Study of a rare medicinal plant *Artemisia nilagirica*: phytochemical screening, antioxidant properties and antimicrobial activities

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Dedicated to

My Parents, My Sister

And Mentor

&

My husband for his unlimited support

DECLARATION

I hereby declare that the research work embodying the results reported in this thesis **entitled ““Study of a rare medicinal plant *Artemisia nilagirica*: phytochemical screening, antioxidant properties and antimicrobial activities”** has been carried out under the supervision of Ms. Jebunnesa Chowdhury, Assistant Professor, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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Abstract

Artemisia nilagirica is an aromatic herbaceous plant belongs to Asteraceae family used in traditional medicine for the treatment of various ailments. Locally the plant is known as Nagdan or Nagdamani. This plant has been reported to be used in treating skin diseases, wounds, ulcers, bronchitis, tuberculosis, epilepsy, and nervous diseases. *Artemisia nilagirica* is a rare plant in Bangladesh, found only in Sylhet district. Research suggests Nagdana also possesses medicinal properties including antimicrobial, antifungal, and anti-carcinogenic activities. Hence, this study was carried out using locally produced organic green tea. Preliminary screening of the extracts was conducted to identify the presence of alkaloids, flavonoids, steroids, saponins, tannins, phenols, cardiac glycosides, amino acids, terpenoids, anthraquinones and carbohydrates. Three organic solvent extract of *Artemisia nilagirica* namely, methanolic, ethanolic and chloroformic were screened for phytochemical profiling. Dried leaves with Inflorescences were used for screening of bioactive constituents by standard methods. Phytochemical analyses were carried out to identify the presence of eleven types of bio-active compounds in leaves and florescence. Antioxidant activity of extracts was determined by DPPH free radical scavenging activity with and without buffer. Methanolic extract in buffered solution showed the highest antioxidant activity (92.25% free radical scavenging activity) followed by ethanolic and Chloroformic extract. The lowest % of free Radical scavenging activity is 56.69% in non-buffered Chloroformic exraction. Comparative analysis of the antimicrobial activity of the extracts was investigated against *Escheriechia coli*, *Shigella flexneri*, *Streptococcus pneumonia*, *Bacillus subtilis* and *Staphylococcus aureus* along with synergistic activities. Ampicillin, ciprofloxacin, tetracyclin, kanamycin and chloramphenicol discs were used as positive control to measure the antimicrobial activity and synergistic activity of tea extracts and antibiotics. *E.coli* showed highest antimicrobial activity by representing 58.33% activity index in Ethanolic extract. *S. aureus* only showed inhibition potency for Chloroformic extracts, while *S. pneumoniae* showed resistance to it. These phytochemical characterization data along with antioxidant and antimicrobial assay would be helpful in authentication of raw material or crude drug of *Artemisia Nilagirica* leaves found in Sylhet district.

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List of Abbreviations

BRAC	Building Resources Across Communities
icddr,b	International Center for Diarrheal Disease Research, Bangladesh
Rpm	Rotation per minute
ºC	Degree Celsius
G	Gram
Mg	Milligram
Mm	Milliliter
Mg	Microgram
Ml	Micoliter
HEPA	High Efficiency Particulate Air
FRSA	Free Radical Scavenging Activity
mM	Milli Moles
v/v	Volume by volume
DMSO	Dimethyl Sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
e.g.	For example
et al.	And others
pH	Negative logarithm of hydrogen ion concentration

Chapter 1

Introduction

1.1. Introduction

Artemisia nilagirica is a wild perennial hardy plant belongs to Asteraceae family. *Artemisia* is one of the largest genus represents more than 800 species. The genus *Artemisia* found throughout northern Europe, North and South America, Africa, and Asia. *Artemisia nilagirica* is a gregarious shrub in Sikkim Hills, also in Bengal and Assam. This is also found throughout the hilly regions of Sylhet district inBangladesh. Commonly, this plant is known as Indian wormwood or mugwort; locally in Bangladesh the plant is known as Nagdana or Nagdamini. It has important medicinal value, to cure human sickness with signs and symptoms associated with some parasitism. *Artemisia* sp bear components that have been shown to possess properties against insects and parasites (protozoa, nematodes and helminthes). *Artemisianilagirica* widely used in Ayurvedic and the homeopathic medicine. Traditionally it is used in the treatment of epilepsy, nervous disorders as diuretic, anti-inflammatory and various skin diseases. It is reported that the flowering tops of *Artemisia nilagirica* were effective in the muscle phase of *T. spiralis* infection. Based on the literature review the oil is reported to possess antifungal, anti-microbial, insecticidal, larvicidal activity. The healing power of this herb is so good that it has trade mark into homeopathic medicine.It is used for the treatment of animals and plants also. However, we have a limited knowledge of its phytochemical constituents and explanation of the pharmacological effects.The present study includes the collected information on the description, chemical constituents and pharmacological actions of *A. nilagirica* which are collected from the works available in various literatures till 2016.

1.2 Background of *Artemisia nilagirica*

*Artemisia nilagirica (*syn. *Artemisia vulgaris L.)* said to have use in flavoring malts in Iron Age (Stika, 2011), but most importantly this plant traditionally been used as folk medicine since ancient times. The use of wormwood in Roman period is well documented by Dioscorides for Pontus and Cappadocia in Turkey (Berendes, 1902, Gunther, 1968). Recipes confirming the use of *Artemisia* sp. during Roman period have been reported by Wittmack (1903). It seems that wormwood was associated during Talmudic period with the use of gall to alleviate the sufferings of condemned and sick persons.China is a country with an old ethno botanical tradition, each 56 nationalities has accumulated a wealth of experience in utilization of plants, including about 500 species of Asteraceae used as medicinal plants. About 100 species are used in Tibetan medicine and those are mainly from the genera *Artemisia* L. (Huang and Ling., 1996). The cultivation and gathering of *Artemisia* species for medicinal and magical purpose

was common among the natives of Canary Islands, during the so called Guanchen period. In Germany, Albertus Magnus recorded the properties of many species of Asteraccae in his book of medicinal plants, including *Artemisia absinthium, A. abrotanum*and, *A. vulgaris* (Biewer, 1992). Shah (1996) described that genus *Artemisia* as one of the largest and most difficult taxa to understand under an ethno botanical point of view. The medicinal use of *Artemisia* spp. was introduced into the Indian Himalayas by different cultural and ethnic groups who entered this region in the past coming from Mediterranean and Arabian regions. *Artemisia nilagirica* (Clarke) Pamp (Nuov., Giorm. 1926. Bot, Ital. 33:425.) is the most common species found in earlier Indian literature. It was used as a decoction and infusion for the relied of nervous and spasmodic afflictions by Himalayan people. The plant used as magical propose; it was traditionally kept under pillow and in front of door to discourage evil spirits.

1.3 Plant description

It is a large aromatic herb/shrub, much branched; the shrub is generally 60–160 cm in height. The herbis erect, hairy, often half-woody. The stems areleafy and branched. Leavesof this plant are 1-12 cm long, upper ones 0.5-10.0 mm broad; they are alternate, large, ovate and lobbed; the matured leaves are deeply pinnatisect with small stipule-like lobes at the base, pubescent above, ash grey or white-tomentose beneath. The upper mostleaves are 3-fid or entire, linear-lanceolate. Theflowers are small and stand in long narrow clustersat the top of the stem, sub-globose heads, in spicateor horizontal panicled racemes. Theyare brownish yellow in colour.Involucres bracts are present as few to many seriate, outer small, ovate, herbaceous with scarious margin, sparsely hairy, innermost wholly scarious. Corolla of female (outer) floret is up to 1.2mm long, glabrous, that of hermaphrodite (inner disc) floret up to 1.5mm long, glabrous. Anther base is slightly sagittate, stylar-arms truncate and penicillate at the tip. Leaves andflowering tops are bitter, astringent and aromatic. The fruit are minute, bracts ovate or oblong. The time for flowering and fruiting is April- November. The percentage of oil constituents and the yield of oilvary with the distribution of the plant and also depend on the growth phases.

The plant is listed in Red book of South African National Biodiversity Institute and marked as rare/endangered by Bangladesh National Herbarium. The plant is planted for preservation in Lawachara National Park of Sylhet.

According to Indian Biodiversity Portal (IBP) Taxonomic Hierarchy

Kingdom Plantae

Subkingdom Viridiplantae

Infrakingdom Streptophyta

Superdivision Embryophyta

Division Tracheophyta

Subdivision Spermatophyta

Class Magnollopsida

Superorder Asteranae

Order Asterales

Family Asteraceae

Genus Artemisia

Species Artemisia nilagirica (Clarke) Pamp.

Synonym: *Artemisia Vulgaris L.var nilagirica* (C.B.Clarke, 1876)

DACB Accession number: 42318

1.4 Therapeutic values of *Artemisia nilagirica*

Some pharmacological effects have been corroborated in experimental biological models such as animals, plants and humans. Of this medicinal herb many interesting pharmacological activities have been described, such as insecticidal, antimicrobial, antiulcer, anticancer, antioxidant and anti-asthmatic.It is considered to be valuable stomachic, emmenagogue, antispasmodic and alternative. It is prescribed for obstructed menses and hysteria, in skin diseases and applied to the head for prevention of convulsions. And leaves and stem apices are administered in nervous and spasmodic affections in asthma and in the disease of brain. A strong decoction is given as a vermifuge, and infusion as a tonic. Leaves are carminative and haemostatic, considered being a remedy for headache, haemorrhagia, blood vomiting and haematuria. It is also recommended for metrorrhagia, dysentery, intestinal and urinary troubles, eczema, herpes and purulent scabies. Volatile oil extracted from the plant is good larvicide and a feeble insecticide.

In Bangladesh this plant is variously used by ethnic communities. Lalong, Patra of Sylhet use decoction of leaves for cough and cold, fresh leaves along with *Momordica charantia* are grounded and made into a paste in order to use it as "voron" for reliving headache. Khaisa community of greater Sylhet district use young the paste of young leaves in livers in liver and body pain and to prevent inflammation of the body. Garo ethnic people of Sunamganj, Netrokona, Mymensingh, Tangail and Sherpur districts apply leaf juice for the treatment of leprosy.*Artemisia nilagirica* has such high therapeutic value because of the presence of secondary metabolites, such as:

1.4.1 Phenols

Phenolics are aromatic benzene ring compounds with one or more hydroxyl groups produced by plants mainly for protection against stress. Phenolics play important roles in plant development, particularly in lignin and pigment biosynthesis. They also provide structural integrity and scaffolding support to plants. Importantly, phenolics secreted by wounded plants, repel or kill many microorganisms, and some pathogens can counteract or nullify these defenses or even subvert them to their own advantage (Bhattacharya *et al*., 2010).

1.4.2 Alkaloids

Alkaloids are a heterogeneous class of naturally occurring secondary metabolites. They have diverse and important physiological effects on humans and other animals. Distinguished alkaloids include morphine, strychnine, quinine, ephedrine, and nicotine. The utilizations of alkaloids are not constrained to natural control of herbivores but rather likewise have pharmacological, veterinary and medicinal significance. Alkaloids are found primarily in plants and are especially common in certain families of flowering plants (Britannica., n. d.).

1.4.3 Cardiac Glycoside

Cardiac glycosides are glycosides of mostly C23-steroidal compounds. They are called cardiac glycosides because they modify heart action and are used to treat congestive heart failure. Cardenolides inhibit the Na+-K+-ATPase pump in mammals. There are about 400 known cardenolides which are derived from steroidal precursors, such as cholesterol, via the intermediacy of pregnenolone or progesterone intermediates (Life., n. d.).Plants containing cardiac steroids have been utilized as toxins and heart drugs at any rate following 1500 B.C. All through history these plants or their concentrates have been differently utilized as bolt toxins, emetics, and diuretics.

1.4.4 Saponins

Saponins are glycosides with a distinctive foaming characteristic. The ability of a saponin to foam is caused by the combination of the nonpolar sapogenin and the water soluble side chain. Normal saponins have been found to have noteworthy against malignancy properties; saponins dependably show hostile to tumorigenic impacts through mixtures of antitumor pathways. Studies have illustrated the beneficial effects on blood cholesterol level; maintain bone health and give stimulation of the immune system (PhytochemicalsInformation., n. d.). Rising consumer demands for natural products coupled with physiochemical properties and mounting evidence on their biological activity has led to the emergence of saponins as commercially significant compounds with expanding applications in food, cosmetics, and pharmaceutical sectors (Güçlü-Ustündağ and Mazza, 2007).

1.4.5 Flavonoids

Flavonoids belong to the polyphenol family. Flavonoids are water soluble polyphenolic molecules containing 15 carbon atoms. The flavonoids consist of 6 major subgroups: chalcone, flavone, flavonol, flavanone, anthocyanins and isoflavonoids. Flavonoids are known for their antioxidant and anti-inflammatory health benefits, as well as their contribution of vibrant color to the many of the foods we eat. Flavonoids are becoming very popular because they have many health promoting effects. Some of the activities attributed to flavonoids include anti-allergic, anti-cancer, antioxidant, anti-inflammatory and anti-viral. The flavonoids quercetin is known for its ability to relieve hay fever, eszema, sinusitis and asthma (Phytochemicals Information., n. d.).

1.4.6 Steroids

In plants, steroids play an important role as constituents of cell membranes, insect deterrents, and growth hormones. Steroids extracted from plants have been used in traditional medicine and over into hormonally dynamic substances that serve as antifertility or prophylactic mixes or as anti-inflammatory medications, for example, cortisone and so forth that are utilized to treat various ailments, for example, joint inflammation and so forth.

1.4.7 Tannins

Tannins or tannic acid are water-soluble polyphenols. Results indicate that tannins reduce the mutagenic activity of a number of mutagens. The anti-carcinogenic and anti-mutagenic potential of tannins may be related to their anti-oxidative property, which is important in protecting

cellular oxidative damage, including lipid peroxidation. The anti-microbial activities of tannins are well documented. The growth of many fungi, yeasts, bacteria, and viruses was inhibited by tannins. Tannins can thus be used to increase the shelf-life of certain foods accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, produce liver necrosis, and modulate immune responses (Chung *et al*., 1998).

1.4.8 Carbohydrate

Carbohydrates are watersoluble, non-starch polysaccharides that are used in practical applications primarily to thicken or gel aqueous systems and to control water. They may also function as adhesives, crystallisation inhibitors, emulsifying agents, emulsion stabilizers, encapsulating agents, film formers, foam stabilizers, suspending agents, suspension stabilizers etc. (BeMiller, 2014).

1.4.9. Anthraquinones

Anthraquinone is a polycyclic aromatic hydrocarbon derived from anthracene or phthalic anhydride. Anthraquinone used in the manufacture of dyes, in the textile and pulp industries, and as a bird repellant. Anthraquinone has been shown in certain studies to help aid in digestion as a laxative, to reduce inflammation in arthritis patients, and to inhibit the growth of cancer cells. These Anthraquinone that are used for medical purposes found to be naturally occurring implants and are not made through chemical reactions. However, even in medical use, the substance still does have side effects (Thegreenbook., n. d.).

1.4.10 Terpenoids

Terpenoid, also known as isoprenoids or Terpens are the largest group of organic compounds found thus far, comprising at least 20,000 distinct molecules. Terpenes are basic hydrocarbons, whereas Terpenoid contain extra functional groups that could be comprised of a range of chemical elements (Medicaljane., n. d.). Terpenoid is used in medicine as an anti-inflammatory, expectorant, bronchodilator and local antiseptic. (Seniseeds., n. d.).

1.5 Assessment of Antimicrobial Activities of *Artemisia nilagirica* Extracts

Bioassays play an important role in the evaluation of a particular bioactivity. A bioassay which is applied to large numbers of initial samples to determine whether or not they have any bioactivity such as antibacterial or antioxidant assay is referred to as a prescreen assay. Hence, the tests must be simple, rapid, reliable, reproducible, sensitive, meaningful and, most

importantly, predictive. The in vitroassessment of antimicrobial susceptibility is done by Agar-Well Diffusion Assay. Brief overview of the selected bacteria on which the antibacterial effects were observed:

1.5.1 *Staphylococcus aureus*

It is named thus for its yellow-pigmented colonies *(aureus*means golden) and occurs in grapelike clusters. It is one of the most common gram positive facultative anaerobic bacteria responsible for food poisoning (Wikipedia., n. d.).

