ANTIOXIDANT, ANTIMICROBIAL AND CYTOTOXIC EFFECTS OF STEM EXTRACT OF *TINOSPORA CORDIFOLIA* (GURJO)

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By

Yadvendra Kumar Shah

Supervisor
Asst. Prof. Hemanta Khanal
Dept. of Biotechnology
Sunsari Technical College T.U.

Co- Supervisor
Lect. Puskar Thapa
Dept. of Biotechnology
Sunsari Technical College T.U.

Regd. No. 11494/056/57

SUNSARI TECHNICAL COLLEGE

(Affiliated to T.U. & CTEVT)

Ref. No.

Date : .....

Recommendation

This is to certify that the **Mr. Yadvendra Kumar Shah** has completed his research work entitled "**Antioxidant, antibacterial and cytotoxic effect of stem extract of *Tinospora cordifolia*(Gurjo)**" has been carried out under our supervision. This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. I, hereby, recommend this thesis for final evaluation.

.....

Asst. Prof.Hemanta Khanal

Supervisor

Dept. of Biotechnology,

Sunsari Technical college,

Dharan, Nepal.

.....

Lecturer Puskar Thapa

Co-Supervisor

Dept. of Biotechnology

Sunsari Technical college,

Dharan, Nepal.

Head Office : Dharan-4 | Sunsari | Nepal | Ph. 025-523262 | Fax: 025-533302 | P.O. Box: 10947
Branch Office : Putali Sadak (In front of Sankerdev Campus | Kathmandu | Phone: 01-4231178
Email: info@stc.edu.np | Web: www.stc.edu.np

Regd. No. 11494/056/57

SUNSARI TECHNICAL COLLEGE

(Affiliated to T.U. & CTEVT)

Ref. No.

Date : .....

Certificate of Evaluation

This is to certify that **Mr. Yadvendra Kumar Shah** thesis entitled **"Antioxidant, antibacterial and cytotoxic effect of stem extract of *Tinospora cordifolia* (Gurjo)"** has been approved for the examination and submitted to Tribhuvan University in partial fulfillment of the requirement for Master of Science in Biotechnology.

.....

Mr. Puskar Thapa

(Head of Department)

Department of Biotechnology

Sunsari Technical College ,Dharan

.....

Prof. Dhan Bahadur Karki, Ph.D

(External Examiner)

Central Campus of Technology,

Dharan

.....

Lecturer. Anish Aacharaya

(Internal Examiner)

Department of Biotechnology

Sunsari Technical College, Dharan

.....

Asst. Prof. Hemanta Khanal

(Supervisor)

Department of Microbiology

Sunsari Technical College, Dharan

.....

Lecturer. Puskar Thapa

(Co-Supervisor)

Department of Biotechnology

Sunsari Technical College, Dharan

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……

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LIST OF ABBREVIATIONS

8-OhdG	:	8-Hydroxy-2' –Deoxy Guanosine
AIDS	:	Acquired Immuno Deficiency Syndrome
ATCC	:	American Type Culture Collection
BHA	:	Butylated Hydroxy Anisole
BHT	:	Butylated Hydroxy Toluene
BPKIHS	:	Bisheswar Prasad Koirala Institute of Health Science
BSLA	:	Brine Shrimp Lethality Assay
CDC	:	Centre For Disease Control and Prevention
CFU	:	Colony Forming Unit
CRE	:	Carbapenem-Resistant Enterobacteriaceae
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribo Nucleic Acid
DPPH	:	2, 2- Diphenyl-1-picrylhydrazyl
ESBL	:	Extended Spectrum Beta Lactamase
HPV	:	Human Papilloma Virus
LC	:	Lethality Concentration
LDL	:	Low Density Lipoproteins
LOOH	:	Lipid Hydroperoxide
MDR	:	Multiple Drug Resistance
MDR-TB	:	Multi-Drug-Resistant Mycobacterium tuberculosis
MHA	:	Mueller-Hinton Agar
MIC	:	Minimum Inhibitory Concentration
MRO	:	Multi-Resistant Organisms
MRSA	:	Methicillin-Resistant Staphylococcus aureus

NCCLS	:	National Committee for Clinical Laboratory Standards
PHGPX	:	Phospholipid Hydroperoxide Glutathione Peroxidase
PPM	:	Part Per Million
ROS	:	Reactive Oxygen Species
SODs	:	Superoxide Dismutases
SPSS	:	Statistical Package For Social sciences
UTI	:	Uinary Tract Infection
VRE	:	Vancomycin-Resistant Enterococcus
WHO	:	World Health Organisation
ZOI	:	Zone Of Inhibition

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ABSTRACT

Tinospora cordifolia, belonging to the Menispermaceae family, has been investigated for extraction of its secondary metabolites and evaluation of biological activities with special emphasis to the antioxidants, antibacterial screening, and cytotoxic study. Phytochemicals were extracted in ethanol as organic solvent. Upon phytochemical analysis in the present study crude extract of *T. cordifolia* showed the presence of following potent bioactive compounds: alkaloids, terpenoids, saponins, flavonoids, phenolics, tannins, carbohydrates, and proteins.The crude ethanolic stem extract of *Tinospora cordifolia* was subjected to antioxidant investigation by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and maximum free radical scavenging activity was observed (71.76%) at 10 mg/ml. Antibacterial activities of crude ethanolic extract were performed by agar well diffusion method against antibiotic-resistant and pathogenic strain of bacteria like vancomycin resistant enterococcus (VRE), methicillin-resistant *S. aureus* (MRSA), *Klebsiella pneumonia*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*. The maximum effect of the extract was observed on MRSA(19.3 mm zone of inhibition) at 100 mg/ml and minimum on VRE(9.7 mm) at 12.5 mg/ml. Results suggest that the ethanolic extract has significant antibacterial activity against tested bacteria. The present study was also conducted to test for in vivo Brine Shrimp Lethality Assay (BSLA) of the ethanolic extracts of Tinospora cordifolia and correlate cytotoxicity results with known pharmacological activities of the plants. Cytotoxicity was evaluated in terms of LC50 (lethality concentration). Ten nauplii were added into three replicates of each concentration of the plant extract. After 24 hours the surviving brine shrimp larvae were counted and LC50 was assessed. Results showed that the extracts of *Tinospora cordifolia* were potent against the brine shrimp with an LC50 value of 150 ppm (µg/mL). It indicated that bioactive components were present in these plants that could be accounted for its pharmacological effects. Thus, the results support the uses of this plant species in traditional medicine.

The phytochemical activities of Tinospora cordifolia like antioxidant, antibacterial and cytotoxic were investigated and showed good result. Further, the molecular mechanism of the potent bioactive constituents present in the crude ethanolic extract remains to be investigated.

Keywords: phytochemical, antioxidant, DPPH, antibacterial, cytotoxicity, BSLA, stem extract*, T. cordifolia*

CHAPTER 1

INTRODUCTION

1.1 Background

Nepal is the underdeveloped country and most of the people are dependent on agriculture. Also, Nepal is a very rich country in the herbal medicinal plant because most of the area is covered with tropical and subtropical forest. These herbal plants are medicinally important as they possess bioactive compounds. Nowadays the use of herbal medicine in the treatment of disease has drawn attention due to increased antimicrobial resistance and a significant side effect of drugs available. Hence since last few decades scientist had paid their attention toward herbal medicine alternative to the synthetic drugs.

Guduchi [*Tinospora cordifolia* (Willd.) Miers ex Hook. F. &Thoms] is a large, smooth, deciduous climbing shrub belonging to the family Menispermaceae (Vani, Rajani, Sarkar, & Shishoo, 1997). It is spread throughout tropical Indian subcontinent, Nepal, and China, ascending to an altitude of 300 m. In Hindi, it is commonly known as Giloya, which is a Hindu mythological term that refers to the heavenly elixir that has saved celestial beings from old age and kept them eternally young and energetic. The stem of *Tinospora cordifolia* is rather succulent with long filamentous fleshy aerial roots from the branches. The bark is creamy white to grey sometimes yellow, deeply left spirally, the space in between being spotted with large rosette-like lenticels. The leaves are membranous and heart shaped. The flowers are small and yellow or greenish yellow. In auxiliary and terminal racemes or racemose panicles, the male flowers are clustered and female is usually solitary. The drupes are ovoid, glossy, succulent, red and pea-sized. The seeds are curved. Fruits are fleshy and single-seeded. Flowers grow during the summer and fruits during the winter (Kirtikar, 1918; Vani et al., 1997). Medicinal plants have been used as a source of herbal medicine with their own personal ways, which have been passed from one generation to another. Due to having their broad applications plant-derived substances have recently become of great interest (Bariş et al., 2006). In recent

years, because of the hazardous effect of synthetic drugs the use of natural antioxidants has been promoted (Shahidi, 2000).

