Antimicrobial and Antioxidant Properties of Various Phytochemicals Extracted from *Tectaria macrodonta*

Thesis Submitted in partial fulfillment of the requirements for the degree of

MASTER OF PHILOSOPHY

in

MICROBIOLOGY

Submitted by

VIABHAV KUMAR UPADHAYAY

Roll No. 12PDMB08

Reg. No. 12SU6401

Department of Microbiology

Sikkim University

Gangtok, Tadong - 737102

2014

सिक्किम विश्वविद्यालय

(भारतीय संसद के अधिनियमद्वारा स्थापित केन्द्रीय विश्वविद्यालय)
गुणवत्तापूर्ण प्रबंधन प्रणाली ISO 9001:2008 हेतु प्रमाणित संस्थान

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DECLARATION

I declare that the thesis entitled **"Antimicrobial and Antioxidant Properties of Various Phytochemicals Extracted from *Tectaria macrodonta*"** submitted by me for the award of **Master of Philosophy (M.Phil) Degree in Microbiology** of Sikkim University is my original work. The content of this thesis is based on the experiments which I have performed myself. This thesis has not been submitted for any other degree to any other University.

Date: 22nd July 2014

Viabhav

Viabhav Kumar Upadhayay
Roll No: 12PDMB08
Regn.No: 12SU6401

We recommend that the thesis be placed before the Examiners for evaluation.

HK

Signature of Head of the Department
Dr H K Tiwari

Bimala Singh

Signature of the Supervisor
Dr Bimala Singh

ASSISTANT PROFESSOR
Department of Microbiology
School of Life Sciences
SIKKIM UNIVERSITY
6th Mile, Samdur P.O. Tadong-Sikkim

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6th Mile, Samdur P.O. Tadong-Sikkim

6 माईल, सामदुर, तादोंग, गगंटोक - 737102 सिक्किम, भारत
दूराभाष : 00-91-3592 - 251067, 251403, फैक्स :- 251067/251757
6th Mile, Samdur, PO Tadong 737102, Gangtok, Sikkim, India
Phones : 00-91-3592-251067, 251403, Fax - 251067/251757
website : www.sikkimuniversity.in/www.sikkimuniversity.ac.in
Email : sikkimuniversity@gmail.com

सिक्किम विश्वविद्यालय

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All the assistance and help received during the course of the investigation have been acknowledged by him.

Place: Gangtok, Sikkim

Date: 22nd July 2014

Dr. Bimala Singh

(Supervisor)

Department of Microbiology

ASSISTANT PROFESSOR
Department of Microbiology
School of Life Sciences
SIKKIM UNIVERSITY
6th Mile Samdur P.O. Tadong-Sikkim

6 माईल, सामदुर, तादोंग, गगंटोक - 737102 सिक्किम, भारत

दूरभाष : 00-91-3592 - 251067, 251403, फैक्स :- 251067/251757

6th Mile, Samdur, PO Tadong 737102, Gangtok, Sikkim, India

Phones : 00-91-3592-251067, 251403, Fax - 251067/251757

website : www.sikkimuniversity.in/www.sikkimuniversity.ac.in

Email : sikkimuniversity@gmail.com

ACKNOWLEDGEMENTS

All praises and thanks to ***Almighty God*** *(most merciful and beneficent) whose grace and special succor enabled me to complete this M. Phil dissertation.*

With deep sense of gratitude, I would like to thank my esteemed supervisor ***Dr. Bimala Singh****, Assistant professor, Department of Microbiology, Sikkim University to whom I owe my entire effort. Without her keen supervision, erudite guidance, constant encouragement, precise discussion and invaluable suggestions, this work would not have been possible. I am deeply indebted to her for her untiring cooperativeness.*

I express my sincere gratitude to ***Prof. (Dr). Jyoti Prakash Tamang****, Dean of School of Life Sciences, Sikkim University for his encouragement and blessing. I take this opportunity to express my sincere gratitude to* ***Dr. Hare Krishna Tiwari****, H.O.D, Department of Microbiology for his valuable suggestions.*

I owe my special words of thanks to my respected teachers ***Dr. Nagendra Thakur*** *and* ***Dr. Buddhiman Tamang,*** *Assistant Professor, Department of Microbiology, Sikkim University for their precious gift of knowledge and invaluable guidance and advice. I would like to offer my sincere thanks to* ***Mrs. Radha Basnet****, Laboratory Assistant, for her kind support.*

I am highly thankful to ***Ms. Pramila Koirala*** *and* ***Ms. Bhumika Poudyali****, Research Scholar, Department of Microbiology, Sikkim University, for providing support and guidance throughout my dissertation work. I would also like to thank my friends for their direct and indirect support during the course of time.*

Viabhav

Viabhav Kumar Upadhayay

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List of Abbreviations used

%	Percent
(v/v)	Volume per volume
(w/v)	Weight per volume
µg	Microgram
µl	Micro-liter
BHT	Butylated Hydroxy Toluene
CFU	Colony forming unit
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylene diamine tetra acetic acid
gm	Gram
h	Hours
MBC	Minimum bactericidal concentration
mg	Milligram
MIC	Minimum inhibitory concentration
ml	Milliliter
mm	Millimeter
nm	Nanometer
pH	Power of H ion concentration
SD	Standard deviation
UV	Ultraviolet
WHO	World health organization

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Chapter 1

INTRODUCTION

1. INTRODUCTION

Plant based drugs have been isolated from natural sources for therapeutic purposes (Kaisar *et al.*, 2013). About 20 – 30% modern drugs and synthetic analog of natural products have been derived from traditional plants used by many Indian communities (Siwach *et al.*, 2013). Plants in traditional medicine contain wide array of substances that are used to treat various chronic and infectious diseases (Bunawan *et al.*, 2013). Most of the synthetic drugs already in use to cure diseases are of concern, as they lead to the development of drug resistance microorganisms and exert other adverse effects in human body (Chandra *et al.*, 2014). To overcome this problem, the antimicrobial substances from plants should always be explored, because plant derived compounds are less toxic, effective in the treatment of infectious diseases and simultaneously mitigate many side effects (Hemalatha *et al.*, 2013). The health beneficial properties of plants are attributed mainly to the secondary metabolites or bioactive compounds such as tannin, flavonoid, steroid and alkaloid (Ahmad *et al.*, 2009), which are synthesized in specific or all parts of plants. These plant based compounds have revealed potential antimicrobial, antioxidant, anticancer and antidiabetic activity (Hemalatha *et al.*, 2013). Most of the bioactive plant metabolites used for curing many diseases like hypertension, cold flu, cancer and eczema for centuries, have been identified and isolated for pharmaceutical uses (Aziz *et al.*, 2003). The beneficial medicinal effects of plant products usually result from the combinations of secondary metabolites or bioactive compounds present in the plant (Donald *et al.*, 2000).

The "bioactive chemicals" are defined as phytochemicals that are present naturally in plants as secondary metabolites and possess several bioactivities such as antibacterial, antioxidant, antivirus, antifungal and anticancer (Aziz *et al.*, 2003). The usage of medicinal herbs for their antioxidant properties against free radicals and to reduce their hazardous health effects is an emerging area under discussion to many researchers (Pourmorad *et al.*, 2006). The other major biological significance of medicinal plants is their antimicrobial activity against pathogenic microorganisms. The rampant usages of commercial antibiotics for the treatment of infectious diseases have led to the emergence of drug resistant pathogens (Wendakoon *et al.*, 2012).

The secondary metabolites or phytochemicals are considered as raw material for the production of medicinal drugs with antimicrobial and disease preventive properties

(Ahmed *et al.*, 2009). Therefore, there is a necessity to screen for medicinal plants for isolation of bioactive compounds for manufacturing pharmaceutical drugs (Purkayastha *et al.*, 2012). The bioactive compounds of plants have been used as drugs, functional foods and dietary supplements (Aziz *et al.*, 2013). During the last 20 years, the reports of various human infections are increasing due to the development of microbial resistance to some of the synthetic antimicrobial drugs (Alavijeh *et al.*, 2012). The resistances of microorganisms towards drugs coupled to the detrimental side effects of several antibiotics (Bhullar *et al.*, 2012) have led to the investigation of new antimicrobial substances from alternative sources such as plants (Devanaboyina *et al.*, 2013). Hence plant based antimicrobial compounds became popular because various plant extracts exhibited antimicrobial (Lamichhane *et al.*, 2014) and potent antioxidant properties due to the presence of secondary metabolites like alkaloids, tannins, flavonoids and phenolic compounds (Venkata *et al.*, 2012).

The plant selected for the present study is *Tectaria macrodonta* locally known as *'Kaalo nigure / Kaalo unew'* and belongs to the Family *Dryopteridaceae*. This is a terrestrial fern, found in the Eastern Himalayas (Dixit *et al.*, 1978). Ferns and their related plants have little economic significance to mankind as compared to other groups, particularly angiosperms; Hence these are understood poorly, overlooked or often ignored for medicinal uses by the society (Sukumaran *et al.*, 2012). Numerous species of *Tectaria* are being used as ethnomedicine to cure different diseases (Hardik *et al.*, 2013). The rhizome decoction of *Tectaria coadunate* or *Tectaria macrodonta* is given to children in stomachache by the local people of Nepal (Joshi *et al.*, 2011), Kumaun Himalaya (Upreti *et al.*, 2009), Darjeeling district (Benniamin, 2011), Amarkantak (Singh *et al.*, 2005) and Ratnagiri district of Maharashtra (Masal *et al.*, 2010). The fronds (leaves) of *Tectaria* possess antibacterial activity (Parihar *et al.*, 2010) and the rhizome powder of the plant is used as potent remedies for cough, cold and fever (Kamble *et al.*, 2010). Decoction of the leaves is given to treat asthma and bronchitis (Parihar *et al.*, 2005). Some species of *Tectaria* are also used as green vegetables (Bhagat *et al.*, 2010) in regions of Terai, Nepal and Madhya Pradesh (Sukumaran *et al.*, 2012).

The methanolic, ethanolic and aqueous extracts of *Tectaria macrodonta* exhibited potential antimicrobial activity against different test microorganisms associated with

diarrhea, gastroenteritis (*Escherichia coli*), pneumonia (*Klebsiella pneumonia),* food borne diseases (*Bacillus cereus*) and general infectious diseases (*Staphylococcus aureus* and *Proteus vulgaris*) (Poudyali, 2013). Numerous reports concluded that the species of *Tectaria* possess various bioactive compounds which may be beneficial in preventing several infections (Sukumaran *et al.,* 2012). The antimicrobial activity of *Tectaria macrodonta* may be attributed to various phytochemicals namely saponins, tannins, anthocyanin, flavonoid, phenol and alkaloid (Masal *et al.,* 2010). The ethanol extract of *Tectaria macrodonta* have exhibited promising antibacterial and antioxidant property (Poudyali, 2013).

Therefore, the present study aims to extract some of the crude phytochemicals (flavonoid, tannin, saponin, alkaloid and steroid) present in rhizome of *Tectaria macrodonta* using various solvents and standard procedures and analyze for their potential antimicrobial and antioxidant properties. Since the phytochemicals extracted from *Tectaria macrodonta* have not been subjected to column purification therefore they have been termed as 'crude phytochemicals'.

1.1 RATIONALE AND SCOPE OF STUDY

The microorganisms acquire resistance to particular drugs and find new ways to strengthen themselves and live longer and further cause various ailments in human (Tchakam *et al.,* 2012). In such cases, plants can serve as a good alternative source for natural medicinal substances to defend against pathogenic bacteria, fungi and helminthes (Singh *et al.,* 2005). Plants are the source of antimicrobial substances as they are used for inhibition of antibiotic resistance bacteria (Verma *et al.,* 2012). Under the condition of stress or microbial infections, plants synthesize secondary metabolites to overcome the stressful environment. These bioactive secondary metabolites can be potential natural sources of drugs having potent antimicrobial and antioxidant properties (Abdallah, 2011). Certain compounds isolated from plants have been screened for antioxidant properties and therefore plants as dietary supplements can be good sources for scavenging of free radicals in human body (Lamichhane *et al.,* 2014). Most of the research has been done on higher plants for screening of drugs but most of lower plant such as pteridophytes is not fully explored for medicinal purposes (Sukumaran *et al.,* 2012). It has been proved that some ferns and other allied

plants exhibit strong antibacterial and antioxidant activities (Parihar *et al.*, 2010). *Tectaria macrodonta* is a fern and found in many parts of India and is used as medicine for prevention of bacterial diseases (Hardik *et al.*, 2013).

Sikkim is well known for its biodiversity hot spot region with a unique combination of nature and ethnic groups (Idrisi *et al.*, 2010). Most of the folk healers and people of various communities rely upon traditional medicines. There are many folk healers with different identity and healing knowledge like '*Baidya*' in Nepali Community, '*Amji*' and '*Pow*' in Bhutia Community and '*Bongthing*' in Lepcha Community, who give treatment for local people by folk medicines (Idrisi *et al.*, 2010). The traditional knowledge about the folk medicines can offer a good platform for the detection of novel plant based drugs (Abdallah, 2011). However, due to the insufficient exploration and poor documentation of traditional medicinal plants used by the folk healers, there is possible loss of this ancient wisdom (Idrisi *et al.*, 2010). Therefore, the present study is an effort to evaluate the antioxidant and antimicrobial properties of crude phytochemicals from rhizome of *Tectaria macrodonta.*

1.2 AIMS AND OBJECTIVES

- To extract and analyze the various crude phytochemicals (Alkaloids, Flavonoids, Saponins, Steroids, Tannins) from *Tectaria macrodonta.*
- To evaluate the antimicrobial property of extracted crude phytochemicals of *Tectaria macrodonta.*
- To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of extracted crude phytochemicals of *Tectaria macrodonta.*
- To evaluate the antioxidant properties of extracted crude phytochemicals of *Tectaria macrodonta.*

Chapter 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Plants have been used as food and also for medicinal purposes since ancient times (Prakash *et al.*, 2005). Ayurveda, Siddha and Unani are the important traditional medication systems in India (Panday *et al.*, 2013). The ancient Indian scripture "*Rig-Veda*" seems to be the most primitive documentation of medicinal herbs having curable properties (Rungsung *et al.*, 2013). In Ayurveda methodology, "Sushruta Samhita" (Dalhancharya, 2009) and "Charak Samhita" describes the use of plants and their parts for curing various infections (Gewali, 2008).

Around 70 - 80% of people rely on traditional methods of treatment by medicinal plants to meet their primary healthcare needs (Prakash *et al.*, 2005). During the last decades, indiscriminate use of antibiotics caused various microbial diseases at an alarming rate, especially in developing countries (Tchakam *et al.*, 2012). Presently, resistance towards synthetic drugs has been shown by many pathogenic microorganisms creating immense problem in the treatment of infectious diseases (Mustapha *et al.*, 2012). The microbial resistance to most antibiotics occurs through the aegis of extremely efficient enzymes, efflux proteins and other transport systems that often are highly specific towards precise antibiotic molecules (Bhullar *et al.*, 2012). Therefore, the chemicals present in plant and herbal extracts can play an important role in combating diseases caused by pathogenic microorganisms. The phytochemicals such as alkaloids, tannins, flavonoids, saponin and phenolic compounds are secondary metabolites (Devanaboyina *et al.*, 2013) present in various plant extracts which have bacteriostatic and bactericidal properties (Tchakam *et al.*, 2012). Most of these secondary metabolites other than possessing antimicrobial activity, can also act as effective antioxidants (Venkata *et al.*, 2012).

Antimicrobial resistance (AMR) is resistance of a microbial pathogen to an antimicrobial drug that was initially effective for curing of infections caused by it (WHO, 2014). The evolution and spread of antibiotic resistance in bacterial pathogens is increasing globally and the resulting failures of antibiotic therapy cause thousands of deaths annually (Palmer *et al.*, 2013). In the last decade, a number of reports have been published about antibiotic resistant bacteria from both environmental and wildlife sources (Guenther *et al.*, 2011). In 2012, approx 450 000 novel cases of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant

tuberculosis (XDR-TB) has been recognized in 92 countries (WHO, 2014). The increased level of antibiotic resistance has been found in indicator microorganisms such as *Escherichia coli* from commercially reared swine, cattle and poultry population in Europe (Johan *et al.*, 2014).

The antibiotic resistance in microorganisms is created by some mechanisms including direct inactivation of the active antibiotic molecule, alteration of the sensitivity to the antibiotic by modification of the target site, efflux pumps and outer membrane permeability, mutation in nucleotide sequences and horizontal gene transfer (Dzidic *et al.*, 2007). It has been reported that *Mycobacterium tuberculosis* attains antibiotic resistance through changes in nucleotide sequences, whereas *Enterobacteriaceae* infections often possess multidrug resistant genetic cassettes (Palmer *et al.*, 2013).

Staphylococcus aureus, a Gram positive bacterium, is responsible for several diseases including folliculitis, furuncle, scalded skin syndrome, osteomyelitis, endocarditis, pneumonia, toxic shock, urinary tract infections and gastroenteritis (Kabir *et al.*, 2014). The antibiotic resistant *Staphylococcus aureus* from normal flora seem to make up a significant pool of antimicrobial gene which can be transferred to other pathogens (Stryjewski *et al.*, 2014) thus spreading the resistance traits among microbial population (Kabir *et al.*, 2014). A PCR based experiment was performed to examine *in vitro* transfer of mecA gene horizontally from methicillin-resistant *Staphylococcus aureus* to methicillin sensitive one (Sabet *et al.*, 2012). The methicillin resistant *Staphylococcus aureus* (MRSA) present a major clinical challenge to the management of serious infections worldwide (Stryjewski *et al.*, 2014). More recently, it has been found that MRSA is becoming resistant to glycopeptides, vancomycin, linezolid and daptomycin (Stryjewski *et al.*, 2014). The technique of whole-genome sequencing (WGS) provided a single platform for developing the genotypic prediction method for antimicrobial resistance of *Staphylococcus aureus* (Gordon *et al.*, 2014).

Bacillus cereus is Gram positive, spore producing, rod shaped bacteria responsible for a type of food poisoning (Pelczar, 2005). Many *Bacillus cereus* acquired resistance to commonly used antibiotics, because detailed study has been done on antibiotic-modifying enzyme (fosfomycin resistance enzymes) from *Bacillus species* and this enzyme catalyze nucleophilic addition of either L-cysteine or bacillithiol to the

antibiotic, resulting in a modified antibiotic with no bactericidal properties (Thompson *et al.*, 2014).

Pseudomonas aeruginosa is a Gram negative bacterium and the causative agent of 9 – 10 % opportunistic nosocomial infections. It has been demonstrated that its intrinsic antibiotic resistance arises from the combination of restricted outer-membrane permeability, energy dependent multidrug efflux and chromosomal encoded periplasmic β-lactamase (Hancock *et al.*, 2000). Moreover, some mutations in fluoroquinolone binding sites in *Pseudomonas aeruginosa* strains, exhibited loss of porin channels and improved beta-lactamase or cephalosporinase production (Lutz *et al.*, 2011).

A report concluded that the antibiotic resistant bacteria may interact with microflora of human colon and transfer antibiotic resistance gene (Dzidic *et al.*, 2007). Abuse of antibiotics may lead to resistance in many pathogenic bacteria (Sukumaran *et al.*, 2012). Antibiotic resistant *Escherichia coli* has been reported in intestinal flora of healthy children (Shakya *et al.*, 2013). *Escherichia coli* is the Gram negative bacterium and is a part of the normal flora of the intestinal tract of animals and human beings. But certain enterotoxigenic strains can cause gastroenteritis by producing heat labile and heat stable toxin (Pelczar, 2005). Commensal *Escherichia coli* are the reservoirs of antibiotic resistance genes in the human gut and such kind of gene might be transferred to other commensal or pathogenic organisms (Shakya *et al.*, 2013).

Beside the problem of antibiotic resistance of bacteria, another problem is the formation of free radicals in human body. In 1956, D. Harman explored the world of free radicals and gave the concept of free radicals in ageing (Oloyede *et al.*, 2013). Free radicals are molecules with unpaired electrons produced inside the body and play a significant role in various patho-physiological conditions (Valko *et al.*, 2006). Oxygen, nitrogen and other heavy metals take part in formation of free radical species and causes oxidative damage leading to carcinogenicity, cytotoxicity and mutagenicity (Oloyede *et al.*, 2013). Reactive oxygen species (ROS), superoxide, hydroxyl radical, singlet oxygen, hydrogen peroxide, nitric oxide and transition metal ions are important free radicals causing potential damage in living system (Valko *et al.*, 2006). Low level of free radicals exert beneficial role in cellular response against

protection from contagious agents or may induce mitogenic response. On the contrary, high amount of free radicals are extremely reactive and causes oxidative damage to proteins, nucleic acids and lipids (Bratic *et al.,* 2013). To overcome the unnecessary effect of free radicals, living beings get natural antioxidant defenses by different types of enzyme such as peroxidase, catalase and glutathione. However, the protective effect of these enzymes may be restricted by huge production of ROS, making cellular oxidative stress as a result of the inequality between antioxidant and oxidant species of living organisms (Gasca *et al.,* 2013). Therefore, the bioactive compounds of plants prevent the diseases related to oxidative stress (Gasca *et al.,* 2013) by maintaining an adequate balance in biological systems (Goyal *et al.,* 2013). Some of the bioactive compounds of plant are water or fat soluble (Gasca *et al.,* 2013) whose potential antioxidant activity have continued to be extensively studied over recent years (Ayoola *et al.,* 2008). Sometimes ultraviolet radiations form skin lesions and the secondary metabolites of plants have proved their effect for protecting skin against ultraviolet mediated oxidative damage (Valko *et al.,* 2006).

The analysis of antioxidant activity can be determined through spectrophotometric techniques based on the reaction of a radical, radical cation or complex with an antioxidant molecule able to give a hydrogen atom (Pisoschi *et al.,* 2011). In The DPPH method, DPPH (2, 2-diphenyl-1- picrylhydrazyl) is used as a stable free radical and when DPPH• reacts with a hydrogen donor, the reduced form of DPPH is generated with the disappearance of the violet color, so, the absorbance reduction depends linearly on the concentration of antioxidant (Molyneux, 2004).

Hydroxyl radicals are considered as highly reactive molecules which can react with sugars, amino acids, nucleotides in cells and therefore initiate cell damage *in vivo* (Sowndhararajan *et al.,* 2013). Consequently, the removal of hydroxyl radical is very important by different extracted compounds of plant (Pisoschi *et al.,* 2011). The technique of hydroxyl radical scavenging relies on the measurement of metal chelating activity of antioxidant compounds (Sowndhararajan *et al.,* 2013) using standard as gallic acid (Pisoschi *et al.,* 2011). The extract of *Bauhinia vahlii* showed hydroxyl radical scavenging activity increased with increasing concentration of sample (Sowndhararajan *et al.,* 2013).

The ABTS cation radical is formed by the loss of an electron by the nitrogen atom of ABTS (2, 2'- azino-bis (3- ethylbenzthiazoline-6-sulphonic acid). In the presence of hydrogen donating antioxidant, the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization (Pisoschi *et al.,* 2011). In ferric reducing antioxidant power (FRAP) method, the antioxidant compounds reduce the complex ferric ion-TPTZ (2, 4, 6-tri (2-pyridyl) - 1, 3, 5-triazine) (Thaipong *et al.,* 2006). The absorbance can be measured to check the quantity of iron reduced and can be correlated with the quantity of antioxidants (Pisoschi *et al.,* 2011).

For evaluating the inhibition of bacterial growth by antimicrobial substances two methods are used namely Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) described by many authors. Minimum inhibitory concentration (MIC) can be described as the lowest concentration of any antibacterial substance that inhibits observable growth of bacteria at overnight incubation (Jorgensen *et al.,* 2009). However, the Minimal bactericidal concentration (MBC) is explained as the lowest concentration of an antibacterial material killing all bacterial inoculums (Sanchez *et al.,* 2010). Agar dilution and broth dilution are the two generally used techniques to find out the Minimal inhibitory concentration (MIC) of antimicrobial substances (Wiegand *et al.,* 2008). Broth dilution method was one of the earliest testing method of antimicrobial susceptibility, where following overnight incubation, the tubes were evaluated for visible bacterial growth as confirmed by turbidity (Jorgensen *et al.,* 2009). For determination of Minimum bactericidal concentration (MBC), various concentrations of plant extracts can be added to tubes with medium containing cells and after incubation the sample which do not show growth can be inoculated on plate having LB medium. If the plate does not show any growth after overnight incubation, then the particular concentration of the crude phytochemicals can be determined as Minimum bactericidal concentration (MBC) (Sanchez *et al.,* 2010).