1.5.2 *Shigella flexneri*

This is the causative agent of shigellosis. A disease found only in humans. Abdominal cramps and fever are some of the symptoms of infection. The bacteria rarely invade the bloodstream. It is quickly developing resistance against antibiotics such as ampicillin, chloramphenicol, streptomycin, trimethoprim-sulphamethoxazol and tetracycline (Reploglet *al*, 2000).

1.5.3 *Streptococcus pneumoniae*

Pneumococcal disease is an infection caused by *Streptococcus pneumoniae* bacteria. These bacteria can cause many types of illnesses, including: pneumonia, ear infections, sinus infections, meningitis, and bacteremia. Pneumococcus bacteria are spread through coughing, sneezing, and close contact with an infected person.

1.5.4 *Bacillus subtilis*

Bacillus subtilis is thought to have a weak ability to cause disease in humans unless the number of bacteria a person consumes is very high or the immune status of the person is very low (Powerofprobiotics., n. d.).

1.5.5 *Escherichia coli*

It is a gram negative bacterium. Although they are commensal gut bacteria, some pathogenic strains produce enterotoxins that cause very serious foodborne diseases and gastrointestinal infections. On entering the urinary tract, *E. coli* can cause urinary tract infections (Tortora*et al*., 2010).

1.6 Synergistic Activity of Nagdana and Antibiotics

Combination chemotherapy, the use of two or more antimicrobial agents, is being employed in medical practice with increasing frequency. The rationale for using a combination of drugs is the expectation that the effective combinations might lower the incidence of bacterial response, reduce host toxicity of the antimicrobial agents (due to lower dosage requirements), or enhance the agents' bactericidal activity. Enhanced bactericidal activity is known as synergism. Synergistic activity is evident when the sum of the effects of the chemotherapeutic agents used in combination is significantly greater than the sum of their effects when used individually. This result is readily differentiated from an additive effect, which is evident when the interaction of two drugs produces a combined effect that is no greater than the sum of their separately measured individual effects (Cappuccino and Natalie, 2013).

1.7. Objectives of the study

1.7.1 General objective:

In this study, three different types of extracts from samples were collected and investigated for the screening of phyto-constituents. These extracts were also used to test their antibacterial activities against 5 strains of bacteria *(E.coli, Bacillus subtilis, Shigella felxneri, Staphylococcus aureus, and Streptococcus pneumoniae*).

1.7.2 Specific objective:

- Collection of plant from Sylhet district.
- Establishment of a suitable extraction process for *Artemisia nilagirica* and collection of extract.
- Phytochemical screening of collected *Artemisia nilagirica* plant extract.
- Comparative analysis of Antioxidant properties of all three kinds of extracts.
- Comparative analysis of plant extracts antibacterial activities against five bacterial strains.

Chapter 2

Materials and Method

2.1 Method and Materials

This study was performed in the Biotechnology Laboratory in the Department of Mathematics and Natural Sciences, BRAC University.

2.2 Plant sample Collection

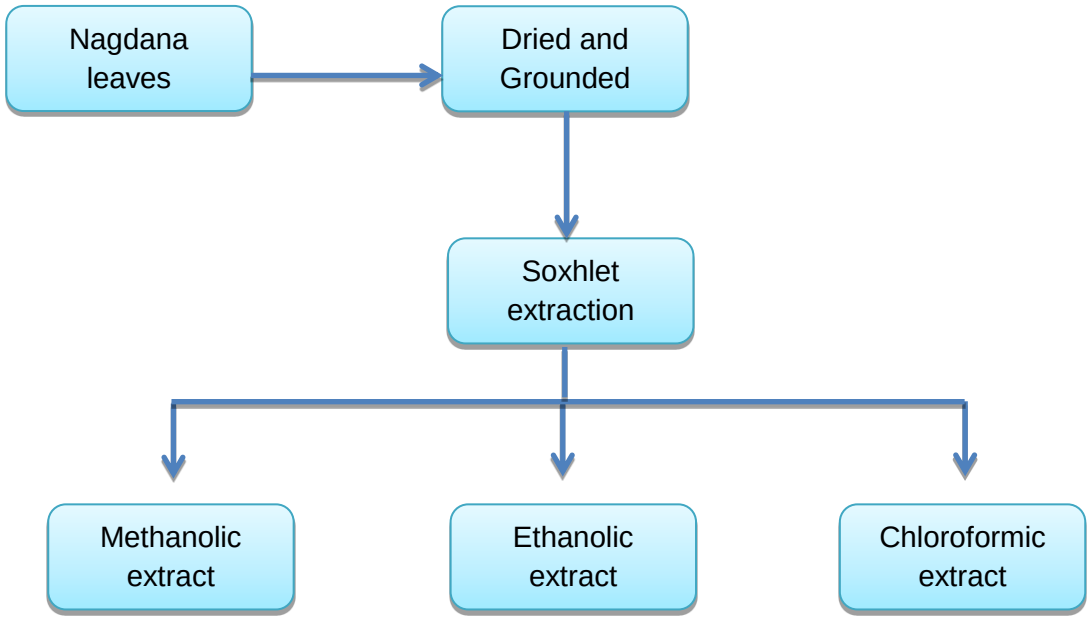
The branches of Artemisia Nilagirica were collected from Sylhet and nurtured in Uttara, Dhaka. The plant was identified and authenticated at '**Bangladesh National Herbarium**', Mirpur, Dhaka. The branches were sun dried and those dried leaves and stems were separately grounded with a blender machine and stored in airtight container.

2.3 Carrying out extraction procedure from the sample

A total of three types of extracts were collected from the sample powders namely methanolic extract, ethanolic extract and chloroformic extract.

2.3.1 Methanolic and Ethanolic and Chloroformic Extracts

For collection of Ethanolic, Methanolic and chloroformic extracts 20g of Nagdana powder was packed in thimble and extracted in Soxhlet's apparatus using 280ml of Methanol and Ethanol respectively. The temperature was kept between 60-80ºC for both the cases. The samples in the thimble of the Soxhlet's apparatus was kept boiling for approximately 2-3 hours till the solution becomes dark and the extract was collected at the bottom of the apparatus. This was then taken to the rotatory evaporator and kept for 40-60 minutes at 70ºC at 150 atm pressure. Then it was transferred in petri dishes and left to dry for 2 hours. The dried extract having a sticky appearance was stored in 25 ml McCartney bottles at temperatures below 10ºC in the refrigerator for further use. The whole process was repeated 3 times for the collection of a substantial amount of extracts for the study. The crude extract were collected using three different solvent, methanol, absolute ethanol and chloroform by soxhlet apparatus.



2.4 Storage and Preservation of Extracts

Once the desired consistency was achieved, the extracts were stored in 25 ml McCartney bottles which were thoroughly cleaned with 70% ethanol prior to storage. Bottles were tightly stoppered and wrapped in foil to protect any photosensitive compounds that maybe present in the extracts from degradation. After proper labeling, the McCartney bottles were stored at 4ºC for preservation and prevention from external contaminants.

2.5 Preparation of Stock Solution for Phytochemical Assays

0.5gm of crude extract was dissolved in 100 ml of respective solvent to make a stock solution of 5µg/µl. This served as the working solution for all the phytochemical tests without any further dilutions.

2.6 Phytochemical Screening of *Artemisia nilagirica*extracts

Qualitative preliminary screening of all three extracts was carried out for identifying the presence or absence of any phytochemicals present in the extracts. All three extracts were tested individually. All tests were conducted in triplicates. Two types of negative control were used; only reagent and only sample.

2.6.1 Test for Alkaloids

There are a various number of tests for alkaloids, of which two were used to detect the presence or absence of alkaloids in the tea extracts.

2.6.1.1 Hager's Test

1 ml of extract was carefully mixed with 3 drops of freshly prepared Hager's reagent (Appendix I) in a test tube. The formation of yellow precipitates showed a positive result and the presence of alkaloids in the extract.

2.6.1.2 Wagner's Test

1 ml of extract was mixed together in a test tube with 3 drops of Wagner's reagent (Appendix I). The formation of dark brown precipitate showed the presence of alkaloids.

2.6.2Test for Phenolic Compounds

1% Ferric Chloride solution and 1% Potassium Ferro-cyanidewas mixed in equal amounts. 3 drops of this freshly prepared solution was added to 2ml of extract. The formation of a blue-black colour is considered positive.

2.6.3 Test for Steroidal Compounds (Salkowaski's Test)

2ml extracts were dissolved in 2ml chloroform in a test tube. 4ml concentrated Sulfuric Acid (97%) was carefully poured from the inner wall of the test tube. Formation of a red colour in the upper layer and Sulfuric Acid layer turning yellow with green fluorescence is indicative of positive for steroids.

2.6.4 Test for Terpenoid Compounds (The Liebermann–Burchard):

2ml extracts were dissolved in 2ml Acetic Anhydride in a test tube. 4ml concentrated Sulfuric Acid (97%) was carefully poured from the inner wall of the test tube. The formation of a green or green-blue colour after a few minutes is indicative of positive for Terpenoids.

2.6.5 Tests for Tannins (Lead Acetate Test)

1ml of freshly prepared 10% Lead Acetate was added to 1ml of extract. Formation of aprecipitate indicates the presence of Tannins.

2.6.6. Tests for Saponins (Froth Test)

5ml of each type of extract was dissolved in 10ml distilled water. The test tube wasstoppered and then shaken vigorously for 30 seconds. It was then allowed to stand for30 minutes.

Formation and retention of honey-comb froth on the surface for 30minutes is considered positive for saponins.

2.6.7 Test for Flavonoids (Caustic Soda Test)

1 ml of freshly prepared 1 M of NaOH was added to 2ml sample extract. Formation of yellowish precipitate indicates the presence of Flavonoids.

2.6.8 Test for Cardiac Glycosidase (Killer-Killani Test)

2ml glacial Acetic Acid was added to 3ml of extract followed by 1 drop of Ferricchloride and 3 ml concentrated Sulfuric Acid. A positive result shows a brown ring atthe interface.

2.6.9 Test for carbohydrates (Molisch's Test)

To 5ml extract, Molisch's reagent (Appendix I) and concentrated Sulfuric acid wereadded drop-wise. Positive result shows a red-violet ring.

2.6.10 Test for Amino Acids (Ninhydrin Test)

Few drops of Ninhydrin reagent (Appendix I) were added to 2 ml of each extract. Formation of purple colour is indicates the presence of Amino Acids.

2.6.11 Test for Anthraquinones

2ml extracts were dissolved in 1ml of Benzene and Ammonia solution in a test tube. 4ml concentrated Sulfuric acid (97%) was carefully poured from the inner wall of the test tube. Formation of a rose pink colour is indicative of positive for steroids.

2.7 Antioxidant Assay: DPPH Scavenging Activity

"Antioxidant is a term widely used but rarely defined" (Halliwell & Gutteridge, 1999), They likewise characterized antioxidant as "Any substance that, when present at low fixation contrasted and those of an oxidizable substrate, altogether delays or forestalls oxidation of that substrate" (Halliwell and Gutteridge, 1999). Antioxidants may offer protection against the oxidative stress by scavenging the free radicals and inhibiting lipid peroxidation that may counteract ailments. Although organisms possess several natural defense mechanisms, such as enzymes and antioxidant nutrients, to prevent ROS production, continuous exposure for a long time could still lead to irreversible oxidative damage.DPPH(2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a

violet solution in methanol. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colorless methanol solution. The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry, so it can be useful to assess various products at a time. This method was developed by Blois (1958) with the viewpoint to determine the antioxidant activity in a like manner by using a stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH; C18H12N5O6, $M = 394.33$). The assay is based on the measurement of the scavenging capacity of antioxidants towards it. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine (Contreras-Guzman and Srong, 1982).DPPH is a stable free radical due to the delocalization of its spare electron over the molecule so that the molecule does not dimerize like most other free radicals. The delocalization of electron gives rise to the characteristic deep violet colour of DPPH solution. When a solution of DPPH is mixed with that of a hydrogen donating substrate, DPPH gets reduced, resulting in the loss of this violet colour. In methanolic or ethanolic solutions of DPPH, the absorbance of this violet colour is generally measured at 517 nm (Alam *et al*., 2013). The antioxidant activity of the extracts was assessed using DPPH free radical scavenging activity by modified method (Prabhune *et al*., 2013).

2.7.1 Preparation of Standard Solution

Ascorbic acid was used as standard by dissolving ascorbic acid in methanol to give the concentration of 15.2, 31.3, 62.5, 125, 250, 500 and 1000µg/ml.

2.7.2 Preparation of Test Sample

Stock solutions of each extract of sample was prepared by dissolving 10 mg extract in 10 ml of respective solvent to make concentration of 1mg/ml. This was further diluted into different concentrations of 15.2, 31.3, 62.5, 125, 250, 500 and 1000µg/ml which were used in the assay.

2.7.3 Preparation of DPPH Solution

0.1 mM concentration of DPPH was freshly prepared by dissolving 4 mg of DPPH in 100 ml methanol. This was wrapped in aluminum foil beforehand in order to protect the reagent from light.

2.7.4 Preparation of Acetic acid (pH 5.5) Solution

1.43 ml of acetic acid was mixed with 12.5 ml of distil water. The pH is adjusted by adding few gram of sodium acetate until it reached pH 5.5. More distil water is added to make the volume 50 ml.

2.7.5 Measuring Absorbance of Control

2.7.5.1 Buffered: For the control, 1ml DPPH was added to 1ml of acetic acid and 2ml of methanol and the absorbance was immediately measured at 517 nm wavelength. This is the control absorbance or blank containing all reagents except the test sample.

2.7.5.2 Non-buffered: For the control, 1ml DPPH was added to 3ml of methanol and the absorbance was immediately measured at 517 nm wavelength. This is the control absorbance or blank containing all reagents except the test sample.

2.7.6 Measuring Absorbance of Standard

2.7.6.1 Buffered: In each test tube 2ml of each concentration of ascorbic acid was added to 1ml of DPPH solution and 1ml of acetic acid. After 30 minutes dark incubation at room temperature, the absorbance was measured at 517nm keeping methanol as blank.

2.7.6.2 Non-buffered: In each test tube 3ml of each concentration of ascorbic acid was added along with 1ml of DPPH solution. After 30 minutes dark incubation at room temperature, the absorbance was measured at 517nm keeping methanol as blank.

2.7.7 Measuring Absorbance of Sample Extracts

2.7.7.1 Buffered: 2ml of each concentration of sample was added to 1ml of DPPH solution along with 1ml of acetic acid in each test tube. After 30 minutes dark incubation at room temperature, the absorbance was measured at 517nm keeping methanol as blank.

2.7.7.2 Non-buffered: 3ml of each concentration of sample was added to 1ml of DPPH solution in each test tube. After 30 minutes dark incubation at room temperature, the absorbance was measured at 517nm keeping methanol as blank.

2.8 Determination of Antiradical Activity

Each experiment was carried out in triplicate and results are expressed as mean % antiradical activity. The free radical scavenging activity (FRSA) (% antiradical activity) was calculated using the following equation (Sanja *et al*., 2009):

$$\%\text{Free Radical Scavenging Activity} = \frac{Control\ absorbance - Sample\ absorbance}{Control\ absorbance} \times 100$$

2.9 Antibacterial Activity of Different Extracts of *Artemisia nilagirica*

The in vitro assessment of antimicrobial susceptibility is done by Agar-Well Diffusion Assay. The antimicrobial activity of the extracts of *Artemisia nilagirica*against the targeted microbes was studied. Standard antibiotic discs were used as positive control and to further examine the synergistic activity of Nagdana and antibiotics. For each organism, nine plates were inoculated having three replicates of each extract. Each test was replicated thrice for validation and accuracy of the results. Ciprofloxacin was used as positive control for *E. coli* and EPEC. Ampicillin served as positive control for S. *aureus,* kanamycin was used for *Bacillus subtilis*. Chloramphenicol used for *S. pneumoiae;* lastly, cefoxitin was positive control for *S. flexneri*.