Guduchi is widely used in veterinary folk medicine ayurvedic system of medicine for its general tonic, antiperiodic, anti-spasmodic, anti-inflammatory, antiarthritic, anti-allergic and anti-diabetic properties (Rose et al., 2007). The plant is used in Ayurvedic, "Rasayanas" to improve the immune system and the body resistance against infections. The root of this plant is known for its antistress, anti-leprotic and anti-malarial activities (Nayampalli, Ainapure, & Nadkarni, 1982; Zhao, Wang, Rimando, & Che, 1991).

A tremendous number of scented, medicinal, spicy and other plants contains chemical composition showing antioxidant properties. Numerous studies were done on some of which medicinal plants, e.g. neem, rosemary, tulsi, capsicum etc which resulted in the development of natural antioxidant formulations for food, cosmetic and other applications. However, scientific knowledge on antioxidant properties of various plants, particularly those that are less broadly used in culinary and medicine is still to be studied. Therefore, the investigation of such properties still remains an interesting and useful task, especially for searching new sources for naturally occurring antioxidants, functional foods, and nutraceuticals. It could be noticed that the free radical scavenging activity of the selected plants was poorly investigated, therefore testing of their antiradical properties is of interesting work, primarily in order to find new promising sources for natural antioxidants, functional drugs, and neutraceuticals food . Free radicals can be evolved by metabolic pathways inside human body tissues; also they could be entered by external sources, with food, drugs, and can be caused by environmental pollution etc. Use of natural antioxidants, as food additives and as an herbal medicine for inactivating free radicals receives a lot of attention nowadays, not only for their free radical scavenging properties, but also because they are natural, non-synthetic products and their appreciation by consumers is very favorable and acceptable.

Antioxidant plays a major role in normal physiological functions in humans by protecting against cell damage by reactive oxygen species and reducing the adverse effects of these

free radicals. Oxidative stress is the major causative factor for producing free radicals and reactive oxygen species (ROS). These free radicals acts as a primary catalysts for initiating oxidation in in vivo and in vitro, for creating oxidative stress which leads to numerous diseases and disorder such as cancer, cardiovascular disease, alcohol-induced liver disease, neural disorder, Alzheimer's disease, Parkinson's disease, ageing and atherosclerosis and they also act as reducing agents, hydrogen donors, metal chelators and singlet oxygen quenchers due to their redox properties.

Cancer is the second leading cause of death in the world after cardiovascular diseases. Deaths arising from cancer constitute 2–3% of the annual deaths recorded worldwide and kill about 3500 million people annually all over the world. Cervical cancer is the most common cause of death (Rackova, Oblozinsky, Kostalova, Kettmann, & Bezakova, 2007). Phenolic compounds act as an antioxidant agent, by scavenging the free radicals due to the presence of hydroxyl group in them (Diplock, 1997; Shahidi, 2000).

Cancer death among women in developing countries and worldwide it is the second most common cancer in women. It is initiated by a change in the epithelial cells, which line the wall of the cervix, and the human Papilloma virus (HPV) is the most common risk factor for this type of cancer. The method of combine conventional therapy with some form of complementary therapy is growing very rapidly. Throughout medical history, plant products have been shown to be valuable sources of novel anticancer drugs. There is widespread use of herbal medicines depicted even in conventional medical history. Out of 121 prescription drugs in use for cancer treatment, 90 are derived from plant species and 74% of these drugs were discovered by investigating a folklore claim (Craig, 1999).

In developing countries, the bacterial disease is extensive due to decreased sanitation and unhygienic conditions. Infectious diseases are the world's primary cause for premature deaths, killing almost 50,000 people every day. Human infections, predominantly those linking with skin and mucosal surfaces constitute a quite serious problem, particularly in tropical and subtropical developing countries (Reid, Jäger, Light, Mulholland, & Van Staden, 2005). The hunt for new effective antimicrobial agents is

necessary to resist bacterial and fungal pathogens due to the occurrence of fatal opportunistic infections associated with AIDS, antineoplastic chemotherapy and transplants. Medicinal plants are an important medicinal aid for various diseases. Scientific experiments on the antimicrobial properties of plant components were first documented in the 19th century (Reid et al., 2005). Ethnobotanical data is helpful in providing new antimicrobial therapy (Olajide, Afolayan, Adewusi, & Adeyanju, 2012). The use of current microbiological techniques demonstrates that medicinal plants normally exhibit significant strength against human bacterial and fungal pathogens (Abi-Ayad, Abi-Ayad, Lazzouni, & Rebiahi, 2011; Palombo & Semple, 2001). In India, medicinal plants are extensively used by ancient people as folk remedies but in this recent year, these herbal drugs are widely using in the pharmaceutical preparations of modern medicines (Kumarasamy, Cox, Jaspars, Nahar, & Sarker, 2002). According to National Health Experts, 2000 different plants are used for medicinal preparations for both internal and external use in India alone. Up to now, only 10 % of higher plants were chemically investigated and about 80% of the world population is dependent herbal drugs. Plants contain numerous biologically active constituents, many of which have been exposed to have antimicrobial properties. Medicinal plants are of important therapeutic aid for various human ailments. Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century. The screening of plant extracts and natural products for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents.

Pseudomonas aeruginosa is an opportunistic pathogen able of causing nosocomial infections. Patients with burns are at high risk of acquiring *P. aeruginosa* infection of the burn wound with subsequent septicemia and death because burn injury weakens both the normal skin barrier and many of the systemic host defense mechanisms, which makes skin susceptible to microbial colonization and reproduction resulting in the development of burn wound sepsis. *P. aeruginosa* is naturally resistant to a large number of antibiotics and this resistance of *P. aeruginosa* to commonly used therapeutic agents has increased in recent years (Porras-Gómez, Vega-Baudrit, & Núñez-Corrales, 2012). In case of burn patients, the burned skin remains vulnerable to invasive microbial infections of all kinds until the complete epithelial repair has occurred. Microbial drug-

resistant emerges as a major problem in the health care industry as microbes involved in the change of their metabolism and genetic structure to acquire resistant against the drugs used in the treatment of infectious disease. MDR can be defined as resistance to at least four classes of antibiotics used during treatment of these infections. The emergence of MDR strains is often may due to the selective pressure of antimicrobial therapy (Porras-Gómez et al., 2012). These drug-resistant pathogens are more pathogenic with high mortality rate than that of wild strain. To overcome microbial drug-resistant, scientists are looking forward to the development of alternative and novel drugs. Herbal medicine is expected to open some new aspects to fight and prevent diseases using controlled and adequate concentration of medicinal compounds. Herbal drugs are currently employed as an approach to explore the darkest avenues of medical sciences because herbal medicine has significant antibacterial activity with a very low side effect on human health.

Urinary tract infections (UTI) are the most common form of bacterial infection affecting people throughout their life. It is one of the common reasons for adults to seek medical help and is one of the most frequently occurring nosocomial infections (Gastmeier et al.,1998). The treatment of UTI has become more complicated because of the appearance of pathogens with increasing resistance to antimicrobial agents (Munakarmi et al., 1995). Such antibiotic-resistant organisms are difficult to eliminate during the course of an infection and are lethal to the human population. In addition, antibiotics in these cases are sometimes associated with adverse effects on the host, which include hypersensitivity, depletion of the beneficial gut and mucosal microorganism, immunosuppressant and allergic reactions (Ahmad et al., 1998). Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases in general and of UTI in particular condition.

