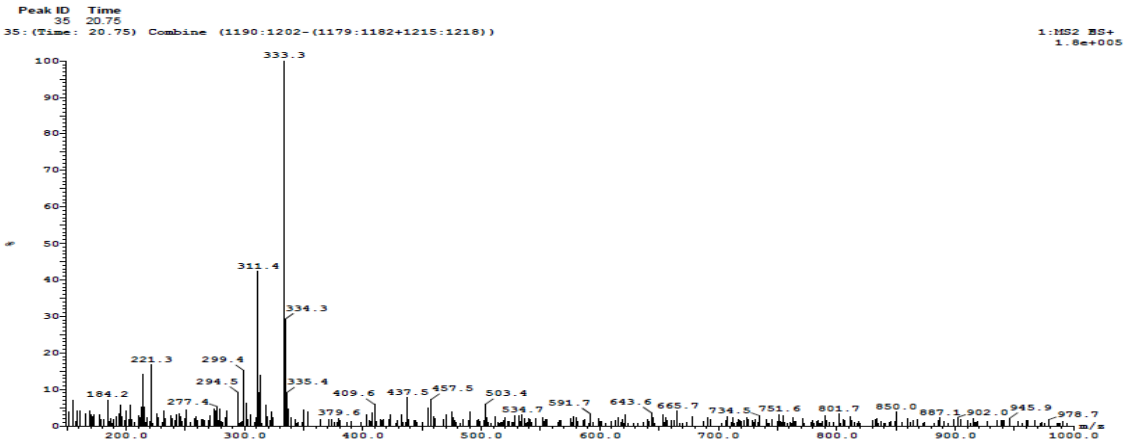
Mass Spectrum at Retention time 10.53 (ESI+)



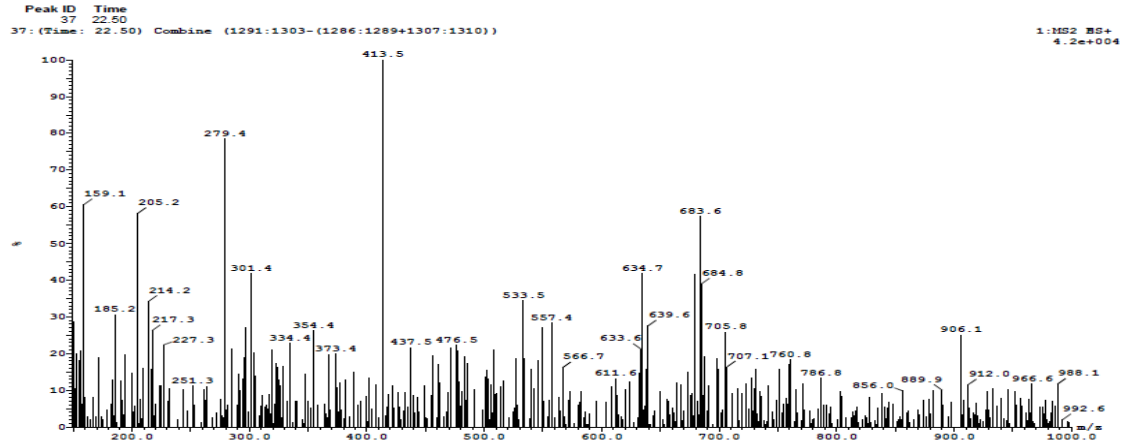
Mass Spectrum at Retention time 10.53 (ESI-)



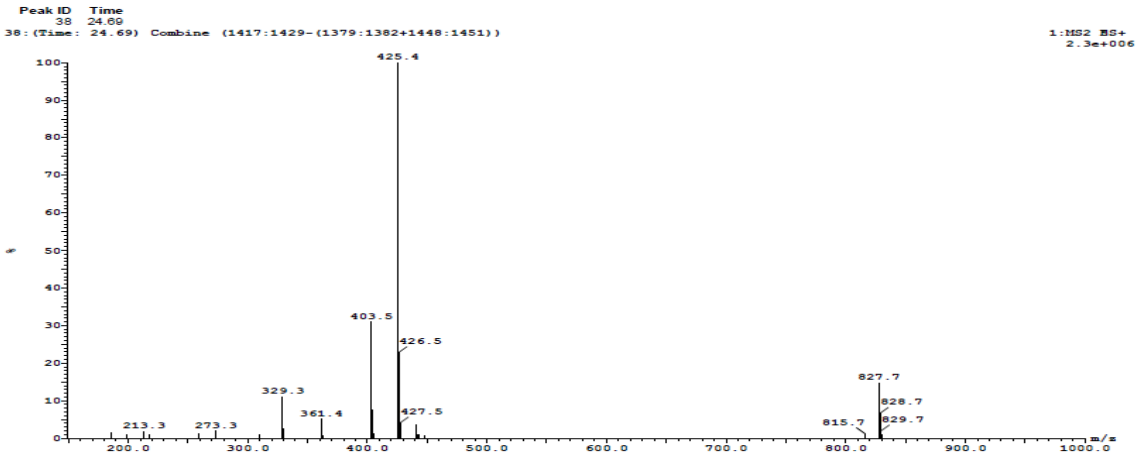
Mass Spectrum at Retention time 15.39 (ESI+)



Mass Spectrum at Retention time 20.75 (ESI+)



Mass Spectrum at Retention time 22.50 (ESI+)



Mass Spectrum at Retention time 24.69 (ESI+)

APPENDIX IV

NAME OF PREPARED MEDIA

A) Nutrient Agar

Ingredients	**Grams per litre(g/l)**
Peptic digest of animal tissue	5gm
Sodium Chloride (NaCl)	5gm
Beef Extract	1.5gm
Yeast Extract	1.5gm
Agar	15gm

Medium preparation: The medium was weighed 28.00 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the medium was sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. The medium was cooled to 40-45^0C and poured in sterilized petriplate.

B) Blood agar

Ingredients	**Grams per litre (g/l)**
Beef Heart Peptone	10 gm
Tryptose	10 gm
Sodium chloride	5 gm
Agar	15.00 gm

Medium preparation: The medium was prepared by weighing 40 grams of blood agar base powder and dissolving it in 1 litre of distilled water. The medium was boiled to completely dissolve it. Then medium was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121˚C. After that medium was cooled to 50˚C, 5-7% sterile defibrinated blood was aseptically added mixed homogenously and poured in sterilized petriplate.

C) MacConkey Agar

Ingredients	**Grams per litre (g/l)**
Peptones (Meat and Casein)	3 gm

Pancreatic digest of gelatin	17gm
Lactose monohydrate	10gm
Bile salts	1.5gm
Sodium Chloride (NaCl)	5gm
Crystal violet	0.001gm
Neutral red	0.030gm
Agar	13.5gm

Medium preparation: The medium was weighed 49.53 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the medium was sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. The medium was cooled to 40-45^0C and poured in sterilized petriplate.

D) Mueller Hinton Agar

Ingredients	**Grams per litre(g/l)**
Meat(Infusion solids)	2 gm
Casein acid hydrolysate	17.5 gm
Starch	1.5 gm
Agar	17.00 gm

Medium Preparation: The medium was weighed 38.00 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the medium was sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. The medium was cooled to 40-45^0C and poured in sterilized petriplate.

E) Mannitol Salt Agar (MSA)

Ingredients	**Grams per litre (g/l)**
Proteose peptone	10.00 gm
Meat extract B	1.00 gm
Sodium Chloride	75.00 gm
D-Mannitol	10.00 gm

Phenol red	0.025 gm
Agar	15.00 gm

Medium Preparation: The medium was weighed 111.02 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the medium was sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. The medium was cooled to 40-45^0C and poured in sterilized petriplate.

F) Nutrient Broth

Ingredients	**Grams per litre (g/l)**
Peptic digest of animal Tissue	5.00 gm
Sodium Chloride	5.00 gm
Beef extract	1.50 gm
Yeast Extract	1.50 gm

Medium Preparation: The medium was weighed 13.00 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the medium was poured in test tube or desired container and sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. The medium was cooled to 40-45^0C and use for the purpose.

G) Eosin Methylene Blue Agar

Ingredients	**Grams per litre (g/l)**
peptone	10.00 gm
Dipotassium hydrogen phosphate	2.00 gm
Lactose	10.00 gm
Eosin Y	0.400 gm
Methylne blue	0.065 gm
Agar	15.00 gm

Medium Preparation: The medium was weighed 37.46 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the

medium was sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. The medium was cooled to 40-45^0C and mix well then poured in sterilized petriplate.

H) Brain Heart Infusion (BHI) Broth

Ingredients	**Grams per litre (g/l)**
HM infusion powder	12.50 gm
BHI powder	5.00 gm
Proteose peptone	10.00 gm
Dextrose(Glucose)	2.00 gm
Sodium chloride	5.00 gm
Disodium hydrogen phosphate	2.50 gm

Medium Preparation: The medium was weighed 37.00 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the medium was poured in blood culture bottle and sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes.The medium was cooled to normal temperature and used for inoculation of blood sample.

I) Xylose- Lysine Deoxycholate (XLD) Agar

Ingredients	**Grams per litre (g/l)**
Yeast extract	3.00 gm
L-Lysine	5.00 gm
Lactose	7.50 gm
Sucrose	7.50 gm
Xylose	3.50 gm
Sodium chloride	5.00 gm
Sodium deoxycholate	2.50 gm
Sodium thiosulphate	6.80 gm
Ferric ammonium citrate	0.80 gm
Phenol red	0.08 gm

Agar	15.00 gm

Medium Preparation: The medium was weighed 56.68 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the medium was sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. The medium was cooled to 40-45^0C and mix well then poured in sterilized petriplate.