2.9.1 Aseptic Conditions

All antibacterial assays were conducted inside the biosafety hood under complete aseptic conditions. The laminar air flow chamber with HEPA filters was cleaned with 70% ethanol.

2.9.2 Test Organism

The pathogens used in this experiment were obtained from clinical isolates from icddr,b and preserved in the Biotechnology Laboratory, Department of Mathematics and Natural Sciences, BRAC University**.** In this study, five bacterial strains *Escherichia coli, Bacillus subtilis, Shigella flexneri, Streptococcus pneumonia* and *Staphylococcus aureus* were used.

2.9.3 Media preparation:

For carrying out the antibacterial activity test, Muller Hinton Agar (MHA) was used. Nutrient Agar (NA) medium was used to prepare fresh cultures.

Composition of Nutrient Agar:

Ingredients	**Amount**
Peptone	0.5%
Beef extract/ Yeast extract	0.3%
Agar	1.5%
NaCl	0.5%
Distilled Water	20 ml
Final pH	7.4±0.2

Composition of Muller Hington Agar:

Ingredients	**Amount**
Beef,infusion form	30%
Casein hydrolysate	1,75%
Agar	1.7%
Starch	0.15%
pH	Neutral

2.9.4 Maintenance and preparation of inoculum

Each organism was cultured on nutrient agar and only fresh cultures were used. Cell suspensions were freshly prepared the day of the test using fresh cultures only. Loop full of the test organisms were taken from fresh cultures incubated 24 hours prior to the test and dissolved in individual autoclaved test tubes, each containing 10ml of 0.9% NaCl to make the respective bacterial cell suspensions. The test tubeswere vigorously mixed using a vortex (Appendix II) to ensure complete dissolution of the bacterial cells within the saline. The cell suspensions were of equal concentration to make sure that there are an equal number of cells in every cell plate. The concentration of each cell suspensions were checked until the turbidity equals or exceeds that of a 0.5 McFarland Standard. The suspensions were adjusted to obtain turbidity optically comparable to that of the 0.5 McFarland. A spectrophotometer (Appendix II) was used to measure an absorbance of 0.110 at 600 nm which is the standard turbidity of 0.5 McFarland Standard.

2.9.5 Preparation of Extract Solution for Antibacterial Activity Test

All three types of the collected crude extracts of *Artemisia nilagirica*were dissolved in 0.25% (v/v) autoclaved dimethyl sulphoxide (DMSO) to make two different concentrations of extract solutions for the antibacterial activity tests. 1.2gm of crude extract was dissolved in 15ml of 0.25% DMSO to prepare 80µg/µl of extract. This was further diluted to 60µg/µl by using 7.5ml of the 80µg/µl stock and further diluting it with 12.5ml DMSO.

2.9.6 Inoculation of Media

Antimicrobial activity was observed by preparing lawn culture after turbidity of bacterial suspension was adjusted to equivalent of 0.5 McFarland on nutrient agar plates. The lid was left slightly ajar to allow inoculum to be absorbed before carrying out agar-well diffusion method.

2.9.7 Agar-Well Diffusion and Application of Disks for Bioassay of Different Concentrations of Extracts

Test plates were previously labeled into four quadrants; one for positive control, second for crude extract, third for 80µg/µl of extract and the fourth for 60µg/µl of extract. Three wells of about 0.6mm diameter were aseptically cut out from the inoculated plates using cork borer allowing 30mm between adjacent wells and the edge of the petri dishes. 60 µl of each extract were then added into the wells. This process was repeated for each extract as every plate contained three concentrations of a single extract. Antimicrobial discs were applied as positive control to the first quadrant of the inoculated plates no more than 15 minutes following inoculation. Flame sterilized bend-forceps were used to impregnate the discs on the agar by tapping them gently to ensure complete contact with the agar surface. Care was taken not to relocate the disks once it has come in contact with the agar surface as the drug diffuses almost instantaneously. Plates were kept in an upright position in an incubator to allow the extracts to diffuse into the agar. The plates were incubated for 24 hours at 37ºC and observed for zone of inhibition (mm) around the wells. The extracts producing significant zone of inhibition were subsequently tested for synergistic activity with standard antibiotic discs.

2.9.8 Measuring Activity Index

The inhibitory effects of the methanolic, ethanolic and Chloroformic extracts were calculated and compared by measuring the activity index using the following formula (Rahman, 2014):

$$Activity\ Index = \frac{\text{Zone of Inhibition of extract}}{\text{Zone of Inhibition of Antibiotics}}\ X\ 100$$

2.10 Synergistic Activity of Nagdana and Antibiotics

In the current study, a disc-agar diffusion technique was performed to demonstrate the synergistic activity of green tea with the respective antibiotics. This technique uses the Kirby-Bauer antibiotic susceptibility test procedure. Bacterial cultures were swabbed on their respective nutrient agar plates from their cell suspensions. Antibiotic discs were soaked in extracts for a few minutes. The plates were divided into three equal sections and labeled as having only antibiotic disk in one segment, antibiotic disc soaked in extract in the second segment and only the respective extract in the last section. Here, both the antibiotic disc and the extract serve as positive controls for comparison of synergistic or additive activity. The two discs representing the drug combination are placed on the inoculated agar plate separated by a distance (in mm) that is equal to or slightly greater than half the sum of their individual zones of inhibition when obtained separately. The plates were placed upright and incubated at 37ºC for 24 hours and synergistic or additive activity was recorded by measuring the zones of inhibition (mm).

Chapter 3

Results

3.1 Phytochemical Screening

Specific tests were conducted to screen for the presence of the following phytochemicals in all three (ethanol, methanol and chloroform) different extracts of *Artemisia nilagirica*. Firstly the presence of alkaloid compounds was screened. On obtaining consistent positive results; Hager's Test, Wagner's Test, Lead Acetate Test, Salkowaski's Test, Froth Test, Ferric Chloride Test and Molisch's Test were performed and their results are recorded in Table 1. Screening result showed the presence of alkaloid in only ethanolic solution (Figure 1), whereas tannins (Figure 4), flavonoid (Figure 7), saponins (Figure 6), steroid (Figure 5) and gum (Figure 8) were present in both ethanolic and methanolic extract. Lastly Terpenoid (Figure 3), phenolic compounds (Figure 12), Amino Acids (Figure 10) and cardiac glycosidase (Figure 9) were assayed and found in all three types of organic extract of *Artemisia nilagirica*. However, Anthraquinone (Figure 11) was absent in the all three types of crude extracts.

Table 1: Result of Phytochemical Assay

Chemical group test	**Specific test**	**Observation**		
		Methanolic Extract	**Ethanolic Extract**	**Chloroformic Extract**
Alkaloids	Hager's test	Negative	Positive	Negative
	Wagner's test	Positive	Positive	Negative
Flavonoids	Caustic soda test	Positive	Positive	Negative
Steroids	Salkowaski's test	Positive	Positive	Positive
Terpenoids	Liebermann burchard test	Positive	Positive	Positive
Saponins	Froth test	Positive	Positive	Negative
Tannins	Lead acetate test	Positive	Positive	Negative
Amino acid	Ninhydrin test	Positive	Positive	Positive
Cardiac glycosidase	Killer-killani test	Positive	Positive	Positive
Carbohydrates	Molisch's test	Positive	Positive	Negative
Phenols	—	Positive	Positive	Positive
Anthraquinones	—	Negative	Negative	Negative

* All tests were performed in triplicate

3.1.1 Test for Alkaloids

3.1.1.1 Hager's Test

1 ml of extract was carefully mixed with 3 drops of freshly prepared Hager's reagent in a test tube. Colour change was observed.

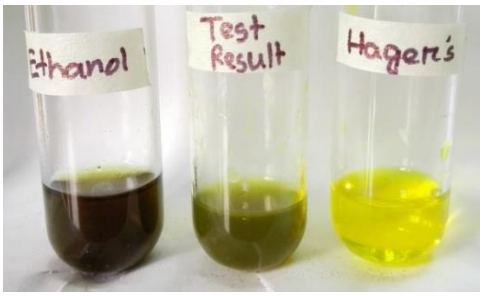
	(a) Significant colour changes observed, so ethanolic extract is positive for Alkaloids.
	(b) No significant colour change is observed; Methanolic extract was negative for Alkaloids.
	(c) No colour change, Chloroformic extract is negative for Alkaloids.

Figure 1: Hager's test for Alkaloid detection (a) Ethanolic extracts (b) Methanolic extract (c) Chloroformic extract

* All tests were performed in triplicate

3.1.1.2 Wagner's Test

1 ml of extract was mixed together in a test tube with 3 drops of Wagner's reagent. The formation of brown precipitate showed the presence of alkaloids.

	(a) Dark brown precipitation is observed, Ethanolic extract is positive for Alkaloids
	(b) Dark brown precipitation is observed, Alkaloid is present in Methanolic extract
	(c) Layers of extract and reagent separated, Chloroformic extract is negative for Alkaloids

Figure 2: Wagner's test for Alkaloids detection (a) Ethanolic extract (b) Methanolic extract (c) Chloroformic extract

* All tests were performed in triplicate

3.1.2 Test for Terpenoids(Liebermann burchard test)

2ml of extract was dissolved in 2 ml of acetic anhydride in a test tube. 4 ml concentrated sulfuric acid(97%) carefully poured from inner wall of the test tube. Formation of green colour indicates the positive of Terpenoids

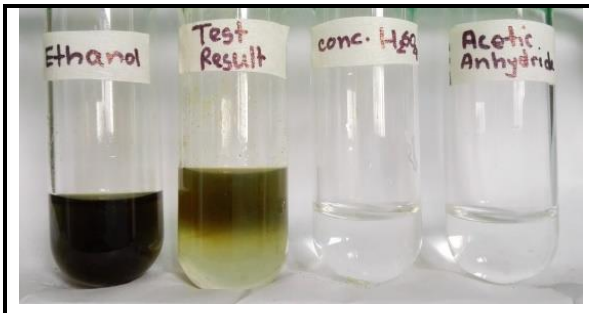
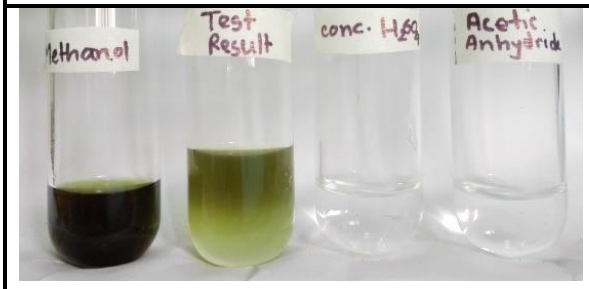
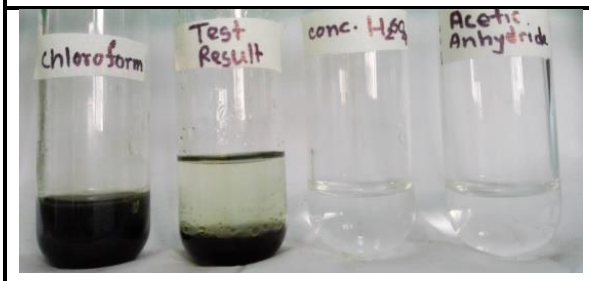
	(a) Formation of fluorescent green indicated presence of Terpenoids in Ethanolic extract
	(b) Formation of fluorescent green indicated presence of Terpenoids in methanolic extract
	(c) Terpenoid is absent in Chloroformic extract as no colour had been formed.

Figure 3: Acetic Anhydride test for Terpenoids (a) Ethanolic extract (b) Methanolic extract (c) Chloroformic extract

* All tests were performed in triplicate

3.1.3 Test for Tannins (Lead Acetate Test)

1ml of freshly prepared 10% lead acetate was added to 1ml of extract. Precipitate indicates the presence of flavonoids.

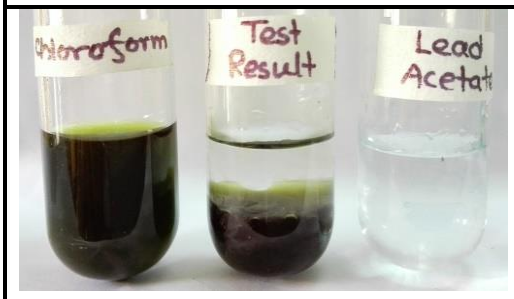
	(a) Lead acetate test for ethanolic extract showing moderately positive results for tannin.
	(b) Lead acetate test for methanolic extract showing strong positive results for tannin.
	(c) Lead acetate test for Chloroformic extract showing negative results for tannin.

Figure 4: Lead Acetate test for Tannins (a) Ethanolic extract (b) Methanolic extract(c) Chloroformic extract

* All tests were performed in triplicate

3.1.4 Test for Steroids (Salkowaski's Test)

2ml extracts were dissolved in 2ml chloroform in a test tube. 4ml concentrated sulfuric acid (97%) was carefully poured from the inner wall of the test tube. Formation of a red colour in the upper layer and yellow in sulphuric acid layer was observed.

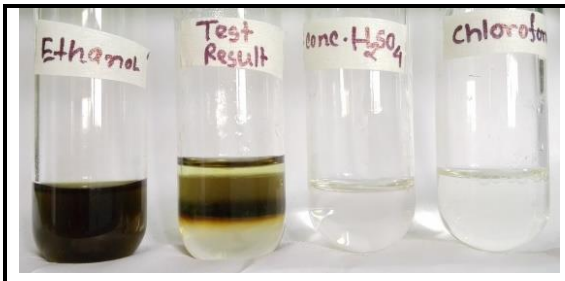
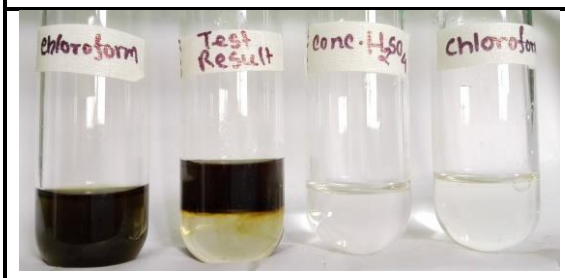
	(a) Salkowaski Test for ethanolic extract showing positive result for steroids.
	(b) Salkowaski Test for methanolic extract showing strong positive result.
	(c) Salkowaski Test for chloroformic extract showing strong positive result.

Figure 5: Salkowaski's test for steroids (a) Ethanolic extract (b) Methanolic extract(c) Chloroformic extract

* All tests were performed in triplicate

3.1.5 Test for Saponins (Froth Test)

5ml of each extract was dissolved in 10ml distilled water. The test tube was stoppered and then shaken vigorously for 30 seconds. Honey-comb froth remained on the surface for 30 minutes.

	(a) Honey-comb froth on the surface of Ethanolic extract showed strong positive result for Saponins.
	(b) Honey-comb froth on the surface of methanolic extract showed strong positive result for Saponins.
	(c) No honey-comb froth is present on the surface of Chloroformic extract showed negative result for Saponins.

Figure 6: Froth test for Saponins (a) Ethanolic extract (b) Methanolic extract (c) Chloroformic extract

* All tests were performed in triplicate

3.1.6 Test for Flavonoid (Caustic soda Test)

1 ml of freshly prepared 1 M of NaOH was added to 2ml sample extract. Formation of precipitate indicates the presence of Flavonoids.