Cancer treatment has been remarkably consistent with the last few years. Surgery, radiation, and chemotherapy have been mostly used conventional treatment. Not surprisingly, the clinical success of these treatment has reached at a plateau (Mayer, 1991). Today, treating cancer has significant importance and numerous multidisciplinary scientific approaches have been applied to treat this disease but the exact method, the

perfect cure is yet to be discovered into the world of medicine. The present western system of medicine is originated with highly unbearable complications and patient in compliance. Hence, a more specific and safe remedy is the need of the present situation. Recent studies have shown that cancer treatment by phytochemicals obtained from vegetables, fruits, spices, teas, herbs, and medicinal plants, is one of the most feasible methods to treat this disease. Plants have been a source of medicinal compounds for thousands of years, and the phytochemical product continues to play an important role in medicine. Medicinal plants have a very long history of use in the treatment of cancer and are still in use (Huang & Williams, 1999). They have been the source of some of the presently available antitumor agents such as vinblastine, vincristine, etoposide, and paclitaxel (Cragg et al., 1997). Among them, one of the best known is so-called vinca alkaloids (vinblastine and vincristine) isolated from the Madagascar periwinkle, Catharanthus roseus (Vinca Rosea L., Apocynaceae). C. roseus was used by the various community for the treatment of diabetes while vinblastine and vincristine were first discovered during an investigation of the plant as a source of potential oral hypoglycemic agents (Newman, & Weiss, 1997). The rich and diverse plant sources in Nepal are likely to provide effective anticancer agents. One of the most effective approaches in the search for anticancer compounds from plant resources is the selection of plants based on ethnomedical leads.

The emergence of resistance to the antibiotic and anticancer drug is a global issue; hence this study was done to discover new herbal medicine with a lower side effect. Also, *T. cordifolia* in Nepali also called gurjo is proven as a storehouse of different types of herbal compound and yet much more to be discovered.

1.2 Rationale of study

In recent years, there has been a great deal of attention toward the field of free radical chemistry. Free radicals reactive oxygen species and reactive nitrogen species are generated by our body by various endogenous systems, exposure to different physiochemical conditions or pathological states. A balance between free radicals and antioxidants is necessary for proper physiological function. If free radicals overwhelm

the body's ability to regulate them, a condition known as oxidative stress ensues. Free radicals thus adversely alter lipids, proteins, and DNA and trigger a number of human diseases. Hence application of an external source of antioxidants can assist in coping this oxidative stress. Synthetic antioxidants such as butylated hydroxy toluene and butylated hydroxy anisole have recently been reported to be dangerous for human health. Thus, the search for effective, nontoxic natural compounds with antioxidative activity has been intensified in recent years. The present review provides a brief overview of oxidative stress-mediated cellular damages and the role of dietary antioxidants as functional foods in the management of human diseases.

Antibiotics and other antimicrobial agents are common chemical agents used against bacterial and other microbial infections and these agents have extreme benefits due to huge researches and discovery of new drugs. However irrational use of antimicrobial agents is generating great threats of the emergence of new microbial strains of bacteria, fungi and other microbial agents. Resistance development is an even bigger problem since the bacterial resistance is often not restricted to the specific antibiotic prescribed, but generally extends to other compounds of the same class. Bacterial resistance and its rapid increase is a major concern of global public health and are emerging as one of the most significant challenges to human health. Treating microbial infections by chemical antimicrobial agents (viz: antibiotics, antifungal, antiviral, antiparasitic and antihelminthic) are useful but their haphazard use has led to a frightening resistance among microorganisms as well as led to re-emergence of old infectious diseases.

Use of natural products from different herbal plants and their extracts may be a novel approach for treatment as antimicrobial agents individually. Their extracts at different concentration therapy against resistant microbes may generate important ideas for future prospective for treatment. Since natural products are not harmful, this study is highly essential for the future prospective of treatments by using herbal medicine to decrease the use of chemical antimicrobial agents.

1.3 Objectives:

1.3.1 General Objectives:

- To evaluate the antioxidant, the antimicrobial and cytotoxic effect of *T.cordifolia* stems extract at different concentration.

1.3.2 Specific Objectives:

- To extract biologically active components from the stem of *T.cordifolia.*
- To study the antimicrobial effect of stem extract of *T. cordifolia* along with different antibiotics on antibiotic-resistant, non-resistant and ATCC culture of bacteria.
- To study the antioxidant effect of stem extract of*. T. cordifolia* at different concentration.
- To study the cytotoxic effect of stem extract of *T. cordifolia* at different concentrations on brine shrimp (*Artimia salina).*

1.4 Hypothesis

1.4.1 Null Hypothesis

- There will be no significant antioxidant, the antimicrobial and cytotoxic effect of *T. cordifolia* stem extract at a different concentration with different organisms.

1.4.2 Alternative Hypothesis

- There will be the significant antioxidant, antimicrobial and cytotoxic effect of *T. cordifolia* stem extract different concentration with different of organisms.

CHAPTER 2

LITERATURE REVIEW

2.1 Antioxidant

An antioxidant is a molecule stable enough to donate an electron to a free radical and neutralize it, thus reducing its capacity to damage the cellular component. These antioxidants delay or inhibit cellular and biomolecular damage mainly through their free radical scavenging property (Halliwell, 1995). These low molecular-weight antioxidants can safely interact with free radicals and end up the chain reaction before vital molecules are damaged. Some of such antioxidants, including glutathione, ubiquinol, and uric acid, are produced during normal metabolism in the cell (Shi, Noguchi, & Niki, 1999). Other low molecular weight antioxidants are found in the diet. Although there are several enzymes system within the body that terminate free radicals, the principle micronutrient (vitamins) antioxidants are vitamin E (α-tocopherol), vitamin C (ascorbic acid), and B-carotene (Elkhateeb & Alshammary, 2017). The body cannot synthesize these micronutrients, so they must be supplemented through the diet.

The term antioxidant originally was used to refer specifically to a molecule that prevented the consumption of oxygen. In the late 19th and early 20th century, an extensive study was devoted to the uses of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines (Mukund, Sivasubramanian, & Kumar, 2013). Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity(German, 1999). The antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption. However, it was the identification of vitamins A, C, and E as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms(Jacob, 1996; Knight, 1998). The possible mechanisms of action of antioxidants were first explored when it was recognized that a

substance with anti-oxidative activity is likely to be one that is itself readily oxidized (Islam & Pervin, 2011). Research into how vitamin E prevents the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidative reactions, often by scavenging ROS before they can damage cells (Wolf, 2005).

2.2 Free Radical

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain common properties that are shared by most radicals. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants (Lobo, Patil, Phatak, & Chandra, 2010). The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical. These are highly reactive species, capable in the nucleus, and in the membranes of cells of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids (Young & Woodside, 2001). Free radicals attack important macromolecules leading to cell damage and homeostatic disruption. Targets of free radicals include all kinds of molecules in the body. Among them, lipids, nucleic acids, and proteins are the major targets.

2.3 Production of free radicals in the human body

Free radicals and other ROS are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals (Bagchi & Puri, 1998).Free radical formation occurs continuously in the cells as a consequence of both enzymatic and nonenzymatic reactions. Enzymatic reactions, which serve as a source of free radicals, include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis, and in the cytochrome P-450 system (Liu, Stern, Roberts, &

Morrow, 1999). Free radicals can also be formed in nonenzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing reactions.

Some internally generated sources of free radicals are (Husain, Kumar, & RADICALS, 2012)

- Mitochondria
- Xanthine oxidase
- Peroxisomes
- Inflammation
- Phagocytosis
- Arachidonate pathways
- Exercise
- Ischemia/reperfusion injury
- Some externally generated sources of free radicals are:
- Cigarette smoke
- Environmental pollutants
- Radiation
- Certain drugs, pesticides
- Industrial solvents
- Ozone

2.4 Free radical in biology

Free radical reactions are expected to produce progressive adverse changes that accumulate with age throughout the body [Table 1]. Such “normal” changes with age are relatively common to all. However, superimposed on this common pattern are patterns influenced by genetics and environmental differences that modulate free radical damage. These are manifested as diseases at certain ages determined by genetic and environmental factors. Cancer and atherosclerosis, two major causes of death, are salient “free radical” diseases. Cancer initiation and promotion are associated with chromosomal defects and oncogene activation. It is possible that endogenous free radical reactions, like those initiated by ionizing radiation, may result in tumor

formation. The highly significant correlation between consumption of fats and oils and death rates from leukemia and malignant neoplasia of the breast, ovaries, and rectum among persons over 55 years may be a reflection of greater lipid peroxidation (Lea, 1966). Studies on atherosclerosis reveal the probability that the disease may be due to free radical reactions involving diet-derived lipids in the arterial wall and serum to yield peroxides and other substances. These compounds induce endothelial cell injury and produce changes in the arterial walls (Harman, 1992).