The medicinal plants can be the best option for overcoming the diseases caused by bacteria and free radicals because they have huge therapeutic potential (Hemalatha *et al.,* 2013). The plant *Tectaria macrodonta* or *Tectaria coadunate,* locally known as '*Kaalo nigure / Kaalo unew*' in Nepali language (Poudyali 2013, Idrisi *et al.,* 2010) and "*Kukkutnakhi*" in Marathi language (Gokhale, 1989), belongs to the Family of

Dryopteridaceae which is a terrestrial ferns, with short rhizome and spore bearing (Hardik *et al.*, 2013) found in the region of Easter Himalayas and other parts of India (Dixit *et al.*, 1978). In several ethnic communities of Nepal and Sikkim, the rhizome of *Tectaria macrodonta* is given for the prevention of dental problem (Idrishi *et al.*, 2010). In a study, it has been reported that this plant can be used in the treatment of dysentery, diarrhea and asthma (Kamble *et al.*, 2010). In past, some of study has been done on rhizome of *Tectaria macrodonta* and preliminary studies showed concentration dependent free radical scavenging activity of rhizome of *Tectaria coadunate* equivalent to that of the positive control ascorbic acid in the respective models (Ghoghari *et al.*, 2006). Several phytochemicals such as phenols, tannins, flavonoids and sterols are found in this pteridophytic fern and transverse section of rhizome present barrel shaped epidermal cells embedded with tannin (Hardik *et al.*, 2013). The methanol, ethanol and aqueous extract of powdered rhizome exhibited the antimicrobial activity against *Bacillus cereus, Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumonia* (Poudyali, 2013). The extract of *Tectaria macrodonta* possesses variety of phytochemicals which showed antioxidant activity through free radical scavenging assay (Ghoghari *et al.*, 2006).

The extracted components of plant are the important source of active natural products which differ generally in terms of their structure and biological properties (Ajaib *et al.*, 2011). The extracted components of plant such as tannins, alkaloids, flavonoids, phenolics and saponins are secondary metabolites possessing antimicrobial and antioxidants potential (Tchakam *et al.*, 2012).

Flavonoids or bioflavonoids, polyphenolics are low weight secondary metabolites (Balasundram *et al.*, 2006) found in barks, seeds or flowers of higher plant species (Kuntic *et al.*, 2014). Among the eight thousand naturally occurring phenolics, mainly flavonoids make up major group of plant phenolics (Balasundram *et al.*, 2006). Generally fifteen carbon atoms arranged in C6–C3–C6 configuration constitute the flavonoid (Balasundram *et al.*, 2006). For extraction of flavonoid from plant sample, commonly used solvents are methanol, ethanol, acetone and ethyl acetate with soxhlet apparatus (Constantine, 2007). The concentration of phenolic compounds in plants is influenced by many factors such as soil, irrigation, and climatic conditions (Chandra *et al.*, 2014). The total flavonoid content (TFC) of plant samples can be determined using the aluminium chloride assay through measuring the absorbance in

spectrophotometer (Samatha *et al.*, 2012). The flavonoids have many therapeutically active traits (Farhan *et al.*, 2012) because of its unique structure (Tsao *et al.*, 2004). For instance, the antioxidant activity of this compound determined by the electron delocalization (Chanda *et al.*, 2009) and stabilization by the resonance effect of the aromatic nucleus (Tsao *et al.*, 2004). Through a variety of mechanisms, such as chelation of metal ions and inhibition of enzymes (Chanda *et al.*, 2009) this scavenges free radicals and prevents the continuation of the free radical chain reactions (John *et al.*, 2010). Some flavonoids including apigenin, flavonol, flavones, galangin, isoflavones, chalcones and flavanones have been exposed to have potent antibacterial activity (Kumar *et al.*, 2013). They present antimicrobial mechanism through disrupting cytoplasmic membrane and reduction in cell respiration (Anandhi *et al.*, 2014). The antimicrobial activity of flavonoid is resolved by inhibition of enzyme DNA gyrase and β- hydroxyacyl-acyl carrier protein dehydratase activities (Zhang *et al.*, 2008) and inhibited the synthesis of DNA and RNA in *Vibrio harveyi* (Anandhi *et al.*, 2014). Several researchers have reported synergistic effect between flavonoids (epicatechin gallate and sophoraflavanone G) and other antimicrobial agents against drug resistant bacteria (Cushnie *et al.*, 2005). A new flavonoid "kaempferol-7,8-diglucoside" was isolated for the first time from the methanol extract of *Farsetia aegyptia Turra* by chromatographic techniques and it showed maximum inhibition against *Klebsiella pneumoniae* in well diffusion method (Atta *et al.*, 2013).

Saponins are made up of sugar moieties connected to a triterpene or steroid aglycone (Moghimipour *et al.*, 2014) and form the soap-like froth when shaken in aqueous solutions (Surendar *et al.*, 2011). Saponins have many applications in food, pharmaceutical and cosmetics industries and exhibit many pharmacological activities such as antibacterial, antiviral, antifungal, antioxidant and anti-inflammatory (Moghimipour *et al.*, 2014). The crude saponins are the mixture of many compounds which are unable to cross cytoplasmic membrane of Gram negative bacteria because of thick microbial coat. So, it is effective mostly against Gram positive bacteria such as *Staphylococcus aureus* and *Bacillus cereus* (Soetan *et al.*, 2006). The saponins can be fractioned from plant sample by using various solvent namely petroleum ether, methanol and acetone, where, petroleum ether is used for defatting of sample and mixture of acetone and methanol precipitated the crude saponin in form of amorphous

powder (Kannabiran *et al.*, 2009). A study evaluated the saponin-rich fraction isolated from *Camellia oleifera* cake and investigated its antimicrobial activity to the target bacteria and fungi in order to avoid causing harm in human (Hu *et al.*, 2012). Saponin from *Solanum anguivi* was administered by gavages to experimental rats at various dosages and investigated its effect on antioxidant enzymes and lipid peroxidation in rats (Elekofehinti *et al.*, 2012). Among the series of concentrations of crude and pure saponin fractions from the leaves of *Gymnema sylvestre* and *Eclipta prostrate*, the pure saponin gave excellent antimicrobial activities against *Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Staphylococcus aureus* and for fungal pathogens *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus flavus* (Khanna *et al.*, 2008). Beside the higher toxicity of saponin to insects, parasite worms, bacteria and fungi, low toxicity was observed in human due to low absorption from intestine (Hu *et al.*, 2012).

Tannins are high molecular weight poly-phenolic compounds found in plant part including bark, wood, leaves, fruits, and roots (Banso *et al.*, 2006). Water is used as solvent for extraction of crude tannin from dried plant sample (Banso *et al.*, 2006) through microwave technique (Cobzac *et al.*, 2005). The extraction with 80% acetone (v/v) with Sephadex LH-20 column chromatography with ethanol and 50% acetone as mobile phases are a valuable method for separation of tannins from tannin-rich plant material (Karamac *et al.*, 2007). Tannin is estimated by Folin-Denis method based on the non- stoichiometric oxidation of the molecule containing phenolic hydroxyl group (Shukla *et al.*, 2009). Huge amount of tannin are present in tea leaves and when tea leaf steeped in boiling water it develops a "tart" (astringent) flavor that is typical feature of tannins (Shukla *et al.*, 2009). The molecular weights of tannin vary from 500 to 3000 dalton (Shukla *et al.*, 2009). These compounds may be divided into two important groups as hydrolysable tannins and condensed tannins (Sharief *et al.*, 2014). In plants, condensed tannins are produced biosynthetically through the condensation of flavanols to form polymeric group and proanthocyanidins is an important example of condensed tannins. However, hydrolyzable tannins are esters of a sugar (mainly glucose) with one or more trihydroxybenzenecarboxylic acids (gallic acid). Examples of important hydrolyzable tannins are corilagin and geranin, isolated from leaves of *Eucalyptus spp* and *Geranium spp*, respectively (Cseke *et al.*, 2006). Condensed tannins have been reported to bind with cell walls of ruminal bacteria, thus, inhibiting

microbial growth and protease activity. On the other hand, it has been found that harmful protozoal population were reduced in rumen of cattles with diet supplemented with plant containing condensed tannin in higher amounts (Anantasook *et al.,* 2014). Acid-treated hydrolyzable tannins showed antibacterial activity against *Helicobacter pylori* by damaging liposomal membranes (Karamac *et al.,* 2007). The important tannin such as "epigallitannin" is considered as inhibitors of HIV replication and "punicalin" and "punicacortein C" inhibited the activity of purified HIV reverse transcriptase (Shukla *et al.,* 2009). The tannin compound have phenolic hydroxyl groups, therefore, it shows greater antioxidant activity through a couple of mechanisms such as scavenging of ROS, quenching of singlet oxygen and inhibition of lipoxygenases (Sharief *et al.,* 2014). "Pedunculagin" is the cytotoxic tannin extracted from leaves of *Pimenta dioica* and act as potent scavenger against the radical DPPH, physiological radicals including OH and robustly inhibited the NO production (Mohamed *et al.,* 2007).

Plants produce basic nitrogenous compounds having one or more heterocyclic rings, known as alkaloids (Garba *et al.,* 2013). Alkaloids are important pharmaceutical agents because of several biological traits such as antioxidant, antimicrobial potential and anti-inflammatory activities (Laghari *et al.,* 2014). In 1805, Morphine was the first medically useful drug and an example of alkaloid, isolated from the *Papaver somniferous* (Sawant *et al.,* 2013). Presence of alkaloids in plant sample can be examined by dragendorff's reagent on TLC plate (Singh *et al.,* 2011), where dragendorff positive substances appear as orange spots on yellowish or brownish colored background (Santos *et al.,* 2013). Alkaloids have the ability to intercalate with DNA of microorganisms, therefore they present bactericidal and anti-diarrheal effect (Garba *et al.,* 2012). The alkaloid fraction extracted from *Prosopis julifora,* identified by GC-MS analysis, has been reported to give excellent antifungal activity against five clinical and five environmental strains of *C. neoformans* by disc diffusion assay (Valli *et al.,* 2014). Alkaloid-enriched extracts from *Prosopis juliflora* pods exhibited antibacterial activity against *Micrococcus luteus, Staphylococcus aureus* and *Streptococcus mutans* with MIC in range of 50 µg/mL, whereas alkaloid extract from the pods served as a potential source for the alternative feed additive that reduces undesired production of CH_4 and CO_2 during ruminal digestion. Hence, reducing their emission into the atmosphere (Santos *et al.,* 2013). "Canthin-6-one" an example of

alkaloid is considered as renowned constituents of the *Rutaceae* and *Simaroubaceae*, displayed MIC against fast-growing *Mycobacterium species* in the range of 8 – 32 µg/ml and against methicillin-resistant strains of *Staphylococcus aureus* in range of 8 – 64 µg/ml (O'Donnell *et al.*, 2007). In a study, the alkaloids "glycoborinine" and "N-p-coumaroyltyramine" extracted from *G. pentaphylla* has been screened for their photo-activated antibacterial activities by TLC overlay assay against *Staphylococcus aureus* and *Bacillus subtilis*, while photo-activated DNA binding activities also assessed by using restriction enzymes and 1.8 kb DNA fragment under UVA irradiation (Yu *et al.*, 2012).

In recent years, intensive research has focused on steroids with the aim of developing the novel, potentially selective antimicrobial and anticancer bio-molecules (Shamsuzzaman *et al.*, 2014). Plants produce wide range of steroidal compounds which show physiological roles in plants and toxic effects towards parasites, fungi and bacteria (Dinan *et al.*, 2001). Steroids are characterized by the presence of a 1, 2-cyclopentenophenanthrene ring system and this ring can be partially reduced or modified (Raaman, 2006). The naturally occurring steroids such as sterols, adrenocortical hormones, bile acids and cardiac glycosides have important function in human physiology (Dinan *et al.*, 2001). Petroleum ether is the choice of solvent for the extraction of steroids and other fatty compounds from plant sample (Tiwari *et al.*, 2011). The steroid, "ergosterol-5,8-endoperoxide" isolated from *Ajuga remota*, displayed high antiplasmodic activity against the chloroquine-sensitive *P. falciparum* with an IC_{50} value of 8.2 µM, whereas steroidal saponins extracted from the leaves of *Vernonia amygdalina* exhibited anti-malarial activity (Ntie-Kang *et al.* 2014). Amino steroids disrupt the outer membrane of gram negative bacteria whereas it depolarizes the membrane of gram positive bacteria (Djouhri-Bouktab *et al.*, 2011). Two steroids namely "tomatidine" and "solasodine" isolated from *Solanum aculeastrum* was characterized through spectroscopic technique and evaluated for high antioxidant potential using DPPH, ABTS and reducing power assay (Koduru *et al.*, 2007). Some steroid derivatives having dihydropyrimidine ring induced antibacterial activity against *Klebsiella pneumonia, Escherichia coli, Salmonella typhi* and *Staphylococcus aureus* in dose dependent manner (Figueroa Valvere *et al.*, 2011). In a study, steroidal pyrazolones were screened for their antimicrobial activities against Gram positive and

Gram negative bacterial strains and these compounds gave excellent MIC values against the pathogenic microorganisms (Shamsuzzaman *et al.*, 2014).

Chapter 3

MATERIALS & METHODS

3. MATERIALS AND METHODS

3.1 Chemicals and culture media used

All the chemicals and compositions of culture media and reagents were obtained from Merck Germany and HiMedia Laboratory Private Limited, Mumbai (India) (Appendix: 1, 2 and 3).

3.2 Test Microorganisms used

	Reference strain	Source
Gram Positive Bacteria	*Staphylococcus aureus*	MTCC – Chandigarh (MTCC- 7443)
	Bacillus cereus	MTCC - Chandigarh (MTCC-6840)
Gram Negative Bacteria	*Escherichia coli*	Subashshree Biotech, Kolkata
	Pseudomonas aeruginosa	MTCC - Chandigarh (MTCC-1034)

3.3 Collection of plant sample

The plant material used for the present study have been identified and authenticated by taxonomist at Botanical survey of India (BSI), Gangtok, Sikkim and were provided by the laboratory of the Department of microbiology, Sikkim University.

3.4 Preparation of crude phytochemical extracts from dried roots of plant

The rhizome of *Tectaria macrodonta* were collected and washed carefully and kept for dry under shade. The dried plant material was made to a fine powder using Waring blender (Cole Parmer, RZ-04245-21). 10 gm of dried plant sample was directly used for the extraction of different crude or total phytochemicals by employing standard protocols. The solvent was evaporated using Rotary evaporator (B.U.chi, Switzerland, R-3) to obtain crude phytochemical extract, which was then stored at refrigerator temperature (4°C) for further use. Different concentrations of the crude phytochemicals were prepared by using of 10% DMSO from the stock concentration

of 500mg/ml and were filtered through 0.45 micron cellulose acetate membrane filter (Sartorious). Further dilutions of the filtered extract were prepared to get the concentration of 20mg/ml, 50mg/ml, 100mg/ml, 150mg/ml, 200mg/ml, 300mg/ml, 350mg/ml and 400mg/ml.

3.5 Extraction of crude phytochemicals

Five crude phytochemicals namely crude flavonoid, crude tannin, crude saponin, crude alkaloid and crude steroid were extracted from dry rhizome powder of *Tectaria macodonta* by employing standard methods.

3.5.1 Extraction of crude flavonoid

10 gram powdered plant material was extracted for 24 hours using methanol (100ml) as a solvent in shaking incubator at 37 °C temperature. After filtration, the methanolic extract was evaporated to dryness. The dry residue was soaked with boiling water and cooled in the refrigerator, filtered and then extracted with diethyl ether (for isolation of phenolic acids). Then, the water solution was extracted by ethyl acetate. The ethyl acetate extract was collected and evaporated to dryness by rotary evaporator. The dry residues were weighed and dissolved in methanol and used for further analysis of antimicrobial and antioxidant activities (Waksmundzka-Hajnos *et al,* 2011).

3.5.2 Extraction of crude tannin

10 gram plant material were weighed and extracted with 100ml distilled water in microwave for three cycles of one minute with two minutes pause. After the filtration, the water extracted material was collected and evaporated to dryness (Cobzak *et al.*, 2005). The dry residue was weighed for determination of total tannin and was dissolved in methanol (Li *et al,* 2011) for further investigation.

3.5.3 Extraction of crude saponin

The powdered sample of plant (10 gram) was defatted by 100 ml petroleum ether for 1 hour at 40°C in water bath. After filtering the petroleum ether, the sample was extracted with 100 ml methanol for 1 hour with gentle heating in water bath. The combined methanol extract was concentrated and methanol extract of sample was obtained. In order to get the crude saponins extract, the sample was dissolved in methanol and acetone was added (1:5 v/v) to precipitate the saponin. The precipitate

was dried under vacuum to a whitish amorphous powder and termed as crude saponin extract (Khanna *et al.*, 2008). The solvent was evaporated to get crude filtrate of saponin and weighed for quantitative analysis for total crude saponin. The residue containing crude saponin was dissolved in methanol and stored in refrigerator for further use (Soetan *et al.*, 2006).

3.5.4 Extraction of crude alkaloid

In 10 gram of powder material of plant, 10% acetic acid in ethanol (100 ml) was added. The mixture was incubated for 4 hours in the dark. After incubation, the extract was filtered and the solution concentrated to $1/4^{th}$ volume in a boiling water bath. To the extract, 25% ammonia was added until a precipitate was formed and was centrifuged at 2500 rpm for 5 minutes. The residue obtained was washed with 1% NH_4OH and filtered. The residue that contained alkaloids were weighed and dissolved in ethanol and stored at 4°C for further use (Harborne, 1973).

3.5.5 Extraction of crude steroid

Air dried plant material (10 gram) was extracted with petroleum ether at 50°C temperature for 1 hour. After filtration, the hot methanol was added to the filtrate. After two to three hours, the methanol was evaporated to reduce the volume for obtaining crude steroid which was then dried and weighed for further analysis (Abdulmalik *et al,* 2011).

3.6 Quantitative analysis of extracted crude phytochemicals.

The extracted crude phytochemicals (Flavonoid, Tannin, Saponin, Alkaloid and Steroid) were dried in rotary evaporator and the percentage weight of the dried residues was determined (Shabbir *et al.*, 2013).

3.7 Phytochemical Analysis

The phytochemical screening was done to evaluate the specific and other related phytochemicals present in crude flavonoid, crude saponin, crude tannin, crude steroid and crude alkaloid extracted from rhizome of *Tectaria macrodonta.*

3.7.1 Detection of Alkaloids:

0.5 gram of crude phytocemical extract was dissolved in 5 % HCl and heated in a water bath for 4 to 5 minutes. The resultant filtrate was subjected to the following tests:

- **Mayer's Test**: Add one to two drops of Mayer's reagent to three ml of filtrate. A white precipitate indicates the presence of alkaloids (Lalithal *et al*, 2012).
- **Wagner's Test**: Two drops of Wagner's reagent were added to three ml of filtrate. A reddish – brown precipitate confirmed the test as positive (Garba *et al*, 2013).
- **Hanger's Test**: To 4ml of filtrate, 3 ml of Hager's reagent (Saturated aqueous solution of picric acid) was added. The presence of prominent yellow precipitate indicates the presence of alkaloids (Tiwari *et al.,* 2011).

3.7.2: Detection of Flavonoids

- **Zinc HCl Test**- In 3 ml crude phytochemical extract, the test solution mixture of zinc dust and concentrated HCl were added. The formation of red color indicated the positive result (Yadav and Agarwal, 2011)
- **H_2SO_4 Test** – 3 ml of dissolved crude phytochemical extract was treated with concentrated H_2SO_4 and the formation of orange color indicated the presence of flavonoid (Lalithal *et al*, 2012)

3.7.3: Detection of Saponins and Tannins

- **Froth Test**: 1ml of crude phytochemical extract was diluted with water and shaken vigorously in a graduated cylinder for 15 min. Formation of layer of froth indicated the presence of saponins (Pandey *et al*, 2013)..
- **Foam Test**: 0.5 gm of crude phytochemical extract was shaken with 2ml of water. Production of foam indicated the presence of saponins (Sujana *et al*, 2013).
- **Gelatin Test**: To the 1 ml of crude phytochemical extract, 1 % gelatin solution containing sodium chloride was added. Formation of white precipitate indicated the presence of tannins (Pandey *et al*, 2013).

3.7.4: Detection of Anthocyanin

Two reactions were set up for the detection of anthocyanin in the crude phytochemical extracts. In one tube 2 ml of filtrate and 1ml of NaOH was added whereas in second tube 2 ml of filtrate and 1ml of HCl was added. The development of different color in both the reaction mixture indicated the presence of anthocyanin (Lalithal *et al*, 2012, Pandey *et al.*, 2013).

3.7.5: Detection of Phenols

- **Ferric chloride Test**: To 1 ml of crude phytochemical extract, 3-4 drops of 1 % ferric chloride solution was added. The formation of bluish black or green or purple color indicated the presence of phenol (Roopashree *et al*, 2008).

3.7.6: Detection of Resins:

- **Acetone Water Test**: The 3 ml crude phytochemical extract was treated with acetone. 2 ml of water was and shaken. Appearance of turbidity indicated positive result (Roopashree *et al*, 2008).

3.7.7: Detection of Carbohydrates: 100 mg of crude phytochemical extract was dissolved in 5 ml of water and the filtrate was subjected to the following tests:

- **Molish's Test**: 2 drops of alcoholic solution of α- napthol was added to 2 ml of filtrate and shaken vigorously. Then 1 ml of conc. sulphuric acid was added and allowed to stand. The presence of violet ring indicated positive test for carbohydrates (Pandey *et al*, 2013).
- **Fehling's Test**: 1 ml of filtrate was boiled on water bath with 1ml each of Fehling solution A and B. A red precipitate indicated the presence of sugar (Sujana *et al*, 2013).
- **Benedict's test**: To 0.5 ml of filtrate, 0.5 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 min. Orange-red precipitate indicated the presence of sugar (Roopashree *et al*, 2008).

3.7.8: Detection of Glycosides:

- **Salkowskis's Test:** 1 ml of crude phytochemical extract was mixed with 2 ml of chloroform, and then 2 ml of concentrated H_2SO_4 was added and shaken

gently. Formation of reddish brown color indicated the presence of steroidal ring (Pandy *et al.*, 2013).

- **Keller- Kilani Test:** 1 ml of crude phytochemical extract was mixed with 2 ml of glacial acetic acid containing 1-2 drops of 2 % $FeCl_3$. The mixture was then poured into another tube containing 2 ml of conc. H_2SO_4. A brown ring at the interphase indicated the presence of cardiac glycoside (Tiwari *et al*, 2011).

3.7.9: Detection of Amino Acid

- **Ninhydrin Test**: To the crude phytochemical extract 0.25 % w/v ninhydrin reagent was added and boiled for 5 min. The formation of blue color indicated the presence of amino acid (Tiwari *et al*, 2011).

3.7.10: Detection of Fixed oils and Fats:

- **Spot Test**: A small quantity of crude phytochemical extract was pressed between two filter papers. Oil stain on the paper indicated the presence of fixed oil (Tiwari *et al*, 2011).

3.8: Test microorganisms

In the present study, two Gram positive bacteria namely *Bacillus cereus, Staphylococcus aureus* and two Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* were used. These test microorganisms were preserved at 4 °C on nutrient agar slants. Before performing the antimicrobial experiment, the test microorganisms were revived by growing in nutrient broth (NB) at 37 °C for 24 hours.

3.8.1: Preparation of bacterial suspension:

The bacterial suspension was prepared by picking up a single colony of bacteria from agar plate and transferred it to freshly prepared and autoclaved nutrient broth. After inoculation, the nutrient broth was placed in bacteriological incubator for overnight incubation at 37 °C. Next day the turbidity of nutrient broth was assessed with the help of Spectrophotometer (Eppendrof Biophotometer) by measuring the absorbance of the suspension. When the turbidity of suspension was more it was adjusted by adding sterile distilled water or when the turbidity was too less then it was balanced by adding prepared bacterial suspension. The bacterial suspension was maintained

approximately in the range of 0.08 - 0.13 at 600 nm (McFarland standard 0.5) (10^8cfu/ml) (Alavijeh *et al.*, 2012; Shihabudeen *et al.*, 2010). The bacterial suspension of 10^8cfu/ml was analyzed further for antimicrobial activities with respect to crude phytochemicals.