J) Sulfide Indole Motility (SIM) medium

Ingredients	**Grams per litre (g/l)**
Peptone	30 gram
HM Peptone	3 gram
Peptonized iron	0.2 gm
Sodium thiosulphate	0.025 gram
Agar	3 gram

Media Preparation: The medium was weighed 36.23 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the medium was dispensed in test tube of approx in amount of 2-3ml then mouth was closed with cotton and sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. The medium was cooled to 40-45^0C and used as desired.

K) Simmons Citrate agar

Ingredients	**Grams per litre (g/l)**
Magnesium sulphate	0.2 gram
Ammonium dihydrogen phosphate	1 gram
Dipotassium Phosphate	1 gram
Sodium Citrate	2 gram
Sodium Chloride	5 gram
Bromothymol blue	0.08 gram
Agar	15 gram

Media Preparation: The medium was weighed 36.23 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the medium was dispensed in test tube of approx in amount of 2-3 ml then mouth was closed with cotton and sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. The medium was tilted to form slant and left for cooling to 40-45^0C and used as desired.

L) Urease Agar base

Ingredients	**Grams per litre (g/l)**
Peptone	1 gram
Dextrose	1 gram
Sodium Chloride	5 gram
Disodium hydrogen phosphate	0.20 gram
Potassium dihydrogen phosphate	0.80 gram
Phenol red	0.012 gram
Agar	15 gram

Media Preparation: The medium was weighed 24.01 gram and dissolved in 950 ml of distilled water then it was heated to boil to dissolve the medium completely. The medium was sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. Then the medium was cooled to 40-45^0 C and added 5 ml of sterile 40% urea solution mix homogeneously and dispensed in test tube of approx in amount of 2-3 ml then mouth was closed with cotton and was tilted to form slant and left for cooling and used as desired.

M) Triple Sugar Iron Agar (TSI)

Ingredients	**Grams per litre (g/l)**
Peptone	10 gram
Casein enzyme hydrolysate	10 gram
Yeast Extract	3 gram
Meat Extract	3 gram
Lactose	10 gram

Sucrose	10 gram
Dextrose	1 gram
Sodium Chloride	5 gram
Ferrous Sulphate	0.20 gram
Sodium Thiosulphate	0.30 gram
Phenol Red	0.024 gram
Agar	12 gram

Media Preparation: The medium was weighed 64.52 gram and dissolved in 1000 ml of distilled water then it was heated to boil to dissolve the medium completely. Then the medium was dispensed in test tube of approx in amount of 3-4 ml then mouth was closed with cotton and sterilized by autoclaving at 15 lbs at 121^0C for 15 minutes. The medium was tilted to form slant and butt (3cm) and left for cooling to 40-45^0C and used as desired.

NAME OF THE PREPARED REAGENTS

A) 3% H_2O_2

The 3% H_2O_2 was prepared by dissolving 3 ml of hydrogen peroxide in 97 ml of distilled water.

B) Plasma with EDTA

Collect whole blood into commercially available anticoagulant-treated tubes e.g., EDTA-treated Cells are removed from plasma by centrifugation for 10 minutes at 1,000–2,000 x g using a refrigerated centrifuge. Centrifugation for 15 minutes at 2,000 x g depletes platelets in the plasma sample. The resulting supernatant is designated plasma. Following centrifugation, it is important to immediately transfer the liquid component (plasma) intoa clean polypropylene tube using a Pasteur pipette. The samples should be maintained at 2–8°C while handling.

C) VP Reagent:

40% KOH: 40 gram of potassium hydroxide was weighed and dissolved distilled water.

5% α-naphthol: 5 gram of α-naphthol was weighed and dissolved in absolute Ethanol

D) Mc Farland Standard:

1.175% anhydrous Barium chloride: 1.175 gm of barium Chloride was weighed and dissolved in 100 ml of distilled water.

1% sulphuric acid: 1 ml of sulphuric acid was diluted with 99 ml of distilled water carefully.

0.05 ml of 1.175% of anhydrous Barium chloride was added to 9.95 ml of 1% sulphuric acid in a test tube.

E) Wagner's reagents:

Iodine: 1.27 gram

Potassium Iodide: 2 gram

Distilled Water: 100 ml

E) 10% sodium Hydroxide:

10 gram of sodium hydroxide was weighed and dissovved in 100 ml of distilled water.

F) 1% Ferric chloride:

1 gram of ferric chloride crystals were weighed and dissolved in 100 ml of distilled water.

G) 1% Picric acid solution:

1 gram of picric acid was weighed and dissolved in distilled water (100 ml).

APPENDIX V

HEAVY METAL ANALYSIS REPORT

NEPAL RESEARCH FOUNDATION PVT. LTD.

Analytical Service Division

REG. OFFICE : [illegible], BIRGANJ, NEPAL

PHONE NO: OFFICE : 977-51-521097,528689,FAX : 00977-51-522084, e-mail: nrfnepal777@yahoo.com

TEST REPORT

Sample Name: **BRP LPU Syrup**	Reg. No.: 0734/077-78	AR No.: NRF-049/P7
	PR. No: Not available	
Received Date: 27.11.2020	Analysis start Date: 30.11.2020	Released Date: 06.12.2020
	Analysis completion date: 04.12.2020	
Mfg. By: Gorkha Ayurved Pvt. Ltd.	Batch No: Not available	Sample Qty.: About 30ml
Supplied by: Bijay Raj Pandit	Mfg. Dt: Not available	Exp. Dt: Not available
Analysis Requested : pH & Heavy metals only	Batch size: Not available	Page No.: 1 of 1

S.No.	PARAMETERS	METHOD	OBSERVATION	LIMIT
1.	**pH**	By pH meter	6.13	-
2.	**Heavy Metals**			
	a. Arsenic	NRF/AAM/As-01/01	<0.001 ppm	Not more than 3.00 ppm
	b. Cadmium	NRF/AAM/Cd-01/01	< 0.005 ppm	Not more than 0.3 ppm
	c. Lead	NRF/AAM/Pb-01/01	<0.1 ppm	Not mare than 10.00 ppm
	d. Mercury	NRF/AAM/Hg-01/01	<0.0005 ppm	Not more than 1.00 ppm

End of report

Results : 1. The sample submitted ~~does not comply~~/complies IP/BP/EP/JP/USP/IHS with the prescribed standards of quality with respect to the tests carried out.
2. In the opinion of the undersigned, the sample referred to above ~~is not of~~/of standard quality for the reasons given above.

DATE : 06.12.2020 ANALYSED BY CHECKED BY AUTHORIZED SIGNATORY

Authorized signatory: Rakesh K. Jha / Santosh K. Patel / ~~Jitendra Tiwari~~
Designation : DGM / Sr. Technical Officer / ~~Sr. Quality Officer~~

TERMS AND CONDITION :

1. Test results presented in this report relate only to the test(s) specified and sample(s) submitted.
2. The test report shall not be reproduced and used as evidence in the court of law and shouldn't be used for any advertisement without prior written permission.
3. Total liability of our Analytical Service Division is limited to the invoiced amount.
4. Samples are not drawn by NRF, ASD unless otherwise stated.
5. Retained samples will be destroyed after three (3) months form the date of issue of test certificates, unless otherwise stated. Perishable samples shall be destroyed immediately after the analysis.

Format No.: NRF/ASD/OP/T-11/F-002/02

APPENDIX VI

Ethical Approval for the Study

Regd No: [illegible]
PAN No: 300516076

NATIONAL MEDICAL COLLEGE

(NATIONAL MEDICAL COLLEGE PRIVATE LIMITED)
Affiliated to Tribhuvan University, Recognized by Nepal Medical Council
Approved by the Ministry of Education, Government of Nepal

Institutional Review Committee
(IRC)

F-NMC/125/2016-2017

Date: 30th Dec, 2016

Mr. Bijay Raj Pandit
Ph.D. Scholar
Department of Microbiology
Lovely Professional University

Ref: Ethical Approval of Research Proposal

Dear Mr. Bijay Raj Pandit,

Thank you for the submission of your research proposal entitled **"Studies on Neonatal Sepsis and Effect of Phytochemicals from selected medicinal plants."** to the Institutional Review Committee, National Medical College. The proposal was ethically reviewed by IRC. We are pleased to inform you that the above mentioned research proposal has been approved from ethical point of view by IRC of National Medical College on 30th December, 2016.

Approval is given for three years. Project which have not commenced within two years of original approval must be re-submitted to IRC. You must inform IRC when the research has been completed. If you are unable to complete your research within three years validation period, you will be required to write to IRC to request an extension or you will need to re-apply.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration must be reported to IRC, and an Ethical amendment Form submitted where appropriate. You are requested to follow the ethical principles for the health and biomedical research.

Thanking you,

Raman
.....
Dr. Tripti Pal Raman
Member secretary
IRC, NMC-TH
Birgunj, Nepal

Birganj-15, Nepal
+977-51-417015, 417320, Fax No.: +977-51-417009
nmcbir@ataenet.com.np, principal@nmcbir.educ.np, mededu@nmcbir.educ.np
www.nmcbir.edu.np

Regd No.: 11155/056/057
PAN No.: 300516076

NATIONAL MEDICAL COLLEGE

(NATIONAL MEDICAL COLLEGE PRIVATE LIMITED)
Affiliated to Tribhuvan University, Recognized by Nepal Medical Council
Approved by the Ministry of Education, Government of Nepal

F-NMC/509076/077 **Institutional Review Committee**
(IRC)

Date: 8th October, 2020

Mr. Bijay Raj Pandit
Ph.D. Scholar
Department of Microbiology
Lovely Professional University

Ref: Ethical Approval of Research Proposal

Dear Mr. Bijay Raj Pandit,

Thank you for the submission of your research proposal entitled **"Oral Acute Toxicity of Polyherbal Syrup in Mice"** to the Institutional Review Committee, National Medical College. The proposal was ethically reviewed by IRC. We are pleased to inform you that the above mentioned research proposal has been approved from ethical point of view by IRC of National Medical College on 6th October, 2020.