2.5 Concept of oxidative stress

The term is used to describe the condition of oxidative damage resulting when the critical balance between free radical generation and antioxidant defenses is unfavorable (Rock, Jacob, & Bowen, 1996). Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids(McCord). Short-term oxidative stress may occur in tissues injured by trauma, infection, heat injury, hypoxia, toxins, and excessive exercise. These injured tissues produce increased radical generating enzymes (e.g., xanthine oxidase, lipoxygenase, cyclooxygenase) activation of phagocytes, the release of free iron, copper ions, or a disruption of the electron transport chains of oxidative phosphorylation, producing excess ROS. The initiation, promotion, and progression of cancer, as well as the side-effects of radiation and chemotherapy, have been linked to the imbalance between ROS and the antioxidant defense system. ROS have been implicated in the induction and complications of diabetes mellitus, age-related eye disease, and neurodegenerative diseases such as Parkinson's disease (Rao, Bharani, & Pallavi, 2006).

2.6 Oxidative stress and human disease

A role of oxidative stress has been postulated in many conditions, including atherosclerosis, inflammatory condition, certain cancers, and the process of aging. Oxidative stress is now thought to make a significant contribution to all inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematous, adult respiratory

diseases syndrome), ischemic diseases (heart diseases, stroke, intestinal ischemia), hemochromatosis, acquired immunodeficiency syndrome, emphysema, organ transplantation, gastric ulcers, hypertension and preeclampsia, neurological disorder (Alzheimer's disease, Parkinson's disease, muscular dystrophy), alcoholism, smoking-related diseases, and many others (Stefanis, Burke, & Greene, 1997). An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structure and functions.

2.6.1 Cardiovascular disease

Heart diseases continue to be the biggest killer, responsible for about half of all the deaths. The oxidative events may affect cardiovascular diseases, therefore; it has the potential to provide enormous benefits to the health and lifespan. Polyunsaturated fatty acids occur as a major part of the low-density lipoproteins (LDL) in blood and oxidation of these lipid components in LDL play a vital role in atherosclerosis (Esterbauer, Puhl, Dieber-rotheneder, Waeg, & Rabl, 1991). The three most important cell types in the vessel wall are endothelial cells; smooth muscle cell and macrophage can release free radical, which affect lipid peroxidation (Neužil, Thomas, & Stocker, 1997). With a continued high level of oxidized lipids, blood vessel damage to the reaction process continues and can lead to the generation of foam cells and plaque the symptoms of atherosclerosis. Oxidized LDL is atherogenic and is thought to be important in the formation of atherosclerosis plaques. Furthermore, oxidized LDL is cytotoxic and can directly damage endothelial cells. Antioxidants like B-carotene or vitamin E play a vital role in the prevention of various cardiovascular diseases.

2.6.2 Carcinogenesis

Reactive oxygen and nitrogen species, such as superoxide anion, hydrogen peroxide, hydroxyl radical, and nitric oxide and their biological metabolites also play an important role in carcinogenesis. ROS induce DNA damage, as the reaction of free radicals with DNA includes strand break base modification and DNA protein cross-links. Numerous investigators have proposed participation of free radicals in carcinogenesis, mutation,

and transformation; it is clear that their presence in biosystem could lead to mutation, transformation, and ultimately cancer. Induction of mutagenesis, the best known of the biological effect of radiation, occurs mainly through damage of DNA by the HO. Radical and other species are produced by the radiolysis, and also by direct radiation effect on DNA, the reaction effects on DNA. The reaction of HO. Radicals are mainly an addition to the double bond of pyrimidine bases and abstraction of hydrogen from the sugar moiety resulting in a chain reaction of DNA. These effects cause cell mutagenesis and carcinogenesis lipid peroxides are also responsible for the activation of carcinogens. Antioxidants can decrease oxidative stress induced carcinogenesis by direct scavenging of ROS and/or by inhibiting cell proliferation secondary to the protein phosphorylation. B carotene may be protective against cancer through its antioxidant function because oxidative products can cause genetic damage. Thus, the photoprotective properties of B-carotene may protect against ultraviolet light-induced carcinogenesis. Immunoenhancement of B-carotene may contribute to cancer protection. B-carotene may also have anticarcinogenic effect by altering the liver metabolism effects of carcinogens (Van Poppel & Goldbohm, 1995). Vitamin C may be helpful in preventing cancer (Glatthaar, Hornig, & Moser, 1986). The possible mechanisms by which vitamin C may affect carcinogenesis include antioxidant effects, blocking of formation of nitrosamines, enhancement of the immune response, and acceleration of detoxification of liver enzymes. Vitamin E, an important antioxidant, plays a role in immunocompetence by increasing humoral antibody protection, resistance to bacterial infections, cell-mediated immunity, the T-lymphocytes tumor necrosis factor production, inhibition of mutagen formation, repair of membranes in DNA, and blocking micro cell line formation (Sokol, 1988). Hence vitamin E may be useful in cancer prevention and inhibit carcinogenesis by the stimulation of the immune system. The administration of a mixture of the above three antioxidants revealed the highest reduction in the risk of developing cardiac cancer.

2.6.3 Free radical and aging

The human body is in a constant battle to keep from aging. Research suggests that free radical damage to cells leads to the pathological changes associated with aging (Ashok &

Ali, 1999). An increasing number of diseases or disorders, as well as the aging process itself, demonstrate link either directly or indirectly to these reactive and potentially destructive molecules (Luke, 2014). The major mechanism of aging attributes to DNA or the accumulation of cellular and functional damage (Cantuti-Castelvetri, Shukitt-Hale, & Joseph, 2000). Reduction of free radicals or decreasing their rate of production may delay aging. Some of the nutritional antioxidants will retard the aging process and prevent disease. Based on these studies, it appears that increased oxidative stress commonly occurs during the aging process, and antioxidant status may significantly influence the effects of oxidative damage associated with advancing age. Research suggests that free radicals have a significant influence on aging, that free radical damage can be controlled with adequate antioxidant defense, and that optimal intake of the antioxidant nutrient may contribute to enhanced quality of life. Recent research indicates that antioxidant may even positively influence lifespan.

2.7 Oxidative damage to biomolecules

2.7.1 Oxidative damage to protein

Proteins can be oxidatively modified in three ways: oxidative modification of specific amino acid, free radical-mediated peptide cleavage, and formation of protein cross-linkage due to reaction with lipid peroxidation products. Protein containing amino acids such as methionine, cysteine, arginine, and histidine seem to be the most vulnerable to oxidation (Freeman & Crapo, 1982). Free radical-mediated protein modification increases susceptibility to enzyme proteolysis. Oxidative damage to protein products may affect the activity of enzymes, receptors, and membrane transport. Oxidatively damaged protein products may contain very reactive groups that may contribute to damage to the membrane and many cellular functions. Peroxyl radical is usually considered to be free radical species for the oxidation of proteins. ROS can damage proteins and produce carbonyls and other amino acids modification including the formation of methionine sulfoxide and protein carbonyls and other amino acids modification including the formation of methionine sulfoxide and protein peroxide. Protein oxidation affects the alteration of signal transduction mechanism, enzyme activity, heat stability, and proteolysis susceptibility, which leads to aging.