3.9: Screening of antimicrobial activity of crude phytochemicals against test microorganisms (Well Diffusion Method)

The antibacterial activity of the crude phytochemicals was determined by well diffusion method. The Muller Hinton Agar (MHA) was prepared according to the requirements and about 20 ml autoclaved medium was poured into the autoclaved petriplates. After solidifying the medium, 100 μl of bacterial suspension (10^8cfu/ml) was added with the help of micropipette and spread consistently with the glass spreader or sterile swab. A sterile cup- borer used for making wells on to the solidified medium. 100 μl of the crude phytochemicals at different concentrations were added to two of the wells, 100 μl antibiotic (Gentamicin) as a positive control was dispensed in one well and 100 μl of the negative control (10 % DMSO) was introduced to the opposite well. The plates were kept for incubation for 24 hours at 37 °C temperature. The antibacterial activity was determined by measuring the diameter of zone of inhibition in mm inclusive of the well size and expressed in mm (Shihabudeen *et al.*, 2010; Parekh *et al.*, 2005).

3.10: Determination of Minimum inhibitory concentration (MIC) by agar dilution method

The lowest concentration of any antimicrobial substance that does not permit the visible growth of microorganisms can be considered as the minimum inhibitory concentration (MIC) (Mayunzu *et al.*, 2011). The Minimum inhibitory concentration of crude phytochemicals was detected through agar dilution method (Amole and Ilori, 2010). Mueller Hinton Agar was prepared and autoclaved at 121°C for 15 minutes and allowed to cool at room temperature. From the stock concentration of the crude phytochemicals extracts, different amount of the extracts (in range of from 10 to 400 mg/ml) were added to 20 ml of agar medium to make the final required concentration of the extracts. The proper shaking was done to thoroughly mix the extracted components with agar medium. Then, the agar medium containing extract was poured

into the sterilized petri plate and allowed to solidify. To the solidified medium, 20µl of bacterial suspension (10^8cfu/ml) was delivered as a spot. For the control experiment, Gentamicin (100µg/ml) was taken as positive control and 10 % DMSO as the negative control (Mayunzu *et al*, 2011, Amole and Ilori, 2010). The plates were kept for proper incubation at 37°C for 24 hours. The lowest concentration of crude phytochemical extract which inhibited the growth of microorganism was considered as the MIC of that particular extracted crude phytochemical (Mayunzu *et al.*, 2011, Amole and Ilori, 2010, Wiegand *et al.*, 2008).

3.11: Determination of Minimum inhibitory concentration (MIC) by Broth Dilution Method

The method followed by Sette *et al.*, (2006) with some modifications was used for evaluation of Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentrations (MBC) using Nutrient Broth in test tubes for bacterial cultures. The stock solutions of all crude phytochemical extracts were inoculated in 2 ml nutrient broth to make the final concentration in the range of 10 mg/ml to 400 mg/ml in different sterile test tubes. The 20 µl of the bacterial suspension was also added into nutrient broth having crude phytochemical extract. Gentamicin (100µg/ml) was used as positive control while 10% DMSO was used as negative control. The test tubes were kept for incubation at 37°C temperature for 24 h. Antibacterial activity was detected by the visibility of bacterial growth in nutrient broth treated with various concentrations of crude phytochemicals. Therefore, MIC was defined as the lowest concentration of extracts that inhibited visible growth (Karsha *et al.*, 2010).

3.12: Determination of Minimum Bactericidal Concentrations (MBC)

The MBC was assessed by aliquoting 0.1 ml of the culture medium from the nutrient broth used for MIC assay exhibiting no visible growth and was subcultured into freshly prepared Mueller Hinton Agar (MHA) Plates. The plates were then kept for incubation at 37°C for 24 hours. The MBC was rcorded as the lowest concentration exhibiting no visible growth on Mueller Hinton Agar plates (Sanchez *et al.*, 2010). By using the values of MIC in broth dilution method, the MIC index values (MBC/MIC) for each crude phytochemical extract were calculated against the test microorganisms (Nowsheri, 2012).

3.13: Mode of action of crude phytochemicals based on leakage of UV_{260} and UV_{280} absorbing material of test bacteria

The test was done to determine the microbicidal effect of crude phytochemicals of *Tectaria macrodonta* on test microorganisms and evaluating the leaking material such as nucleic acid and protein from bacterial cells in response to crude phytochemicals at different time intervals. The bacterial suspension was prepared by growing cells overnight in 200 ml nutrient broth with continuous shaking at 37°C. After appearance of visible growth, the bacterial cells were harvested, washed with 10 mM EDTA (pH 8). After washing in EDTA, the pellate of cells were washed twice in distilled water by centrifugation each time at 6000 rpm for 15 min at 25 °C and resuspended such that the absorbance of the final suspension was 2 at A_{450}. After incubation for 30 min at room temperature, the crude phytochemicals was added to suspension at value of MIC. At regular intervals of 15 min, aliquot of the sample was centrifuged and absorbance of supernatant was recorded at UV_{260} (nucleic acids) and UV_{280} (proteins) for determine any leaked materials such as proteins and nucleic acid from the bacterial cells (Heipieper *et al.,* 1992, Karsha *et al.,* 2010).

3.14 Antioxidant activity assay

3.14.1: Ferric Reducing Power Assay (FRAP)

Various concentrations of crude phytochemical extracts of the plant sample and as ascorbic acid as standard (10 - 100 mg/ml) were mixed with 2.5ml, 1 % w/v of potassium ferricyanide and 2.5 ml of (0.2M, pH 6.6) phosphate buffer. Then the mixture was kept for incubation at 50 °C for approx 20min into the water bath. After cooling the reaction mixture, 2.5 ml of TCA (10 %) w/v was added to prevent the reaction and centrifuged at 3000 rpm for 10 min. After centrifugation, 2.5 ml of the upper most fraction of the solution was taken in test tube and mixed with 2.5 ml distilled water. After this, 0.5 ml 0.1 % w/v $FeCl_3$ was also added. Control was also prepared without adding crude phytochemical extract. The color intensity of reaction mixture was measured in Lambda 25 UV/Vis Spectrophotometer (Perkin Elmer, L600-00BB) at 700 nm. Higher absorbance of the reaction mixture designated greater reducing power capacity (Duh *et al.*, 1998, Kostic *et al.*, 2010). All tests were performed in triplicates and its mean value was recorded.

3.14.2: DPPH scavenging assay by Spectrophotometer Method

For antioxidant assay various concentrations from 10- 100 μg/ml was prepared from the stock concentration of (1000 μg/ml) of the crude phytochemical extracts. For the standard, ascorbic acid was used in different concentrations from (10-100 μg/ml). Methanol was used for the preparation of 0.1mM solution of DPPH. For the reaction mixtures contained 3ml of different concentrations of crude phytochemical extract and 1ml of DPPH was added to different test tubes. 3 ml of deionized water and 1ml of DPPH with no crude phytochemical extract was used for the preparation as control. The reaction mixtures were incubated for 30 minutes in the dark and recording of absorbance was done at 517nm by using Lambda 25 UV/Vis Spectrophotometer (Perkin Elmer, L600-00BB). The calculation of percentage inhibition (% IP) was performed by using the reduction in optical density of DPPH on addition of crude phytochemical extract in relation to control. IC_{50} value is considered as the concentration of the crude phytochemical extract which exhibits 50% loss of DPPH activity. All experiments were performed in triplicates and its mean value was represented.

% of DPPH Scavenging = $(A_a - A_b / A_a) \times 100$

Where, A_a is the absorption of control, A_b is absorbance of sample at various concentartions (Braca *et al.*, 2001).

3.14.3: Hydroxyl Radical Scavenging Activity (HRSA)

The hydroxyl radical scavenging activity was performed by the method described by (Klein *et al.*, 1991). Different concentrations (10,20,30,40,50,60,70,80,90,100 mg/ml) of the crude phytochemical extracts were taken in capped tubes to which 1ml of iron – EDTA solution (0.13 % ferrous ammonium sulphate and 0.26 % EDTA), 0.5ml of 0.018 % EDTA and 1ml of 0.85 % DMSO (in 0.1M phosphate buffer , pH7.4) were added, followed by addition of 0.5ml of 0.22 % ascorbic acid . The tubes were capped tightly and incubated at 85 °C on a water bath for 15 min to 20min. Post incubation, the tubes were uncapped and immediately 1ml of ice cold TCA (17.5 % w/v) was added. Then, 3ml of Nash reagent was added to all tubes and kept for incubation at room temperature for 15 min. The absorbance of reaction mixture was taken at 412 nm (Klein *et al.*, 1981; Guha *et al.*, 2009). The percentage hydroxyl radical

scavenging activity (% HRSA) was calculated by using the formula given below. The entire test was performed in triplicates and its mean value was recorded.

% HRSA = (A_a-A_b / A_a) x 100

Where, A_a is absorbance of the control, A_b is the absorbance of individual sample at different concentrations.

3.14.4: Scavenging assay of Hydrogen peroxide radicals

Scavenging of hydrogen peroxide was analyzed according to the method given by Natarajan *et al.*, (2013). A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4) and mixed with ascorbic acid. Samples of various crude phytochemical extracts (10µg/ml–100µg/ml in distilled water) were added to hydrogen peroxide solution (0.6 ml, 40mM). After 20 minutes, absorbance of hydrogen peroxide was measured at 230 nm against 50 mM phosphate buffer as a blank. The percentage of hydrogen peroxide scavenging activity was calculated by using the formula given below.

% HRSA = (A_a-A_b / A_a) x 100

Where, A_a is absorbance of the control, A_b is the absorbance of individual sample of different amount.

The entire test was performed in triplicates and its mean value was recorded.

3.15: Total Flavonoid Content

The total Flavonoid content in the crude flavonoid extract was estimated by Aluminium chloride colorimetric method illustrated by Chang *et al.*, (2002) using Rutin as a standard reference. To estimate the total flavonoid content in the crude flavonoid extract (1000 µg/ml), 0.5ml of sample was mixed with 1.5 ml of methanol (70 % v/v), and 0.1ml of aluminium chloride (10 % w/v). After 3 min of incubation at room temperature, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water was added and incubated for 20 - 30 min at room temperature. The absorbance of samples was recorded at 415 nm using Lambda 25 UV/Vis Spectrophotometer (Perkin Elmer, L600-00BB). Rutin was used as a standard to obtain the calibration curve at various concentrations (10 – 100 µg/ml). The result was expressed as rutin equivalent (mg/g

of extracted compound) (Chang *et al.*, 2002). The entire test was done in triplicates and their mean value was represented.

3.16: Total tannin content

The content of tannin was estimated by Folin Denis Method based on the non-stoichiometric oxidation of the molecules having a phenolic hydroxyl group. Stock solution of tannic acid standard is formed by dissolving 100mg tannic acid in 100 ml of distilled water. For making working standard solution 5 ml of stock solution was diluted to make the concentration from 10µg/ml to 100µg/ml. 1 ml of crude tannin extract of plant was added with 75 ml water, 5 ml of Folin- Denis reagent, 10 ml of sodium carbonate solution and diluted to 100 ml with water. After shaking, the absorbance was measured at 700 nm after 30 min. Blank was prepared with water instead of sample. Standard graph was prepared by using 10-100 µg tannic acids (Sharief *et al.*, 2014).

3.17: Statistical Analysis

The statistical analysis was done using student t-test through Graph Pad Prism version 5.01. The statistical analysis was done after calculating the mean and SD values using Microsoft Office Excel 2007. A p-value <0.05 was considered to be statistically significant. All experiments were performed in triplicates.

Chapter 4

RESULTS

4. RESULTS

4.1: Identification of the selected plants

The plant sample was collected from the forest area of East Sikkim and identified by taxonomist of Botanical Survey of India, Gangtok.

Identified Plant	Common name	Family	Accession number	Place of Collection
Tectaria macrodonta or *Tectaria coadunate*	*Kaalo ningure* or *Kukkutnakhi*	*Dryopteridaceae*	07	Pademchey, East Sikkim

4.2: Quantitative and qualitative estimation of phytochemicals

Various methods were followed to determine the presence of phytochemical constituents quantitatively and qualitatively of *Tectaria macrodonta.*

Table 1: Quantitative estimation of extracted dry and crude constituents of *Tectaria macrodonta*

Phytochemicals	Color	Quantity in grams	Quantity in percentage
Flavonoids	Burgundy	1.653±0.01	16.55±0.13
Tannins	Dark Maroon	2.533±0.59	25.334±5.95
Saponins	Light Maroon	1.444±0.12	14.448±1.26
Alkaloids	Reddish grey	0.26±0.05	2.656±0.51
Steroids	Yellowish	0.525±0.04	5.25±0.41

Initial weight of dried powder of each part was 10 g, each value represents mean ± SD (n=3).

The quantity and percentage yield of various phytochemicals extracted from *Tectaria macrodonta* with color characteristics is shown in Table 1 and figures 1 and 2. The crude tannin has shown maximum yield (2.53 gm) followed by crude flavonoid (16.55%), crude saponin (14.44%) and steroid (5.25%). The lowest yield determined of crude alkaloid which was only 2.65% of total 10 g powdered material. The extracted phytochemicals exhibited various color characteristics. Crude flavonoid was burgundy in color. Crude tannin extract was in dark maroon while saponin had light maroon color. Other phytochemicals namely crude alkaloids and crude steroid exhibited reddish gray and yellowish coloration, respectively.

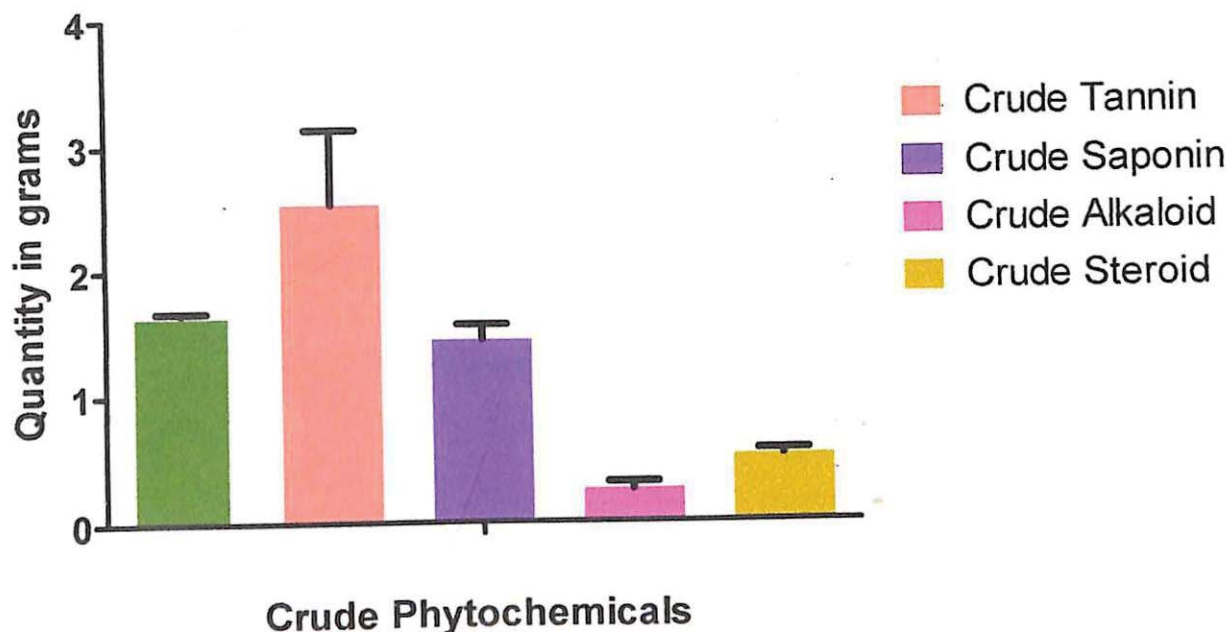

Figure 1: The quantitative estimation of crude phytochemicals extracted from *Tectaria macrodonta.*

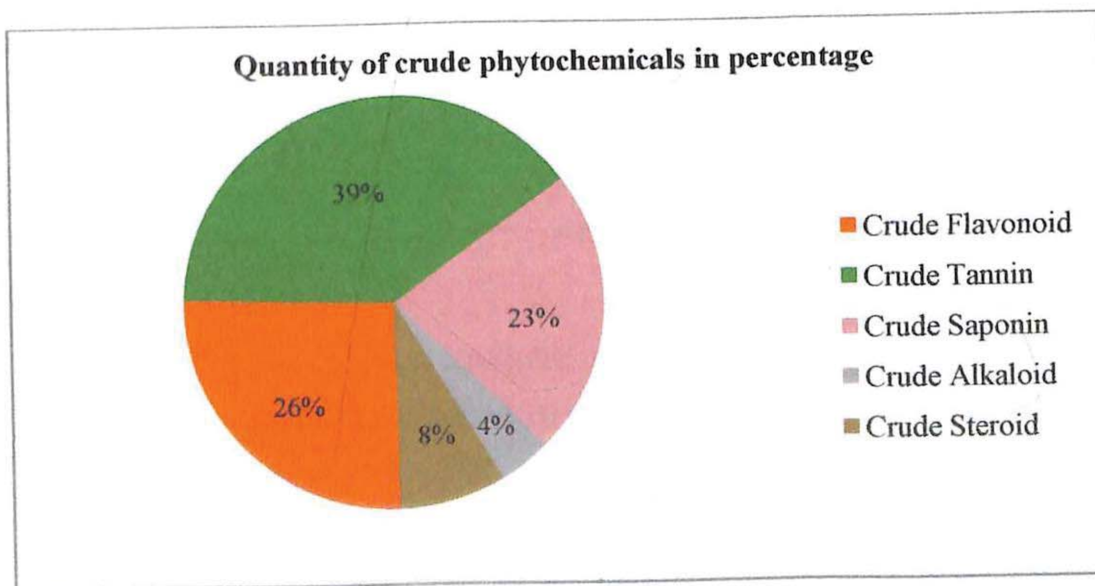

Figure 2: Percentage of crude phytochemicals extracted from *Tectaria macrodonta.*

Table 2: Qualitative estimation of crude phytochemicals extracted from *Tectaria macrodonta*

Phytochemical Test	Crude Flavonoid	Crude Tannin	Crude Saponin	Crude Alkaloid	Crude Steroid
SAPONIN TEST					
a)Foam Test	++	++	+++	–	–
b)Froth Test	++	++	+++	–	–
TANNIN	+	+	+	–	–
ANTHOCYANIN	+	+	+	–	–
ALKALOID					
a)Mayer's Test	–	–	–	–	–
b)Wagner's Test	–	–	–	–	–
c)Hagner's Test	–	–	–	+	–
FLAVONOID					
a) H_2SO_4 test	+	+	–	–	–
b) Zinc HCl test	+	+	–	–	–
RESIN	–	–	–	–	–
PHENOL	+	+	+	–	–
CARBOHYDRATE					
a) Molish's Test	+	+	+	–	–
b) Fehling's Test	+	+	+	–	–
c) Benedict's Test	+	+	+	–	–
PROTEINS AND AMINO ACIDS					
a)Ninhydrin Test	–	–	–	–	–
GLYCOSIDASE					
a)Salkowski's Test	+	+	+	–	+
b)Keller- kilani Test	–	+	–	–	+
FATS & OIL	–	–	–	–	+

'+' indicates present; '-'indicates absent, '++' indicates minimal amount of saponin; '+++' indicates excess amount of saponin.

The qualitative phytochemical screening of crude phytochemicals extracted from *Tectaria macrodonta* was done for detection of specific and related compounds into particular crude phytochemical was extracted by following standard procedure. Apart from the crude saponin extract, saponin was also found to be detected in the crude flavonoid extract, crude tannin extract but was absent in crude extract of alkaloid and steroid. The crude tannin extract of plant material was analyzed for the presence of tannin compound. Beside the crude tannin extract, the tannin was found to be present in crude residues of flavonoid and saponin. Anthocyanin was found in crude extract of

flavonoid, tannin and saponin but was absent in crude alkaloid and crude steroid extract. The analysis of alkaloids were also performed by various tests. Only Hagner's test determined the presence of alkaloid in crude alkaloid extract while other test showed negative result for the alkaloid in other crude phytochemicals. The presence of flavonoid was detected in the crude flavonoid and tannin extract but was absent in other crude phytochemicals. Resin was absent from all extracted crude phytochemicals. Tannin and flavonoids are mainly phenolics, therefore, the phenols have been found in crude tannin and crude flavonoid extract and was present in crude saponin extract. Fat and oil were detected ionly in the crude steroid extract. These results are shown in Table 2.

4.3: Antibacterial activity of crude phytochemicals extracted from *Tectaria macrodonta*

The crude flavonoid extract of *Tectaria macrodonta* was found to show highest antibacterial activities against *Staphylococcus aureus* and *Bacillus cereus*. The highest zone of inhibition (19.67±0.5 mm) was recorded for *Staphylococcus aureus* at the concentration of 400mg/ml followed by the diameter of zone of inhibition as (12±0) for *Bacillus cereus* at concentration of 20mg/ml. The crude tannin extract exhibited antibacterial effect at higher concentration against *Bacillus cereus* (Photo: 8) and *Staphylococcus aureus* with the zone diameter of 11.6±0.5 mm (at conc. 100mg/ml) and 11.33±0.70 mm (at conc. 300mg/ml), respectively. The diameter of zone of inhibition was found to be 17.33±1.1 mm for *Bacillus cereus* with the crude saponin at the concentration of 400mg/ml. The crude alkaloid and crude steroid extract did not exhibit any zone of inhibition against tested bacterial strains. The crude flavonoid, crude tannin and crude saponin did not show any antibacterial activity against *Pseudomonas aeruginosa* and *Escherichia coli*. The phytochemicals extracted from *Tectaria macrodonta* did not exhibit any antimicrobial activity against *Escherichia coli* and *Pseudomonas aeruginosa*. The increase in diameter of zone of inhibition was found to be concentration dependent. The results are shown in Table: 3 - 4, Figures: 3 - 4 and Plates: 5 - 8.

Table 3: Antibacterial activity against *Staphylococcus aureus* by various crude phytochemicals extracted from *Tectaria macrodonta*.

Concentration of crude phytochemicals (mg/ml)	Diameter of zone of inhibition by various crude phytochemicals				
	Flavonoid	Tannin	Saponin	Alkaloid	Steroid
20	–	–	–	–	–
50	11.33±0.57	–	–	–	–
100	14.66±0.57	–	10.33±0.5	–	–
150	15±1.0	–	11.66±0.5	–	–
200	15.66±1.52	–	12.33±0.5	–	–
250	17.66±0.57	–	13±0.0	–	–
300	18.33±0.57	11.33±0.70	13.36±1.52	–	–
350	19±0.00	11.66±0.5	14±0.00	–	–
400	19.67±0.5	12±0.00	15±1.0	–	–
Negative control	–	–	–	–	–
Positive Control	25±0.5				

All values are represented as Mean ± SD, All experiments were perfomed in triplicates, '-': Absence of zone of inhibition, Negative control: 100 μg/ml 'DMSO' (0.25%), Positive control: 'Gentamicin' (100 μg/ml). Zone of inhibition: measured with the well diameter of 8 mm.

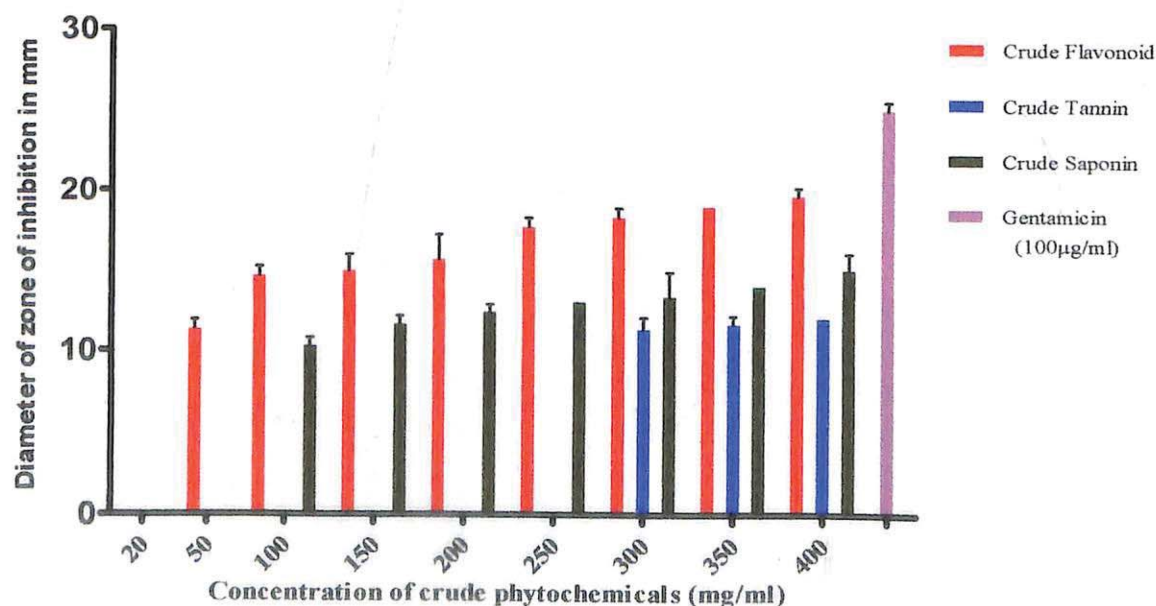

Figure 3: Diameter of zone of inhibition in mm at various concentrations of crude phytochemicals of *Tectaria macrodonta* against *Staphylococcus aureus*.