Approval is given for three years. Project which have not commenced within two years of original approval must be re-submitted to IRC. You must inform IRC when the research has been completed. If you are unable to complete your research within three years validation period, you will be required to write to IRC to request an extension or you will need to re-apply.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration must be reported to IRC, and an Ethical amendment Form submitted where appropriate. You are requested to follow the ethical principles for the health and biomedical research.

Thanking you,

Raman.
.....
Dr. Tripti Pal Raman
Member secretary
IRC, NMC-TH
Birgunj, Nepal

Birgunj-15, Nepal
+977-51-417015, 417320, Fax No.: +977-51-417009
nmc@nmcbir.edu.np
www.nmcbir.edu.np

APPENDIX VII

Methods of qualitative detection of secondary metabolites

Secondary Metabolites	Name of Test	Reactant	Result	References
1. Alkaloids	Dragendroff's test	Potassium bismuth iodide solution	Appearance of Red precipitate	Surendra *et al.*, (2016)
	Mayer's test	Potassium mercuric iodide	Yellow colour Precipitate observed	Bhandari *et al.*, (2017)
	Wagner's test	Iodine in Potassium iodide	Brown/reddish precipitate will appear	Surendra *et al.*, (2016)
	Hager's test	Saturated Picric acid solution	Yellow colour precipitate will seen	Surendra *et al.*, (2016)
2.Coumarins	Opened loop-closed loop response test	sodium hydroxide, hydrochloric acid	Cloudy solution	Cai *et al.*, (2011)
	Fluorescence response test	Ammonium Hydro-oxide(NH_4 OH)	Blue green fluorescence	Bhandari *et al.*, (2017)
	Sublimation test	alcoholic potassium hydroxide, HCl, chloroform, sodium sulphate	UV absorption in 250–350 nm region	Cai *et al.*, (2011)
	NaOH Test	Sodium hydro-oxide(NaOH)	Yellow colouration	Yadav *et al.*, (2014)
3. Quinones		Conc. H_2SO_4	red color	Bhandari *et al.*, (2017)
4. Phenol	Ferric chloride	Ferric chloride	intense green color	Surendra *et al.*,

	Test	solution	formation or bluish black colour appearance	(2016)
	Iodine test	Dilute iodine solution	Transient Red colour	Singh and Kumar (2017)
5. Flavonoids	Alkaline Reagent Test	Sodium hydroxide solution	intense yellow colour developed, changes to colourless after addition of dilute acid	Surendra *et al.*, (2016)
	Lead acetate Test	Lead acetate solution	Formation of yellow colour precipitate	Yadav *et al.*, (2014)
	Aluminum chloride test	aluminum chloride,	Appearance of yellow-green fluorescence under UV	Cai *et al.*, (2011)
	Ammonia test	ammonia	yellow-green fluorescence under UV light seen	Cai *et al.*, (2011)
	Shinoda's test	Ethanol, magnesium Chips,conc. HCl	crimson Red, pink, orange-red to purple coloration	Surendra *et al.*, (2016)
6. Terpenoids	Liebermann-Burchardt test	acetic anhydride, chloroform, conc. H_2SO_4	Green, Pink or red colouration appearance	Cai *et al.*, (2011)
	Salkowski test	Chloroform, conc. H_2SO_4	Red, purple or Reddish brown precipitate formation	Bhandari*et al.*, (2017)

			at the interface	
7. Tannins	Gelatin Test	1% gelatin solution, NaCl	Formation of white precipitate	Pandey and Tripathi (2014)
	Braymer's test	Ferric Chloride	Bluishblack or green color appear	Bhandari *et al.*, (2017)
	Ferric chloride - potassium ferricyanide test	ferric chloride-potassium ferricyanide	Blue color	Cai *et al.*, (2011)
	Vanillin-hydrochloric acid test	vanillin-hydrochloric acid	Red color seen	Cai *et al.*, (2011)
	Mitchell's test	Iron, ammonium citrate plus sodium tartrate	Development of precipitate in ammoniumacetate solution	Rahman *et al.*, (2013)
	Gold Beater's skin test	hydrochloric acid, distilled water, ferrous sulfate	Skin changed to brown or black	Periyanayagam and Mubeen (2018)
8. Steroids	Salkowski Test	Chloroform, of conc. H_2SO_4	Upper layer of tube turned red and acidic layer becomes yellow with green fluorescence	Bhandari *et al.*, (2017)
	Liebermann–Burchard test for steroids	acetic acid, conc. H_2SO_4.	violet to blue or bluish-green colour	Al—Daihan *et al.*, (2013)
9. Saponins	Froth Test	water	1 cm layer of foam	Bhandari *et al.*, (2017)

	Foam Test	water	foam produced persists for ten minutes	Yadav *et al.*, (2014)
	Haemolysis test	normal saline	Blood hemolysis	Deepak *et al.*, (2014)
10. Cardiac glycosides	Keller-Kiliani's test	Glacial acetic acid ferric chloride.Con. H_2SO_4	Greenish blue color	Surendra *et al.*, (2016)
	Kedee's test	3,5-dinitrobenzoic acid, NaOH	Appearance of Purple to violet color	Jagessar (2017)
	Baljet's test	sodium picrate	Yellow to orange color seen	Rahman *et al.*, (2013)
	Legal's test	sodium nitropruside and NaOH	pink color turns to blood red color	Surendra *et al.*, (2016)
	Raymond's test	1,3 dinitrobenzene, potassium hydroxide	Blue color	Periyanayagam and Mubeen (2018)
Glycosides	Modified Borntrager's test	Ferric chloride, benzene, ammonia solution	Development of rose-pink colour in the ammonical layer	Surendra *et al.*, (2016)
11. Diterpenes	Copper acetate test	Copper acetate solution.	Emerald green color seen	Pandey and Tripathi (2014)
12. Anthraquinone	Borntrager's test	Benzene, NH_3	Pink, Violet or Red coloration in ammonical layer	Yadav *et al.*, (2014)
13. Anthocyanins		HCl, NH_3	Pinkish red to bluish violet coloration	Yadav *et al.*, (2014)

APPENDIX VIII

PRESENTATION AND PUBLICATION

Presentation

1. Poster presentation at **106th Indian Science Congress** titled "Bacteriological Profile and Antibiotic Sensitivity Pattern of Neonatal Sepsis in a Tertiary Care Hospital in Southern Nepal"held between 3 to 7th January 2019 at Lovely Professional University, Phagwara, Jalandhar, India.

2. Oral Presentation in title "Neonatal Septicemia in Southern Part of Nepal: Early and Late Onset"at National conference on Trends in Biochemical and Biomedical Sciences held on 2nd -3rd March 2019 organized by Department of Biochemistry, Aligarh Muslim University, Aligarh, India.

Publication:

1. Pandit BR, Vyas A. 2020. Efficacy of Anti-bacterial action on Seven Medicinal Plants extract against Neonatal Sepsis causing bacteria – an *In Vitro* Study. Plant archives 20 (Supplement 2): 2487-2494. Indexed in Scopus.

2. Pandit BR, Vyas A. 2020. Clinical Symptoms, Pathogen Spectrum, Risk factors and Antibiogram of Suspected Neonatal sepsis cases in tertiary Care Hospital of southern Part of Nepal: A descriptive Cross- sectional Study. Journal of Nepal Medical Association 58(232):976-982. Doi:10.31729/jnma.5094. Indexed in Scopus, Web of science and Pubmed.

3. Pandit BR. Vyas A. 2021. Tentative identification of Bioactive compounds from aqueous extract of *Punica granatum* peel by HPLC/LC-MS method. Proceedings of National Academy of Sciences, India Section B: Biological Sciences. 91(4):947-958. https://doi.org/10.1007/s40011-021-01294-3. Indexed in Scopus

APPENDIX IX

CONSENT FORM

Name of research study: **"STUDIES ON NEONATAL SEPSIS AND EFFECT OF PHYTOCHEMICALS FROM SELECTED MEDICINAL PLANTS ON NEONATAL SEPSIS CAUSING BACTERIA"**

Name of Institutions:

1. Lovely Professional University, Phagwara, Punjab, India
2. National Medical College and Teaching Hospital (NMCTH), Birgunj, Nepal

My son/daughter was requested to participate in the research study. I have been provided information about the study and also got opportunity to ask questions regarding research. I found the answers satisfactory. So, I hereby give the consent to participate in this study by providing blood specimen of my children for culture and sensitivity.

I understand that my children can quit the participation at any time.

Name of participant: …….

Address: …….

Name of Guardian: ……..…

Signature: ……...

Date: …….....

Right	Left

Thumb Stamp

Note: For illiterate, participant's guardian must give their stamp of right hand thumb in the box provided after understanding the contents written above.

I have clearly explained about the study to the participant's guardian, I hereby verify that guardian of participant was not forced for blood sample of their neonates. Guardians understand that for diagnosis of sepsis blood sample is required and he/she gave this consent feeling freely.

Investigator

Name: ……

Signature: ……..

Doctor/ Nurse

Name: …….