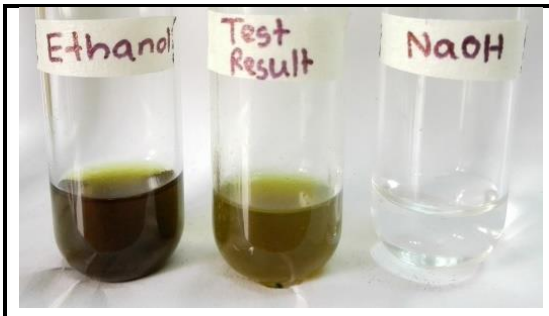
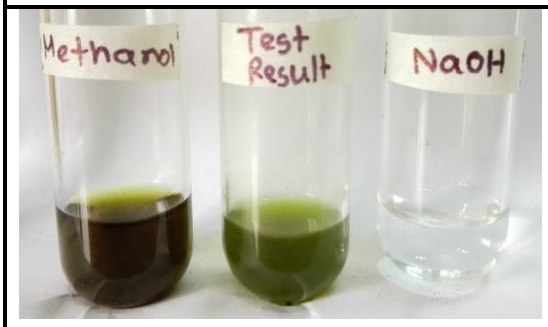
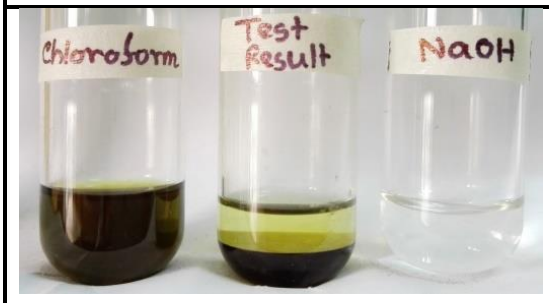
	(a) Flavonoid is present in Ethanolic extract.
	(b) Flavonoid is present in Methanolic extract.
	(c) Flavonoid is absent in Chloroformic extract.

Figure 7: Caustic soda test for Flavonoids (a) Ethanolic extract (b) Methanolic extract (c) Chloroformic extract

* All tests were performed in triplicate

3.1.7 Test for Carbohydrates (Molisch's Test)

To 5ml extract, Molisch's reagent and concentrated sulphuric acid were added dropwise. Positive result shows a red-violet ring only in the aqueous extract.

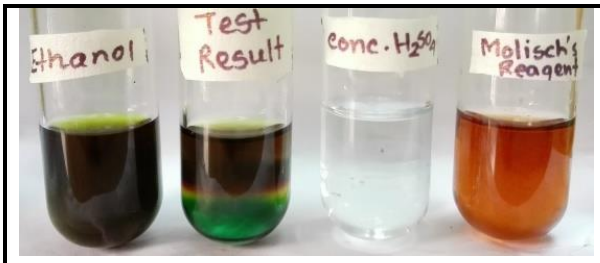
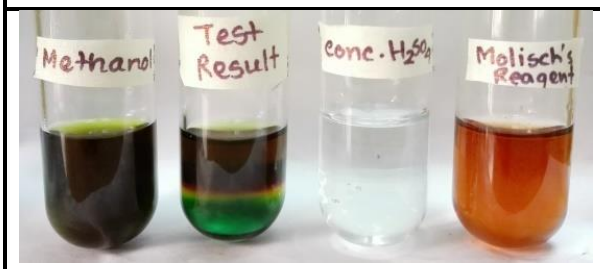
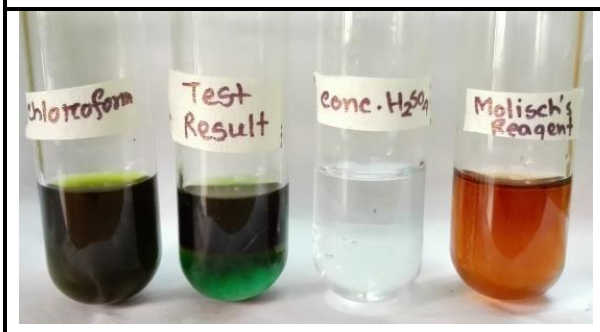
	(a) Red violet ring in the interface showed the presence of carbohydrates in Ethanolic extract
	(b) Red violet ring in the interface showed the presence of carbohydrates in methanolic extract
	(c) No Red violet ring in the interface is observed, carbohydrates are absent in chloroformic extract

Figure 8: Molisch's test for Carbohydrates (a) Ethanolic extract (b) Methanolic extract(c) Chloroformic extract

* All tests were performed in triplicate

3.1.8 Test for Cardiac glycosidases(Killer-killani Test)

2 ml glacial acetic acid was added to 5 ml of extract followed by 1 drop of 1% ferric chloride. 4 ml of concentrated sulfuric acid is added. Brown ring at interface showed positive result.

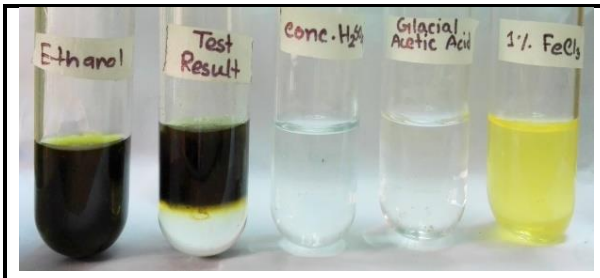
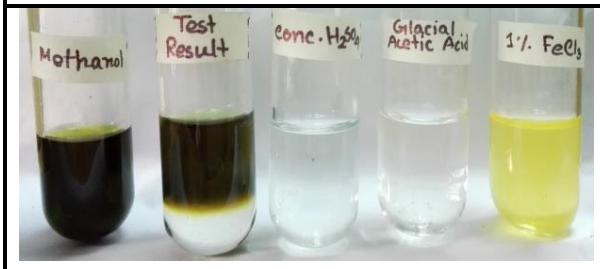
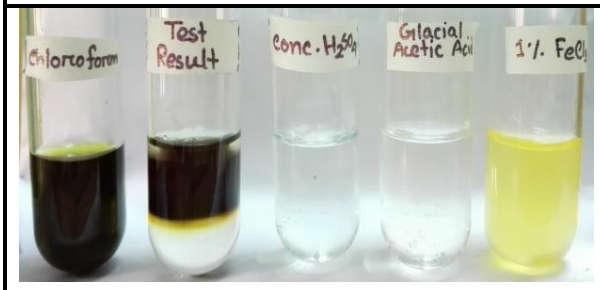
	(a) Brown ring at interface showed the presence of cardiac glycosidase on Ethanolic extract
	(b) Brown ring at interface showed the presence of cardiac glycosidase on methanolic extract
	(c) Brown ring at interface showed the presence of cardiac glycosidase on Chloroformic extract

Figure 9: Killer-Killani test for Cardiac Glycosidases (a) Ethanolic extract (b) Methanolic extract (c) Chloroformic extract

* All tests were performed in triplicate

3.1.9 Test for Amino Acids

Few drops of Ninhydrin reagentwere added to 2 ml of extract. Formation of purple colour indicates the positive result.

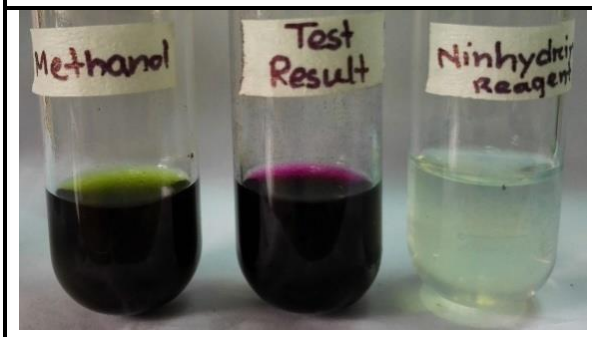
	(a) Purple colour revealed the presence of Amino acids in Ethanolic extract
	(b) Purple colour revealed the presence of Amino acids in methanolic extract
	(c) Separated layers indicated negative result for Chloroformic extract.

Figure 10: Ninhydrin test for Amino acids (a) Ethanolic extract (b) Methanolic extract (c) Chloroformic extract

* All tests were performed in triplicate

3.1.10 Test for Anthraquinones

2ml extracts were dissolved in 1ml of benzene and ammonia solution in a test tube. 4ml concentrated sulfuric acid (97%) was carefully poured from the inner wall of the test tube. No colour formation was observed.

	(a) No pink colour has formed, Anthraquinone are absent in Ethanolic extract.
	(b) No pink colour has formed, Anthraquinone are absent in methanolic extract.
	(c) No pink colour has formed, Anthraquinone are absent in Chloroformic extract.

Figure 11: Test for Anthraquinones (a) Ethanolic extract (b) Methanolic extract (c) Chloroformic extract

* All tests were performed in triplicate

3.1.11 Test for Phenols

1% ferric chloride solution and 1% potassium ferrocyanide mixed in equal amount, 3 drops of freshly prepare mixture added to 2ml of extracts. When colour changes to blue black then it is positive.

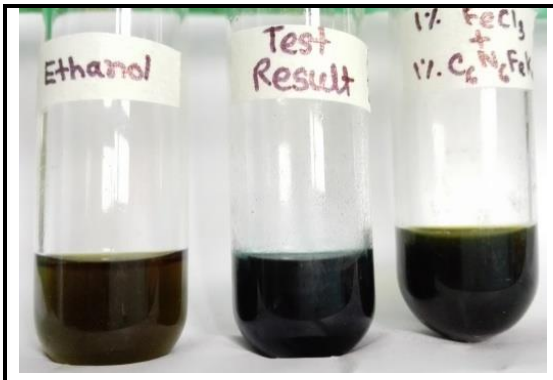
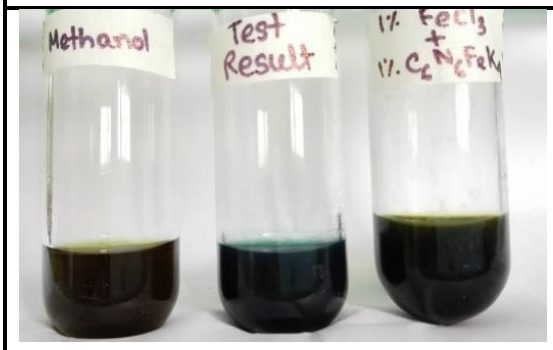
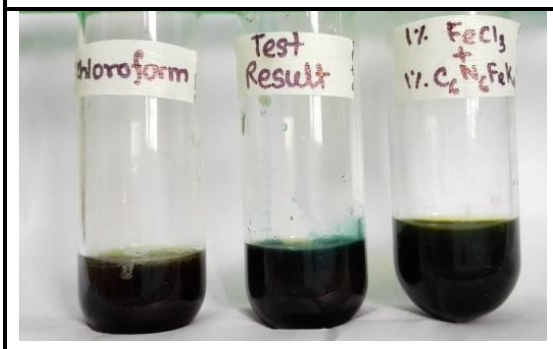
	(a) Ethnolic extract showed positive for phenols by changing the colour to blue black.
	(b) Methnolic extract showed positive for phenols by changing the colour to blue black.
	(c) Chloroformic extract showed positive for phenols by changing the colour to blue black.

Figure 12: Test for Phenol (a) Ethanolic extract (b) Methanolic extract (c) Chloroformic extract

* All tests were performed in triplicate

3.2 Study of Antioxidant Activity in Three Different Extracts of *Artemisia nilagirica*

Evaluation of DPPH free radical scavenging activity of *Artemisia nilagirica* extracts was conducted. Current study establishes the strong presence of antioxidants in locally grown *Artemisia nilagirica*. DPPH assay was conducted by both buffered and non-buffered method. Compared to the standard ascorbic standard in buffered method which gave an average free radical scavenging activity (FRSA) of 78.925% at 15.2µg/ml and 94.293% at 1000µg/ml, methanolic extract showed the highest antioxidant activity with 69.892% at 15.2µg/ml and 92.247% at 1000µg/ml. Ethanolic extract followed suit with 61.761% at 15.2µg/ml and 83.412% at 1000 µg/ml and lastly Chloroformic extract exhibited the least amount of antioxidant activity, having 61.162% at 15.2µg/ml and 81.971% at 1000µg/ml. On the other hand, in non-buffered method the standard ascorbic acid gave an average free radical scavenging activity of 71.673% at 15. 2µg/ml and 93.915% at 1000µg/ml. Methanolic extract showed antioxidant activity with 62.962% at 15.2µg/ml and 90.247% at 1000µg/ml. Ethanolic extract followed suit with 59.542% at 15.2µg/ml and 79.992% at 1000 µg/ml and lastly Chloroformic extract exhibited the least amount of antioxidant activity, having 56.691% at 15.2µg/ml and 76.537% at 1000µg/ml. Absorbances of the extracts are shown in Figure 13 and Figure 14 and the antioxidant activities are shown in Figure 15 and Figure 16. The maximum extent of both buffered and non-buffered DPPH radical scavenging was elicited by the Methanolic extract.