Table 4: Antibacterial activity against *Bacillus cereus by* various crude phytochemicals extracted from *Tectaria macrodonta*

Concentration of crude phytochemicals (mg/ml)	Diameter of zone of inhibition by various crude phytochemicals				
	Flavonoid	Tannin	Saponin	Alkaloid	Steroid
20	12±0.0	–	–	–	–
50	13±1.0	–	10.3±0.5	–	–
100	13.66±0.5	11.6±0.5	10.66±0.5	–	–
150	14.33±0.5	12±1.0	12±0.0	–	–
200	14.66±1.5	12.6±0.5	12.66±0.5	–	–
250	16±1.7	13.6±0.5	14±1.0	–	–
300	16.66±1.1	14±0.0	14.66±1.1	–	–
350	18.33±0.5	14.66±0.5	15.33±0.5	–	–
400	18.67±1.5	15.33±0.5	17.33±1.1	–	–
Negative control	–	–	–	–	–
Positive Control	26±1.0				

All values are represented as Mean ± SD, All experiments were perfomed in triplicates, '-': Absence of zone of inhibition, Negative control: 100 μg/ml 'DMSO' (0.25%), Positive control: 'Gentamicin' (100 μg/ml). Zone of inhibition: measured with the well diameter of 8 mm.

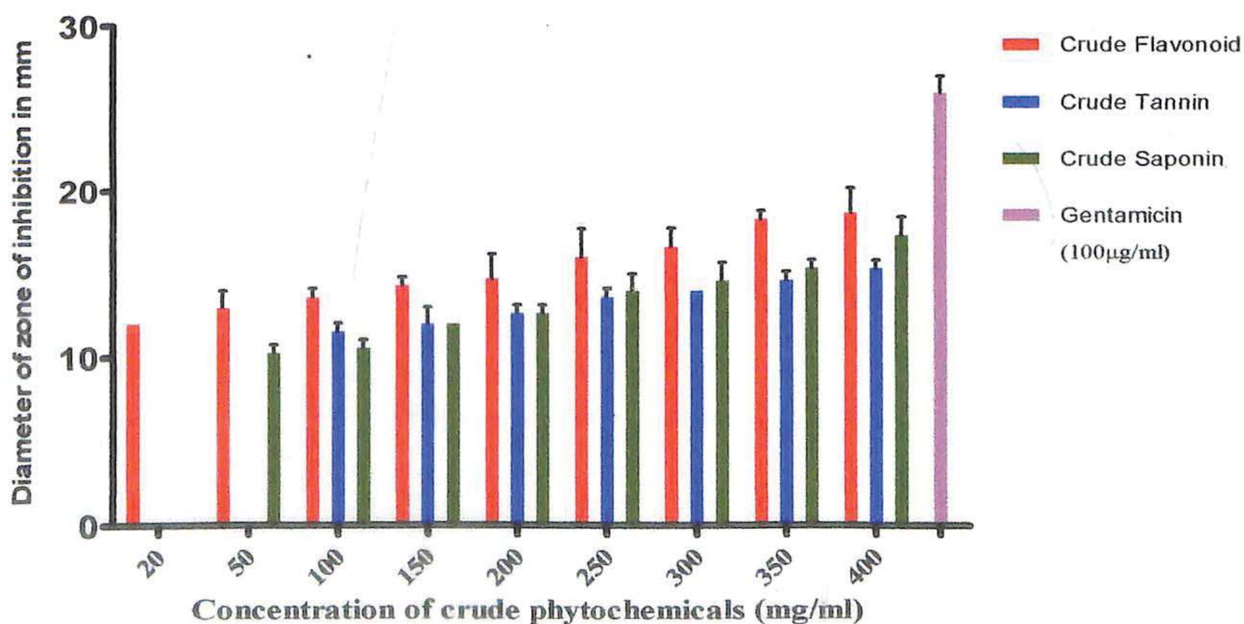

Figure 4: Diameter of zone of inhibition in mm at various concentrations of crude phytochemicals of *Tectaria macrodonta* against *Bacillus cereus.*

4.4: Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC were determined for the crude phytochemicals which exhibited antibacterial properties against the test bacteria. Among the test bacteria, *Bacillus cereus* and *Staphylococcus aureus* were found to be inhibited by the three crude phytochemicals of *Tectaria macrodonta* as illustrated in Table 5. MIC was determined by two methods namely (a) agar dilution and (b) broth dilution methods. Similar MIC values were obtained by employing these methods. Gentamicin was used as positive control while 0.25 % Aqueous DMSO was taken as negative control in each experiment. The crude flavonoid extract inhibited the growth of *Bacillus cereus* and *Staphylococcus aureus* at the concentration of 20mg/ml and 50 mg/ml, respectively. The MBC value of crude flavonoid for *Staphylococcus aureus* (Plate: 11) and *Bacillus cereus* was recorded as 75mg/ml. The crude saponin showed MIC value as 50mg/ml for *Bacillus cereus* and MBC value was 75mg/ml. For *Staphylococcus aureus*, the MIC value was 50mg/ml and MBC value was determined as 100mg/ml. In case of crude tannin extact, MIC and MBC values were recorded as 75mg/ml and 100mg/ml for *Bacillus cereus*, respectively. However, the crude tannin exhibited MIC at the value of 325mg/ml and MBC at 350mg/ml against *Staphylococcus aureus*.

Table 5: MIC and MBC of crude phytochemicals extracted from *Tectaria macrodonta* against test bacterial strains

Strains	Values	Crude phytochemicals		
		Crude Flavonoid (mg/ml)	Crude Tannin (mg/ml)	Crude Saponin (mg/ml)
Bacillus cereus	MIC	20	75	50
	MBC	75	100	75
	MIC Index	3.75	1.33	1.5
Staphylococcus aureus	MIC	50	325	75
	MBC	75	350	100
	MIC Index	1.5	1.076	2.0

'MIC' and 'MBC' assay was performed against test organisms, 'MIC index' = MBC/MIC.

4.5: Determination of mode of action of crude phytochemicals through leakage of UV_{260} and UV_{280} absorbing material from bacteria

In this study, the crude phytochemicals which exhibited antibacterial activity was further determined the mode of action on bacterial membrane leakage. The membrane leakage of UV_{260} and UV_{280} absorbing material (mainly nucleic acid and proteins) was examined over a period of 180 minutes (3 hrs).

Table 6: Determination of membrane leakage of UV_{260} and UV_{280} absorbing material from *Bacillus cereus* spectrophotometrically

Time in minutes	Flavonoid		Saponin		Tannin	
	UV_{260}	UV_{280}	UV_{260}	UV_{280}	UV_{260}	UV_{280}
15	0.0006±0.0012	0.0029±0.001	0.0005±0.0006	0.00293±0.001	0.0003±0.0005	0.0024±0.002
30	0.0013±0.001	0.0036±0.0013	0.0010±0.0008	0.0042±0.0007	0.0009±0.0008	0.0031±0.003
45	0.0016±0.0031	0.0053±0.0027	0.0019±0.0014	0.0043±0.0019	0.0020±0.0012*	0.0032±0.0004
60	0.0018±0.0017	0.014±0.0051*	0.0025±0.0007*	0.0049±0.0026	0.0022±0.0006	0.0033±0.001
75	0.0031±0.0015*	0.0222±0.0054*	0.0033±0.0023	0.0081±0.0028*	0.0027±0.0010*	0.0058±0.008
90	0.0072±0.0028*	0.0226±0.0109*	0.0041±0.006*	0.014±0.0017*	0.0029±0.0036*	0.0071±0.0025
105	0.0124±0.0025*	0.0254±0.0032*	0.0071±0.0027*	0.0187±0.0032*	0.0051±0.0012	0.0086±0.0022*
120	0.0225±0.0022*	0.042±0.0119*	0.0122±0.0025*	0.0308±0.0102*	0.0058±0.0061*	0.0261±0.0133*
135	0.0292±0.0007*	0.0621±0.0097*	0.0209±0.0090*	0.0531±0.0021*	0.0075±0.0302	0.0327±0.0116*
150	0.0418±0.02312*	0.1005±0.0574*	0.0479±0.0053*	0.0662±0.0062*	0.0462±0.0280	0.0406±0.0258
165	0.0954±0.0212*	0.1902±0.0118*	0.0579±0.0084*	0.0852±0.0074*	0.0780±0.0537*	0.0963±0.0649
180	0.1159±0.0114*	0.3060±0.0621*	0.0893±0.0105*	0.1103±0.0202*	0.0812±0.0005	0.0965±0.0130*

All values are represented as Mean ± SD; all experiments were done in triplicate, * Significant changes against lowest time interval of 15 minutes ($p < 0.05$).

The concentration of crude phytochemicals (crude flavonoid, crude tannin and crude saponin) which showed MIC against *Bacillus cereus* further selected for determination of leakage of UV_{260} and UV_{280} absorbing material. The leaking

materials from bacterial cells were estimated by taking absorbance at UV_{260} and UV_{280} at various time intervals. The crude flavonoid treated sample at 15 min exhibited absorbance as 0.0006 (UV_{260}) and 0.0029 (UV_{280}) and the absorbance significantly increased as 0.0031±0.0015 (UV_{280}) at 60 min and as 0.0031±0.0015 (UV_{260}) at 75 min against lowest time intervals. The bacterial suspension treated with crude saponin at 15 min showed absorbance 0.0005 (UV_{260}) and 0.00293 (UV_{280}) which were significantly increased at 60 min as 0.014±0.005 (UV_{260}) and at 75 min as 0.0081±0.0028 (UV_{280}). In the same way, the crude tannin also exerted its membrance damging activity of bacterial cells as the leaking material significantly determined as 0.0020±0.0012 (UV_{260}) at 45 min and 0.0086±0.002 (UV_{280}) at 105 min. The crude flavonoid was responisible for higher leakage followed by crude saponin and crude tannin. There was a significant ($p<0.05$) increase in absorbance at increased time intervals as compared to the lowest time interval of 15 min. The results are shown in Table: 6 and Figures: 5, 6 and 7.

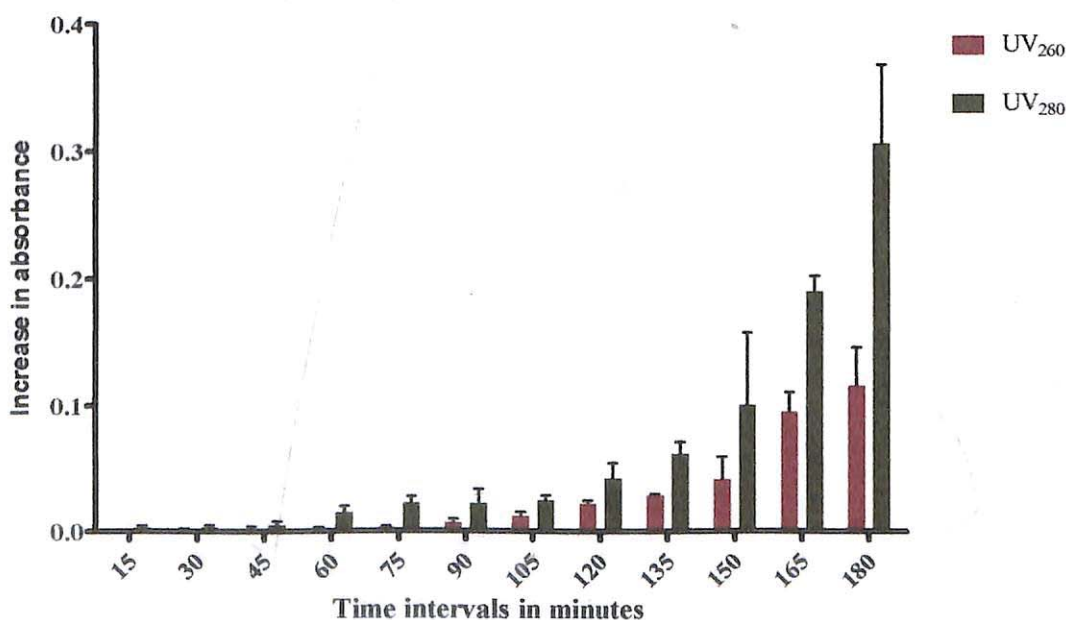

Figure 5: Determination of membrane leakage of UV_{260} and UV_{280} absorbing material from cell suspension of *Bacillus cereus* treated with crude flavonoid at different time intervals.

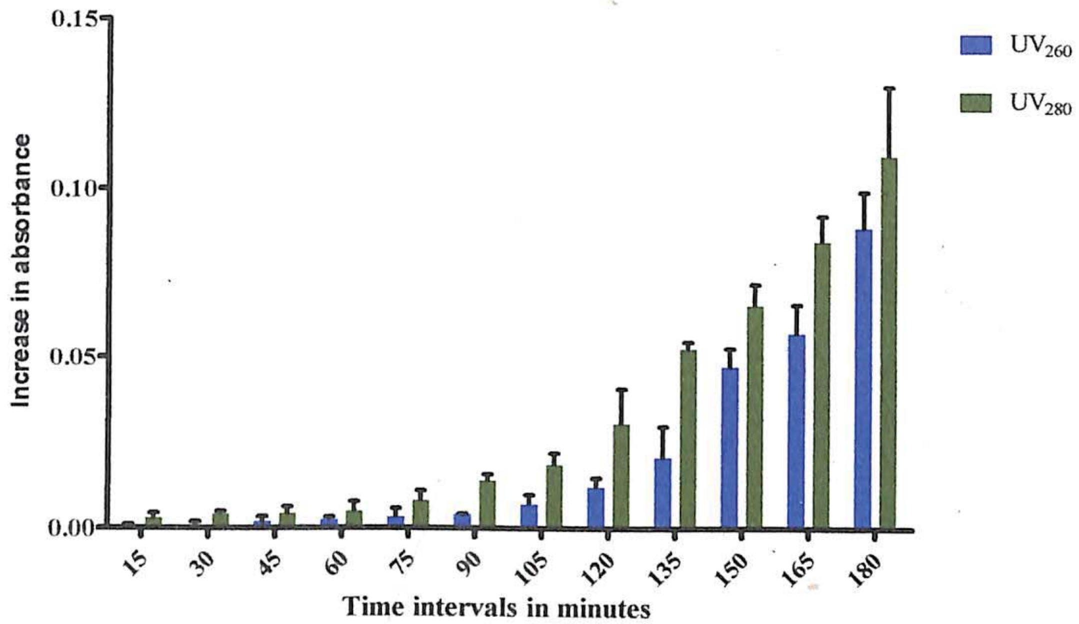

Figure 6: Determination of leakage of UV_{260} and UV_{280} absorbing material from cell suspension of *Bacillus cereus* treated with crude saponin at different time intervals.

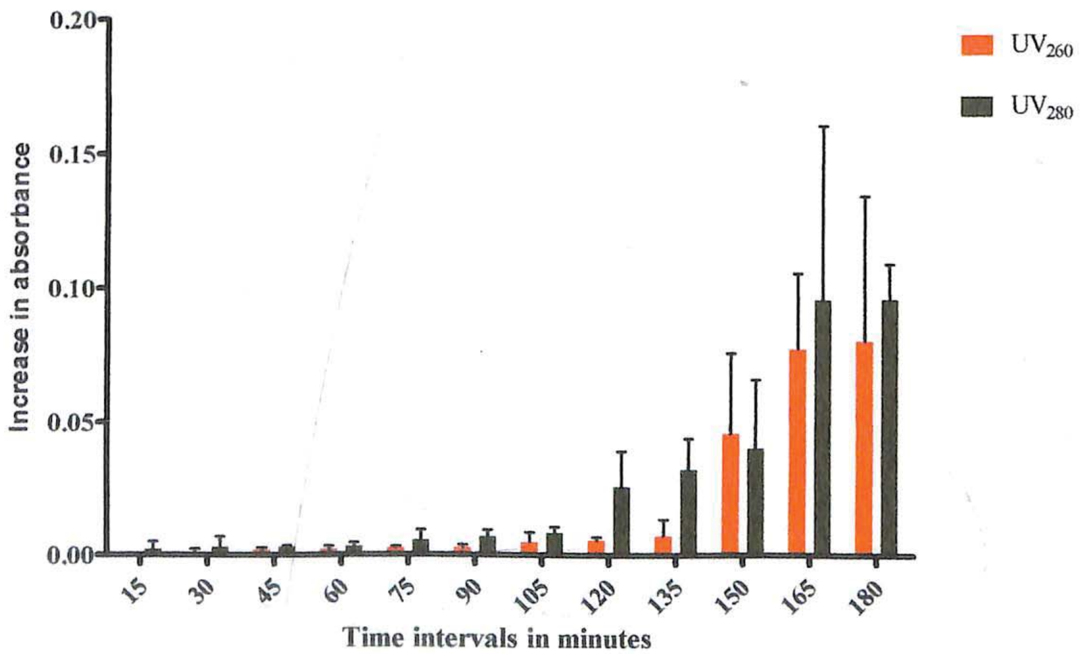

Figure 7: Determination of leakage of UV_{260} and UV_{280} absorbing material from cell suspension of *Bacillus cereus* treated with crude tannin at different time intervals.

Table 7: Determination of leakage of UV_{260} and UV_{280} absorbing material from *Staphylococcus aureus*.

Time in minute	Flavonoid		Saponin		Tannin	
	UV_{260}	UV_{280}	UV_{260}	UV_{280}	UV_{260}	UV_{280}
15	0.0004±0.0006	0.0028±0.001	0.001±0.0017	0.0043±0.0033	0.0009±0.001	0.0017±0.002
30	0.0006±0.001	0.0031±0.002	0.0015±0.0014	0.0045±0.0027	0.0011±0.001	0.0062±0.002
45	0.0010±0.001	0.0038±0.0001	0.0022±0.0019	0.0056±0.0039	0.0016±0.001	0.0063±0.004
60	0.0032±0.0024	0.0079±0.001*	0.0022±0.0002	0.0073±0.0060	0.0019±0.002	0.0065±0.001*
75	0.011±0.0182	0.0182±0.019	0.0024±0.0012	0.0075±0.0038	0.0022±0.0002	0.0068±0.002*
90	0.0361±0.0492	0.0445±0.0368	0.0027±0.0009	0.1186±0.0772	0.0028±0.001	0.0092±0.005
105	0.05±0.0264*	0.0616±0.008*	0.0058±0.0037	0.2042±0.1662	0.0124±0.019	0.0364±0.034
120	0.053±0.0147*	0.0836±0.018*	0.0259±0.024	0.2535±0.3962	0.0341±0.048	0.0828±0.1
135	0.0598±0.0156*	0.1646±0.094*	0.0656±0.0135*	0.259±0.1609	0.0578±0.04	0.1162±0.05*
150	0.1309±0.068*	0.1984±0.1046*	0.0688±0.0381*	0.2978±0.1469*	0.0580±0.039	0.17±0.11
165	0.1699±0.043*	0.2497±0.0951*	0.0841±0.0304*	0.3198±0.1352*	0.1066±0.083	0.1977±0.09*
180	0.1833±0.072*	0.3449±0.0711*	0.0867±0.0145*	0.3282±0.0790*	0.1397±0.053*	0.2816±0.071*

All values are represented as Mean ± SD, All experiments were done in triplicates, * Significant changes against lowest time interval of 15 minutes ($p < 0.05$).

The MIC concentration of crude flavonoid, crude tannin and crude saponin against *Staphylococcus aureus* further selected for determination of mode of action of extract on bacterial cells. The leaking materials from bacterial cells were determined by increase in absorbance at UV_{260} and UV_{280} at different time intervals (15 min – 180 min). The crude flavonoid treated sample at 15 min exhibited absorbance as 0.0004 (UV_{260}) and 0.0028 (UV_{280}) and at 180 min the absorbance increased upto 0.1833 (UV_{260}) and 0.3449 (UV_{280}). The bacterial suspension treated with crude saponin at 15 min showed absorbance 0.0001 (UV_{260}) and 0.0043 (UV_{280}) which were increased at

180 min as 0.0867 (UV_{260}) and 0.3282 (UV_{280}). Similarly, the crude tannin treated bacterial at 180 min exhibited absorbance 0.0812 (UV_{260}) and 0.0965 (UV_{280}). There was a significant ($p<0.05$) increase in absorbance at increased time intervals as compared to the lowest time interval of 15 min. The results are shown in Table: 7 and Figures: 8, 9 and 10.

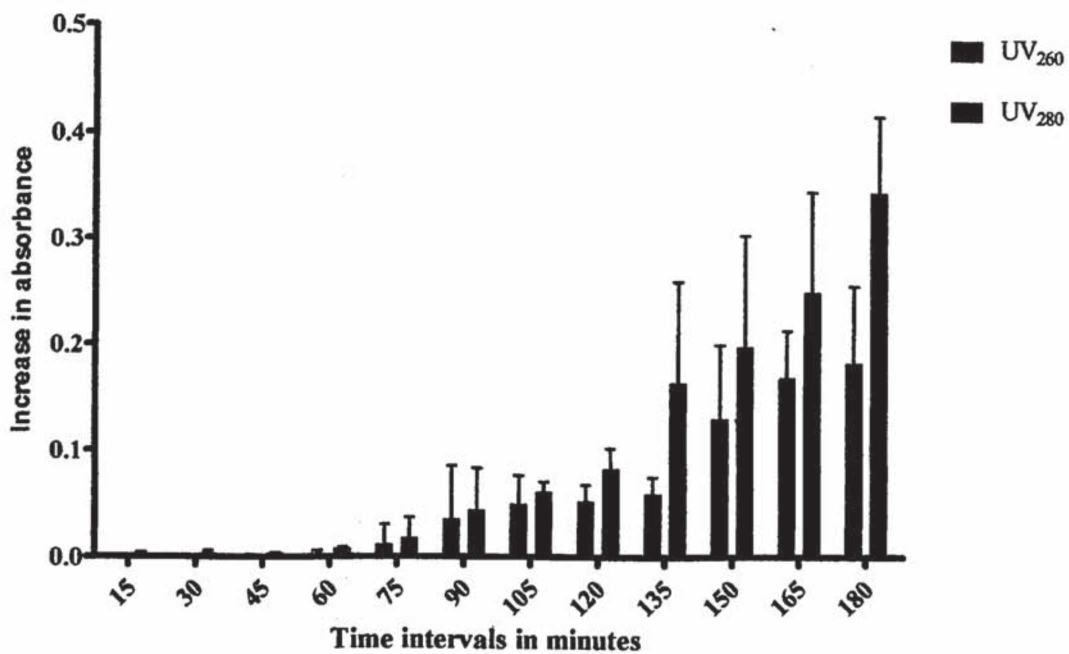

Figure 8: Determination of leakage of UV_{260} and UV_{280} absorbing material from cell suspension of *Staphylococcus aureus* treated with crude flavonoid at different time intervals.

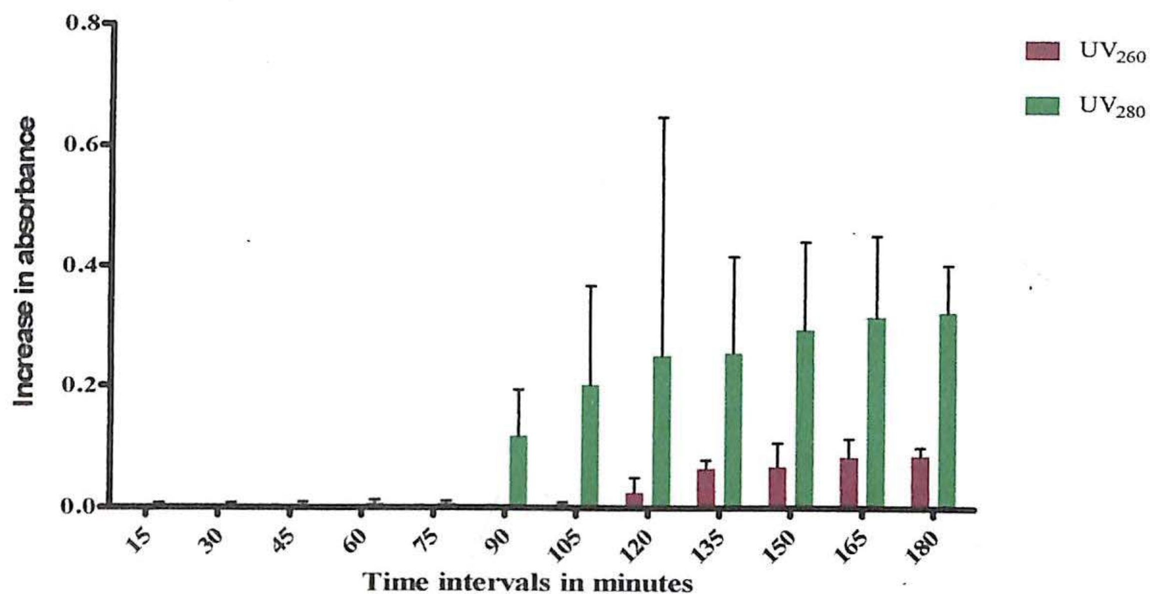

Figure 9: Determination of leakage of UV_{260} and UV_{280} absorbing material from cell suspension of *Staphylococcus aureus* treated with crude saponin at different time intervals.