Signature: ……

APPENDIX X

QUESTIONNAIRE

CLINICAL AND MICROBIOLOGICAL PROFILE OF PATIENT

Clinical Profile Patient ID:

Date: Baby of: Age/sex: Gestational age: <37W / ≥37W

Symptoms:

Mode of Del.: SVD / CS Place of del.: Home/HospitalBirth weight: <2500 / ≥2500

Microbiological profile Lab Ref. No.:

Culture Report: Growth/No growth Growth on: 2^{nd} /4^{th} /6^{th}

Colony Characteristics:

Gram stain: GPC / GNB Biochemical test:

Bacteria isolated:

AST:

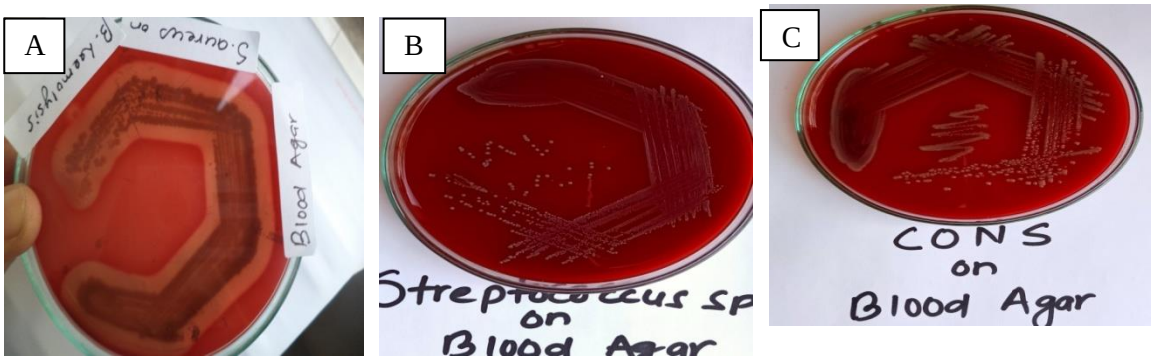
APPENDIX XI

IMAGES OF WORK DONE DURING THE PERIOD OF RESEARCH

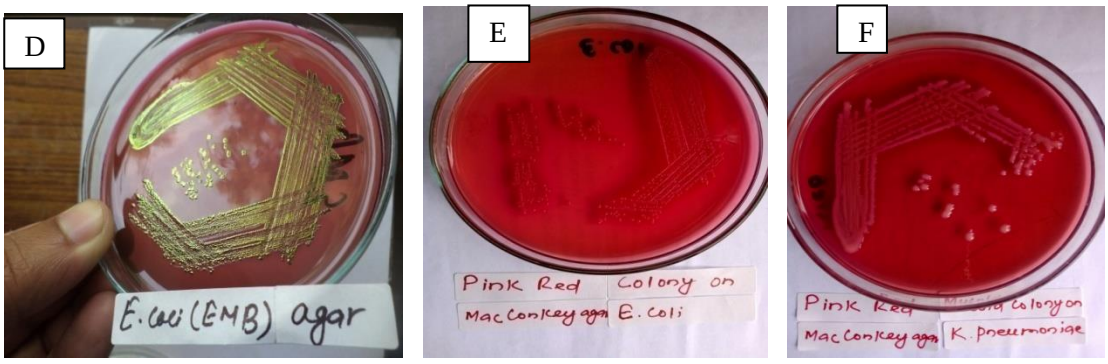
Medicinal Plants used in this Study

Blood Culture bottle (Pediatric) containing Brain Heart Infusion (BHI) Broth with blood sample

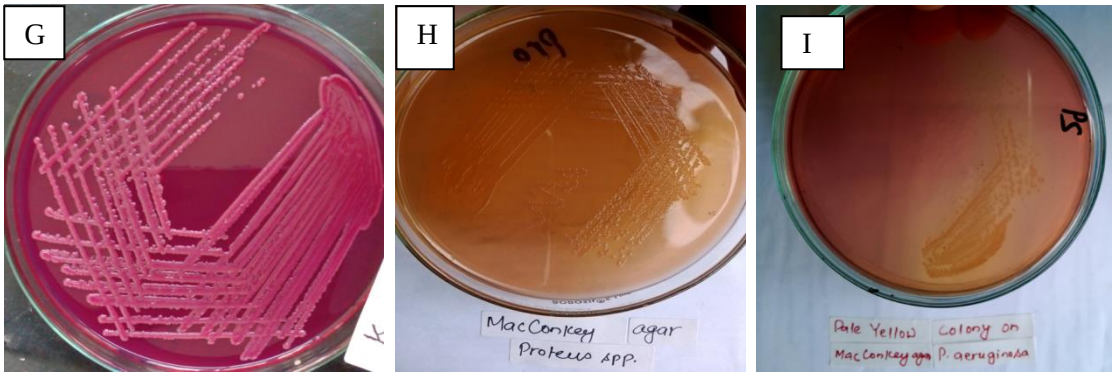
BACTERIAL ISOLATIONS



A: β-haemolysis in Blood agar of *S. aureus*; B: Creamy white small colony of *Streptococcus* spp. in Blood agar; C: Glistening white colony of CoNS on Blood agar

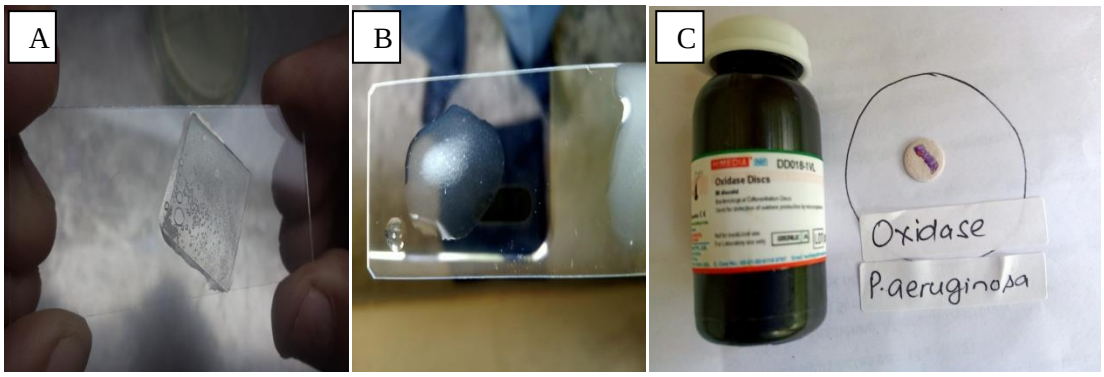


D: Greenish Metallic colony of *E. coli* on EMB agar; E: Pink red colony of *E. coli* on MacConkey agar; F: Pink red mucoid colony of *K. pneumoniae* on MacConkey agar

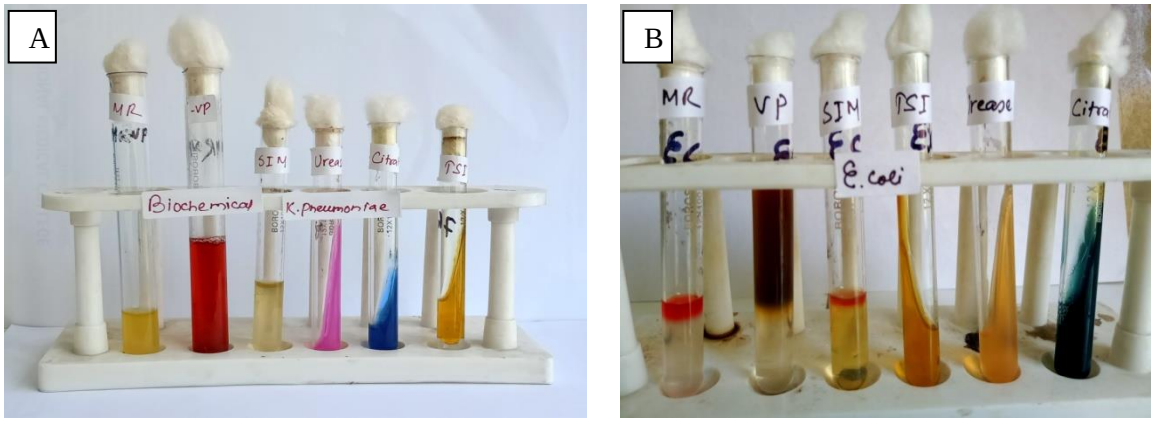


G: Pinkish white mucoid colony of *Enterobacter* spp. in MacConkey agar; H: Faint Yellow colony of *Proteus* spp in MacConkey agar; I: Faint yellowish flat colony of *P. aeruginosa* in MacConkey agar