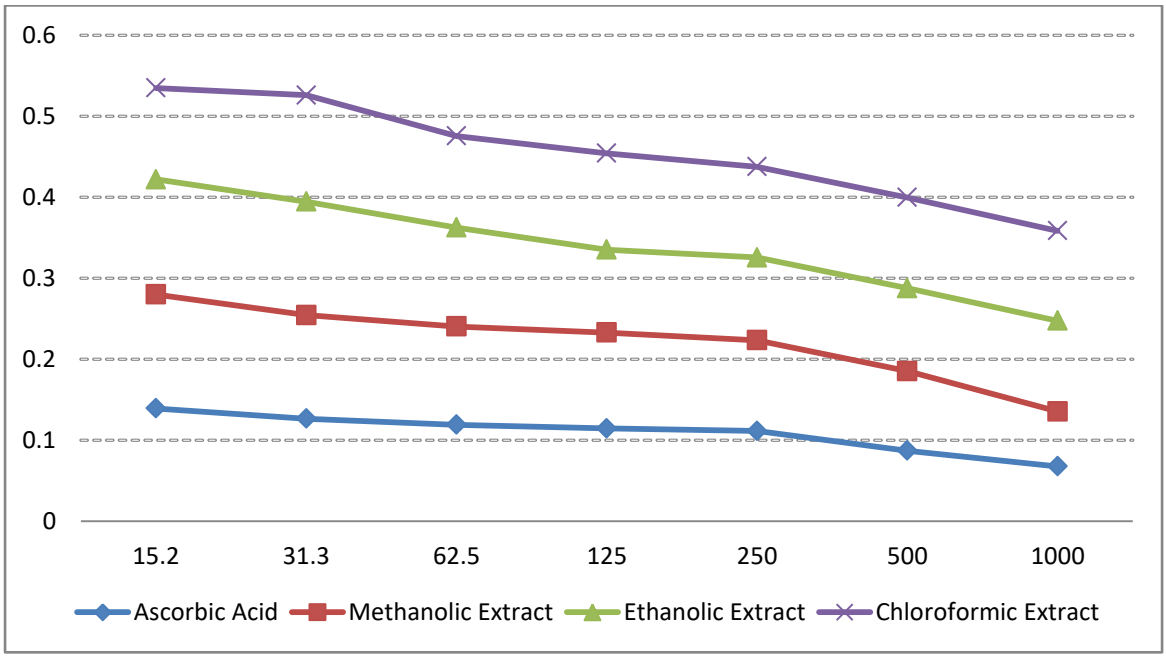


Figure 13: Graphical representation of Absorbance of Buffered Samples

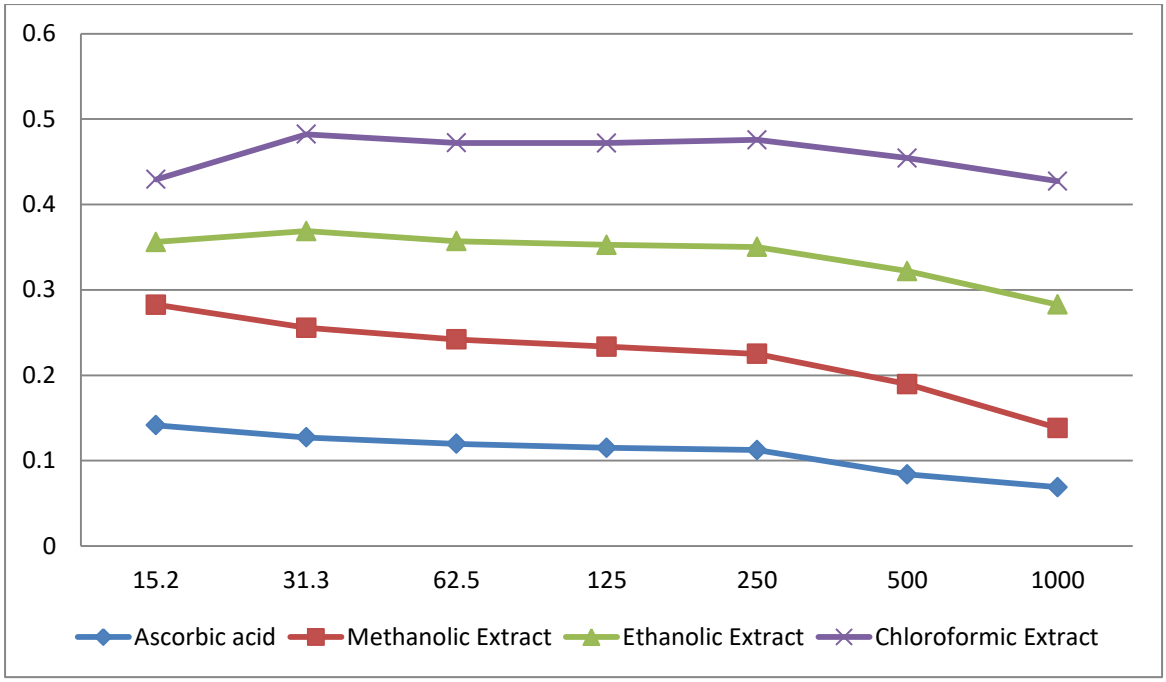


Figure 14: Graphical representation of Absorbance of Non-Buffered Samples

* All tests were performed in triplicate

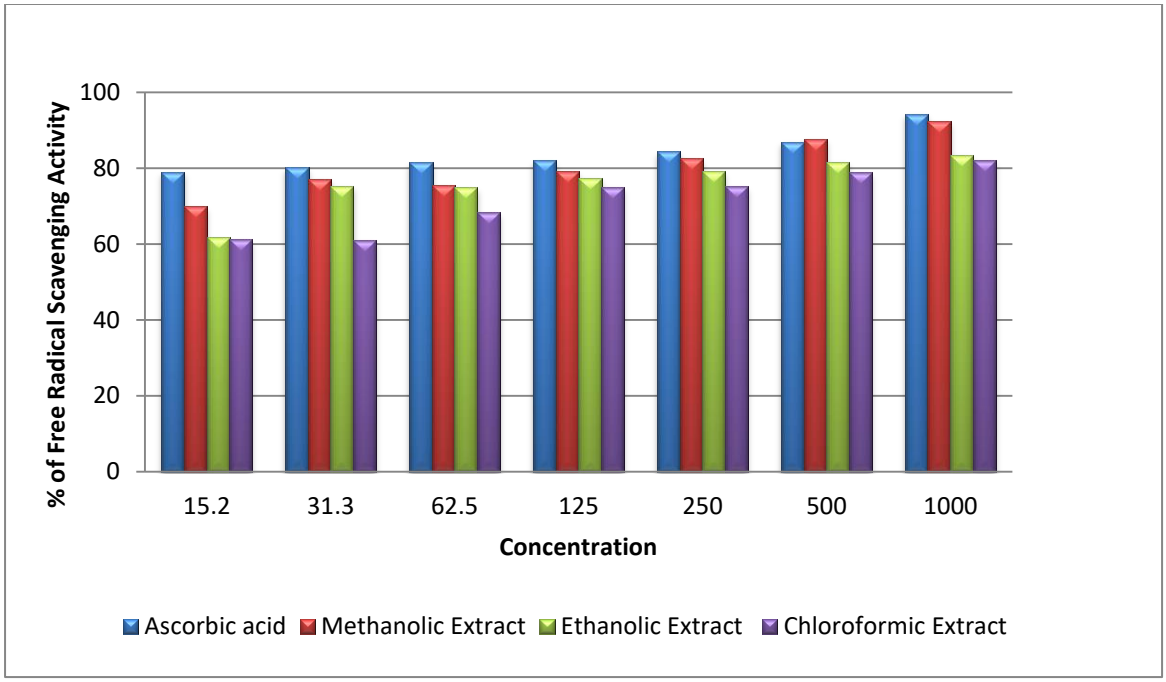


Figure 15: Graphical representation of Free Radical Scavenging Activity of Buffered Samples

* All tests were performed in triplicate

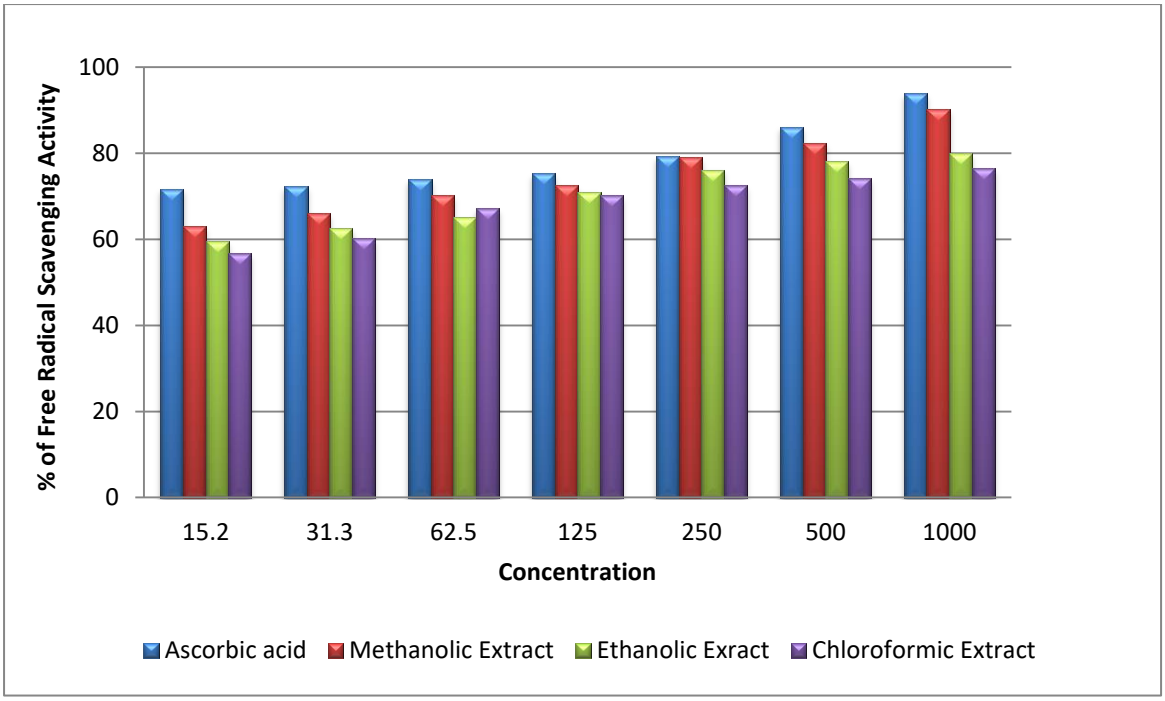


Figure 16: Graphical representation of Free Radical scavenging activity of non-buffered samples

* All tests were performed in triplicate

3.3 Antibacterial Activity of Different Concentrations of Methanolic, Ethanolic and Chloroformic Extracts of *Artemisia nilagirica*

Antibacterial activity test for all three types of extract ethanolic, methanolic and chloroform extracts were performed using agar diffusion method. The zone of inhibitions measured in millimeters (mm) and compared with the zone of inhibitions of antibiotics which were used as positive control. Antibacterial activity of the three different extracts with positive control has been shown in Table 2, table 3 and table 4; the comparison of zone of inhibition of extracts and antibiotics has been graphically presented in Figure 20, Figure 21 and Figure 23. In vitro antimicrobial screening of ethanolic, methanolic and Chloroform extracts of *Artemisia nilagirica* were carried out using crude extracts and subsequently diluted concentrations of 80µg/µl and 60µg/µl to compare the extent of antimicrobial activity that the extracts possess. Ampicillin was used as positive control for *Staphylococcus aureus*. Cefoxitin served as positive control for *Shigella flexneri*, Kanamycin was served as positive for *Bacillus subtilis* whereas Chloramphenicol was served as positive control for *Streptococcus pneumoniae*. Ciproflaxin was served as positive for *E.coli*. Results of the antimicrobial assay of ethanolic (Figures 17 and 21), methanolic (Figures 18 and 20) and chloroform(Figures 19 and 22) extracts are shown in Tables

2, 3 and 4 respectively. Crude methanolic extract exhibits highest antimicrobial activity against *E.coli*and *S.flexneri*. Against *Bacillus subtilis* and *S.pneumoniae* crude methanolic and ethanolic extracts exhibit similar antimicrobial activity. Only Chloroformic extracts exhibits antimicrobial activity against *Staphylococcus aureus* (Table 4). However, 80µg/µl concentration of Methanolic extracts (Figure 20) had higher antimicrobial activity than 80µg/µl concentration of ethanolic extract (Figure 21) for all test strains. 60µg/µl did not prove to be an effective concentration to prevent microbial growth of *S. pneumonia* alone.

Table 2: Effect of different concentrations of methanolic extract of Nagdana on the mean diameter inhibition of test organisms

Test Organism	**Average zone of inhibition(mm)**			
	Positive control	**Crude extract**	**80µg/µl**	**60µg/µl**
Staphylococcus aureus	**23.5**	**0**	**0**	**0**
Bacillus subtilis	**17.6**	**10**	**8**	**0**
Shigella flexineri	**20**	**10.7**	**7.6**	**5**
Streptococcus Pneuminiae	**23**	**11.2**	**8**	**0**
Escherichia coli	**25.2**	**14**	**14**	**6**

* All tests were performed in triplicate

Table 3: Effect of different concentrations of ethanolic extract of Nagdana on the mean diameter inhibition of test organisms

Test Organism	**Average zone of inhibition(mm)**			
	Positive control	**Crude extract**	**80µg/µl**	**60µg/µl**
Staphylococcus aureus	**27**	**0**	**0**	**0**
Bacillus subtilis	**15.3**	**8**	**8**	**4**
Shigella flexineri	**19**	**10**	**9**	**0**
Streptococcus Pneuminiae	**19.5**	**10**	**7.8**	**0**
Escherichia coli	**24**	**14**	**12**	**8**

* All tests were performed in triplicate

Table 4: Effect of different concentrations of Chloroformic extract of Nagdana on the mean diameter inhibition of test organisms

Test Organism	**Average zone of inhibition(mm)**			
	Positive control	**Crude extract**	**80µg/µl**	**60µg/µl**
Staphylococcus aureus	**23**	**9**	**7**	**4**
Bacillus subtilis	**15.6**	**6**	**8**	**3.8**
Shigella flexineri	**20**	**8**	**10**	**0**
Streptococcus Pneuminiae	**22.4**	**0**	**0**	**0**
Escherichia coli	**25.5**	**8**	**8**	**0**

* All tests were performed in triplicate

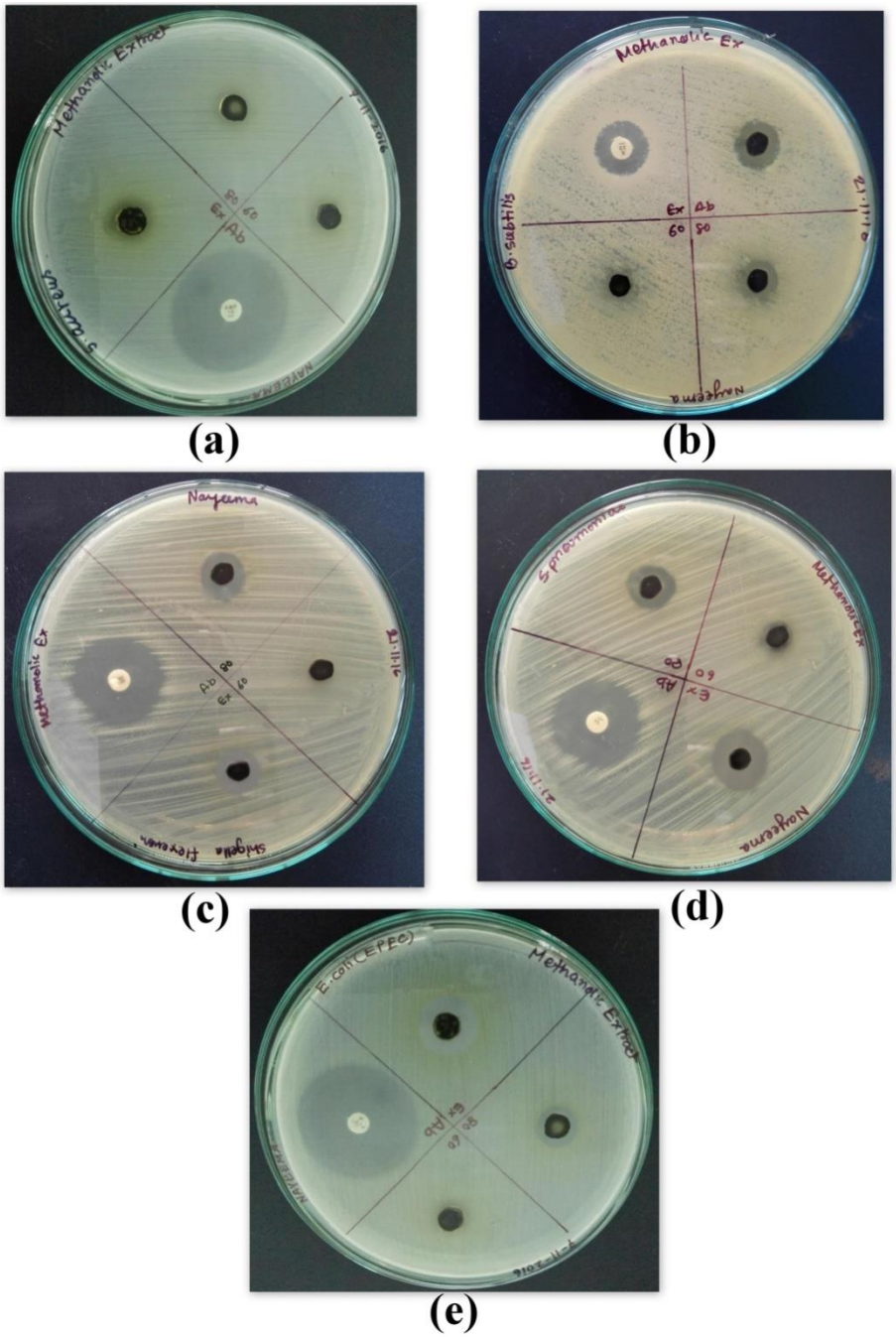


Figure 17: Antibacterial Activities of different concentration of methanol extract (a) *S.aureus*(b) *Bacillus subtilis*(c) *S. flexneri*(d) *S. pneumoniae*(e)*E.coli*