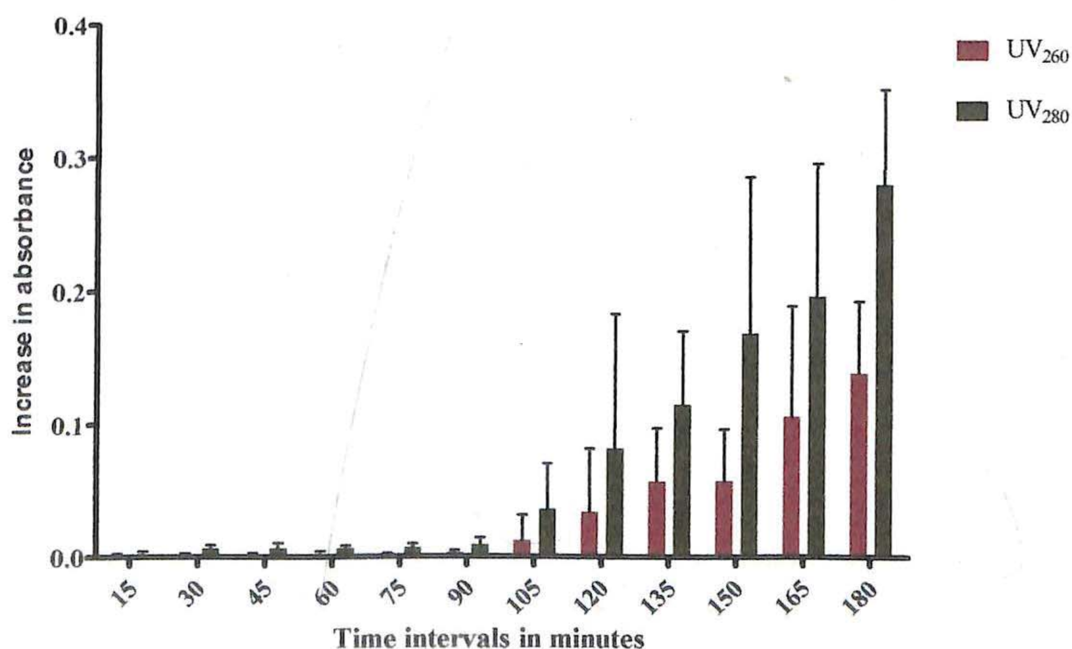

Figure 10: Determination of leakage of UV_{260} and UV_{280} absorbing material from cell suspension of *Staphylococcus aureus* treated with crude tannin at different time intervals

4.6: Antioxidant activity assay

Antioxidant activity assay were done by employing four methos including the DPPH radical scavenging activity, ferric reducing power assay, hydroxyl scavenging and the hydrogen peroxide scavenging were used to assess the antioxidant properties of crude phytochemicals of *Tectaria macrodonta.* The results showed that scavenging property was concentration dependent. The concentrations of crude phytochemicals were in range of 10 -100 μg/ml. Ascorbic acid and BHT were used as control in antioxidant assays.

4.6.1: Ferric reducing power assay (FRAP)

The antioxidant activity of the crude phytochemicals of plant was determined by FRAP method (Table: 8) and compared with ascorbic acid as standard (Figures: 11, 12, 13, 13, 14 and 15). The crude flavonoid extract of the plant sample exhibited maximum reducing power than other crude phytochemicals such as tannin, saponin, steroid. The crude flavonoid at conc. of 10μg/ml exhibited absorbance value as 0.1262± 0.005 while at concentration of 20μg/ml it was 0.1347± 0.002, which showed significant increases in ferric reducing ability of the crude phytochemicals (crude flavonoid) extracted from plants. However, the crude extracts of steroid and alkaloids exhibited less ferric reducing ability as compared to the ascorbic acid (standard) and crude flavonoid. The tannin and saponin exhibited significant increase in ferric reducing ability as 0.0296± 0.001 (30μg/ml) and0.0439± 0.010 (20μg/ml), respectively. There was a significant ($p< 0.05$) increase in the reducing ability of the crude phytochemicals when compared with the least concentration of 10 μg/ml.

Table 8: The antioxidant activity of the extracted crude phytochemicals by FRAP Method

Concentration µg/ml	Flavanoids	Saponins	Tannins	Alkaloids	Steroids	Ascorbic acid
10	0.1262± 0.005	0.0268± 0.004	0.0296± 0.001	0.019± 0.0003	0.0136± 0.004	0.139± 0.001
20	0.1347± 0.002*	0.0439± 0.010*	0.031± 0.0001	0.0251± 0.004	0.0247± 0.004*	0.2303± 0.002
30	0.1453± 0.004*	0.055± 0.0006*	0.0354± 0.001*	0.0342± 0.003*	0.0308± 0.002*	0.2758± 0.002
40	0.155± 0.003*	0.0604± 0.001*	0.037± 0.0006*	0.0421± 0.005*	0.0397± 0.002*	0.343± 0.003
50	0.1664± 0.003*	0.068±0.0002*	0.0452± 0.003*	0.0466± 0.003*	0.0509± 0.002*	0.399± 0.011
60	0.1855± 0.004*	0.0758± 0.004*	0.0535± 0.004*	0.0511± 0.002*	0.0526± 0.005*	0.4536± 0.002
70	0.1932± 0.001*	0.08± 0.002*	0.0552± 0.003*	0.0560± 0.002*	0.0534± 0.003*	0.5054± 0.003
80	0.2177± 0.002*	0.0867± 0.002*	0.0633± 0.002*	0.0609± 0.001*	0.0617± 0.002*	0.5519± 0.002
90	0.2366± 0.002*	0.0921± 0.002*	0.0727± 0.003*	0.0614± 0.002*	0.0648± 0.002*	0.6403± 0.004
100	0.2448± 0.004*	0.0980± 0.003*	0.0764± 0.002*	0.0671± 0.004*	0.0667± 0.004*	0.6988± 0.007

All the values represents mean ± SD (n=3), Experiment were done in triplicates, * Significant changes against lowest concentration of 10 µg/ml of crude phytochemicals ($p < 0.05$).

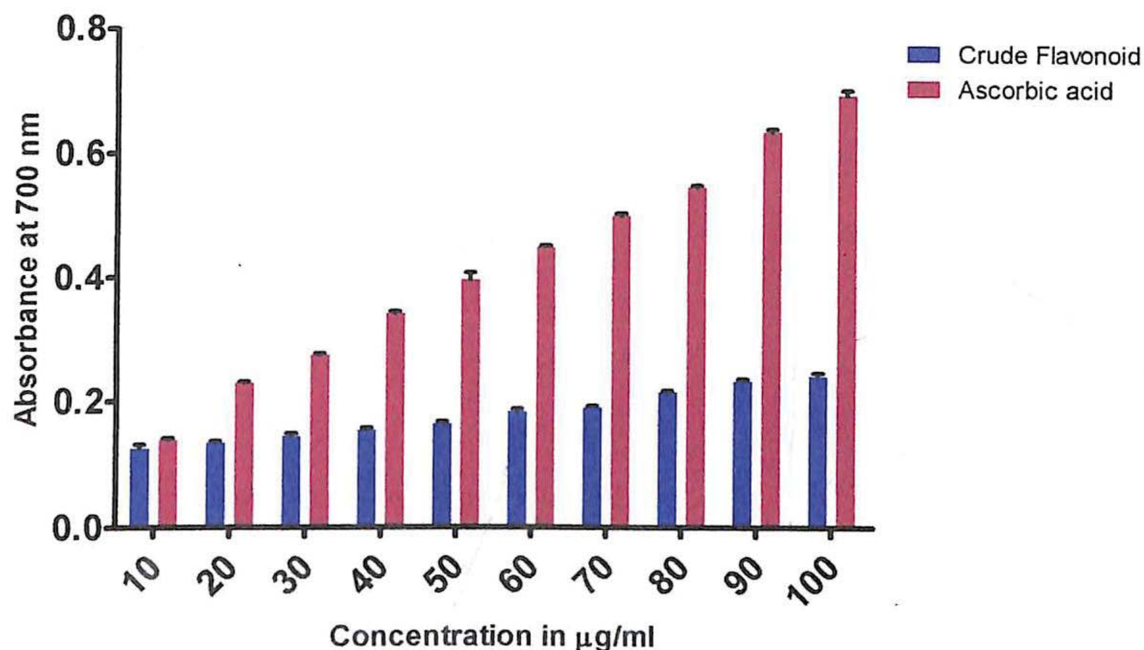

Figure 11: FRAP activity of crude flavonoid of *Tectaria macrodonta* and standard ascorbic acid.

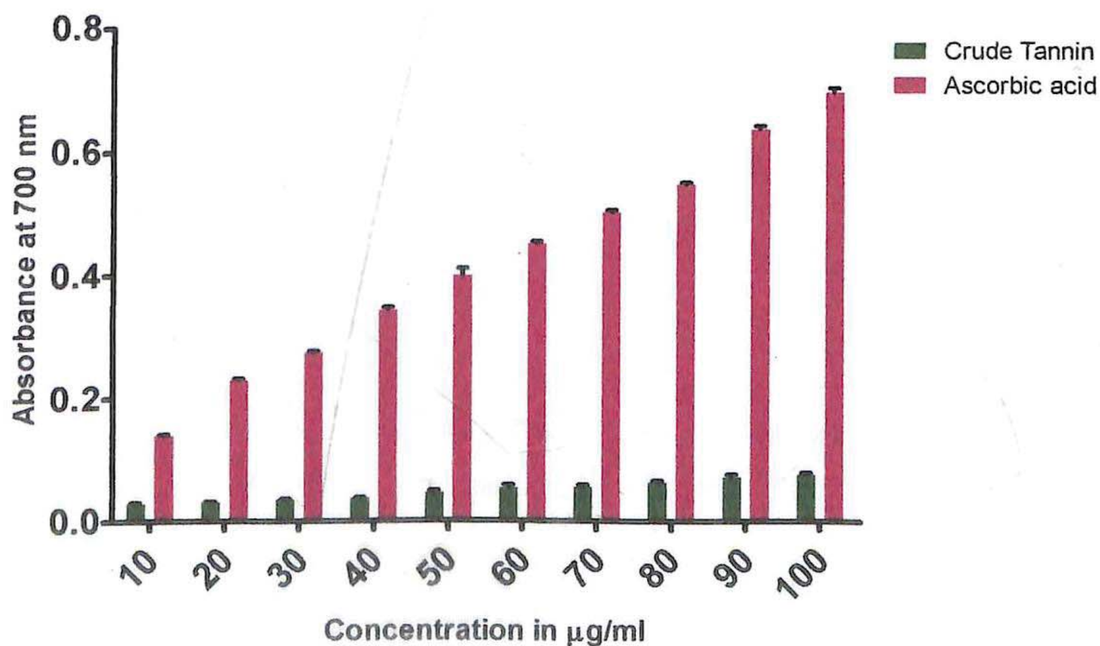

Figure 12: FRAP activity of crude tannin of *Tectaria macrodonta* and standard ascorbic acid.

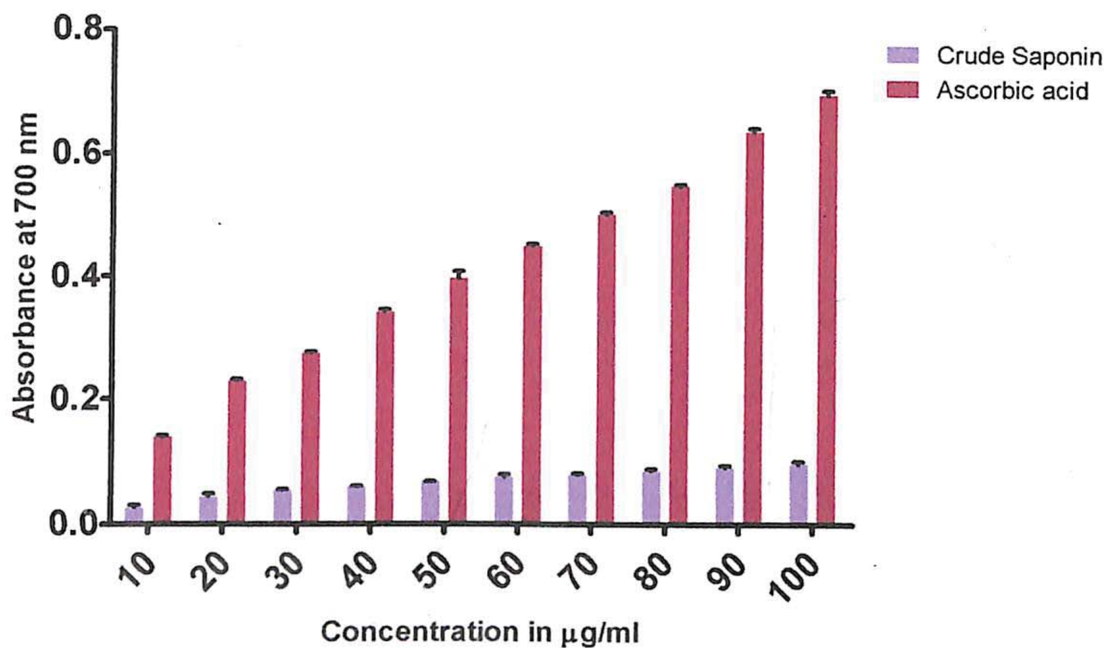

Figure 13: FRAP activity of crude saponin of *Tectaria macrodonta* and standard ascorbic acid.

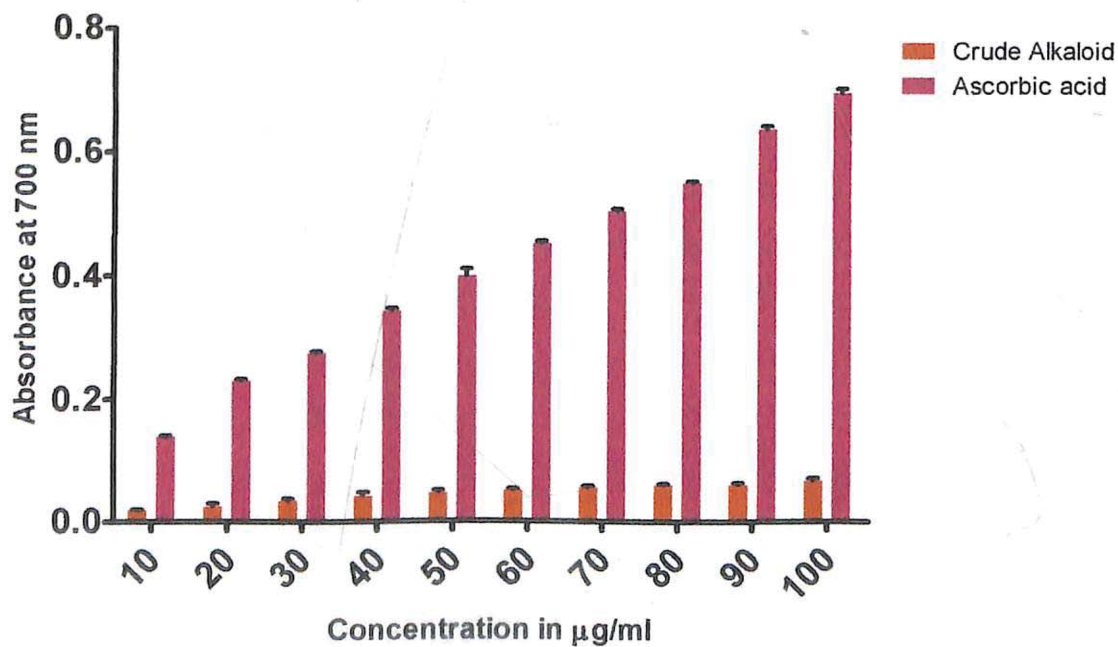

Figure 14: FRAP activity of crude alkaloid of *Tectaria macrodonta* and standard ascorbic acid.

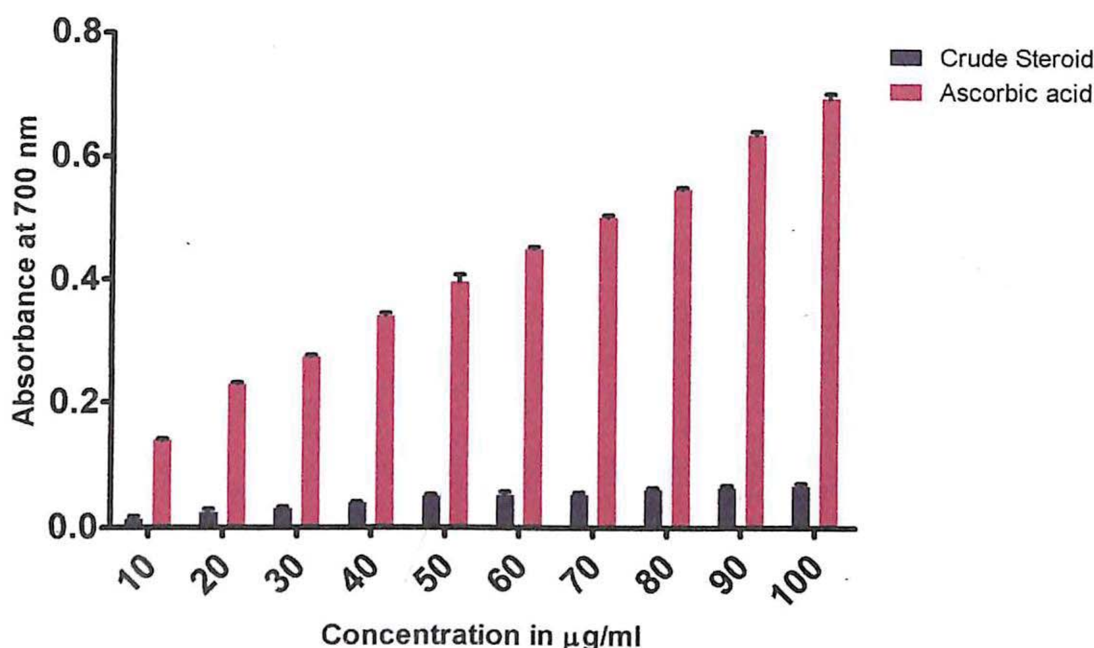

Figure: 15 FRAP activity of crude steroid of *Tectaria macrodonta* and standard ascorbic acid.

4.6.2: DPPH scavenging assay

The antioxidant activity of the crude extracted phytochemicals was evaluated by DPPH radical scavenging activity and compared with ascorbic acid as standard. Free radical scavenging potential of crude phytochemical extracts at various concentrations was detrmined by DPPH method. The antioxidant found in extract react with DPPH (a stable free radical) and converts it to 1, 1 diphenyl-2-picrylhydrazine. Table 9 illustrates the DPPH scavenging assay of all five extracted crude phytochemicals. Highest inhibition of 58.23% and 54% was recorded for crude flavonoid and crude tannin at 100 µg/ml, respectively. However, the control (ascorbic acid) showing 94% inhibition at higher concentration (100 µg/ml). The crude saponin and steroid at highest concentration exhibited 50% inhibition but crude alkaloids even at higher concentration (100µg/ml) could not show 50% inhibition of free radicals. There was a significant ($p<0.05$) DPPH scavenging activity of crude phytochemicals when the increasing concentration was compared with the lowest concentration of 10 µg/ml. The results are shown in Table 9 and Figures from 16 to 20.

Table 9: The antioxidant activity of the extracted crude phytochemicals by DPPH method

Concentration µg/ml	Flavanoids	Saponins	Tannins	Alkaloids	Steroids	Ascorbic acid
10	31.27±0.046	29.73±0.043	43.11±0.011	17.48±0.34	27.94±0.186	36.06±0.038
20	35.15±0.043*	31.66±0.577*	45.40±0.037*	17.99±0.001	30.40±0.526*	43.03±0.966
30	41.27±0.038*	33.73±0.578*	46.30±0.055*	18.69±0.43*	32.36±0.552*	47.08±0.070
40	46.24±0.041*	36.74±0.613*	48.70±0.005*	20.04±0.03*	34.07±0.015*	54.04±0.003
50	50.37±0.215*	39.53±0.949*	49.98±0.960*	22.29±0.17*	37.01±0.632*	62.03±0.031
60	52.14±0.066*	44.66±2.052*	50.97±0.852*	23.37±0.068*	40.09±0.053*	68.67±0.517
70	55.76±0.287*	48.47±1.74*	52.33±0.789*	23.97±0.03*	43.99±1.118*	75.96±0.939
80	57.05±0.065*	50.12±2.85*	52.58±0.823*	25.32±0.62*	46.09±0.702*	83.29±0.612
90	57.96±0.045*	50.55±0.48*	53.07±2.55*	26.59±0.529*	48.13±0.160*	87.33±0.322
100	58.23±0.033*	52.33±0.60*	54.95±0.058*	27.92±0.20*	50.02±0.075*	94.14±2.35

All the values represents mean ± SD (n=3), Experiments were done in triplicates, * Significant changes against lowest concentration of 10 µg/ml of crude phytochemicals ($p < 0.05$).

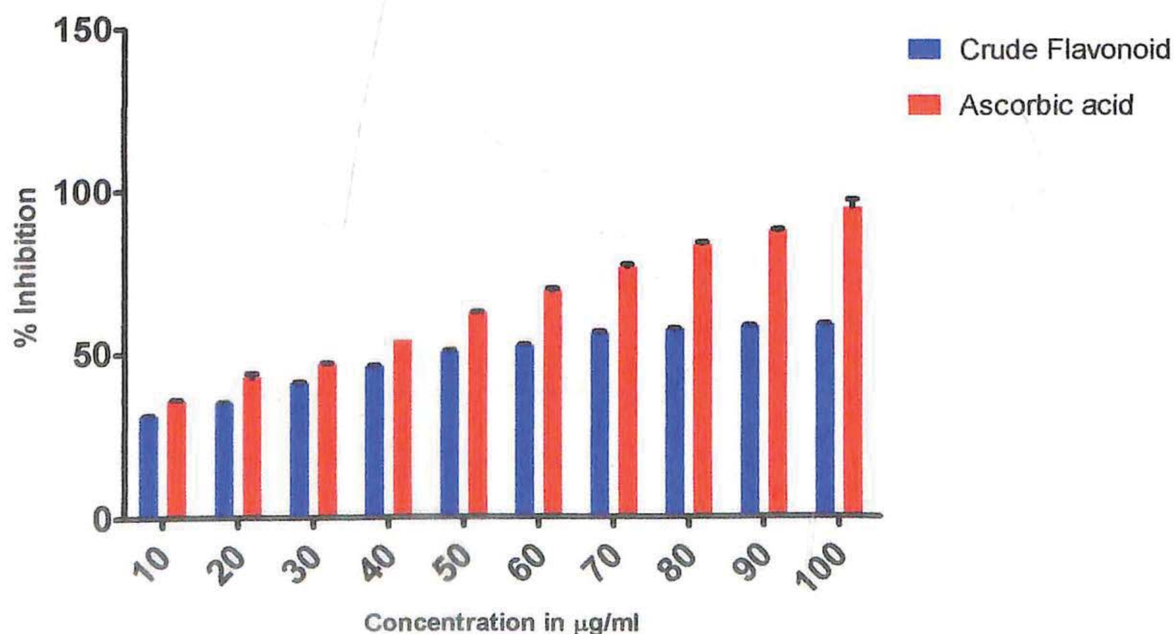

Figure 16: DPPH scavenging activity of crude flavonoid of *Tectaria macrodonta*.