2.7.2 Oxidative damage to DNA

Many experiments clearly provide evidence that DNA and RNA are susceptible to oxidative damage. It has been reported that especially in aging and cancer, DNA is considered a major target (Woo, McLure, Lees-Miller, Rancourt, & Lee, 1998). Oxidative nucleotide as glycol, dTG, and 8-hydroxy- 2-deoxyguanosine is found to be increased during oxidative damage to DNA under UV radiation or free radical damage. It has been reported that mitochondrial DNA is more susceptible to oxidative damage that has a role in many diseases including cancer. It has been suggested that 8-hydroxy-2-deoxyguanosine can be used as a biological marker for oxidative stress (Hattori et al., 1996).

2.7.3 Oxidative damage to lipid

Oxidative stress and oxidative modification of biomolecules are involved in a number of physiological and pathophysiological processes such as aging, atherosclerosis, inflammation and carcinogenesis, and drug toxicity. Lipid peroxidation is a free radical process involving a source of secondary free radical, which further can act as the second messenger or can directly react with another biomolecule, enhancing biochemical lesions. Lipid peroxidation occurs on polyunsaturated fatty acid located on the cell membranes and it further proceeds with a radical chain reaction. Hydroxyl radical is thought to initiate ROS and remove hydrogen atom, thus producing lipid radical and further converted into diene conjugate. Further, by addition of oxygen, it forms a peroxyl radical; this highly reactive radical attacks another fatty acid forming lipid hydroperoxide (LOOH) and a new radical. Thus lipid peroxidation is propagated. Due to lipid peroxidation, a number of compounds are formed, for example, alkanes, malondialdehyde, and isoprostanes. These compounds are used as markers in lipid peroxidation assay and have been verified in many diseases such as neurogenerative diseases, ischemic reperfusion injury, and diabetes (Lovell, Ehmann, Butler, & Markesbery, 1995).

2.8 Antioxidant defense system

Antioxidants act as a radical scavenger, a hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, an enzyme inhibitor, synergist, and metal chelating agents. Both enzymatic and nonenzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS (Frei, Stocker, & Ames, 1988).

2.9 Mechanism of action of antioxidants

Two principle mechanisms of action have been proposed for antioxidants (Rice-Evans & Diplock, 1993). The first is a chain- breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems. The second mechanism involves removal of ROS/reactive nitrogen species initiators (secondary antioxidants) by quenching chain-initiating catalyst. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation (Krinsky, 1992).

2.9.1 Level of antioxidant action

The antioxidants acting in the defense systems act at different levels such as preventive, radical scavenging, repair and de novo, and the fourth line of defense, i.e., the adaptation.

The first line of defense is the preventive antioxidants, which suppress the formation of free radicals. Although the precise mechanism and site of radical formation *in vivo* are not well elucidated yet, the metal-induced decompositions of hydroperoxides and hydrogen peroxide must be one of the important sources. To suppress such reactions, some antioxidants reduce hydroperoxides and hydrogen peroxide beforehand to

alcohols and water, respectively, without the generation of free radicals and some proteins sequester metal ions. Glutathione peroxidase, glutathione-s-transferase, phospholipid hydroperoxide glutathione peroxidase (PHGPX), and peroxidase are known to decompose lipid hydroperoxides to corresponding alcohols. PHGPX is unique in that it can reduce hydroperoxides of phospholipids integrated into biomembranes. Glutathione peroxidase and catalase reduce hydrogen peroxide to water.

The second line of defense is the antioxidants that scavenge the active radicals to suppress chain initiation and/or break the chain propagation reactions. Various endogenous radical-scavenging antioxidants are known: some are hydrophilic and others are lipophilic. Vitamin C, uric acid, bilirubin, albumin, and thiols are hydrophilic, radical-scavenging antioxidants, while vitamin E and ubiquinol are lipophilic radical-scavenging antioxidants. Vitamin E is accepted as the most potent radical-scavenging lipophilic antioxidant.

The third line of defense is the repair and *de novo* antioxidants. The proteolytic enzymes, proteinases, proteases, and peptidases, present in the cytosol and in the mitochondria of mammalian cells, recognize, degrade, and remove oxidatively modified proteins and prevent the accumulation of oxidized proteins. The DNA repair systems also play an important role in the total defense system against oxidative damage. Various kinds of enzymes such as glycosylases and nucleases, which repair the damaged DNA, are known. There is another important function called adaptation where the signal for the production and reactions of free radicals induces formation and transport of the appropriate antioxidant to the right site (Niki, 1993).

2.9.2 Types of antioxidants

2.9.2.1 Enzymatic

Cells are protected against oxidative stress by an interacting network of antioxidant enzymes (Sies, 1997). Here, the superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with

superoxide dismutases catalyzing the first step and then catalases and various peroxidases removing hydrogen peroxide (Ho, Magnenat, Gargano, & Cao, 1998).

A) Superoxide dismutase

Superoxide dismutases (SODs) are a class of closely related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide (Bannister, Bannister, & Rotilio, 1987; Zelko, Mariani, & Folz, 2002). SOD enzymes are present in almost all aerobic cells and in extracellular fluids (Johnson & Giulivi, 2005). There are three major families of superoxide dismutase, depending on the metal cofactor: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese), and finally the Ni type which binds nickel (Wuerges et al., 2004).In higher plants, SOD isozymes have been localized in different cell compartments. Mn-SOD is present in mitochondria and peroxisomes. Fe-SOD has been found mainly in chloroplasts but has also been detected in peroxisomes, and CuZn-SOD has been localized in the cytosol, chloroplasts, peroxisomes, and apoplast (Corpas, Barroso, & del Río, 2001; Corpas et al., 2006). In humans (as in all other mammals and most chordates), three forms of superoxide dismutase are present. SOD1 is located in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular. The first is a dimer (consists of two units), while the others are tetramers (four subunits). SOD1 and SOD3 contain copper and zinc, while SOD2 has manganese in its reactive center (Cao et al., 2008).

B) Catalase

Catalase is a common enzyme found in nearly all living organisms, which are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani, Fita, & Loewen, 2004). Hydrogen peroxide is a harmful by-product of many normal metabolic processes: to prevent damage, it must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules (Gaetani, Kirkman, Mangerini, & Ferraris, 1994).All known animals use catalase in every organ, with particularly high concentrations occurring in the liver (Eisner & Aneshansley, 1999).

C) Glutathione systems

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione S transferases. This system is found in animals, plants, and microorganisms (Cantin, Hubbard, & Crystal, 1989). Glutathione peroxidase is an enzyme containing four selenium cofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals (Brigelius-Flohé, 1999). Glutathione peroxidase 1 is the most abundant and is a very efficient scavenger of hydrogen peroxide, while glutathione peroxidase 4 is most active with lipid hydroperoxides. The glutathione S-transferases show high activity with lipid peroxides. These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism.

2.9.2.2 Non-Enzymatic

A) Ascorbic acid

Ascorbic acid or “vitamin C” is a monosaccharide antioxidant found in both animals and plants. As it cannot be synthesized in humans and must be obtained from the diet, it is a vitamin (Mukund et al., 2013). Most other animals are able to produce this compound in their bodies and do not require it in their diets. In cells, it is maintained in its reduced form by reaction with glutathione, which can be catalyzed by protein disulfide isomerase and glutaredoxins (Meister, 1994). Ascorbic acid is a reducing agent and can reduce and thereby neutralize ROS such as hydrogen peroxide (Padayatty et al., 2003). In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the antioxidant enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants (Shigeoka et al., 2002).

B) Glutathione

Glutathione is a cysteine-containing peptide found in most forms of aerobic life (Vandeputte, Guizon, Genestie-Denis, Vannier, & Lorenzon, 1994). It is not required in the diet and is instead synthesized in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other

metabolites and enzyme systems as well as reacting directly with oxidants (Meister, 1988). Due to its high concentration and central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants (Mukund et al., 2013). In some organisms, glutathione is replaced by other thiols, such as by mycothiol in the actinomycetes, or by trypanothione in the kinetoplastids (Fairlamb & Cerami, 1992).