* All tests were performed in triplicate

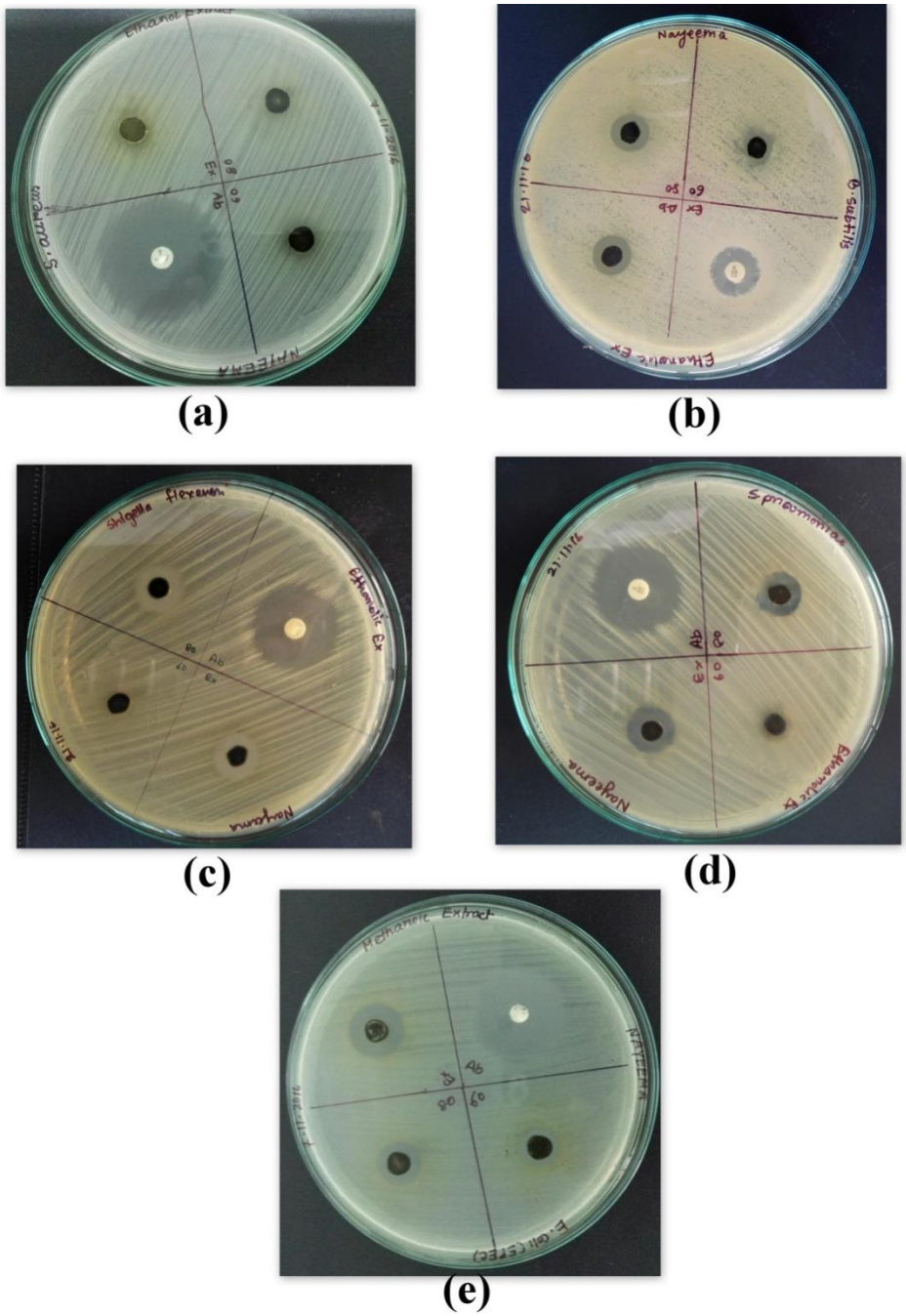


Figure 18: Antibacterial Activities of different concentration of ethanol extract (a) *S.aureus* (b) *Bacillus subtilis* (c) *S. flexneri*(d) *S. pneumoniae* (e) *E.coli*

* All tests were performed in triplicate

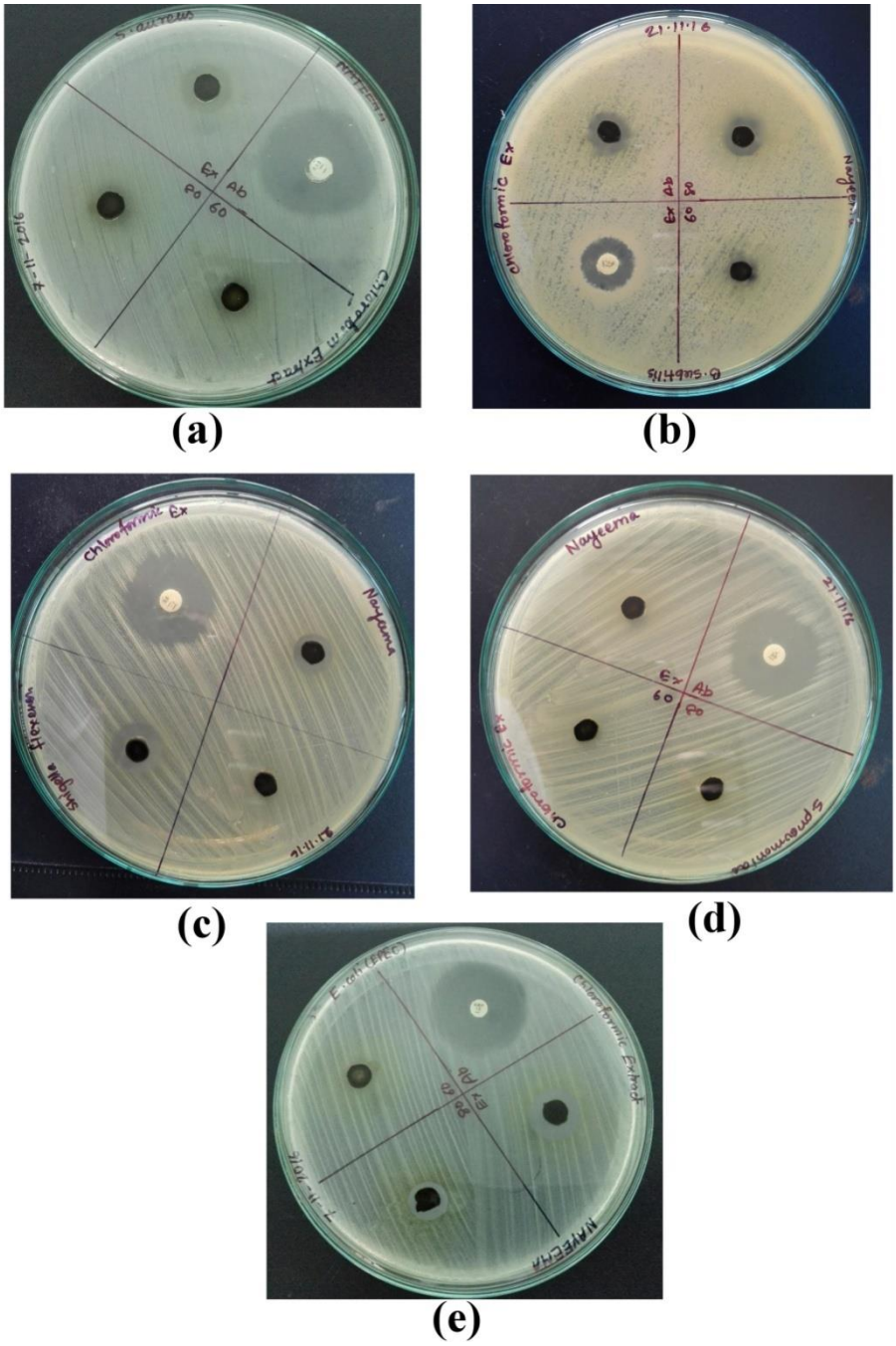


Figure 19: Antibacterial Activities of different concentration of Chloroform extract (a) *S.aureus* (b) *Bacillus subtilis* (c) *S. flexneri* (d) *S. pneumoniae* (e) *E.coli*

* All tests were performed in triplicate

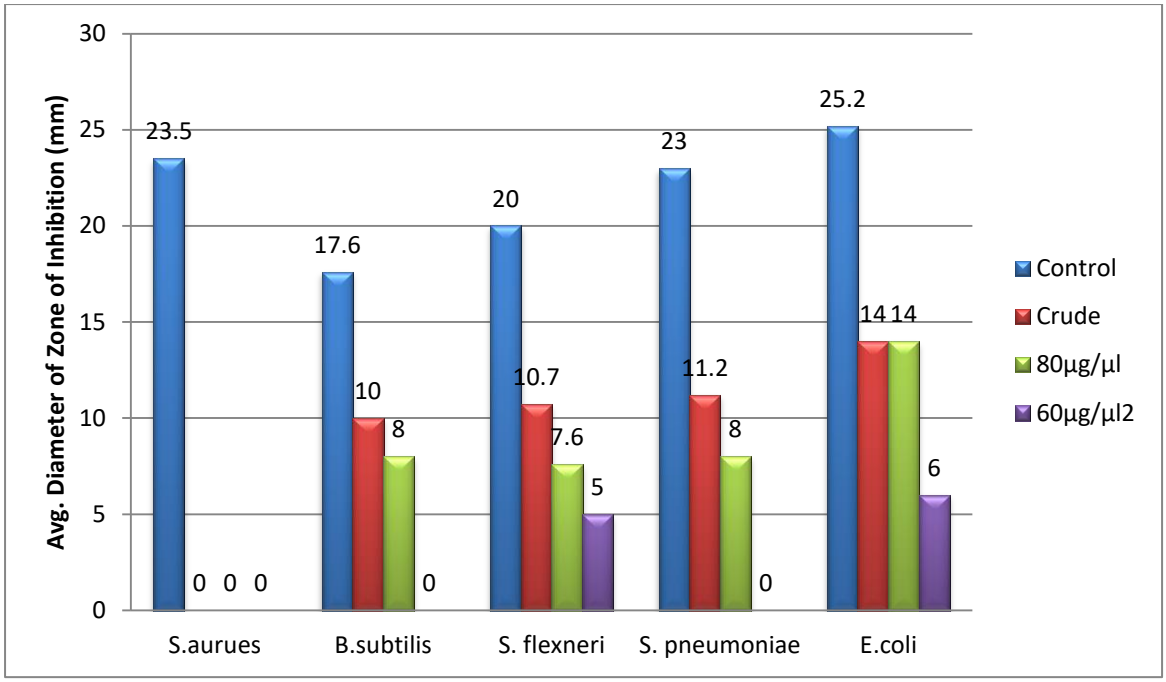


Figure 20: Graphical representation of antimicrobial activity of different concentrations of Methanolic extract

*All measurements are means of individual data obtained from triplicate

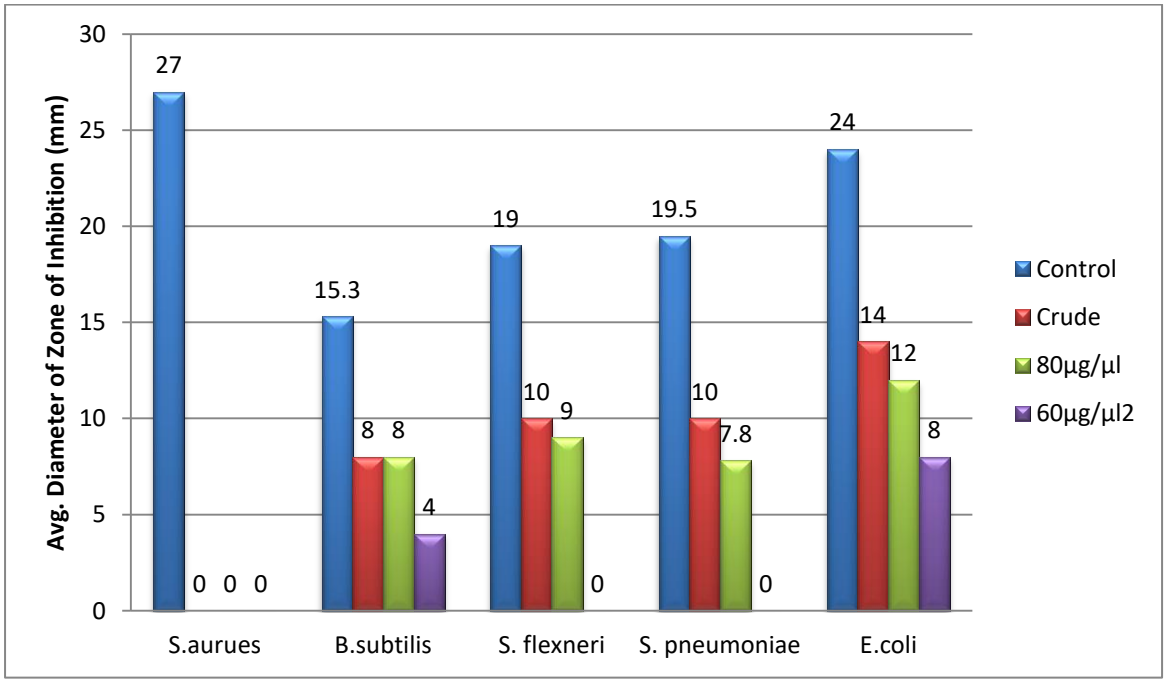


Figure 21: Graphical representation of antimicrobial activity of different concentrations of Ethanolic extract

*All measurements are means of individual data obtained from triplicate

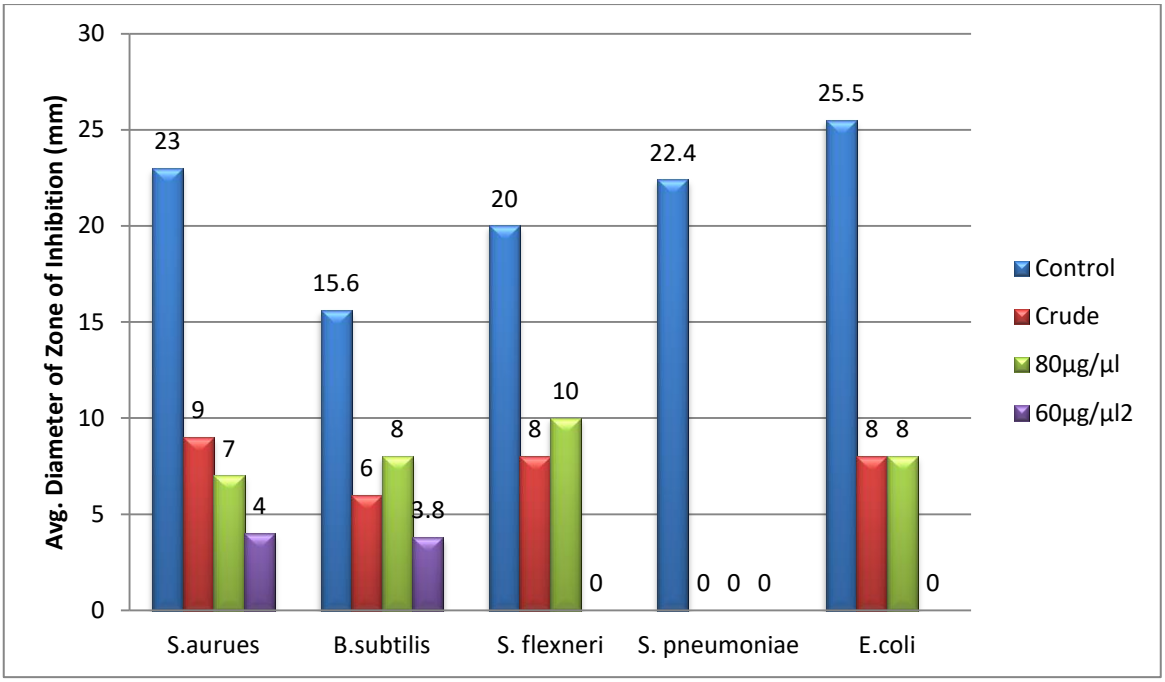


Figure 22: Graphical representation of antimicrobial activity of different concentrations of Chloroformic extract

*All measurements are means of individual data obtained from triplicate

3.3.1 Activity Index of Different Concentrations of *Artemisia nilagirica* Extracts

The activity index is a ratio of the average diameter of zone of inhibition to the average diameter of zone of inhibition of antibiotic. The activity index of the sample gives a quantifiable data for the identification of the level of antibacterial activity possessed by the sample. The bar chart below shows the comparison between the clear zones between the different types of extracts and the antibiotics used fig 20. The zone of inhibition was measured in the X-axis and the extract types along with the organisms were kept in Y-axis. Different colored bars are used to indicate the concentrations of extracts and the antibiotics.