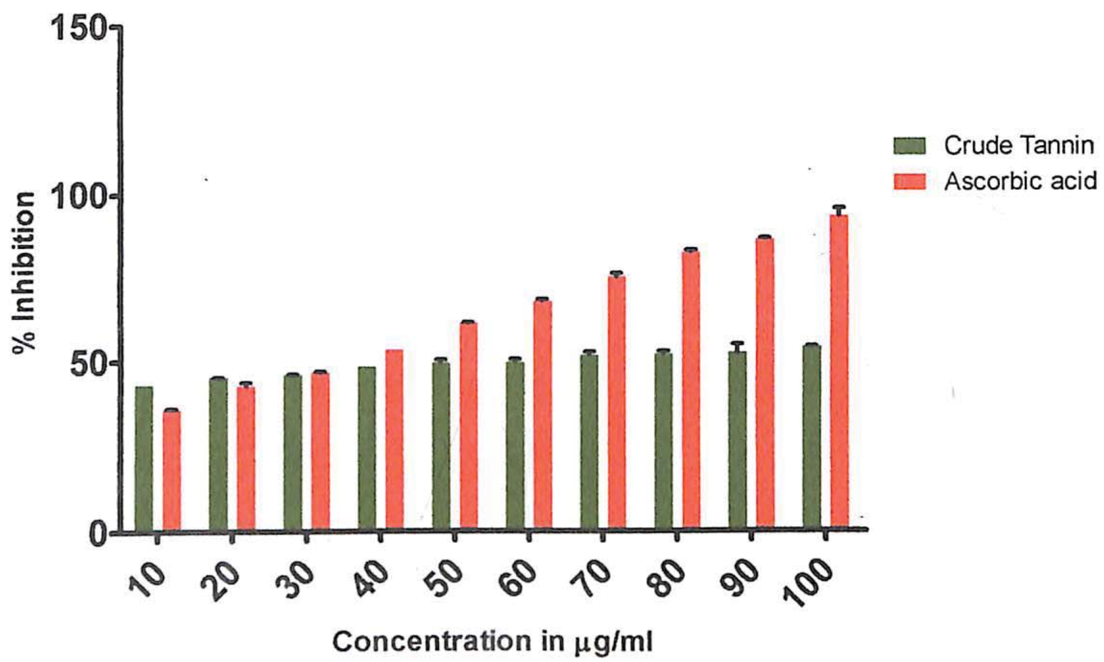

Figure 17: DPPH scavenging activity of crude tannin of *Tectaria macrodonta*.

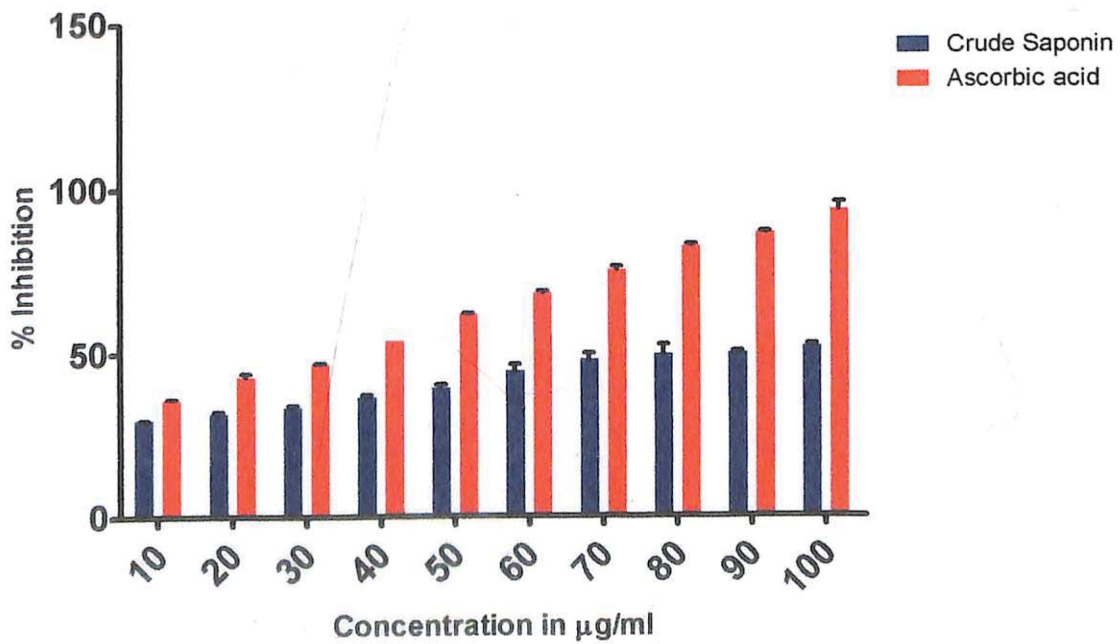

Figure 18: DPPH scavenging activity of crude saponin of *Tectaria macrodonta*.

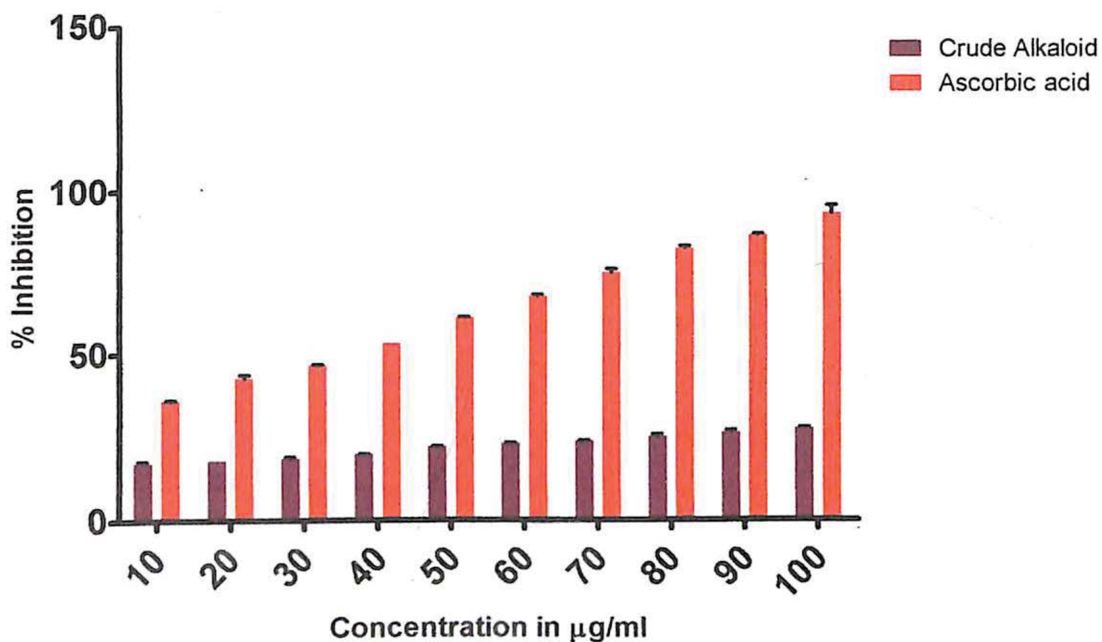

Figure 19: DPPH scavenging activity of crude alkaloid of *Tectaria macrodonta*.

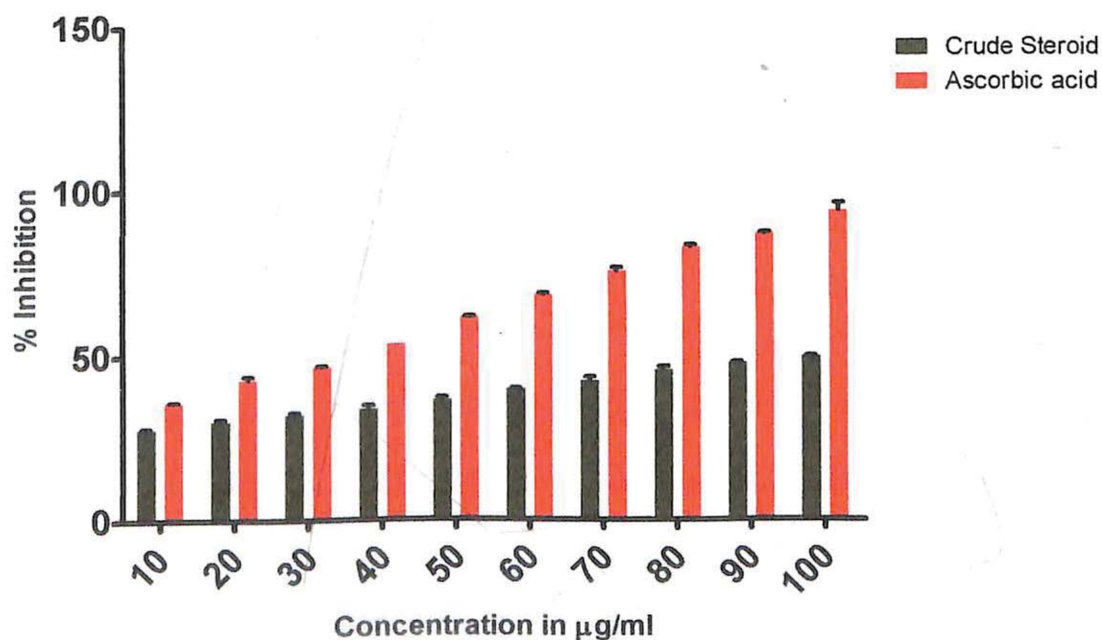

Figure 20: DPPH scavenging activity of crude steroid of *Tectaria macrodonta*.

4.6.3: Hydroxyl (OH) scavenging assay

OH scavenging activity for crude flavonoid at 100µg/ml was 26.05±0.26% followed by 31.20±0.86% for crude tannin, 23.48±0.034% for crude saponin, 23.21± 0.11% for crude steroid and 22.25± 0.87% for crude alkaloid. All the crude phytochemicals showed significant increased in percent inhibition at the concentration of 20µg/ml. The crude flavonoid extract exhibited higher OH scavenging activity at highest concentration (100 µg/ml). On the contrary the standard (BHT) has better activity (76.88±0.67%) at higher concentration 100 µg/ml. There was a significant ($p<0.05$) OH scavenging activity when the increasing concentration was compared with the lowest concentration of 10 µg/ml (Table: 10).

Table 10: The antioxidant activity of the extracted crude phytochemicals evaluated by Hydroxyl (OH) scavenging assay method and compared with BHT as standard

Concentration µg/ml	Flavanoids	Saponins	Tannins	Alkaloids	Steroids	BHT
10	7.38±0.42	5.43±0.29	4.38± 0.05	2.80± 0.63	3.48± 0.47	27.30±1.06
20	8.52±0.28*	6.6±0.398*	7.39±0.52*	4.19± 0.24*	5.05± 0.03*	32.69±0.50
30	11.13±0.92*	8.13±0.49*	9.56±0.41*	5.44± 0.48*	7.52±0.77*	37.15±0.16
40	14.19±0.21*	10.28±0.225*	12.71±0.42*	7.43± 0.52*	10.44± 0.59*	43.81±1.64
50	16.40±0.36*	12.92±0.725*	14.67±0.19*	9.37± 0.12*	12.53± 0.14*	51.38±1.08
60	18.11±0.01*	13.71±0.592*	17.51±0.51*	13.13± 0.18*	15.33± 0.22*	55.34±0.64
70	19.66±0.49*	15.17±0.124*	21.61±0.46*	15.57± 0.43*	18.26± 0.22*	61.84±0.65
80	22.40±0.3*	17.60±0.46*	24.42±0.30*	16.23± 0.23*	20.51± 1.05*	66.16±0.135
90	25.19±0.48*	20.47±0.71*	27.53±0.39*	18.95± 0.21*	21.76± 0.44*	73.94±0.019
100	26.05±0.26*	23.48±0.34*	31.20±0.86*	22.25± 0.87*	23.21± 0.11*	76.88±0.67

All the values represents mean ± SD (n=3), Experiments were done in triplicates, * Significant changes against lowest concentration of 10 µg/ml of crude phytochemicals ($p< 0.05$).

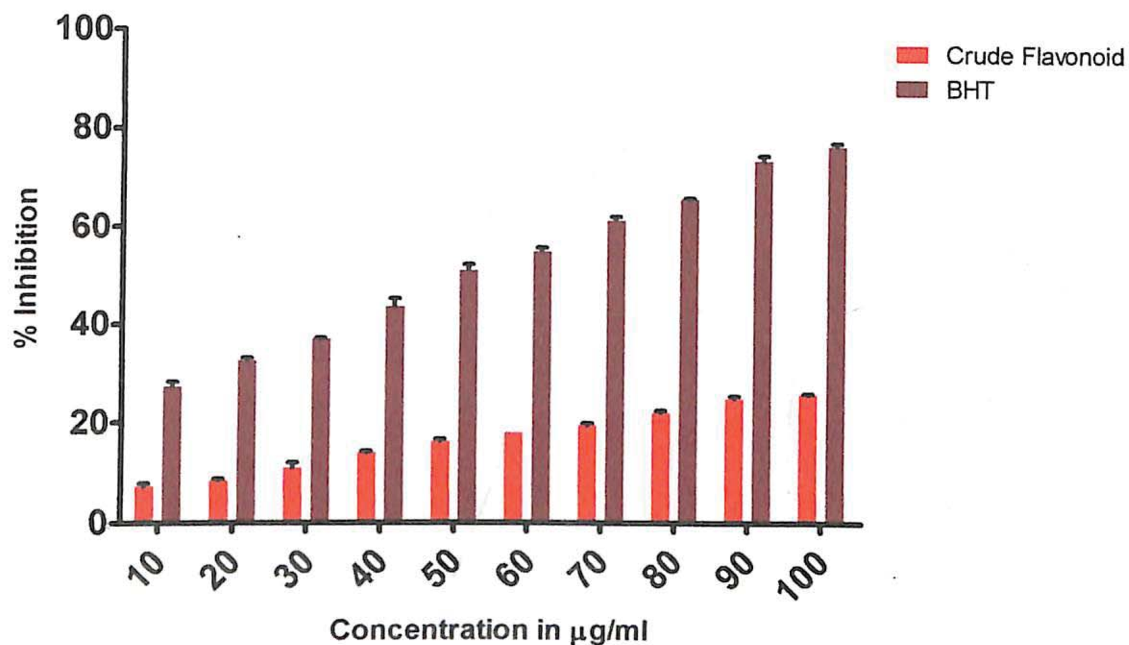

Figure 21: OH radical scavenging activity of crude flavonoid of *Tectaria macrodonta* and standard BHT.

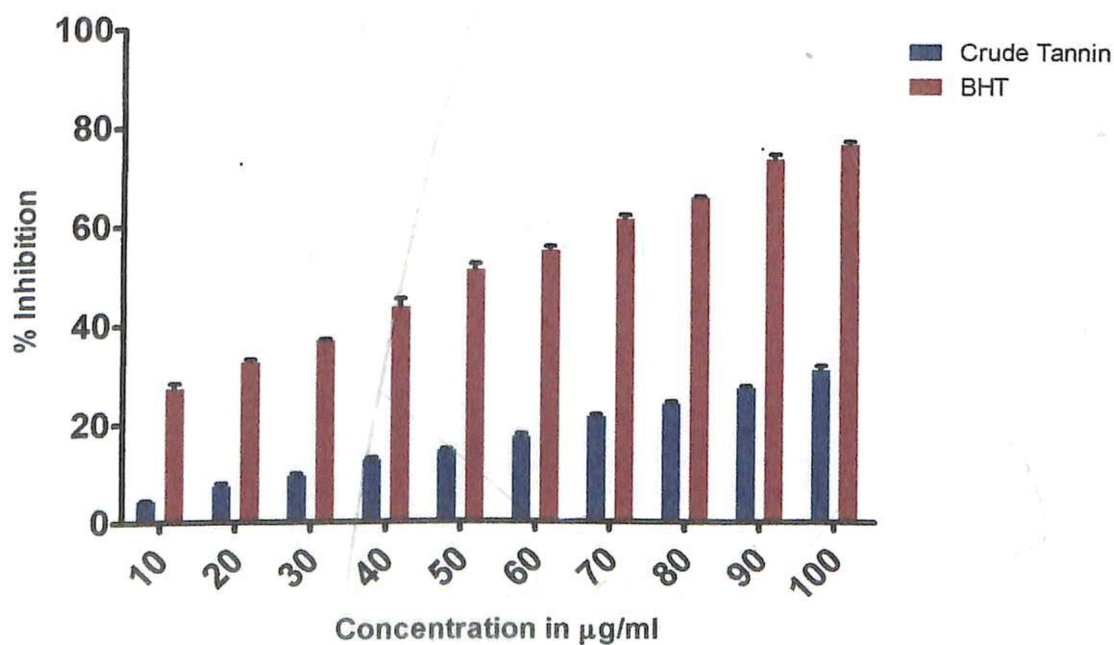

Figure 22: OH radical scavenging activity of crude tannin of *Tectaria macrodonta* and standard BHT.

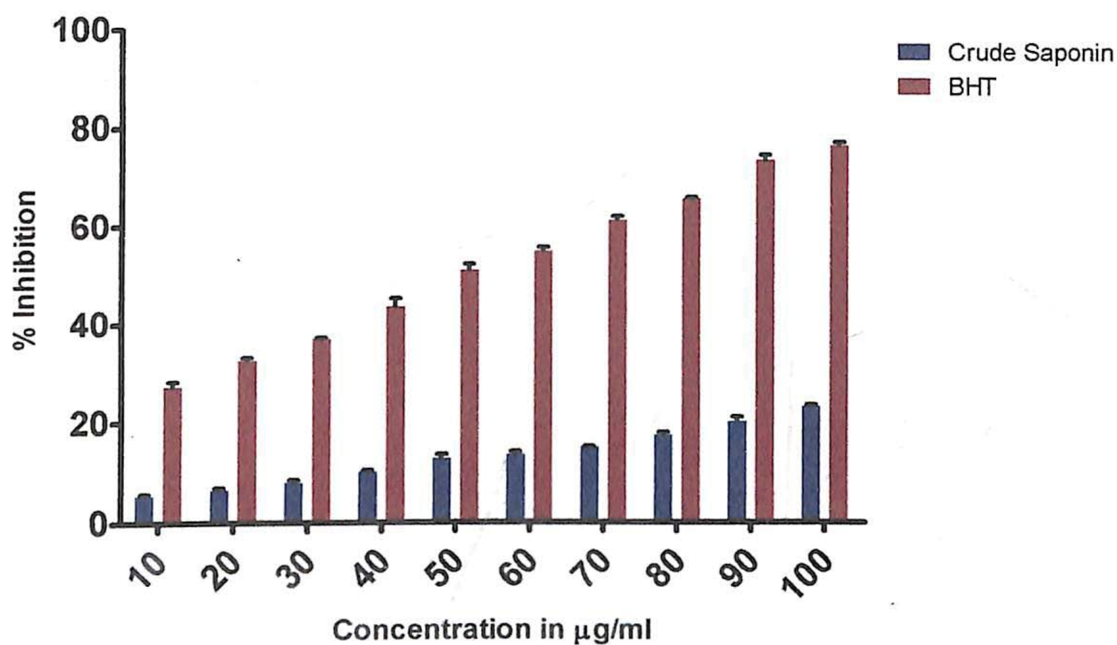

Figure 23: OH radical scavenging activity of crude saponin of *Tectaria macrodonta* and standard BHT.

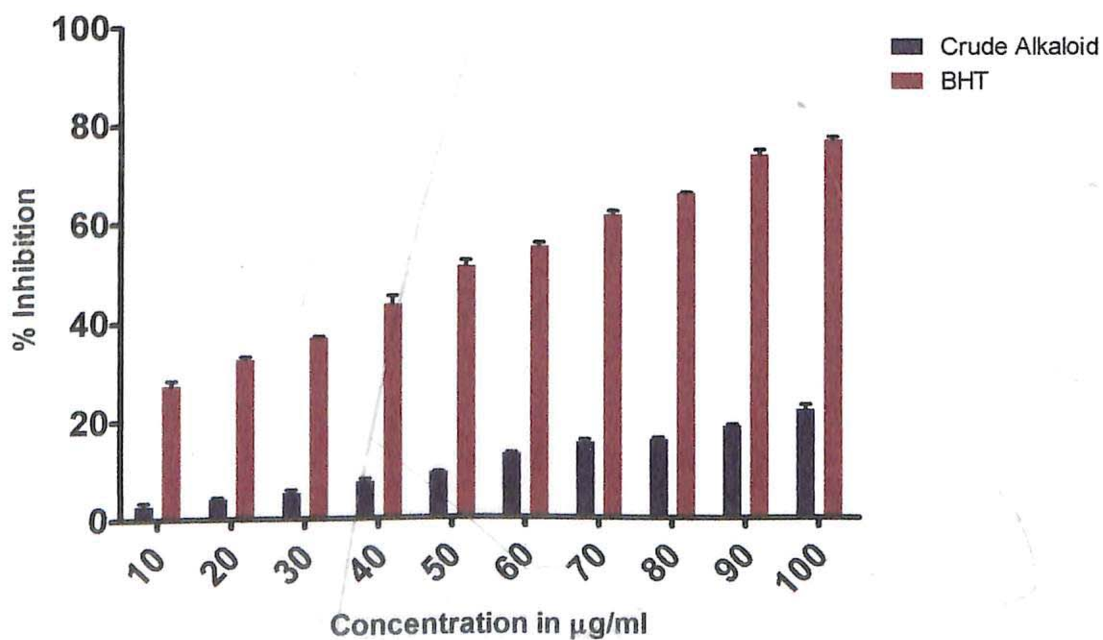

Figure 24: OH radical scavenging activity of crude alkaloid of *Tectaria macrodonta* and standard BHT.

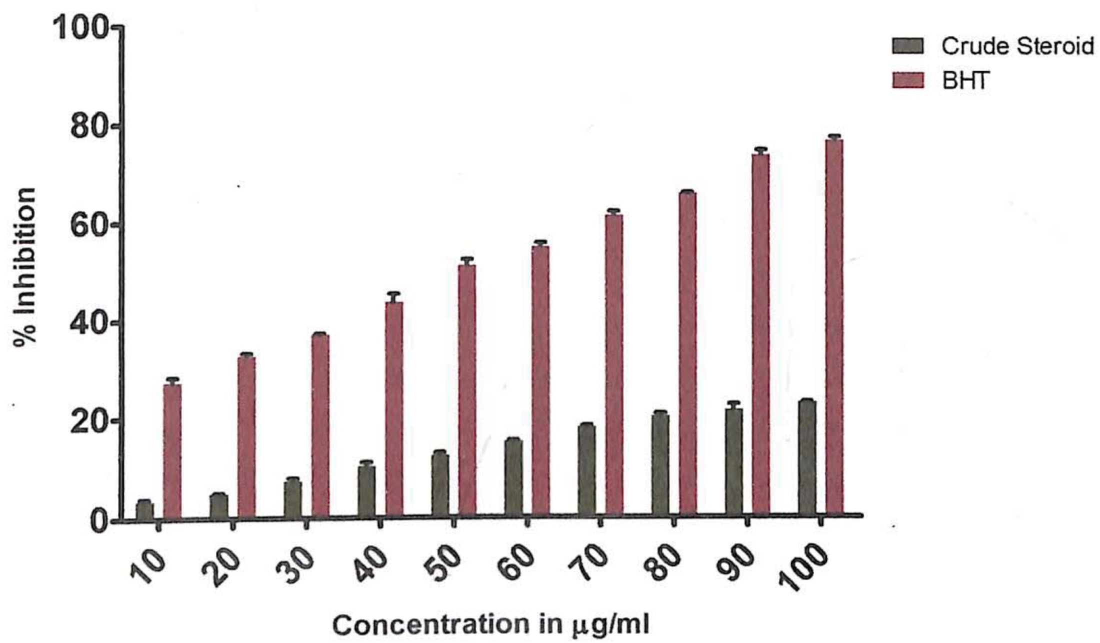

Figure 25: OH radical scavenging activity of crude steroid of *Tectaria macrodonta* and standard BHT.

4.6.4: Hydrogen peroxide scavenging

Various crude phytochemical extracts were used in different concentration for determination of hydrogen peroxide scavenging activity. Hydrogen peroxide scavenging activity of different crude phytochemicals at highest concentration (100μg/ml) can be compared as crude flavonoid (53.55±0.404%) > crude steroid (50.68±1.500%)> crude tannin (33.16±0.886%)> crude saponin (31.05±1.007%)> crude alkaloid (25.71±0.530%). The crude flavonoid and crude steroid were capable of scavenging hydrogen peroxide approx 50% at concentration of 100 μg/ml. There was a significant ($p<0.05$) Hydrogen peroxide scavenging activity when the increasing concentration was compared with the lowest concentration of 10 μg/ml. The results are shown in Table: 11 and Figure: 26.