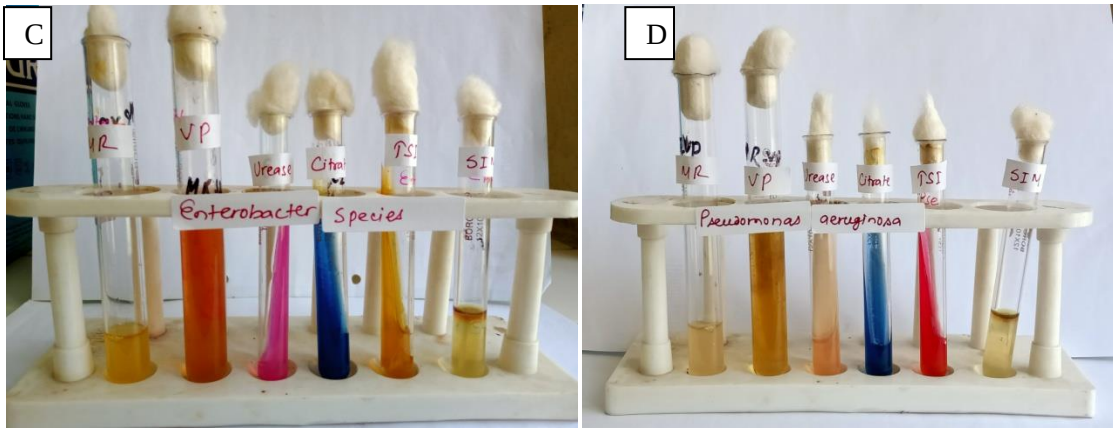
BACTERIAL IDENTIFICATION



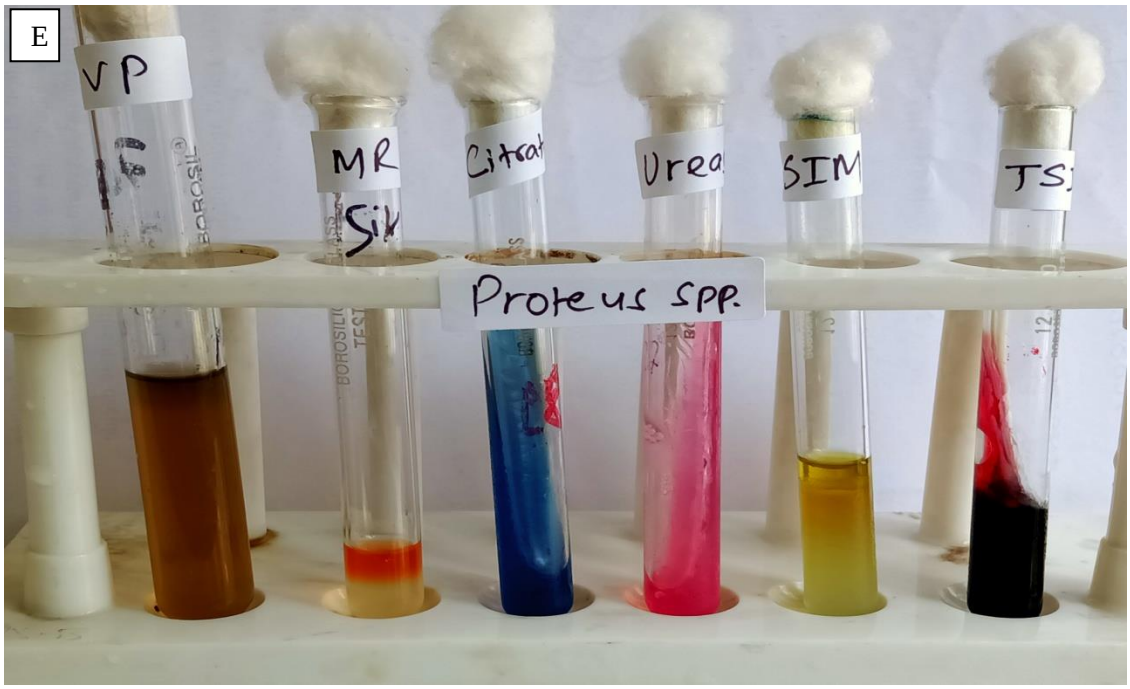
A: Catalase test (Positive); B: Coagulase test (Positive); C: Oxidase test (Positive)



A: Biochemical test result for *K. pneumoniae*; B:Biochemical test result for *E.coli*

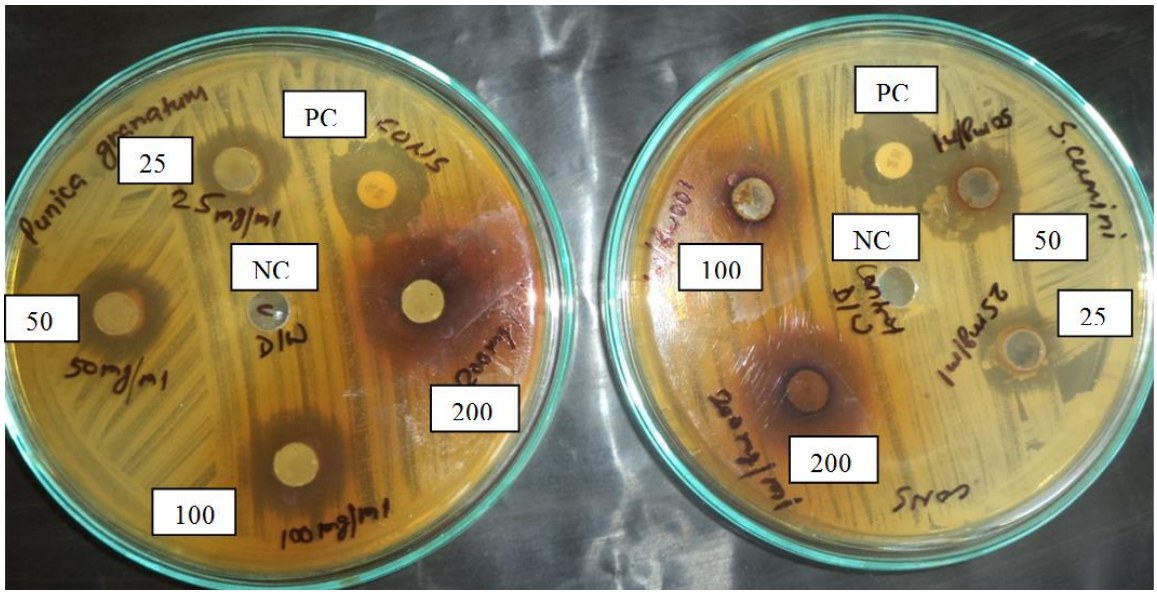


C: Biochemical test result for *Enterobacter* spp.; D: Biochemical test result for *P. aeruginosa*

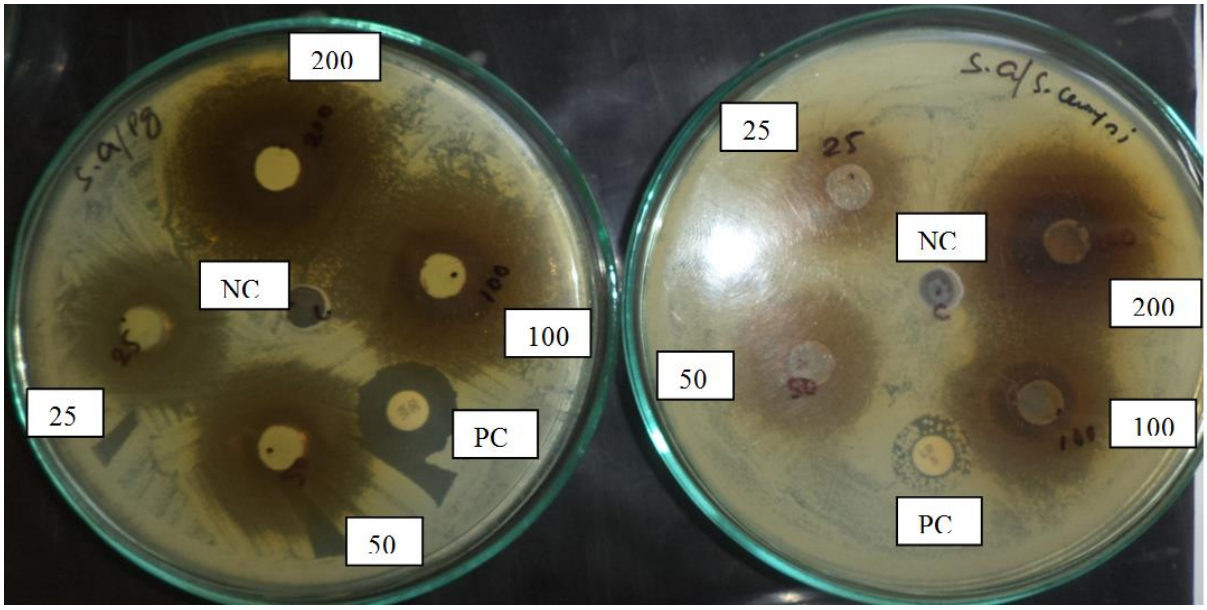


E: Biochemical test result for *Proteus* spp.

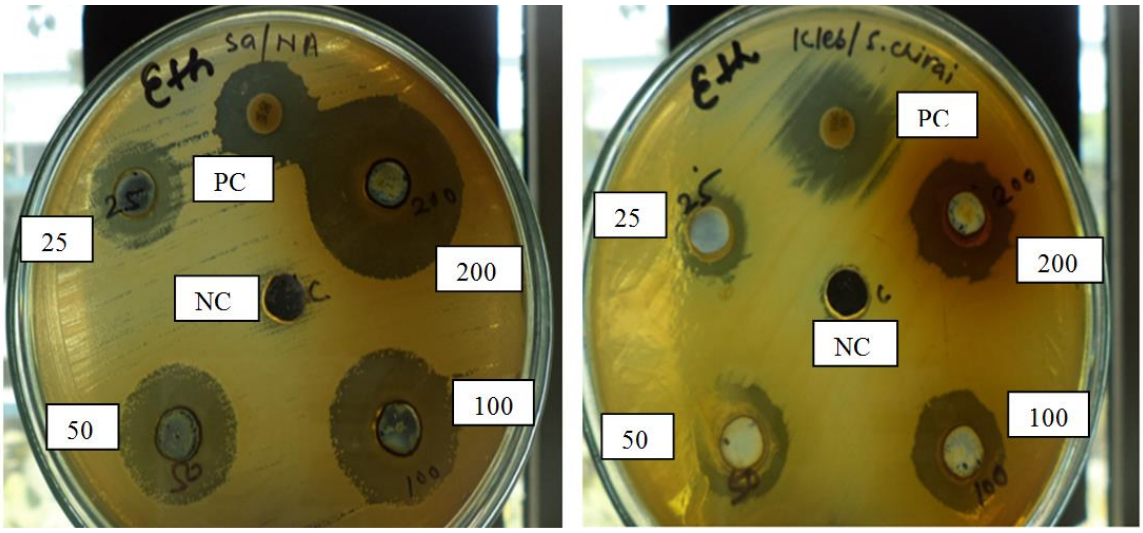
ANTIBACTERIAL EVALUATION OF STUDIED MEDICINAL PLANTS BY AGAR WELL DIFFUSION METHOD