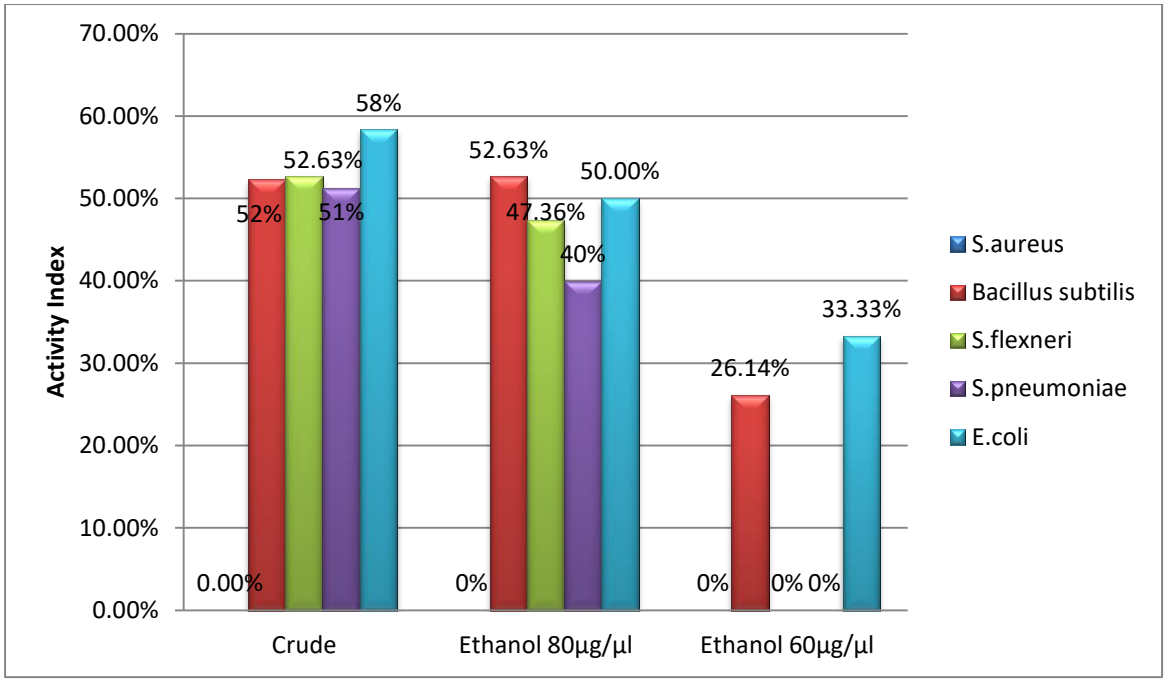


Figure 23: Graphical representation of the activity index of Ethanolic Extract of *Artemisia nilagirica*

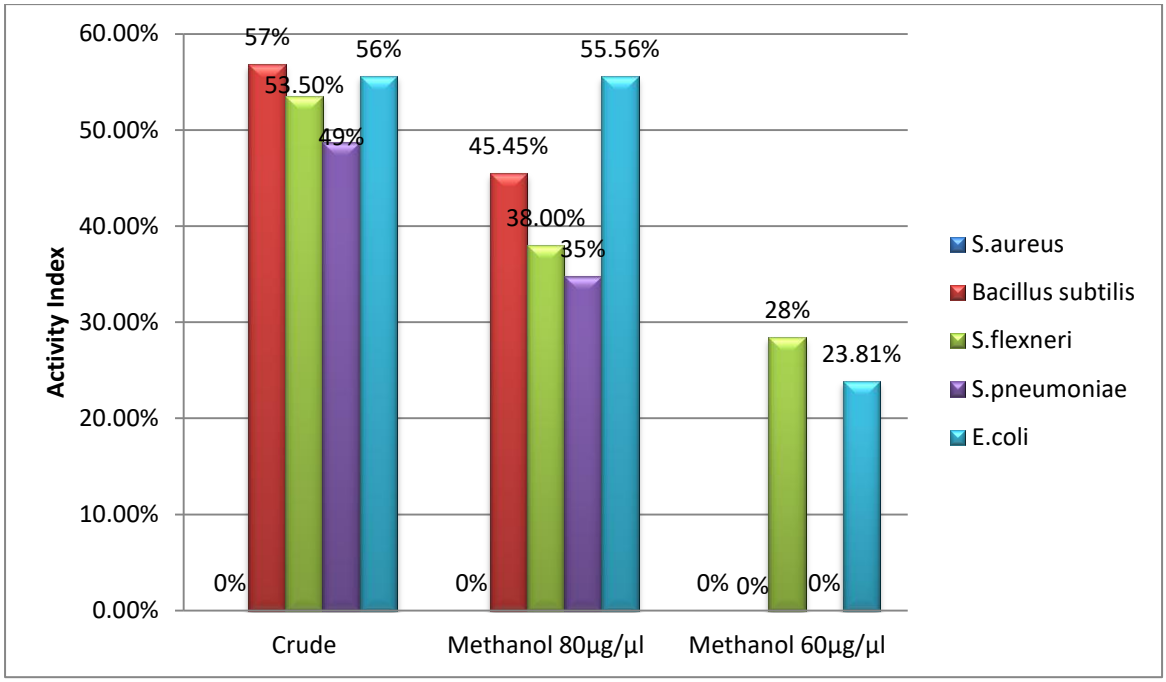


Figure 24: Graphical representation of the activity index of Methanolic Extract of *Artemisia nilagirica*

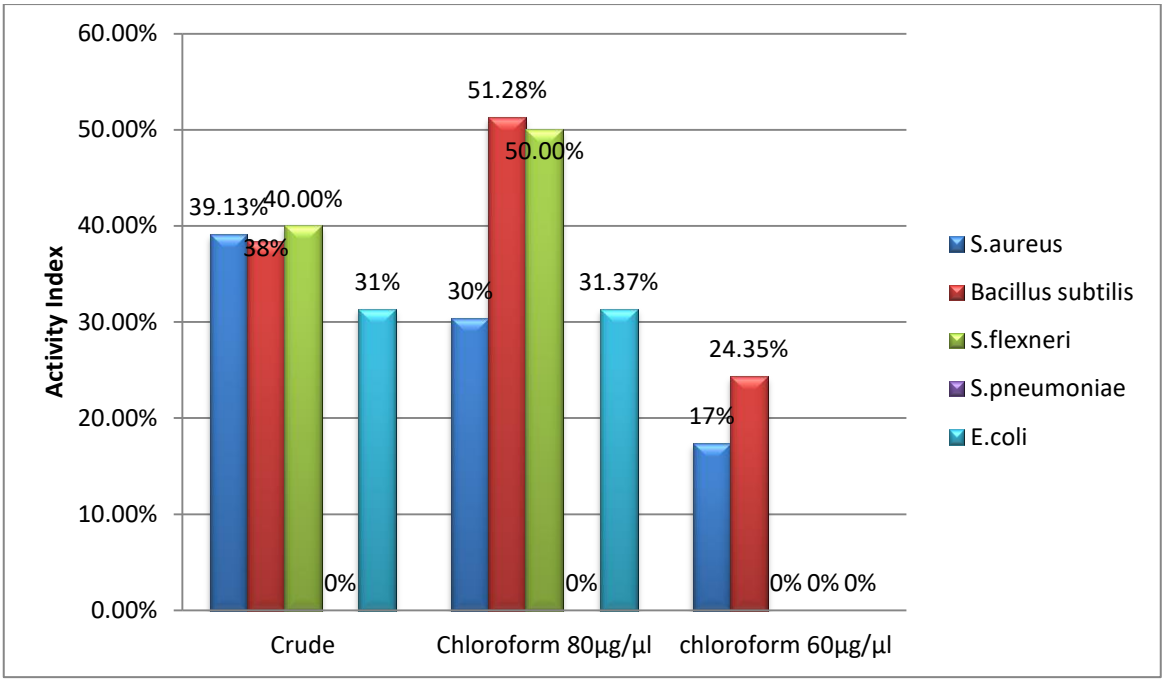


Figure 25: Graphical representation of the activity index of Chloroformic Extract of *Artemisia nilagirica*

3.3.2 Synergistic Activity of Extracts and Selected Antibiotics against Test Organisms

Synergism is tested using a combination of drugs to increase efficiency and reduce dosage as well as side effects. Synergistic activity of the crude extracts were tested with five broad-range antibiotics namely ciprofloxacin, Ampicillin, tetracyclin, kanamycin and chloramphenicol. The crude extracts did not show any synergistic activity with any of the drugs. Results of ethanolic extract are shown in Table 6 and Figure 27. Table 5 and Figure 26 showed results of methanolic extract and the results of aqueous extract are displayed in Figure 28 and Table 7.

Table 5: Average diameter of clear zone for synergistic activity of crude methanolic extract and antibiotic

Name of Organism	Average Zone of Inhibition (mm)		
	Antibiotic	**Crude Extract**	**Extract in antibiotic disc**
Staphylococcus aureus	**30**	**0**	**0**
Bacillus subtilis	**17.5**	**9**	**3.5**
Shigella flexneri	**19.5**	**8**	**8**
Straptococcus Pneumoniae	**26**	**14**	**12**
Escherichia coli	**24.5**	**13**	**12**

*All measurements are means of individual data obtained from triplicate

Table 6: Average diameter of clear zone for synergistic activity of crude ethanolic extract and antibiotic

Name of Organism	Average Zone of Inhibition (mm)		
	Antibiotic	**Crude Extract**	**Extract in antibiotic disc**
Staphylococcus aureus	**30**	**0**	**0**
Bacillus subtilis	**18**	**10**	**10.5**
Shigella flexneri	**21**	**12**	**10**
Straptococcus Pneumoniae	**22**	**10**	**0**
Escherichia coli	**25**	**12**	**12**

*All measurements are means of individual data obtained from triplicate

Table 7: Average diameter of clear zone for synergistic activity of crude chloroformic extract and antibiotic

Name of Organism	Average Zone of Inhibition (mm)		
	Antibiotic	Crude Extract	Extract in antibiotic disc
Staphylococcus aureus	28	10	0
Bacillus subtilis	18	11	0
Shigellaflexneri	20	11.5	8
StraptococcusPneumoniae	19	0	0
Escherichia coli	23	8	4.5

***All measurements are means of individual data obtained from triplicate**

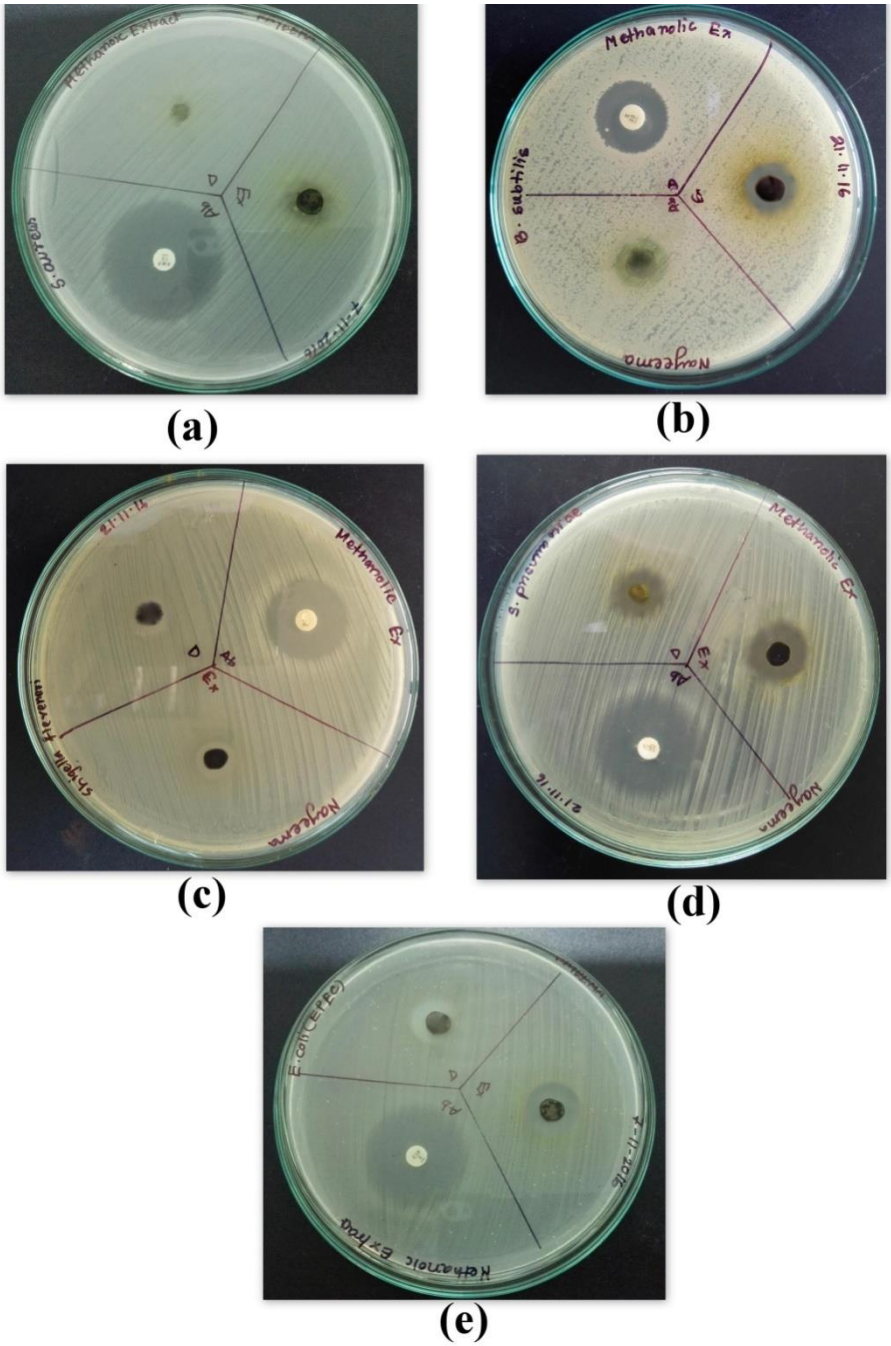


Figure 26: Synergistic activity of crude methanolic extract of Nagdana against (a) *S.aureus*; (b) *B.subtilis*; (c) *S.flexneri*; (d) *S.pneumiae*; (e) *E.coli*

*All measurements are means of individual data obtained from triplicate

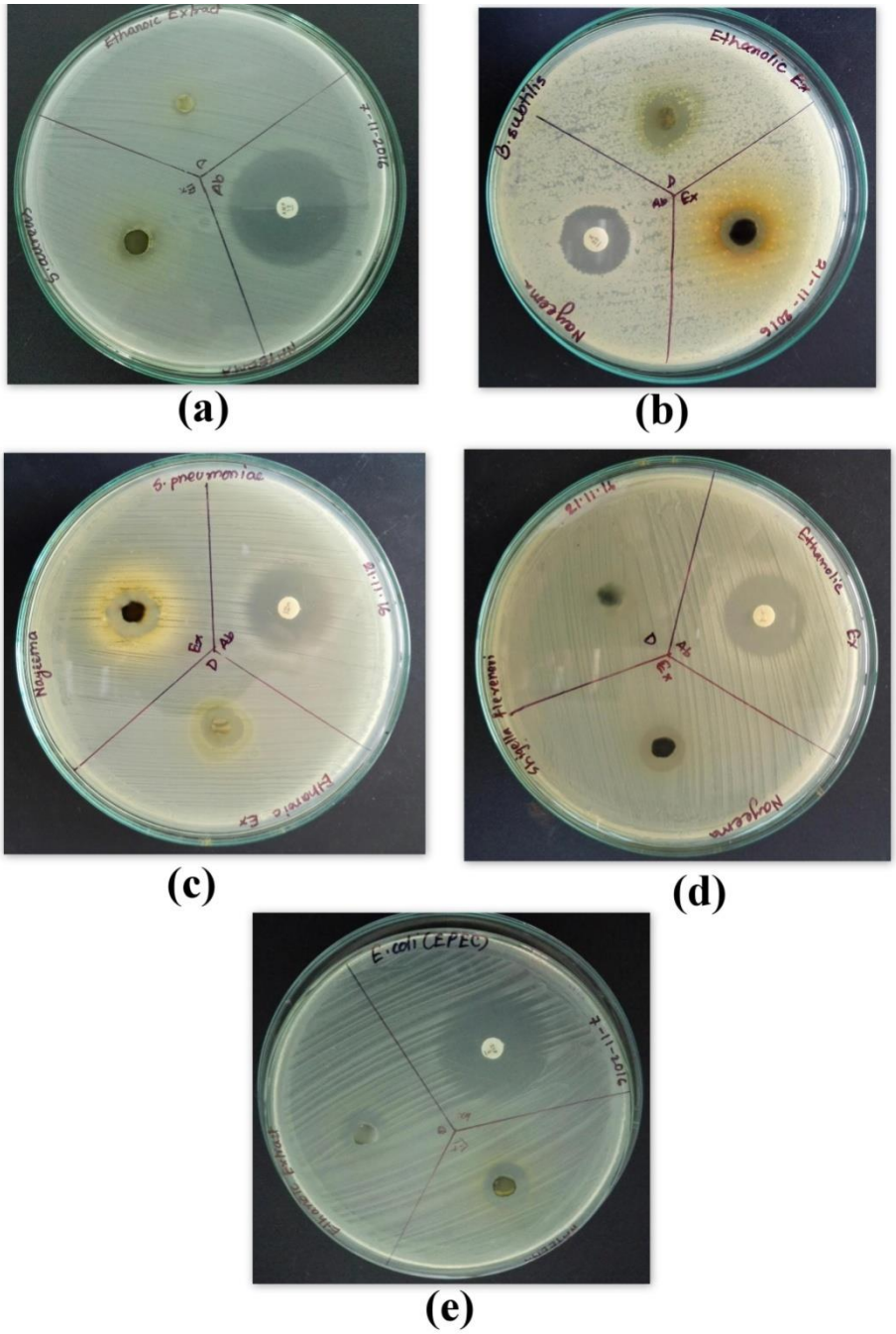


Figure 27: Synergistic activity of crude ethanolic extract of Nagdana against (a)*S.aureus*; (b) *B.subtilis*; (c)*S.flexneri*; (d)*S.pneumiae*; (e) *E.coli*