C) Tocopherols and tocotrienols (Vitamin E)

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties (Herrera & Barbas, 2001). Of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolizing this form (Brigelius-Flohe & Traber, 1999). It has been claimed that the α-tocopherol form is the most important lipid-soluble antioxidant and that it (Jagetia & Rao, 2006)protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Traber & Atkinson, 2007). This removes the free radical intermediates and prevents the propagation reaction from continuing. This reaction produces oxidized α-tocopheroxyl radicals that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol, or ubiquinol (X. Wang & Quinn, 1999).

2.10 Plants as a source of antioxidants

Synthetic and natural food antioxidants are used routinely in foods and medicine especially those containing oils and fats to protect the food against oxidation. There are a number of synthetic phenolic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) being prominent examples. These compounds have been wide using as antioxidants in the food industry, cosmetics, and therapeutic industry. However, some physical properties of BHT and BHA such as their high volatility and instability at elevated temperature, strict legislation on the use of synthetic food additives, carcinogenic nature of some synthetic antioxidants, and consumer preferences have shifted the attention of manufacturers from synthetic to natural antioxidants(Papas, 1999). In view of increasing risk factors of human to various deadly

diseases, there has been a global trend toward the use of natural substance present in medicinal plants and dietary plats as therapeutic antioxidants. It has been reported that there is an inverse relationship between the dietary intake of antioxidant-rich food and medicinal plants and the incidence of human diseases. The use of natural antioxidants in food, cosmetic, and therapeutic industry would be a promising alternative for synthetic antioxidants in respect of low cost, highly compatible with dietary intake and no harmful effects inside the human body. Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers (Brown & Rice-Evans, 1998). Attempts have been made to study the antioxidant potential of a wide variety of vegetables like potato, spinach, tomatoes, and legumes (Furuta, Nishiba, & Suda, 1997). There are several reports showing the antioxidant potential of fruits (H. Wang, Cao, & Prior, 1996). Strong antioxidants activities have been found in berries, cherries, citrus, prunes, and olives. Green and black teas have been extensively studied in the recent past for antioxidant properties since they contain up to 30% of the dry weight as phenolic compounds (Lin, Lin, Liang, Lin-Shiau, & Juan, 1998).Apart from the dietary sources, Indian medicinal plants also provide antioxidants and these include (with common/ayurvedic names in brackets) *Acacia catechu* (kair), *Aeglemarmelos* (Bengal quince, Bel), *Allium cepa*(Onion), *A. sativum*(Garlic, Lahasuna), *Aleovera* (Indainaloe, Ghritkumari), *Amomumsu bulatum* (Greater cardamom, Barielachi), *Andrographis paniculata* (Kiryat), *Asparagus recemosus* (Shatavari), *Azadirachtaindica* (Neem, Nimba), *Bacopamonniera*(Brahmi), *Buteamonosperma*(Palas, Dhak), *Camellia sinensis* (Green tea), *Cinnamomumverum*(Cinnamon), *Cinnamomum tamala*(Tejpat), *Curcma longa* (Turmeric, Haridra), *Emblica officinalis*(Indian gooseberry, Amlaki), *Glycyrrhizaglapra* (Yashtimudhu), *Hemidesmus indicus*(Indian Sarasparilla,Anantamul), *Indigoferatinctoria*, *Mangifera indica*(Mango, Amra), *Momordica charantia*(Bitter gourd), *Murraya koenigii*(Curry leaf), *Nigella sativa* (Blackcumin), *Ocimum sanctum* (Holy basil, Tusil), *Onosma echioides* (Ratanjyot), *Picrorrhiza kurroa* (Katuka), Piper beetle, *Plumbago zeylancia* Chitrak), *Sesamum indicum*, *Sida cordifolia, Spirulina fusiformis*(Alga), Swertia decursata, Syzigium cumini (Jamun), *Terminalia ariuna*(Arjun), *Terminalia bellarica* (Beheda), *Tinospora cordifolia* (Heart left moonseed, Guduchi), *Trigonella foenum-graecium* (Fenugreek), *Withania somifera* (Winter cherry, Ashwangandha), and *Zingiber officinalis* (Ginger) (Devasagayam et al., 2004).

2.11 DPPH free radical scavenging activity

In 1958 Marsden Blois, working at Stanford University evidently introduced the DPPH method. The model of scavenging the stable DPPH radical model is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was considered due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The nitrogen-centered radical 2, 2- diphenyl-1-picrylhydrazyl has been extensively employed in kinetic studies of hydrogen atom abstractions from carbon, nitrogen, sulfur, and oxygen, particularly from phenols (da Silva et al., 2012).

It is monomeric in solution, air stable, commercially available and strongly colored. This last property allows the course of the reaction to be monitored using conventional UV-Vis spectrophotometer. Regarding the pH level, in the original Blois paper, it was suggested that the system should be maintained at a pH in the range 5.0 to 6.5 by using acetate buffers. However, this precaution seems to have been abandoned in current practice. Indeed there is great uncertainty in the meaning of pH values in these predominantly organic (methanol or ethanol) media. This solution is kept in the fridge wrapped in foil when not in use, to reduce its degradation (light induced). The solution degrades at a rate of 2-4 % each week and is remade weekly if necessary or if the absorbance at 515 nm, is significantly changed. Before use it must be taken out from the fridge and allowed to reach the room temperature otherwise the concentration will be higher due to volume contraction (Karthik, Nithiya, & Jayabharathi, 2011).

The odd electron in DPPH free radical gives a strong absorption at 517 nm and is purple in color. DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole so that the molecules do not dimerize as would be the case with most other free radicals. The delocalization also gives rise to the deep violet color characterized by an absorption band in ethanol solution centered at about 520 nm. It should be evident that the method is a constant-volume colorimetric titration although the slowness of the overall reaction (with mixtures having to be left

for 30 minutes before the absorbance reading is taken) complicates the experimental procedure.

When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color (although there would be expected residual of pale yellow color from the picryl group still present) (Mansouri, Movahedian, Rostami, & Fassihi, 2012). The resulting decolorization is stoichiometric with respect to a number of electrons captured. Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is

Z• + AH = ZH + A•

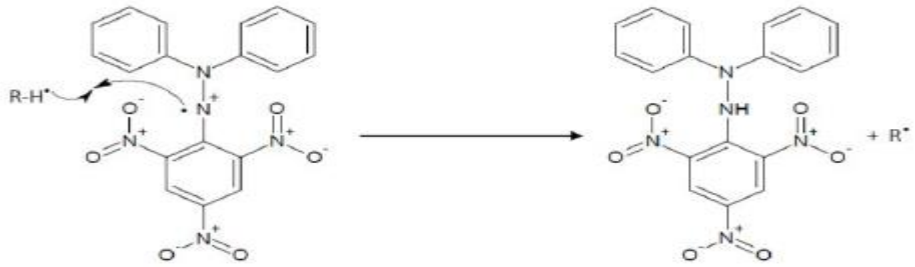


Fig. 1: Mechanism of DPPH action

2.12 Antibacterial activity

Natural products have been traditionally accepted as remedies for many diseases. The beneficial medicinal effects of plant products typically result from the combinations of secondary metabolites present in the plants. The most important of these bioactive constituents are phenolics, flavonoids, alkaloids, and tannins(Bellini et al., 2006). Plant extracts have been known since antiquity to possess notable biological activities, including antibacterial, antioxidant, and anticancer properties. It is a popular belief that they present minor side effects. Infectious diseases are the leading cause of death worldwide. The ever increasing resistance of pathogens to antibiotics as well as the undesirable side effects of certain antimicrobial agents has necessitated the discovery of novel bioactive compounds (Mishra, Mishra, Kehri, Sharma, & Pandey, 2009). There has been an increasing interest in medicinal plants as a natural alternative to synthetic drugs. Several members of Enterobacteriaceae are responsible for causing severe infections. Many reports have been published in recent years on the antimicrobial activity of essential oils and crude extracts

derived from plants against etiological agents of infectious diseases and food-borne pathogens (Shashank Kumar & Pandey, 2013).

2.13 Antimicrobial resistance

Antibiotic / Antimicrobial resistance is the ability of microbes to resist the effects of drugs – that is, the germs are not killed, and their growth is not stopped. Although some people are at greater risk than others, no one can completely avoid the risk of antibiotic-resistant infections. Infections with resistant organisms are difficult to treat, requiring costly and sometimes toxic alternatives. Bacteria will inevitably find ways of resisting the antibiotics developed by humans, which is why aggressive action is needed now to keep new resistance from developing and to prevent the resistance that already exists from spreading.