Table 11: The antioxidant activity of the extracted crude phytochemicals by hydrogen peroxide scavenging method

Concentration μg/ml	Flavanoids	Saponins	Tannins	Alkaloids	Steroids
10	16.21±0.056	10.39±0.612	12.02±0.025	6.10±0.104	21.06±0.052
20	17.46±0.602*	11.62±0.464*	15.69±0.531*	7.44±0.429*	24.76±0.870*
30	20.06±0.485*	14.85±0.462*	17.52±0.467*	9.22±0.211*	27.19±0.295*
40	23.66±0.702*	17.49±0.417*	20.31±0.366*	12.51±0.430*	29.61±0.537*
50	28.94±0.604*	19.71±1.09*	22.68±0.56*	13.14±0.107*	32.24±0.251*
60	34.04±1.68*	20.59±0.04*	25.08±0.109*	16.17±0.044*	35.81±0.011*
70	38.74±1.04*	23.36±0.31*	26.04±0.152*	18.63±0.540*	40.97±2.668*
80	44.06±1.69*	26.61±0.453*	28.78±0.559*	19.10±0.012*	44.48±1.008*
90	49.44±0.479*	28.93±1.757*	31.10±0.164*	23.64±0.703*	48.11±0.1718
100	53.55±0.404*	31.05±1.007*	33.16±0.886*	25.71±0.530*	50.68±1.5*

All the values represents mean ± SD (n=3), Experiment were done in triplicates. * Significant changes against lowest concentration of 10 μg/ml of crude phytochemicals ($p< 0.05$).

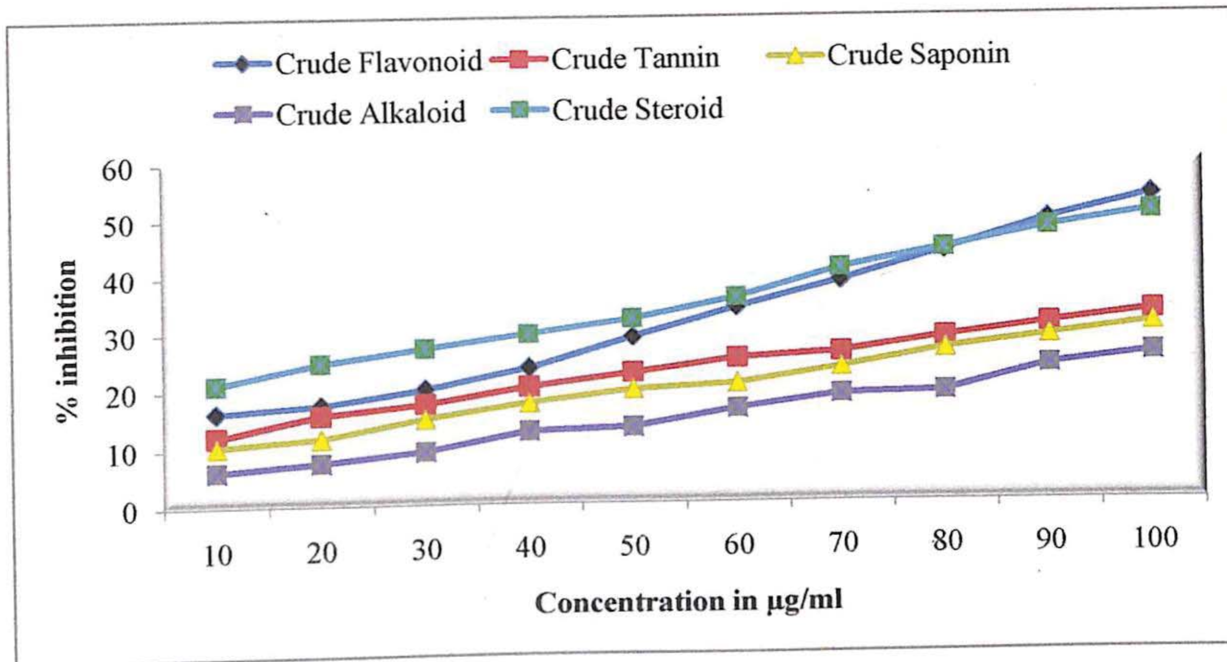

Figure 26: Hydrogen peroxide (H_2O_2) scavenging activity of crude phytochemicals of *Tectaria macrodonta.*

4.7: Total flavonoid content in crude flavonoid extract

The total flavonoid content in crude flavonoid extract of *Tectaria macrodonta* was measured by Aluminium chloride colorimetric method and expressed in terms of Rutin equivalent / mg of the sample. The concentration of crude flavonoid extract taken was 1000 µg/ml.

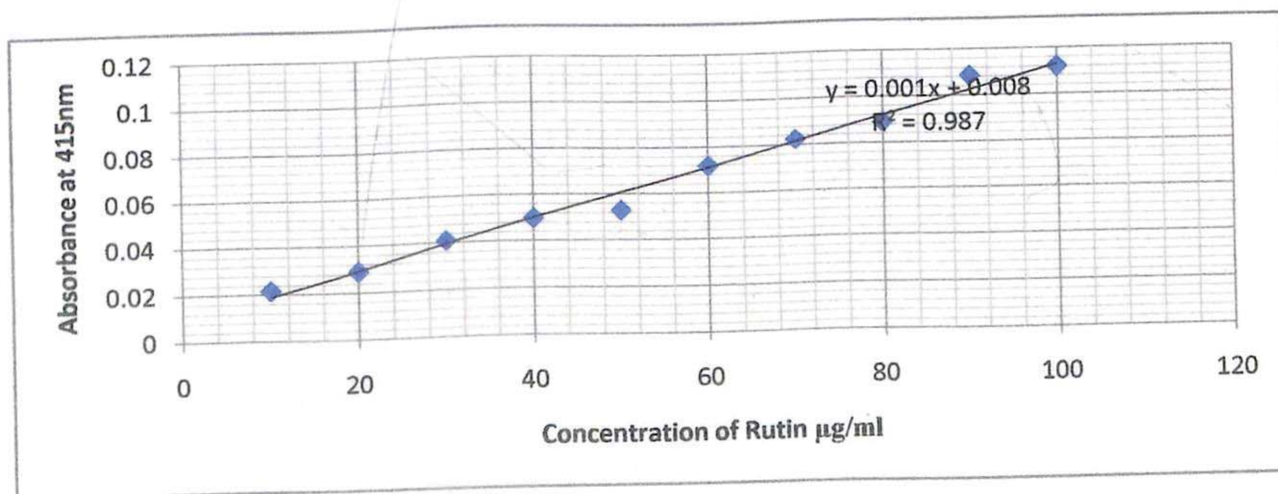

Figure 27: Calibration curve obtained by using Rutin as standard for estimation of total flavonoid content in crude flavonoid extract.

The quantity of total flavonoid compounds present in the crude flavonoid extracts of plant sample was calculated from the regression curve ($y = 0.001x + 0.0083$, $R^2 = 0.9872$) as shown in Figure 27. The amount of flavonoid content in crude flavonoid extract was determined as 64 mg/g in RE.

4.8: Total tannin content in crude tannin extract

The total tannin content in crude tannin extract of *Tectaria macrodonta* was measured and expressed in terms of tannic acid equivalent / mg of the sample. The concentration of crude tannin extract taken was 1000 μg/ml.

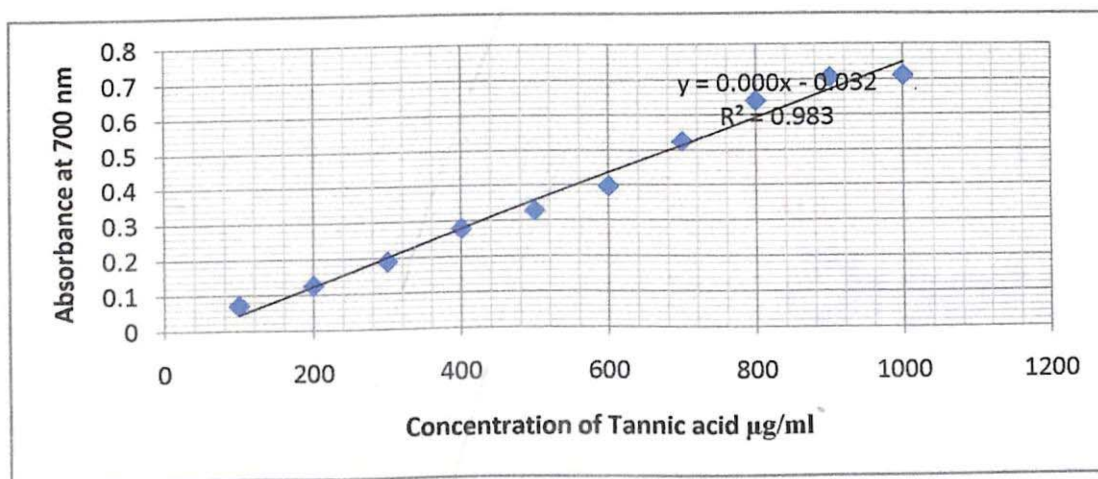

Figure 28: Calibration curve obtained by using tannic acid as standard for estimation of total tannin content in crude flavonoid extract.

The quantity of total tannin compounds present in the crude tannin extracts of *Tectaria macrodonta* was calculated from the regression curve ($y = 0.008x + 0.00325$, $R^2 = 0.9837$) as shown in Figure 28. The amount of tannin content in crude tannin extract was determined as 400 mg/g in TAE (tannic acid equivalent).

Chapter 5

DISCUSSION

solvent for the extraction of dry powdered of rhizome through microwave. The microwave assisted extraction is used for the extraction of wide range of compound, but, for the tannin extraction microwave, hotplates and water bath can be used with appropriate solvent (Cobez *et al.*, 2005). The high extraction efficiency of tannin has been analyzed in microwave assisted extraction procedure, where it has given higher amount of tannin than ultra sound assisted extraction, when tannin estimated quantitatively through spectrophotometer (Cobzac *et al.*, 2005). In present study, warm water as solvent utilized for tannin extraction because in microwave extraction there is more chance to extraction of tannin. This crude tannin further analyzed for phytochemical screening and it has been found that crude tannin also contained flavonoid, saponin and anthocyanin apart from tannin. Flavonoids, anthocyanin and tannin are phenolic compounds (Sharief *et al.*, 2014) therefore there is strong possibility of presence of all these phenolics and their relative compounds in crude tannin extract. The methanol, aqueous and ethanol extracts of *Tectaria macrodonta* revealed the presence of flavonoid in preleiminary phytochemical testing (Poudyali, 2013). Therefore, the present study was done for the extraction and partial obtaining the crude flavonoids from rhizome powder of *Tectaria macrodonta*. For the extraction of crude flavonoid, methanol and ethyl acetate were used since the ethyl acetate fraction contained flavonoids. Various researches have been done that exhibited the presence of highest quantity of flavonoid in ethyl acetate fraction (Djeridane *et al.* 2006). The plant *Malva parviflora L.* extracted by methanol and after extraction concentrated residue were defatted by ether for removal of fatty compounds. Then upper phase of ethyl acetate fraction was evaporated to dryness using a rotary evaporator (40°C) and such dry residue was considered as crude flavonoid or crude phenolics (Farhan *et al.*, 2012).

Saponin makes an important chemical constituent of *Tectaria macrodonta* has been proved by phytochemical characterization in earlier studies (Parihar *et al*, 2010). The saponin was present in *Tectaria macrodonta* when it was extracted through water, methanol and ethanol as solvent (Poudyali, 2013). In present study, the crude saponin was qualitatively analyzed (saponin test) by formation of froth and foam in water. (Table: 2). The saponin extraction was based on the use of various solvents such as petroleum ether, methanol and acetone which produced maroon coloration powder form. The same procedure was followed by Kannabiran *et al.*, (2009) for extraction of

crude saponin from *Solanum Xanthocarpum* and *Centella asiatica* in form of whitish amorphous powder (Kannabiran *et al.*, 2009). The crude saponin subjected for further phytochemical determination and revealed that it also contained tannin, flavonoid and anthocyanin as phenolic compounds. The petroleum ether was used for the defatting of powdered root of plant (Moghimipour *et al.*, 2003) followed by extraction with combination of methanol and acetone to the formation of precipitate turning to white amorphous powder named as crude saponin (Kinnabiran *et al.*, 2009). However, to obtain the purified saponin, the fractionation of crude saponin was carried out with chloroform: methanol: water (70:30:10) through column and first fraction was evaporated under reduced temperature (Khanna *et al.*, 2008). The crude saponin extracted from *Sorghum bicolor* with the hexane, instead of petroleum ether, for removing of lipids and then extraction with methanol exposed the occurrence of low molecular weight materials such as sugars, phenolic compounds and flavonoids (Soetan *et al.*, 2006). The saponin from *Vernonia amygdalina* extracted with methanol also contained phenolic compounds because methanol helps in extraction of various phenolics (Igile, 1995). These studies support that the crude saponin may contain other compounds, as saponins of *Tectaria macrodonta* have certain other phenolic compounds namely tannin and flavonoid, confirmed by phytochemical testing.

The alkaloids are nitrogenous compound found in many parts of plant such as rhizome, bark and leaves (Manske, 1965). The earliest reports on *Tectaria macrodonta* showed the absence of alkaloid in all solvent extraction estimated by qualitative phytochemical investigation (Poudyali, 2013; Sukumaran *et al.*, 2012). The presence of alkaloid in crude alkaloid extract was determined by Hagner's test. But other tests were negative for presence of alkaloids (Table: 2). Therefore, on the basis of phytochemical investigation the *Tectaria macrodonta* has been found to contain very less form of alkaloids. Alkaloids are absent or infrequently found in pteridophytic plants or ferns (Harborne, 2007), thus there is very less possibility of extraction yield of crude alkaloids that may be mixture of various substances.

The processing of the crude phytochemicals done in present study is not similar to the traditional method when the plant practitioners or folk healers use hot water for extraction.

Plants are important sources of antimicrobial compounds and have huge therapeutic potential without any side effect as often seen with synthetic antimicrobial drugs (Singh *et al.*, 2011). There are many reports that describe the antibacterial, antifungal, antiviral, antiprotozoal properties of plants. In the present study, the test strains of bacteria obtained from MTCC Chandigarh and tested for susceptibility against various crude phytochemicals of *Tectaria macrodonta* by well diffusion method. Different amounts of each crude phytochemicals (20, 50, 100, 150, 200, 250, 300, 350 and 400 µg/ml) were used against test microorganisms. The various concentrations of crude phytochemicals were prepared by dissolving in 0.25% DMSO and diameter of inhibition zone was measured with the help of antibiotic scale. Gentamicin was used as positive control while 0.25% DMSO used as negative control in each plates. The DMSO is used because it increases the absorption and penetration of dissolved drugs and other chemicals into tissues (Leake, 1967).

The result showed that the crude extracted phytochemicals exhibited significant antibacterial activities against Gram positive bacteria namely *Staphylococcus aureus* and *Bacillus cereus*. Comparative analysis reveals that the crude flavonoid was found to exhibit highest antibacterial activity against *Staphylococcus aureus* and *Bacillus cereus* whereas no antibacterial activity was developed against Gram negative bacteria *Esherichia coli* and *Pseudomonas aeruginosa*. However, as the concentration of crude phytochemical was increased the diameter of zone of inhibition also increased for *Staphylococcus aureus* and *Bacillus cereus*. The crude flavonoid at concentration of 20mg/ml gave the zone in range of 12 mm against *Bacillus cereus* (Table: 4) but at the same concentration there was no zone of inhibition recorded against *Staphylococcus aureus* (Table: 3, Figure: 3). In previous reports, it was found that the best antibacterial property of flavonoid was reported against *Staphylococcus aureus* and purified flavonoids have given excellent results of antimicrobial properties between the concentrations of 3.9 µg/ml to 15.6 µg/ml (Cushnie *et al.*, 2005).

After crude flavonoid, the highest diameter of zone of inhibition was found for crude saponin against *Staphylococcus aureus* and *Bacillus cereus* by taking different concentrations. The lowest concentrations (20 and 50 mg/ml) did not show any antibacterial activity (Table: 3 and 4), but at higher concentration the zone of inhibition was recorded as 10.33±0.5 and 11.66±0.5 mm against *Staphylococcus*

aureus (Table: 3). At lower concentration (50 mg/ml) the zone of inhibition was recorded for *Bacillus cereus* as 10.3±0.5 mm (Table: 3). The antibacterial activity of crude saponin was limited only to Gram positive bacteria but not against Gram negative tested strains (*Escherichia coli* and *Pseudomonas aeruginosa*). These results were accordance with reports by Soetan *et al.*, (2006). The crude saponin extract prepared from *Sorghum bicolor* exhibited antibacterial activity against Gram positive bacteria but was not inhibitory to the growth of Gram negative bacteria (Soetan *et al.*, 2006). This is not surprising because most of the Gram negative bacteria and fungi have been shown to be resistant to antibiotics and drugs (Hugo and Russell, 1983). It is interesting that the crude saponins of *Tectaria macrodonta* were active against *Staphylococcus aureus*, a pathogen which is responsible for several infections of human and animals. Additionally, the crude saponin extract is a mixture of several saponins and other compounds and each of which could be as effective as or even more efficient than the popular antibiotics presently are being used (Soetan *et al.*, 2006).

Among the crude phytochemicals, the crude tannin extract also exhibited antibacterial activity at higher concentrations. At the lower concentration range from 20 – 50 mg/ml no zone of inhibition was observed against test strains (Table: 3 and 4). Crude tannin at a concentration of 100 mg/ml exhibited antibacterial properties against *Bacillus cereus* while 300 mg/ml was determined for *Staphylococcus aureus* as antibacterial in well diffusion assay (Figures: 3 and 4). However, the higher concentration of crude tannin did not inhibit the growth of Gram negative test bacteria. The previous reports have revealed that the tannin has highest water solubility than other compounds and such trait of water solubility of crude tannin may be important for their antimicrobial action (Yoshida *et al.*, 2000). The increase in antibacterial efficiency observed with raising the concentration of crude tannins is in accordance with the work of Banso *et al.*, (2007), who reported that higher concentrations of antimicrobial components showed significant growth inhibition of pathogenic bacterial strains (Banso *et al.*, 2007). Several plants that are rich in tannins have exhibited antibacterial activities against several microorganisms (Banso *et al.*, 2007). For example, the methanol and acetone extracts of *Tectaria macrodonta* contained tannin and showed the antibacterial effect against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* (Parihar *et al.*, 2010).

The crude alkaloid and crude steroid were also tested for antimicrobial activities but they were not effective against any test bacterial strains even at higher concentration (400mg/ml). The results of present study showed absence of antimicrobial properties of crude alkaloid is in agreement with study of Maatalah *et al.*, (2012) reported that the alkaloid extract of *Anabasis articulata* had no antimicrobial activity. Phytochemical analysis revealed the infrequently presence of alkaloid in *Tectaria* species (Parihar *et al.*, 2010) and other allied ferns (Harborne, 2007) can be the most prominent reason of its as non antimicrobial (Maatalah *et al.*, 2012).

The crude extracts that possess phenolic compounds such as flavonoid and tannin showed antibacterial activity. The previous research focused on solvent extract of *Tectaria macrodonta* and the extract which had phenolic compound revealed antibacterial properties (Poudyali, 2013). The crude extract of various phytochemicals did not show antibacterial effect against Gram negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa.* The resistance shown by Gram negative strains could due to certain mechanisms such as inactivation of enzyme, modification in target site or reduction in accumulation of drug inside cells (Lutz *et al.*, 2011). The outer membrane of Gram negative bacterial cell is rich with lipo-polysaccharides (Shakya *et al.*, 2013) that limit diffusion of the bioactive compounds present in the plant extract (Sharief *et al.*, 2014). Marino *et al.*, (2001) has concluded that plant extracts usually have more inhibitory activity against Gram positive bacteria than Gram negative bacteria (Marino *et al.*, 2001).

The antibacterial screening through well diffusion method served as an indication of the selection of microbial strains that showed antibacterial activity for further determining the MIC and MBC of crude phytochemicals of plant. The MIC value is defined as the lowest concentration of drug that absolutely inhibited the bacterial growth as determined by visual observation. However, the MBC value has been explained as the lowest concentration at which 99.9% of the test bacterial strains were killed and no growth was observed after incubation of plates (Wiegand *et al.*, 2008). The values of the MIC were determined after 24 hours incubation at 37°C, but, MBC value was estimated by streaking or swabbing a sample on Mueller Hinton Agar (MHA) and kept for incubation for 24 hours at 37°C. Gentamicin was used as positive

control in experiment for determination of MIC and MBC and its value was found very low for each test bacterial strain as compared with various concentrations of crude phytochemicals of *Tectaria macrodonta.*

As far as MIC is concerned, the crude flavonoid inhibited the growth of *Staphylococcus aureus* and *Bacillus cereus* at lower concentration (20mg/ml and 50mg/ml) than the any other crude phytochemicals. *Bacillus cereus* was inhibited by the low concentration of crude flavonoid of *Tectaria macrodonta* with MIC and MBC value as 20mg/ml and 75mg/ml respectively followed by crude saponin, with MIC and MBC value as 50mg/ml and 75mg/ml respectively (Table: 5). The crude tannin exhibited MIC and MBC values of 75mg/ml and 100mg/ml respectively, against *Bacillus cereus*. The growth of *Staphylococcus aureus* was also affected by crude flavonoid in comparison with other crude phytochemiclas. At the MIC value of 50mg/ml, the growth of *Staphylococcus aureus* was not visualized and at 75 mg/ml concentration was considered as MBC value for crude flavonoid (Plate: 10). However, the flavonoid extracted from *Chromolaena species* exhibited MIC value of 100μg/ml to 100μgml against *Staphylococcus aureus* (Taleb-Contini *et al.*, 2003). The crude saponin showed MIC and MBC value of 75mg/ml and 100mg/ml respectively against *Staphylococcus aureus.* The crude saponin extracted from *Sorghum bicolor* exhibited MIC values of 25mg/ml and MBC value of 100mg/ml against *Staphylococcus aureus* (Soetan *et al.*, 2006). In other report, the crude saponin isolated from *Gymnema sylvestre* exhibited MIC values in concentration from 1000 mg/l to 12000mg/l for tested bacterial pathogen (Khanna *et al.*, 2008). The higher concentration of crude tannin of *Tectaria macrodonta* in range of 325mg/ml and 350mg/ml are considered for MIC and MBC respectively against *Staphylococcus aureus.* On the other hand, the MIC of tannin extracted from *Dichrostachys cinerea* against the test organisms ranged between 4.5 and 5.0 mg/ml while the MIC ranged between 5 and 6 mg/ml (Banso *et al.*, 2007).

The alcoholic extract of *Tectaria macrodonta* gave the low MIC value against test strains of bacteria (Poudyali, 2013) but in present study the crude phytochemicals exhibited the MIC values at higher concentration (in range of 20mg/ml to 325mg/ml) only against two Gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*). The MIC values of various solvent extracts of *Tectaria macrodonta* were determined

(Parihar *et al.*, 2010, Poudyali, 2013) but MBC values were not determined, although present study examined the MBC values and as well MIC values of crude phytochemicals against test bacterial strains.

The MIC and MBC values of crude phytochemicals (crude flavonoid, crude saponin and crude tannin) of *Tectaria macrodonta* were comparatively lower as compared to methanol and acetone extract of *Tectaria macrodonta* performed by Poudyali, (2013). Organic fractions were found highly active than crude fraction of phytochemicals (Satdive *et al.*, 2003). This proves the fact that majority of mixture of active constituents are present in solvent extract rather than a particular fraction of crude constituents such as crude flavonoid, crude saponin and crude tannin. However, purified phytochemicals can exert better antimicrobial activity in comparison with crude phytochemicals as the purified saponin was more potent than crude saponin for antibacterial activity against *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* (Khanna *et al.*, 2008). Many substances of plant extract can be antimicrobial, but only few of them exhibit therapeutic potential against various diseases (Sivakumar and Alagesaboopathi, 2006).

The mode of antibacterial action of crude phytochemicals was studied on membrane leakage of UV_{260} and UV_{280} absorbing materials spectrophotometrically. UV_{260} absorbed mainly for nucleic acids while UV_{280} absorbed for proteins (Karsha *et al.*, 2009). The absorbance at 280 nm (proteins) was increased at regular time intervals when compared to the absorbance at 260 nm (nucleic acid) (Table: 6 and 7). These results suggested that crude phytochemicals of *Tectaria macrodonta* probably altered the membrane permeability resulting in the leakage of the UV_{260} and UV_{280} absorbing materials. Leakage of intracellular material might cause cell inhibition or cell death, which seems to be one of the modes of bactericidal activity of crude phytochemicals. This finding was similar with work done by Karsha *et al.*, (2009) where it was reported that the antibacterial activity of black pepper (*Piper nigrum Linn.*) against *Staphylococcus aureus* and the mode of action was studied on membrane leakage of UV_{260} and UV_{280} absorbing material spectrophotometrically (Karsha *et al.*, 2009).