*All measurements are means of individual data obtained from triplicate

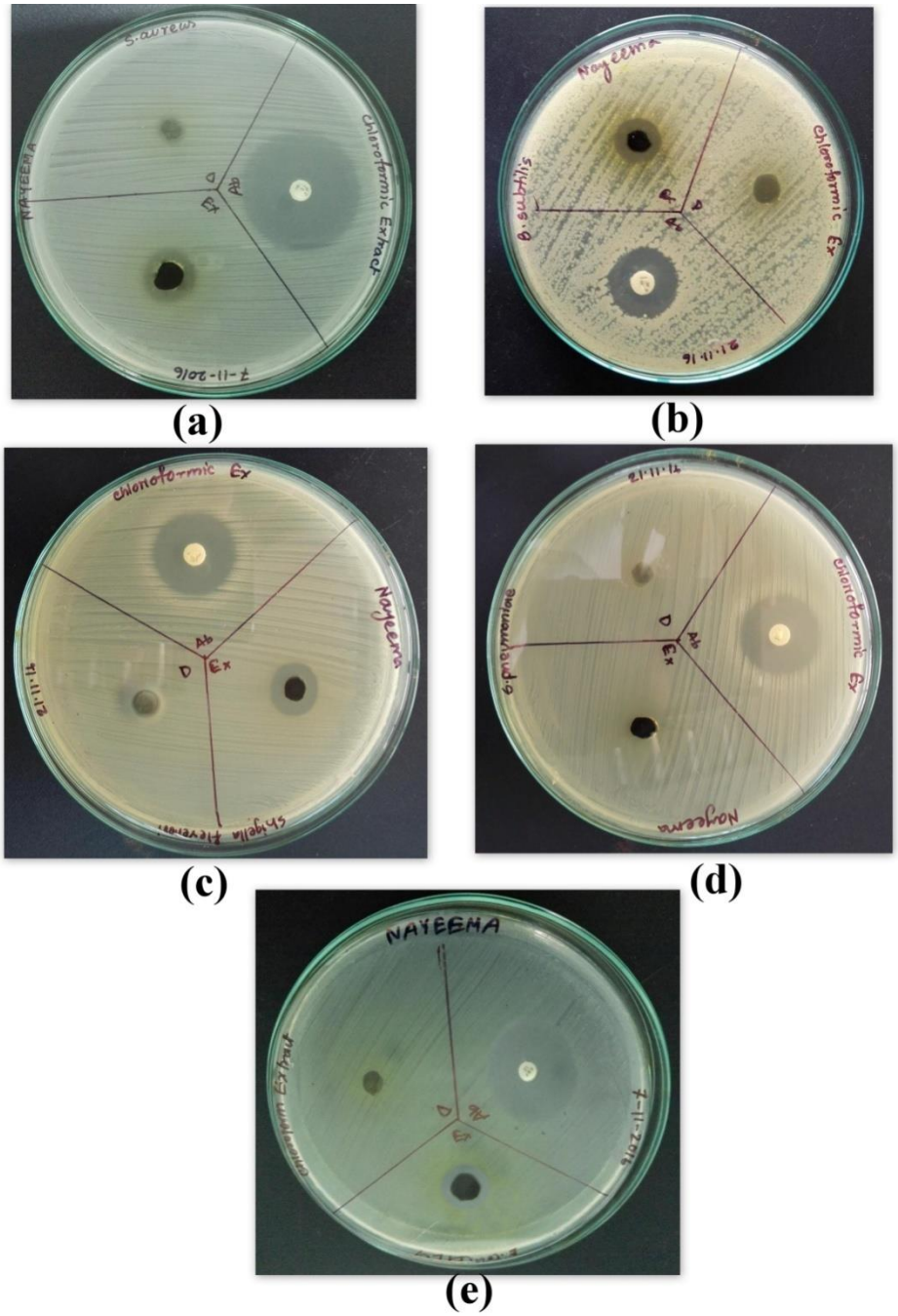


Figure 28: Synergistic activity of crude chloroformic extract of Nagdana against (a) *S.aureus*; (b) *B.subtilis*; (c) *S.flexneri*; (d)*S.pneumiae*; (e) *E.coli*

*All measurements are means of individual data obtained from triplicate

Chapter 4

Discussion

Traditional medicinal plants have the ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Chemical substances that produce a definite physiological action on the human body decides the medicinal values of plants of which the most important bioactive compounds of plants can be divided into several categories that include alkaloids, flavonoids, flavonols, quinines, essential oils, lectins, polypeptides, phenolics, polyphenols, tannins and terpenoids. The results of preliminary phytochemical screening of *Artemisia nilagirica* confirmed the presence of major classes of phytochemicals in the extracts of Nagdana. These polyphenols produced either from phenylalanine or from its precursor shikmic acid, are important dietary antioxidants because they possess an ideal structural chemistry for free radical scavenging activity. Various in vitro studies have shown their antioxidant potential in the prevention of numerous diseases (Matkowski *et al*., 2008).Phytochemical studies of *Artemisia Nilagirica* leaves revealed the presence of alkaloids, amino acids, gums, flavonoids, cardiac glycosides, tannins, phenolic compound, terpenoids, steroids and saponins in various extracts. Pavala Rani did similar analysis in 2012 and revealed the presence of alkaloids, amino acids, carbohydrates, flavonoids, glycosides, terpenoids, steroids, saponins, essential oils, tannins and phenol in various extracts. These phytochemical characterization data would be helpful in authentication of raw material or crude drug of *Artemisia Nilagirica* leaves found in Sylhet district.

It is well known that there is a relationship between antioxidant activity and the phenolic content of the plant extracts. Antioxidant properties of the methanolic extracts can be attributed to the phenolic and flavonoid contents of the *Artemisia* species. However, further studies should be performed for isolation and identification of the compounds of the extracts. Plant phenolics are important secondary metabolites with antioxidant properties due to their redox potential, which plays an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Mishra *et al*., 2011). Plant phenolics are one of the main compounds working as primary antioxidants or free radical scavengers. They work as singlet oxygen scavengers, reducing agents and hydrogen atom donators (Karaman *et al*., 2010); (Rice-Evans *et al*, 1996).The DPPH radical is commonly used in studies of antioxidants due to its stability and simple ESR signal. The DPPH radical is paramagnetic and it can react with radicals or other molecules to become part of a stable diamagnetic molecule by accepting an electron or hydrogen radical or undergo a bond forming reaction. The reaction mechanism between an antioxidant and DPPH varies depending on the antioxidant structure and the reaction media. In most cases, the reaction rate is slow and the mechanism is complex (Bonder et al., 1997). Multiple pathways are involved in DPPH reactions with antioxidants. Different

reaction products and covalent adducts have been reported (Abe et al., 2000; Takebayashi et al., 2007; Osman, 2011).

Antioxidants are believed to intercept the free radical chain of oxidations and to contribute hydrogen from the phenolic hydroxyl groups themselves. Among the examined extracts, alcoholic extracts of Nagdana exhibited the highest free radical scavenging activity. Altogether 29 constituents were identified by Tripathi in 2015 on the basis of GC-FID analysis of essential oil relates the antioxidant activity of this plant. The order of absorbance is highest in buffered reaction solution. The absorbance profile of methanol, ethanol and chloroform and no-buffered ascorbic acid is shown Figure 13and 14 where the buffered ascorbic acid gave better free radical scavenging activity than non-buffered ascorbic acid. Similarly all of the extract gave better result in buffered solution rather than non-buffered solution. Compared to the standard ascorbic standard, methanolic extract showed the highest antioxidant activity followed by ethanolic extract and lastly chloroform extract exhibited the least amount of antioxidant activity. The highest value of free radical scavenging activity were showed by 1000 µg/ml (92.24%) in buffered methanolic extract followed by ethanolic and chloroformic extract. So it can be said that in buffered reaction mixture DPPH radical scavenging activity is influenced by the polarity of the reaction medium and the pH of the mixture, so showed more percentage in inhibition of DPPH. Methanolic extracts of leaves of *Artemisia nilagirica* possessed the highest DPPH scavenging activity.

The expanding bacterial resistance to antibiotics have become a growing concern worldwide (Gradam, 2000). Intensive care physicians consider antibiotic resistant bacteria a significant or major problem in treat of patients (Lepape et al, 2009). Increasing bacterial resistance is prompting resurgence in research of the antimicrobial role of herbs against resistant strains (Alviano, 2009; Hemaiswarya et al, 2008). A large number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds. Medicinal plant extracts offer considerable potential for the development of new agents effective against infections currently difficult to treat (Wendakoonn et al, 2011). Tannins possess strong, broad spectrum antibacterial properties (Turkmen *et al*., 2007; Amarowicz *et al*., 2008; Min *et al*., 2008; Doss *et al*., 2009). Tannins affect bacterial growth by inhibiting extracellular microbial enzymes, depriving them of the substrates required for microbial growth or by the inhibition of oxidative phosphorylation (Scalbert, 1991). All three extracts showed positive for tannins in phytochemical assay. The results obtained also showed the antimicrobial properties of *A. nilagirica.* Agar diffusion method was followed and the zones of inhibition were measured in

millimeters. Ampicillin, Cefoxitin, Kanamycin, Chloramphenicol and Ciproflaxin were used as positive controls. The largest clear zone was seen in *E.coli* (14mm) for 80µg/µl Methanolic extract. For *S. flexneri* the clear zones were 9 mm at concentrations of 80µg/µl for ethanolic extract. *S. aureus* (7 mm) showed clear zone against 80µg/µl Chloroformic extract while they are resistant to Methanolic and Ethanolic extracts. *S. pneumonia* (8 mm) showed clear zone against both alcoholic extracts but resistant to Chloroformic extract. Similar study was done and Arokiyaraj in 2012 reveled that *Artemisia nilagirica* showed significant antibacterial effect against *Bacillus subtilis* (12 mm), *Staphylococcus aureus* (12 mm), *Klebsiella pneumonia* (16 mm), *Proteus mirabilis* (12 mm), *Escherichia coli* (11.5 mm). The current study showed the highest activity index for *E.coli* and lowest activity index for *S.aureus*. The antimicrobial efficacy of its essential oil was evaluated against nine pathogenic bacterial strains by Prakash Goswami et al. in 2015. The net zone of inhibition of the essential oil against these pathogenic bacterial strains was found to be in the range of 5 to 14 mm, As per the antimicrobial results, it is concluded that essential oil of *A. nilagirica* showed good activity against *Staphylococcus aureus* (MTCC 96), *Staphylococcus aureus* (MTCC 2940),and *Bacillus subtilis*, while it exhibited moderate antibacterialactivity against *Staphylococcus epidermidis, Streptococcus mutans, Klebsiella pneumoniae, Escherichia coli*, and *Salmonella typhimurium.*

Chapter 5

Conclusion

Artemisia nilagirica is one of the ancient medicinal plants that have high therapeutic value. But unfortunately the plant is listed as endangered plant according to red data book of South African National Biodiversity Institute (SANBI). Extracts of *A. nilagirica* showed the broad spectrum of antibacterial activity on the tested microorganisms. Methanol and ethanol extract exhibited high activity index against phytopathogens and methanol extract showed maximum inhibition against clinical pathogens except *S. aureus* and pneumonia, whereas Chloroform extract showed inhibitory potency against all four organisms except *S.pneumoniae*. The phytochemical analysis showed the presence of effective biological compounds like alkaloids, amino acids, flavonoids, phenols, tannins and terpenoids, amino acids, cardiac glycoside, carbohydrates but absence of Anthraquinone. These derivatives could be potential alternatives to the traditional chemical control of clinical pathogen and phytopathogenic bacteria. The presence of Flavonoids indicated antioxidant activity which was assessed by DPPH scavenging activity. The DPPH radical is paramagnetic and it can react with radicals or other molecules to become part of a stable diamagnetic molecule by accepting an electron or hydrogen radical or undergo a bond forming reaction. The reaction mechanism between an antioxidant and DPPH varies depending on the antioxidant structure and the reaction media. In this study it is clear that buffered reaction mixture DPPH radical scavenging activity is influenced by the polarity of the reaction medium and the pH of the mixture, so showed more percentage in inhibition of DPPH. Furthermore, the development of natural antimicrobials will help to decrease the negative effects of synthetic drugs. Fractionation and characterization of these active compounds will be the future work to investigate.

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Appendix-I

Reagents and Chemicals

1. **Hager's Reagent**

1% Picric Acid: dissolve 1g of picric acid in 100ml distilled water

2. **Wagner's reagent**

Iodo-potassium iodide: dissolve 2g of iodine and 6g of potassium iodide in 100ml distilled water

3. **1. 1% Ferric chloride**

1 g of Ferric chloride in 100 ml distilled water

4. **1% Potassium Ferrocyanide**

1 g of Ferric chloride in 100 ml distilled water

5. **10% Lead Acetate**

1 g of lead acetate in 100 ml distilled water

6. **10% Ferric chloride**

1 g of Ferric chloride in 10 ml distilled water

7. **5% Ferric chloride**

1 g of Ferric chloride in 20 ml distilled water

8. **Molisch's Reagent**

1-Naphol: dissolve 15g of 1-napthol in 100ml of alcohol or chloroform

9. **Ninhydrin Reagent:**

Dissolve 0.2gm ninhydrin in 100ml ethanol

10. **DPPH (2, 2-diphenyl-1-picrylhydrazyl)**

0.1 mM DPPH: 4 mg of DPPH in 100 ml methanol

APPENDIX-II

Instruments

The important equipments used through the study are listed below:

Autoclave SAARC

Freeze (-20°C) Siemens

Incubator SAARC

Micropipette (100-1000µl) Eppendorf, Germany

Micropipette (20-200µl) Eppendorf, Germany

Oven, Model : MH6548SR LG, China

pH meter, Model: E-201-C Shanghai Ruosuaa

Technology Company, China

Refrigerator (4oC), Model: 0636 Samsung

Safety cabinet

Class II Microbiological

SAARC

Shaking Incubator, Model: WIS-20R Daihan Scientific, Korea

Vortex Mixture VWR International

Weighing balance ADAM

EQUIPMENTTM,

United Kingdom