Microbes are organisms too small for the eye to see and are found everywhere on Earth. There are many types of microbes: bacteria, viruses, fungi, and parasites. While most microbes are harmless and even beneficial to living organisms, some can cause disease among humans, other animals, and plants. These disease-causing microbes are called pathogens; sometimes they are referred to as “germs” or “bugs.” All types of microbes have the ability to develop resistance to the drugs created to destroy them, becoming drug-resistant organisms (CDC 2015).

2.14 Antibiotic-resistant bacteria

Antibiotic-resistant bacteria are bacteria that are not controlled or killed by antibiotics. They are able to survive and even multiply in the presence of an antibiotic. Most infection-causing bacteria can become resistant to at least some antibiotics. Bacteria that are resistant to many antibiotics are known as multi-resistant organisms (MRO).

Antibiotic resistance is a serious public health problem. It can be prevented by minimizing unnecessary prescribing and overprescribing of antibiotics, the correct use of

prescribed antibiotics, and good hygiene and infection control.Some bacteria are naturally resistant to some antibiotics. For example, benzylpenicillin has very little effect on most organisms found in the human digestive system (gut).

Some bacteria have developed resistance to antibiotics that were once commonly used to treat them. For example, Staphylococcus aureus ('golden staph' or MRSA) and Neisseria gonorrhoeae (the cause of gonorrhea) are now almost always resistant to benzylpenicillin. In the past,these infections were usually controlled by penicillin (Guilfoile&Alcamo,2007).

The most serious concern with antibiotic resistance is that some bacteria have become resistant to almost all of the easily available antibiotics. These bacteria are able to cause serious disease and this is a major public health problem. Important examples are:

- methicillin-resistant Staphylococcus aureus (MRSA)
- vancomycin-resistant Enterococcus (VRE)
- multi-drug-resistant Mycobacterium tuberculosis (MDR-TB)
- carbapenem-resistant Enterobacteriaceae (CRE) gut bacteria

2.15 Cytotoxic effect of an extract of *T. cordifolia*

Chemotherapy is a major treatment modality for cancer and some of the plants like *Catharanthus roseus*, *Podophyllum peltatum*, *P. emodii*, *Taxus brevifolia*, *Ochrosia elliptica,* and *Campototheca acuminata*, have provided active principles which are in clinical use for controlling advanced stages of malignancies (Kinghorn & Balandrin, 1993). However, most of these chemotherapeutic agents exhibit severe normal toxicity, resulting in undesirable side effects. Moreover, many of the active molecules sold for the treatment of cancer, are highly expensive, mutagenic, carcinogenic and teratogenic. Hence, there is a need to find alternative drugs, which are highly effective at non-toxic doses, inexpensive and accessible to common man. A need is therefore felt to search

newer remedies, which are cheaper economically and do not have severe side effects of pure compounds. Medicines derived from plants have played a pivotal role in health care of ancient and modern cultures. Ayurveda, the Indian system of medicine mainly uses plant-based drugs or formulations to treat various ailments including cancer. Recent surveys suggest that one in three Americans uses dietary supplements daily and the rate of usage is much higher in cancer patients, which may be up to 50% of patients treated in cancer centers (Richardson, Sanders, Palmer, Greisinger, & Singletary, 2000).

A variety of constituents have been isolated from *T. Cordifolia* and their structures elucidated. They belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds, and polysaccharides. Leaves of *T. Cordifolia* are rich in protein(11.2%) and are fairly rich in calcium and phosphorus (S Kumar, Verma, Pande, & Srivastava, 2000; Zhao, Wang, Rimando, & Che, 1991). Alkaloids like berberine, palmatine, tembetarine, and magnoflorine have been isolated from the stem of *T. cordifolia*.The roots of *T. Cordifolia* are also reported to contain other alkaloids like chorine, tinosporin, isocolumbin, palmatine, tetrahydropalmatine and magnoflorine (Jagetia & Rao, 2006). Our preliminary studies on the stem extracts of *T. cordifolia* have shown a promising response in cultured human cancer cells, where various extracts of guduchi were found to reduce cell survival in a dose-dependent manner. However, dichloromethane extract was found to be the most promising one and has been found to be non-toxic *in vivo* up to1.2 g/b. wt.11,19) The studies on the antineoplastic action of dichloromethane extract of *T. cordifolia in vivo* are lacking. This stimulated us to investigate the antineoplastic activity of dichloromethane extract of *T. Cordifolia in* mice transplanted with Ehrlich ascites carcinoma.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Collection and Identification of plant material

The stem part of *Tinospora cordifolia* (Wild.) Hook. F. And Thomas of the family Menispermaceae were collected from the Saptari district of Nepal. The taxonomic identification was carried out by Prof. Dr. Shiva Kumar Rai taxonomist of Postgraduate college Biratnagar Tribhuvan University department of botany. Plant material was collected in the month of May climbing on the mango tree.

From the collected plant materials stems were separated and washed, dried in shade and crushed to coarse powder.

3.2 Preparation of Extract

The plant stem was washed thoroughly with tap water followed by sterile distilled water and shade dried at room temperature for 10-15 days. Then for complete dry, the plant material was kept in an oven at 45℃ overnight. The dried stem was finely powdered with the help of mortar and pestle and followed by the electric grinder.

The organic constituents from dried plant (Stem) material were obtained by continuously extracting the powdered material in soxhlet apparatus with 90% of ethanol as an organic solvent for 16 hours at 55**℃** until complete exhaustion of the material and the solution become clear. After completion of extraction, the extracts were passed through Whatman No.1 filter paper and the filtrate was concentrated by keeping filtrate on a heating plate at 30-40℃ in order to reduce the volume. The paste-like extracts were stored in labeled screw-capped bottles and kept in a refrigerator at 4**℃** (Natarajan & Francis Xavier, 2003).

3.2.1 Preparation of hot water extract

5 gm of dried finely powdered plant material was taken in a beaker and 200 ml of distilled water was added. The mixture was heated on a hot plate with continuous stirring at 30^{o}-40^{o}C for 20 minutes. Then the water extract was filtered through filter paper and the filtrate was used for the phytochemical analysis. The water extract was kept in the refrigerator when not in use (Yadav & Agarwala, 2011).

3.3 Sterility checking

Prior to subjecting the extracts to antibacterial assay, they were checked for sterility by inoculating on nutrient agar and incubating at 37**°**C.

3.4 Preparation of media and solution

3.4.1 Nutrient Broth

1.3 grams of nutrient broth media was weighed and suspended in 100 ml of distilled water. The media was heated to dissolve the medium completely. Then medium was dispensed into tubes about 5 ml each tube and lastly sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

3.4.2 Mueller Hinton agar (MHA)

38.0 grams of nutrient broth media was weighed and suspended in 1000 ml of distilled water. Heated to boiling to dissolve the medium completely. Then medium is sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then it was allowed to Cool to 45-50°C. The medium was well mixed and poured into sterilized Petri plates.

3.4.3 Preparation of DPPH solution

394.0 mg of DPPH was weighed and dissolved in 100 ml of ethanol to make 1 M solution as a stock solution and the further working solution was made by dissolving 1 microlitre of stock into 0.1mM solution (Om P. Sharma et al, 2009).

3.4.4 Materials and Reagents

The materials and reagents required are listed in appendix B.

3.5 Qualitative phytochemical analysis

The extract was tested for the presence of bioactive compounds by using the following standard method (Yadav & Agarwala, 2011)

3.5.1 Test for proteins

3.5.1.1 Ninhydrin test

Crude extract when boiled with 2ml of 0.2% solution of Ninhydrin, the violet color appeared suggesting the presence of amino acids and proteins.

3.6 Test for carbohydrates

3.6.1 Fehling's test

An equal volume of Fehling A and Fehling B reagents were mixed together and 2ml of it was added to the crude extract and gently boiled. A brick red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

3.6.2 Benedict's test

Crude extract when mixed with 2ml of Benedict's reagent and boiled, a reddish brown precipitate formed which indicated the presence of the carbohydrates.