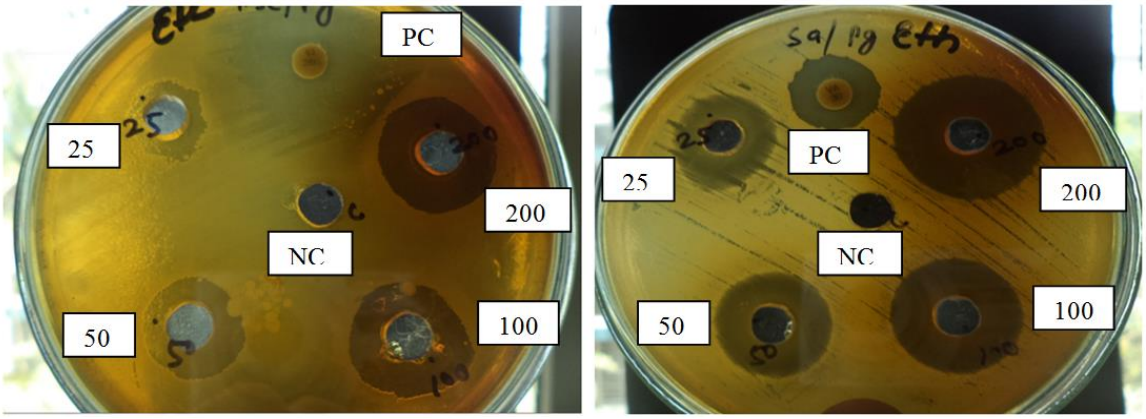
Antibacterial effect of aqueous extract of *P. granatum* and *S. cumini* against CoNS by Agar well diffusion method (NC: Negative Control; PC: Positive Control)



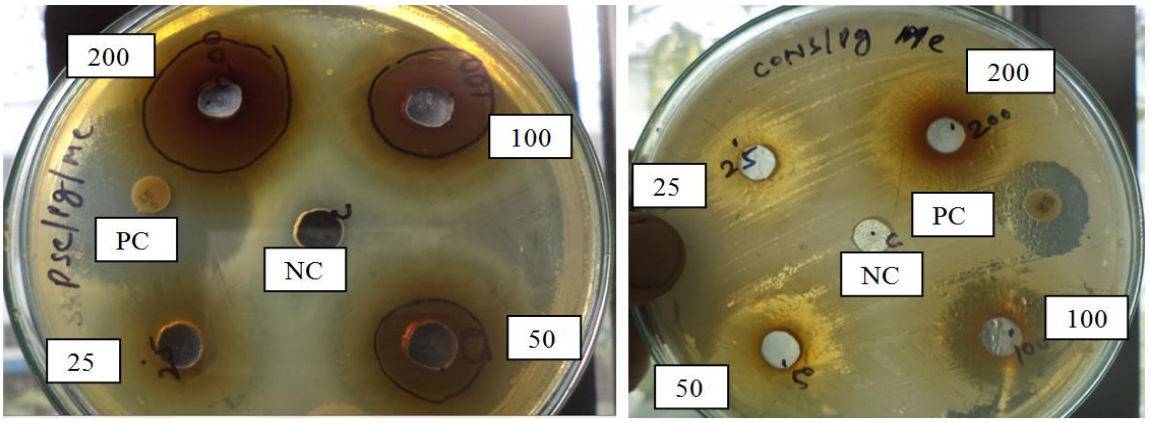
Antibacterial effect of aqueous extract of *P. granatum* and *S. cumini* against *S. aureus* by Agar well diffusion method (NC: Negative Control; PC: Positive Control)



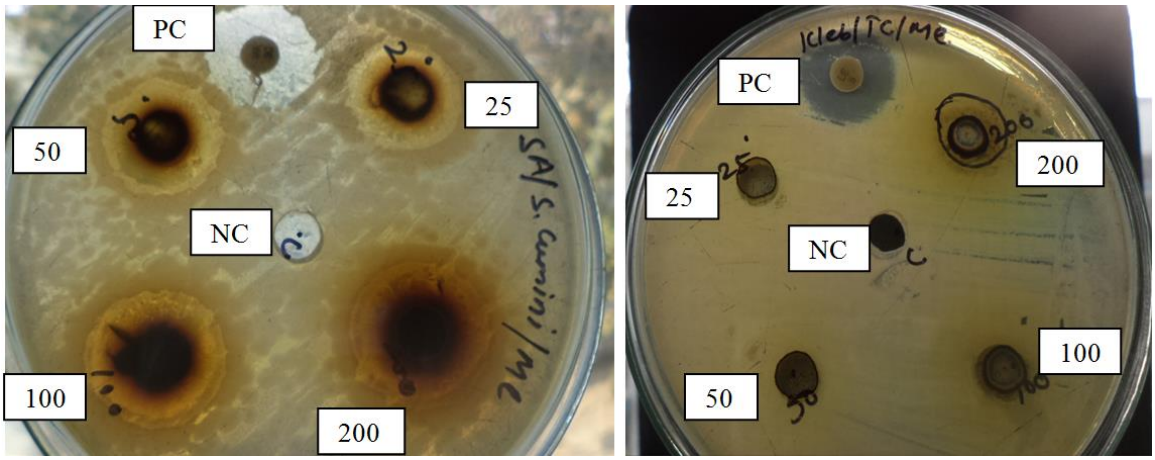
Antibacterial effects of 80% ethanol extract of *N. arbortristis* and *S. chirata* against *S. aureus* and *K. pneumoniae* by Agar well diffusion method (NC: Negative Control; PC: Positive Control)



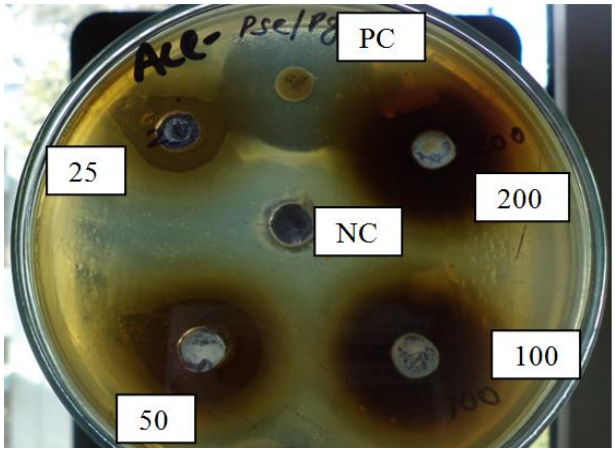
Antibacterial effects of 80% ethanol extract of *P. granatum* against *P. aeruginosa* and *S. aureus* by Agar well diffusion method (NC: Negative Control; PC: Positive Control)

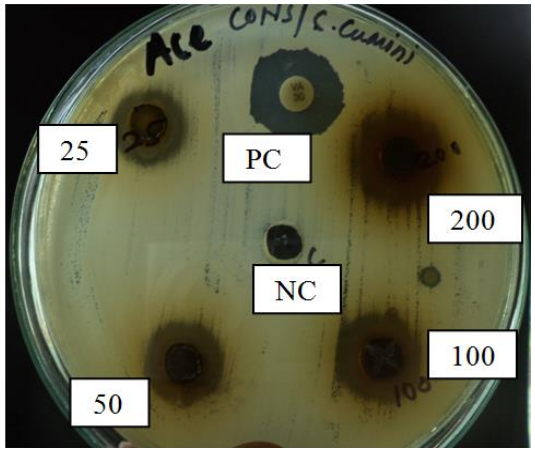


Antibacterial effects of Methanol extract of *P. granatum* against *P. aeruginosa* and CoNS by Agar well diffusion method (NC: Negative Control; PC: Positive Control)

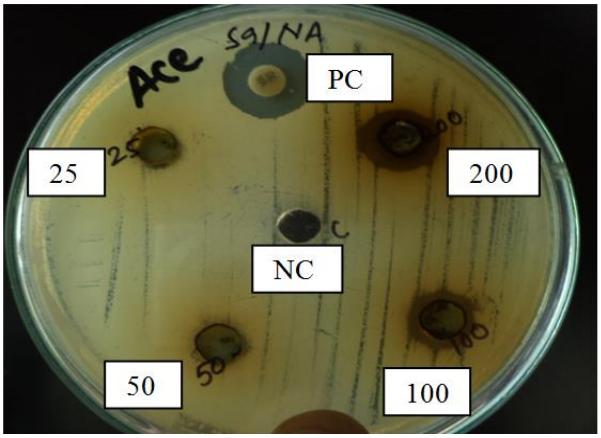


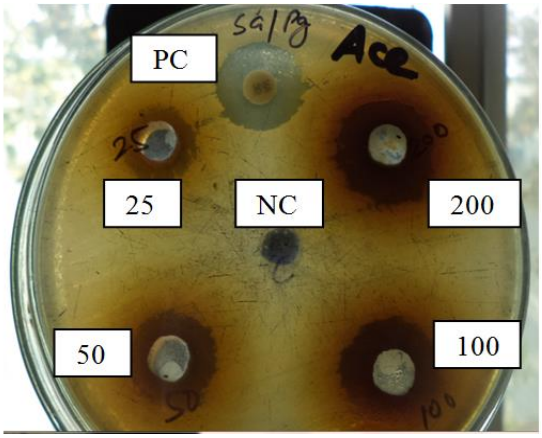
Antibacterial effects of Methanol extract of *S. cumini* and *T. cordifolia* against *S. aureus* and *K. pneumoniae* by Agar well diffusion method (NC: Negative Control; PC: Positive Control)