As stated by Cowan (1999), the antimicrobial phytochemicals generally inhibit the growth of microorganisms through various mechanisms. Phenolics and polyphenols

(usually tannin and flavonoid) present in the plant extracts are responsible for phenolic toxicity to bacteria and include inhibition of enzyme by the oxidized compounds probably through reaction with sulfhydryl groups or making nonspecific interactions with the proteins (Anandhi *et al.*, 2014). The antibacterial activity of flavonoids is due to their ability to complex with extracellular proteins and cell walls (Garba *et al.*, 2013). The phenolics and essential oils have been reported to disrupt the the membrane of Gram positive bacteria (Johan *et al.*, 2014). In a study, the antibacterial activity of phenolics and essential oils (thymol and carvacrol) has been analyzed to disrupt the membrane of *Pseudomonas aeruginosa* and *Staphylococcus aureus* by the leakage of ions and cellular materials (Lambert *et al.*, 2001).

Antioxidant activity of various crude phytochemicals of *Tectaria macrodonta* were determined by four *in-vitro* tests namely DPPH radical scavenging activity, ferric reducing power assay, hydroxyl scavenging and hydrogen peroxide scavenging assay. Together all the assays provide a better estimation of antioxidant properties and the results revealed that antioxidant activity was concentration dependent of the phytochemical extracts of *Tectaria macrodonta.*

FRAP analysis is based on the capacity of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)- s-triazine (TPTZ) and forming an intense blue colored Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm (Chanda *et al.*, 2009). The crude flavonoid gave the higher absorbance (0.2448± 0.004) at 100μg/ml exhibiting the ferric reducing capacity (Figure: 11). All the crude phytochemicals of *Tectaria macrodonta* showed significant increment in ferric reducing capacity with an increase in concentration of extract (Table: 8). However, the crude alkaloid extract exhibited low ferric reducing capability as compared with other crude phytochemicals.

Free radical scavenging potential of crude fractions of various phytochemicals at different concentrations (from 10μg/ml to 100μg/ml) was tested by the DPPH method. Antioxidant compounds of extract reacts with DPPH and converts it to 1, 1-diphenyl-2- picrylhydrazine. The level of discoloration indicates the scavenging potential of the antioxidant compound of extracts. The scavenging assay of DPPH radicals is dependent on the reduction of DPPH solution in the presence of electron or

hydrogen donating antioxidant, leading to the formation of reduced or non radical form of DPPH-H and color change from purple to yellow (Wen *et al.*, 2014). The highest radical scavenging activity (58.23%) was observed for crude flavonoid extract of *Tectaria macrodonta* (Figure: 16) followed by crude tannin (Figure: 17) and crude saponin (Figure: 18) extracts showing about 55% and 53% inhibitory activity respectively compared to the standard i.e. (Ascorbic acid) at the highest concentration (100µg/ml) (Table: 9). The IC_{50} value of crude flavaonoid was determined as 50.37% at 50µg/ml while for crude tannin and crude saponin the IC_{50} value was recorded at concentration of 60µg/ml and 80µg/ml respectively. Previous reports indicated that the DPPH scavenging activity of phenolics such as flavonoid and can be determined by numbers and position of hydroxyl group (Cai *et al.*, 2006) that are most important structural features for the antioxidant activity (Wen *et al.*, 2014). The crude steroid exhibited scavenging effect at 100µg/ml with the IC_{50} value of 50%. However, the crude alkaloid was unable to exert any higher inhibitory effect even at higher concentration (100µg/ml).

Hydroxyl radicals are extremely reactive species formed in human and animal body and it is capable of damaging almost every biomolecule found in living cells (Sowndhararajan *et al.*, 2013). This free radical has the ability to cause strand breakage in DNA. which lead to mutagenesis and carcinogenesis. However, in present study the crude phytochemicals did not show considerable scavenging activity as shown by standard BHT (76.88%) at the concentration of 100µg/ml (Table: 10). Only the crude tannin showed up to 31% scavenging efficiency at the concentration of 100µg/ml (Figure: 22). The IC_{50} value for all the crude phytochemicals was not observed at any test concentration (10 to 100µg/ml) in OH scavenging assay. It was reported by Poudyali (2013) that crude methanolic, ethanolic and aqueous extract of *Tecteria macrodonta* could not exhibited scavenge hydroxyl radical activity > 25% (at 100µg/ml) (Poudyali, 2013).

The various concentrations from 10µg/ml to 100µg/ml of crude phytochemicals were used for detection of scavenging efficiency of hydrogen peroxide. The percent of inhibition was highest in crude flavonoid and crude steroid at concentration of 100µg/ml whereas the crude alkaloid exhibited lowest inhibition (Table: 11). In all the antioxidant assay, crude flavonoid extract exhibited prominent antioxidant activity in

four antioxidant methods employed for determination of antioxidant efficiency of crude phytochemicals (crude flavonoid, crude saponin, crude tannin, crude steroid and crude alkaloid) extracted from *Tectaria macrodonta* (Figure: 26). The antioxidant activity was found to increase with an increase in the concentration of the crude phytochemicals extracted from *Tectaria macrodonta*. In earlier studies, it has been proved that phenolic compounds namely flavonoid and tannin are responsible for antioxidant properties (Ouattara *et al.*, 2011). In a study, several concentrations (5 to 400µg/ml) of the crude ethanolic extract and pure flavonoid fraction of *Cissus quadrangularis (Linn)* were exhibited free radical scavenging properties with an increasing concentration of test compounds in all *in vitro* models (Vijyalakshmi *et al.*, 2013).

For the extraction, mainly methanol and water are used for crude phytoconstituents but some non polar solvent is also used for non polar compounds (Anandhi *et al.*, 2014) However, mostly polar compounds are active constituents for effective antimicrobial and antioxidant properties (Chanda *et al.*, 2009).

Chapter 6

SUMMARY & CONCLUSION

6. SUMMARY AND CONCLUSION

In the present study the crude phytochemicals namely flavonoid, saponin, tannin, steroid and alkaloid have been extracted by employing various methods. After extraction, the crude phytochemicals were evaluated by phytochemicals testings.

In vitro antibacterial activity against *Bacillus cereus, Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* were done by using different crude phytochemicals of the test plant at different concentrations (mg/ml) by well diffusion method. The crude tannin, crude flavonoid, crude saponin, crude alkaloid and crude steroid of *Tectaria macrodonta* were used for the screening of antibacterial activity against the test microorganisms. The crude flavonoid, crude tannin and crude saponin inhibited the growth of *Bacillus cereus* and *Staphylococcus aureus.* The crude flavonoid at the concentration of 20mg/ml inhibited the growth of *Bacillus cereus* whereas crude tannin inhibited at 100mg/ml. The crude saponin at the concentration of 50mg/ml inhibited the growth of *Bacillus cereus*. The growth of *Staphylococcus aureus* is inhibited by crude flavonoid and crude saponin at the concentrations of 50mg/ml and 100mg/ml, respectively. However, the crude tannin inhibited the growth of *Staphylococcus aureus* at the concentration of 300mg/ml. The crude alkaloid and crude steroid extracts were not effective against the test microorganisms. The crude phytochemicals from *Tectaria macrodonta* were effective only against Gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*). The MIC of the crude phytochemicals of plant was estimated by both agar dilution and broth dilution method. The MIC value of crude flavonoid was 20mg/ml for *Bacillus cereus* and 50mg/ml for *Staphylococcus aureus.* The crude tannin showed MIC at higher concentration of 75mg/ml for *Bacillus cereus* and 325mg/ml for *Staphylococcus aureus.* The crude saponin exhibited MIC at the concentration of 50mg/ml for *Bacillus cereus* and 75mg/ml for *Staphylococcus aureus.* Beside the MIC values, the MBC of crude phytochemicals was also determined against test bacterial strains. The MBC value of crude flavonoid and crude saponin was 75mg/ml for *Bacillus cereus* whereas crude tannin showed MBC at the concentration of 100mg/ml. The MBC values of crude tannin, crude saponin and crude flavonoid against *Staphylococcus aureus* were at concentrations of 350mg/ml, 100mg/ml and 75mg/ml respectively.

The crude phytochemicals at their Minimum inhibitory concentration was also analyzed for membrane leakage of bacterial cells. The cells of bacteria were treated with crude phytochemicals for 3hrs (180 min) and the absorbance was measured at UV_{260} and UV_{280} at each 15 min interval. Increased UV_{260} and UV_{280} absorbing materials were detected through spectrophotometer at increasing time intervals of 15 minutes. This possibly caused cell wall or cell membrane leakage of bacterial cells.

All the crude phytochemicals of the test plant were subjected for antioxidant analysis by DPPH, FRAP, OH and H_2O_2 method. Ascorbic acid was used as a standard reference for DPPH, FRAP, and BHT used as standard for OH. The antioxidant activity of crude phytochemicals was concentration dependent and there was an increase in antioxidant property with an increase in concentration of crude phytochemicals. The 50% inhibition of DPPH free radicals was recorded for all the crude phytochemicals except crude alkaloid. None of the crude phytochemicals exhibited more than 50% inhibition in OH assay, while crude flavonoid and crude steroid exhibited more than 50% inhibition for H_2O_2 free radicals. The highest FRAP activity was exhibited by crude flavonoid extract. The total tannin content was determined by using tannic acid as a standard reference in crude tannin extract while the total flavonoid content was determined in crude flavonoid extract by using rutin as a standard reference.

The conclusion of the present study are as follows:

- The rhizome of *Tectaria macrodonta* was determined to have the highest amount of crude tannin content.
- The crude flavonoid extract showed promising antibacterial activity against the test Gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*).
- The *Bacillus cereus* and *Staphylococcus aureus* were found to be most susceptible towards crude flavonoid and crude saponin with MIC and MBC values exhibited at lower concentrations.
- The crude flavonoid exhibited highest absorbance of UV_{260} and UV_{280} absorbing material with an increase in time intervals probably from membrane leakage of bacterial cells.
- The crude flavonoid, crude saponin, crude tannin and crude steroid exhibited promising antioxidant properties.

Chapter 7

PHOTOGRAPHIC PLATES

7. PHOTOGRAPHIC PLATES

Plate 1. Plant of *Tectaria macrodonta*

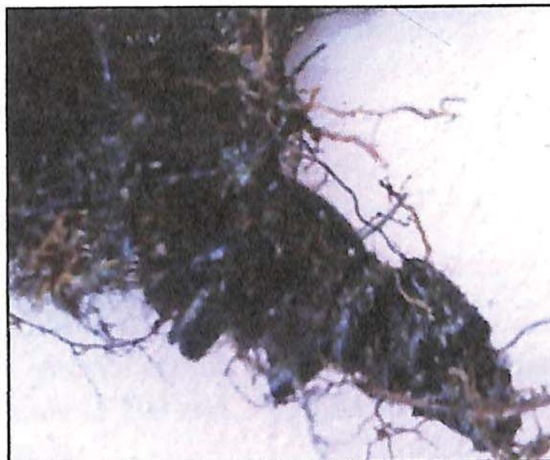

Plate 2. Rhizome of *Tectaria macrodonta*

Plate 3. Crushed rhizome of *Tectaria macrodonta*

Plate 4. Rhizome powder of *Tectaria macrodonta*

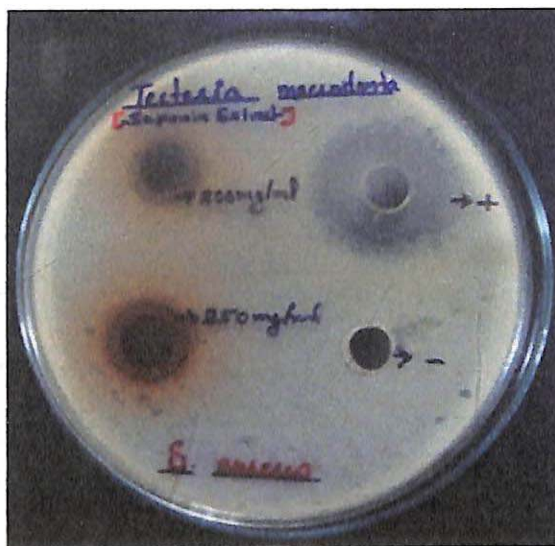

Plate 5. Zone of Inhibition formed by crude saponin extract of *Tectaria macrodonta* against *Staphylococcus aureus* at a concentration of 200 and 250 mg/ml, '+' indicates positive control and '-' indicates negative control.

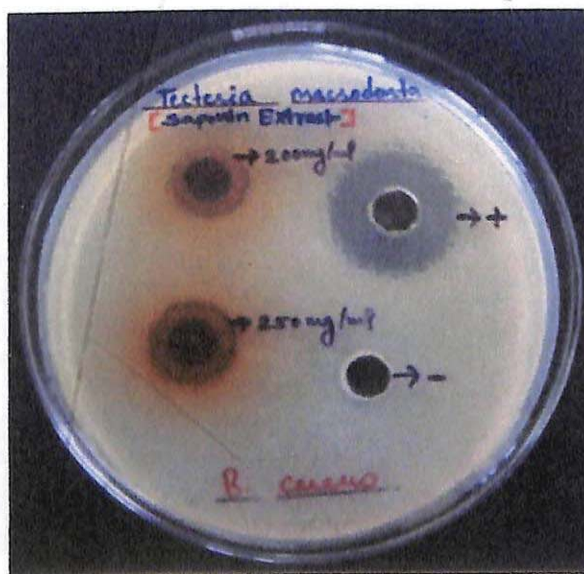

Plate 6. Zone of Inhibition formed by crude saponin extract of *Tectaria macrodonta* against *Bacillus cereus* at a concentration of 200 and 250 mg/ml, '+' indicates positive control and '-' indicates negative control.

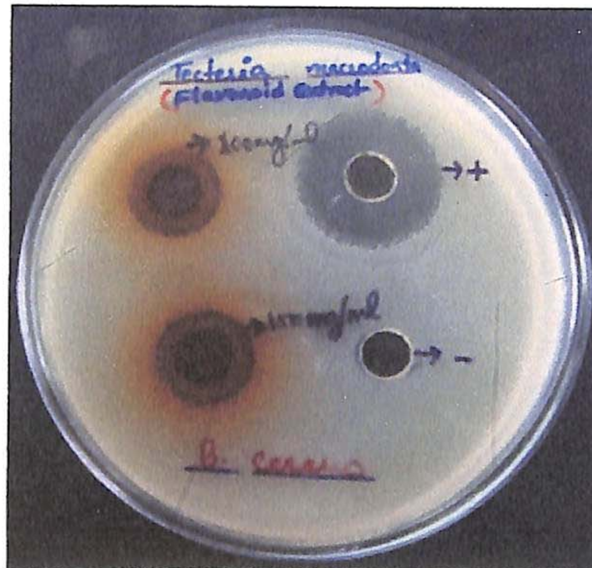

Plate 7. Zone of Inhibition formed by crude flavonoid extract of *Tectaria macrodonta* against *Bacillus cereus* at a concentration of 100 and 150 mg/ml, '+' indicates positive control and '-' indicates negative control.

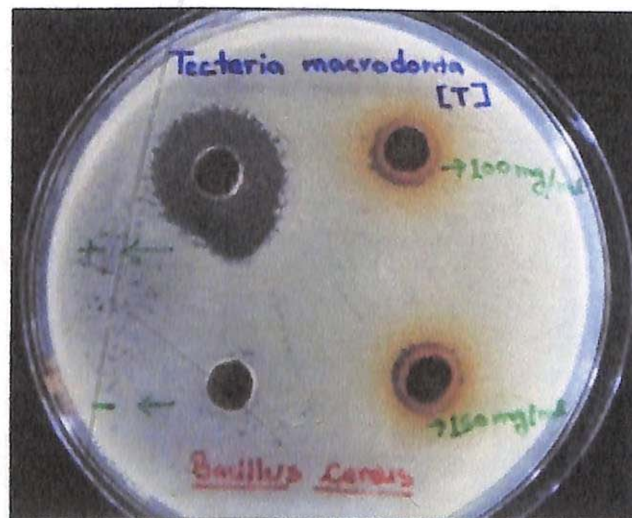

Plate 8. Zone of Inhibition formed by crude tannin extract of *Tectaria macrodonta* against *Bacillus cereus* at a concentration of 100 and 150 mg/ml, '+' indicates positive control and '-' indicates negative control.

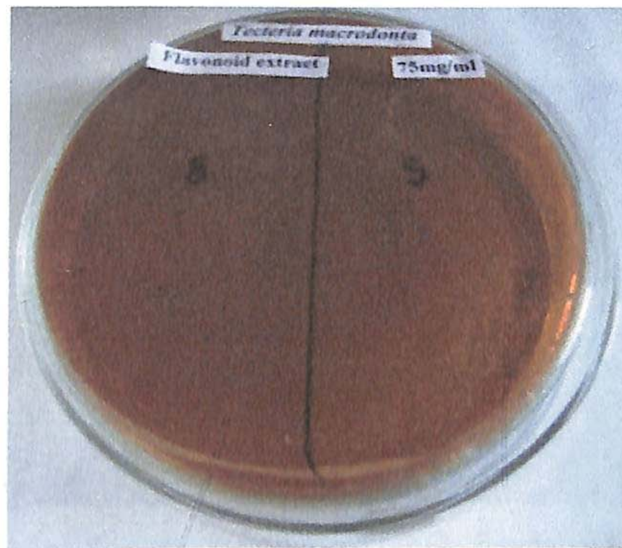

Plate 9. MIC assay of *Tectaria macrodonta* with the crude flavonoid extract. At concentration of 75mg/ml the crude flavonoid extract inhibited growth of two test bacteria (*Staphylococcus aureus* and *Bacillus cereus*). In photographic plate 'S' indicates *Staphylococcus aureus*, 'B' indicates *Bacillus cereus.*

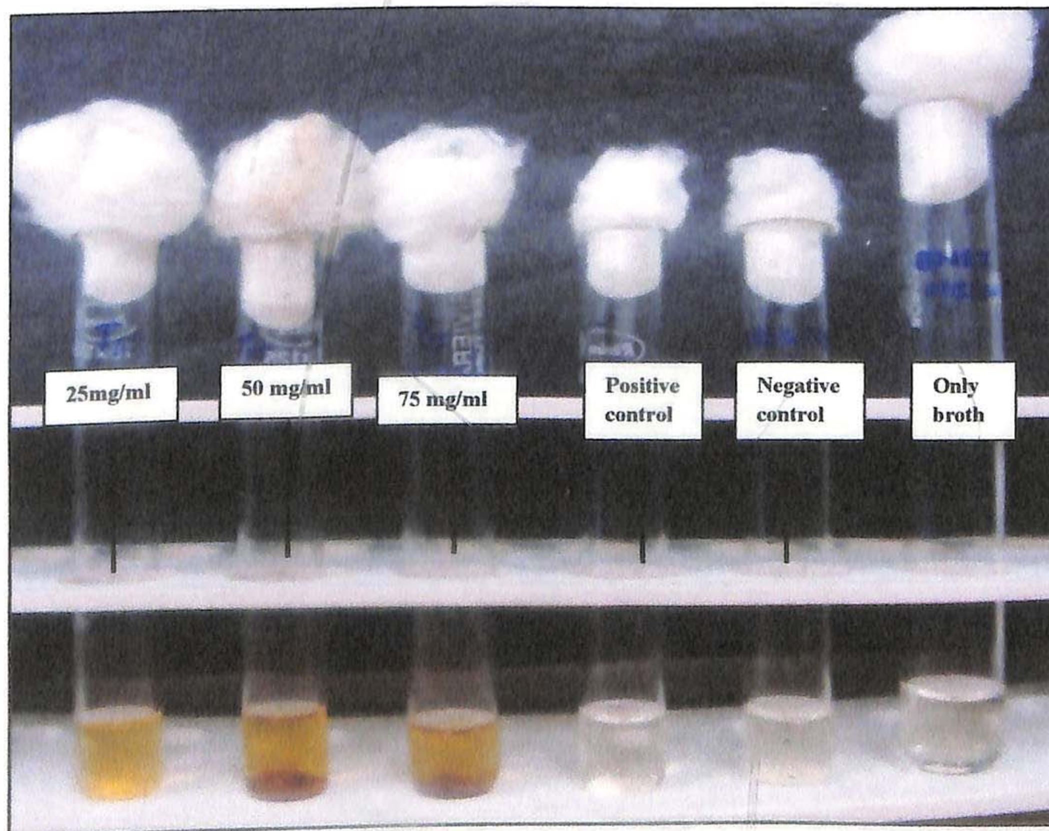

Plate 10. MIC assay of *Tectaria macrodonta* (crude flavonoid) by broth dilution method. At concentration of 50 mg/ml the crude flavonoid extract inhibited growth of *Staphylococcus aureus.* In positive control no growth of bacteria was observed.

Plate 11: MBC assay of *Tectaria macrodonta* (crude flavonoid). At concentration of 75 mg/ml the crude flavonoid extract inhibited growth of *Staphylococcus aureus* but at 50 mg/ml growth of bacteria was observed. In negative control growth of bacteria was observed.

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APPENDIX

APPENDIX

Appendix 1: Culture Media

(A) Nutrient broth

Composition:	Grams/lit
Sodium chloride (Merck, ME9M591006)	5
Peptone (HiMedia, RM001)	5
Beef extract (HiMedia, RM002)	3

(B) Nutrient agar

Composition:	Grams/lit
Sodium chloride (Merck, ME9M591006)	5
Peptone (HiMedia, RM001)	5
Beef extract (HiMedia, RM002)	3
Agar (HiMedia, RM666)	15

(C) Mueller Hinton Agar (HiMedia, M173-500G)

Composition:	Grams/lit
Casien acid hydrolysate	17.50
Beef heart infusion	2.0
Starch soluble	1.5
Agar	17

Appendix 2: List of Chemicals used

1. Acetyl acetone : Merck, SJ0S600642
2. Acetone : Merck, SF8F580421
3. Aluminium chloride : Thomas Baker
4. Ammonium acetate : Merck, MK8M581737
5. Ascorbic acid : HiMedia, RM1014
6. Benzene GR : Merck, SA1SF01075
7. Chloroform GR : Merck, SA7SF57060
8. Copper sulphate : Merck, MK0M603125
9. DPPH : HiMedia, RM5169
10. Dimethyl sulfoxide : Merck, SL0S600706
11. Ethanol : Bengal Chemical, Kolkata
12. EDTA : Merck, MCOM600628
13. Ethyl acetate : Thomas Baker, 72677
14. Ferric chloride : Merck, MK9M592642
15. Folin-Ciocalteu reagent : Merck, AC9A590103
16. Gallic acid : HiMedia, RM233
17. Gelatin : Merck, MD7MS71167
18. Glacial acetic acid : SRL
19. Hydrochloric acid : Merck, HI6H560677
20. Hydrogen peroxide solution : Merk, HI6H560662
21. Iodine : Merck, MJ8G560501
22. Mercuric chloride : Merck, ML0M603894
23. Methanol : SRL, 11528235
24. Ninhydrin : Merck, MH8M581782
25. Petroleum ether : Merck, SL3S530560
26. Picric acid : Merck, MJ0M603126
27. Potassium ferricyanide : HiMedia, RM1034
28. Potassium iodide : Merck, MA1M603829
29. Potassium sodium tartarate : Merck, ME7M571334
30. K_2HPO_4 : HiMedia- RM 168
31. KH_2PO_4 : HiMedia- RM 3943-5004
32. Rutin : HiMedia RM 7464
33. Sodium carbonate : Merck, MD8M580784
34. Sodium citrate : Merck, MJ8M583263
35. Sodium hydroxide : Merck, ML0M603751
36. Sulphuric acid : Merck, HG8H580592
37. Tannic acid powder : HiMedia, RM7541-250G
38. Trichloroacetic acid : HiMedia, RM7570
39. Zinc : Merck, M18M581932

Appendix 3: Reagents

1. Benedict's reagent: 173 gm of Sodium citrate and 100 gm of Sodium carbonate was dissolved in 800 ml distilled water; to it 100 ml of 17.3 gm copper sulphate was added.

2. Fehling's reagent:

Solution A: 34.66 gm of copper sulphate was dissolved in distilled water and made up to 500 ml.

Solution B: 173 gm of Potassium sodium tartarate and 50 gm of Sodium hydroxide was dissolved in distilled water and made up to 500 ml

3. Hagner's reagent: 1 gm of Picric acid was dissolved in 100 ml of distilled water.

4. Mayer's reagent: 1.36 gm of Mercuric chloride dissolved in 60 ml of distilled water and 5 gm Potassium iodide dissolved in 10 ml of distilled water. The two solutions was mixed and made up to 100 ml.

5. Molish's reagent: 15 gm of α – naphthol was dissolved in 100 ml of chloroform.

6. Wagner's reagent: 1.27 gm of Iodine and 2 gm of Potassium iodide was dissolved in 5 ml of distilled water and made up to 100 ml.