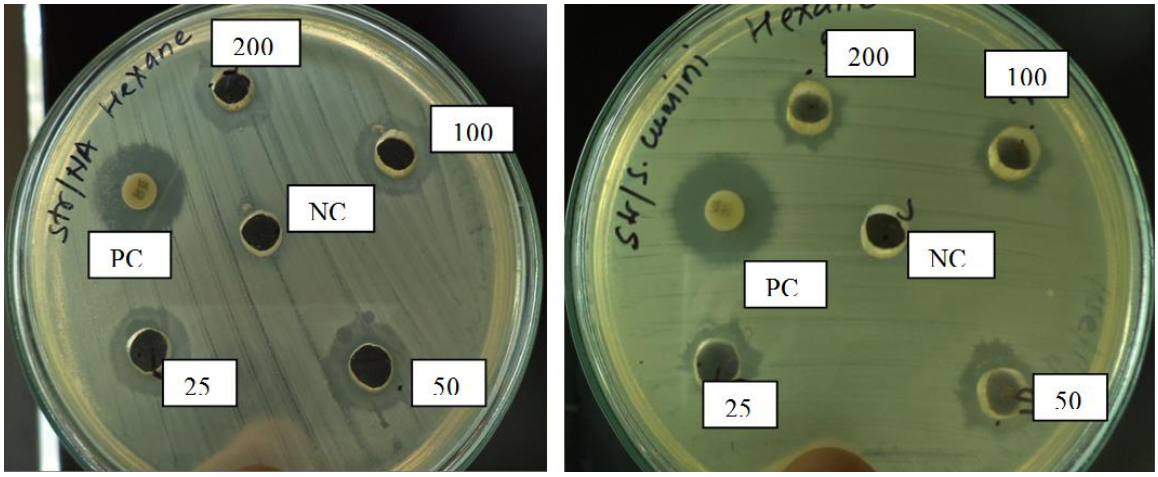


Antibacterial effects of Acetone extract of *P. granatum* and *S. cumini* against *P. aeruginosa* and CoNS by Agar well diffusion method (NC: Negative Control; PC: Positive Control)

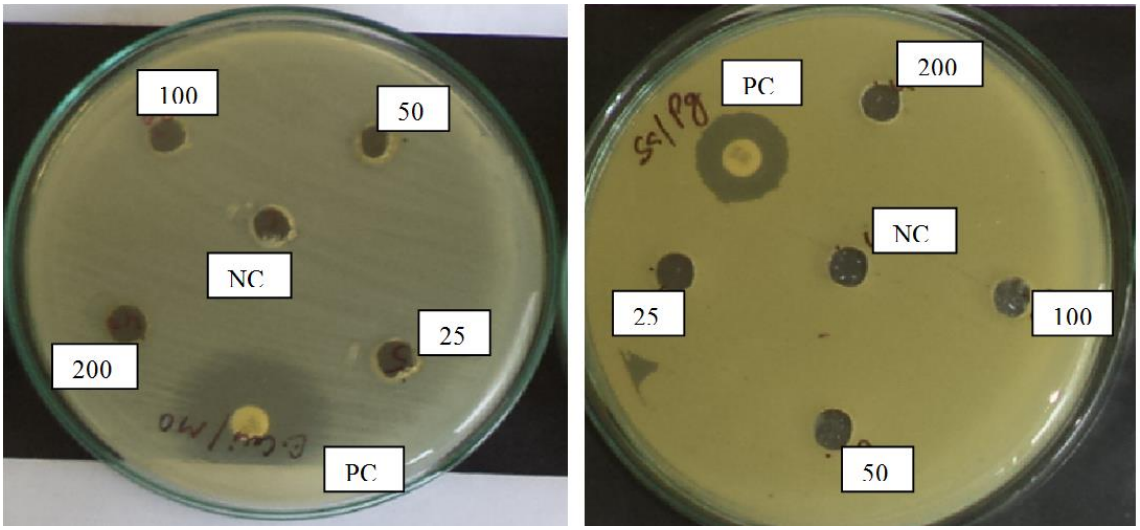




Antibacterial effects of Acetone extract of *N. arbortristis* and *P. granatum* against *S. aureus* by Agar well diffusion method (NC: Negative Control; PC: Positive Control)

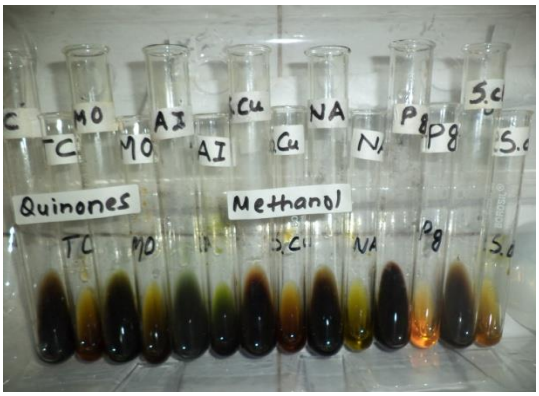


Antibacterial effects of Hexane extract of *N. arbortristis* and *S. cumini* against *Streptococcus* spp. by Agar well diffusion method (NC: Negative Control; PC: Positive Control)

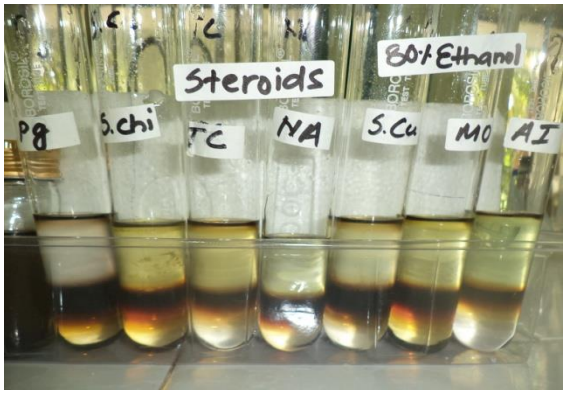


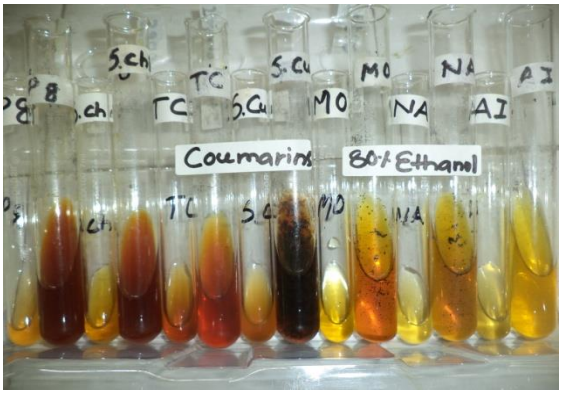
Antibacterial effects of Hexane extract of *M. oleifera* and *P. granatum* against *E. coli* and *S. aureus* by Agar well diffusion method (NC: Negative Control; PC: Positive Control)

PHYTOCHEMICAL SCREENING OF STUDIED MEDICINAL PLANTS

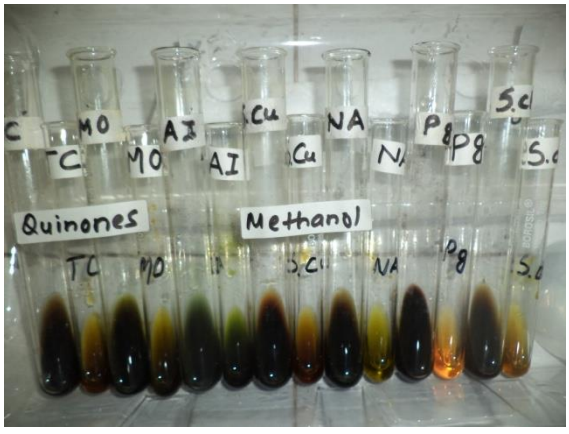


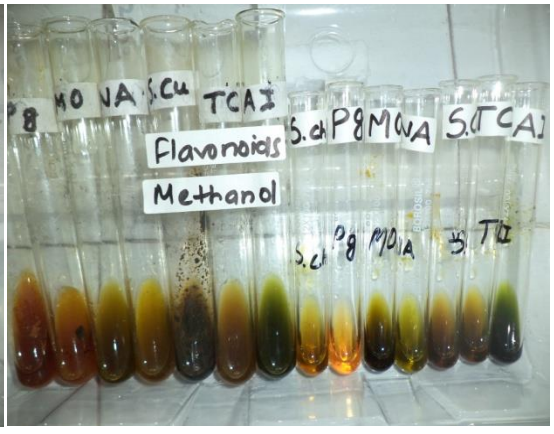
Phytochemical screening of studied plants for Alkaloid and Quinones (Aqueous extract)



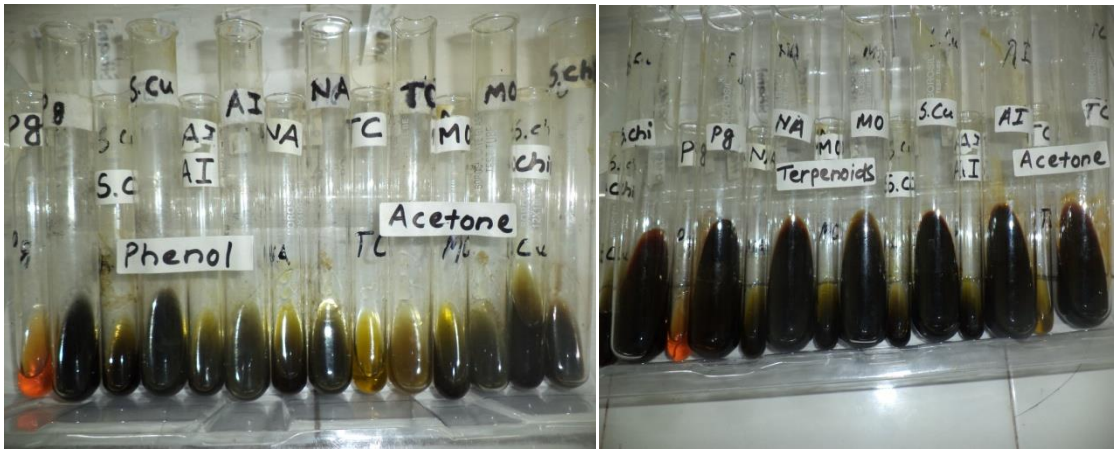


Phytochemical screening of studied plants for Steroids and Coumarins (80% Ethanol extract)

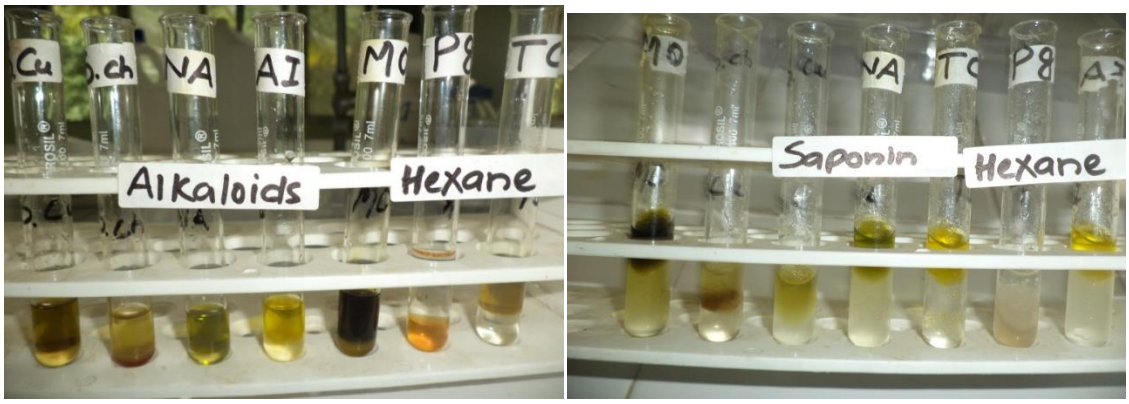




Phytochemical screening of studied plants for Quinones and Flavonoids (Methanol extract)

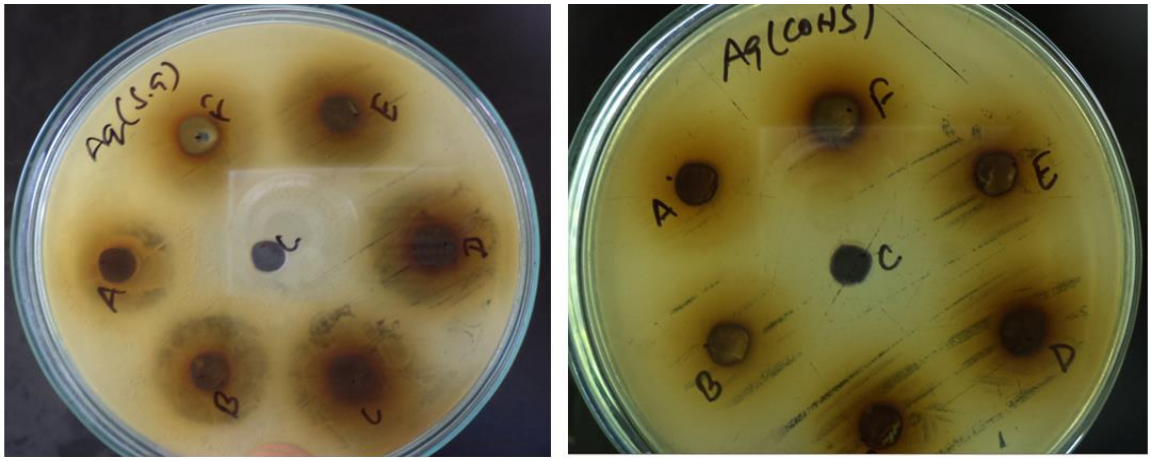


Phytochemical screening of studied plants for Phenol and Terpenoids (Acetone extract)

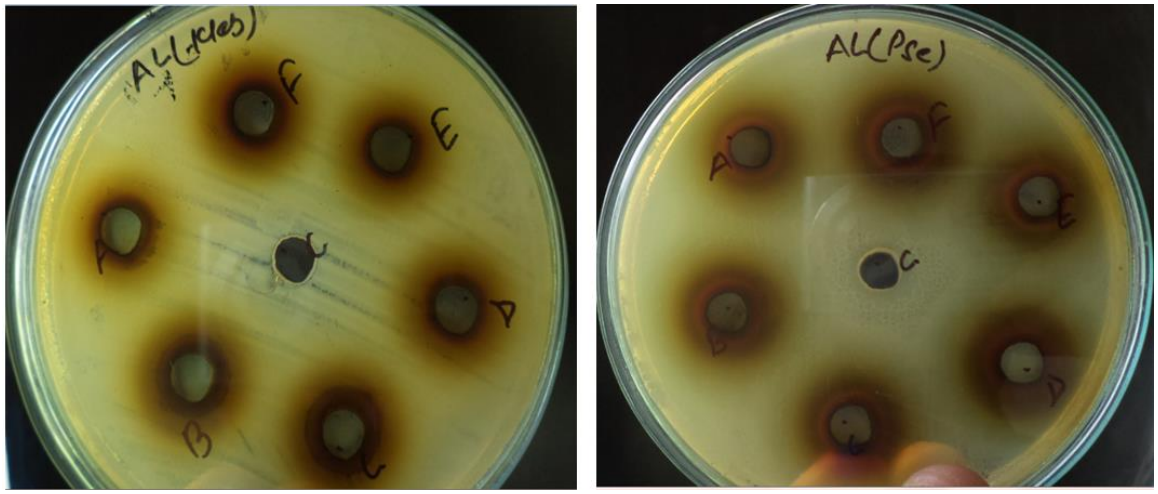


Phytochemical screening of studied plants for Alkaloids and Saponin (Hexane extract)

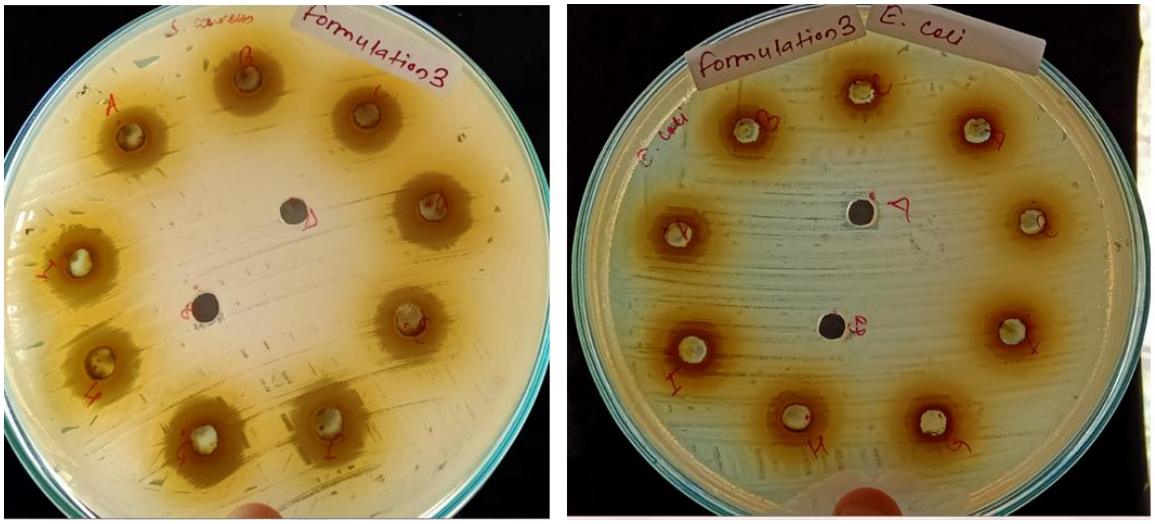
ANTIBACTERIAL EVALUATION OF DIFFERENT FORMULATION IN FOUR PHASES



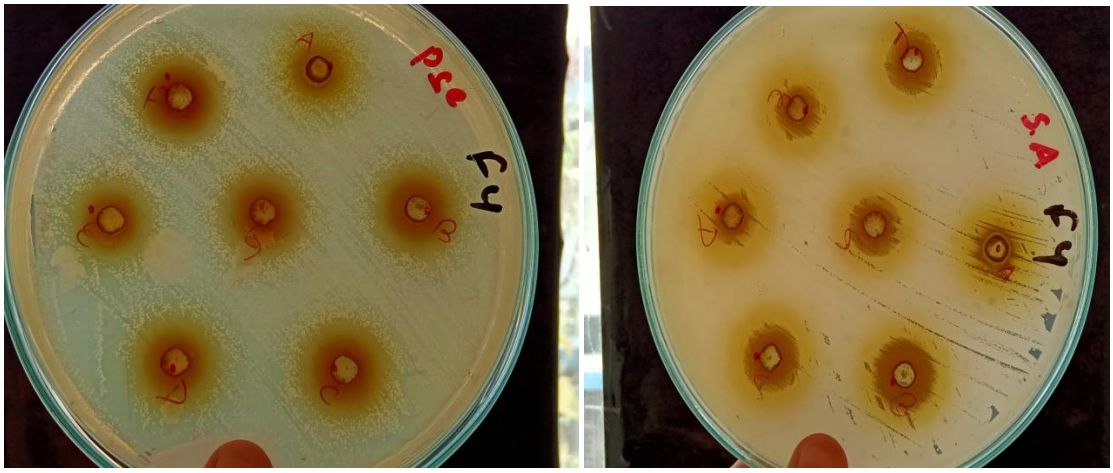
ZOI of Formulation phase I (Different composition from A to F) from aqueous extract against *S. aureus* (SA) and CoNS by Agar well diffusion method



ZOI of Formulation phase II (Different composition from A to F) from 80% Ethanolic extract against *K. pneumoniae* and *P. aeruginosa* by Agar well diffusion method



ZOI of Formulation phase III (Different composition from A to I) from 80% Ethanolic extract against *S. aureus* and *E. coli* by Agar well diffusion method



ZOI of Formulation phase IV (Different composition from A to G) against *P. aeruginosa* (Pse) and *S.aureus* (SA) by Agar well diffusion method

ZOI of Formulation phase IV (Different composition from A to G) against *E. coli* and *K. pneumoniae* by Agar well diffusion method

ACUTE ORAL TOXICITY

Mice ready to feed the formulation

Mice feeding with formulation by oral gavage