3.6.3 Molisch's test

The crude extract was mixed with 2ml of Molisch's reagent and the mixture was shaken properly. After that, 2ml of concentrated H_2SO_4 was poured carefully along the side of the test tube. The appearance of a violet ring at the interphase indicated the presence of carbohydrate.

3.6.4 Iodine test

The crude extract was mixed with 2 ml of iodine solution. A dark blue or purple coloration indicated the presence of the carbohydrate.

3.7 Test for phenols and tannins

The crude extract was mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

3.8 Test for flavonoids

3.8.1 Shinoda test

The crude extract was mixed with few fragments of magnesium ribbon and concentrated HCl was added drop wise. Pink scarlet color appeared after few minutes which indicated the presence of flavonoids.

3.8.2 Alkaline reagent test

The crude extract was mixed with 2ml of 2% solution of NaOH. An intense yellow color was formed which turned colorless on the addition of few drops of diluted acid which indicated the presence of flavonoids.

3.9 Test for saponins

The crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of spooning.

3.10 Test for glycosides

3.10.1 Liebermann's test

The crude extract was mixed with each of 2 ml of chloroform and 2 ml of acetic acid. The mixture was cooled in ice. Carefully concentrated H_2SO_4 was added. A color change from violet to blue to green indicated the presence of a steroidal nucleus, i.e., glycogen portion of the glycoside.

3.10.2 Salkowski's test

The crude extract was mixed with 2 ml of chloroform. Then 2 ml of concentrated H_2SO_4 was added carefully and shaken gently. A reddish brown color indicated the presence of the steroidal ring, i.e., glycone portion of the glycoside.

3.11 Test for steroid

The crude extract was mixed with 2 ml of chloroform and concentrated H_2SO_4 was added sidewise. A red color produced in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing the crude extract with 2ml of chloroform. Then 2 ml of each of concentrated H_2SO_4 and acetic acid was poured into the mixture. The development of a greenish coloration indicated the presence of steroids.

3.12 Test for terpenoids

The crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. To this, 2 ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A grayish color indicated the presence of terpenoids.

3.13 Antioxidant assay

3.13.1 DPPH free radical scavenging assay

The scavenging activity of *Tinospora cordifolia* bark extracts was determined using DPPH assay with some minor modification (Choi et al., 2002; Upadhyay, Ganie, Agnihotri, & Sharma, 2014). This method depends on the reduction of purple DPPH (Sigma- Aldrich) to a yellow colored diphenyl picrylhydrazine. The determination of the disappearance of free radicals was done using a spectrophotometer. The remaining DPPH which showed maximum absorption at 518 nm was measured. Each plant extract sample's stock solution (10 mg/ml) was diluted to final concentrations of (9, 8, 7, 6, 5, 4, 3, 2 and 1 mg/ml) in DMSO (Merck) (Dimethyl sulfoxide). One ml of a 0.3 mM DPPH DMSO solution was added to 2.5 ml of sample solution of different concentrations. These test solutions. Ascorbic acid (HiMedia) was used as positive control and prepared in the same manner as above. As DPPH is sensitive to light, it is exposed to the minimum possible light. These solutions were allowed to react at room temperature for 30 minutes. The absorbance values were measured at 518 nm and converted into the percentage antioxidant activity using the following equation:

$$\text{Free radical scavenging activity}(\%) = \frac{\text{Abs(Control)} - \text{Abs(Sample)}}{\text{Abs(control)}} \times 100$$

Where, (Abs = Absorbance)

The test was done in triplicate.

3.14 Evaluation of Antibacterial activity

3.14.1 Bacterial cultures

The standard pathogenic bacterial cultures were procured from BPKIHS, Nepal and used in the present study (Table 1). The bacterial cultures were rejuvenated in Mueller-Hinton broth (Hi-media laboratories, Mumbai, India) at 37℃ for 18h and then stocked at 40℃ in Mueller-Hinton Agar. The inoculums size of the bacterial culture was standardized according to the National Committee for Clinical Laboratory Standards (NCCLS, 2002) (Standards, 2003) guideline. The pathogenic bacteria culture was inoculated into the sterile Nutrient broth and incubated at 37℃ for 3h until the culture attained turbidity of 0.5 McFarland units. The final inoculums size was standardized to 10-5 CFU/mol with the help of spectrophotometer.

Table 1: Bacterial cultures used in study collected from BPKIHS pathology laboratory (Nepal).

S.N	**Name of the bacteria**
1.	*Staphylococcus aureus*
2.	Vancomycin-resistant *enterococcus*(VRE)
3.	*Klebsiella pneumonia*
4.	*Pseudomonas aeruginosa*
5.	Methicillin-resistant *S.aureus*(MRSA)

3.14.2 Screening for antibacterial properties

Antibacterial activities of plant extracts were tested by agar well diffusion method (Khyade & Vaikos, 2009). The culture plates were prepared by pouring 20 ml of sterile Muller Hinton agar (MHA). 1 ml inoculums suspension was spread uniformly over the agar medium using a sterile glass rod to get uniform distribution of bacteria. A sterile cork borer (6 mm) was used to make wells in each plate for extracts. These plates were labeled and each plant extracts (at a concentration of 100, 50, 25, 12.5 mg/ml) were

added aseptically into the well. Also, 5% DMSO and chloramphenicol (10 µg) were used as negative and positive control respectively. Plates containing drug were left for one hour in order to diffuse properly in media and to get dry. Then the plates were incubated for 24 h at 37^{o}C during which the activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated three times and the antibacterial activity was expressed as the mean of the diameter of the inhibition zones (mm) produced by the plant extracts when compared to the controls.

3.15 Cytotoxic effect of stem extract of *T. cordifolia*

3.15.1 Brine Shrimp Lethality Assay (BSLA)

Brine shrimp eggs were obtained from the Marine aquatics Nepal (Kathmandu) sample for the research work. Filtered, artificial seawater was prepared by dissolving 38 g of sea salt in 1 liter of distilled water for hatching the shrimp eggs. The seawater was put in a small plastic container (hatching chamber) with a partition for dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber while the lamp above the other side (light) will attract the hatched shrimp. Two days were allowed for the shrimp to hatch and mature as nauplii (larva). After two days, when the shrimp larvae are ready, 4 mL of the artificial seawater was added to each test tube and 10 brine shrimps were introduced into each tube. Thus, there were a total of 30 shrimps per dilution (Olowa & Nuñeza, 2013). Then the volume was adjusted with artificial seawater up to 5 mL per test tube. The test tubes were left uncovered under the lamp. The number of surviving shrimps were counted and recorded after 24 hours. Using probit analysis, the lethality concentration (LC50) was assessed at 95% confidence intervals. LC50 of less than 100 ppm was considered as potent (active) (Tolulope, 2007). As mentioned by Meyer and others (Pimenta, Pinto, Takahashi, & Boaventura, 2003), LC50 value of fewer than 1000 µg/mL is toxic while LC50 value of greater than 1000 µg/mL is non-toxic. The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.

CHAPTER 4

RESULTS

4.1 The phytochemical compound

The phytochemical characteristics of *T. cordifolia* stem extract tested were summarized in Table 2. The results revealed the presence of medically bioactive compounds in the stem of plant studied. From the table, it could be seen that proteins, carbohydrates, phenols, tannins, flavonoids, steroids, terpenoids, alkaloids, and saponin were present. However, the test for glycosides showed negative test hence glycosides were absent in the ethanolic extract of *T. Cordifolia* stem extract.

Table 2. Phytochemical constituents of stem extract of *T. cordifolia.*

Phytochemical constituent	Proteins	Carbohydrates	Phenols/ Tannin	Flavonoids	Saponins	Glycosides	Steroids	Terpenoids	Alkaloids
Tinospora cordifolia(Stem)	+	+	+	+	+	-	+	+	+

Where, +: = Presence of phytochemical

-: = Absence of phytochemical

4.2 Antioxidant activity assay

4.2.1 DPPH free radical scavenging activity of stem extract

All the concentration of test solution showed more or less antioxidant activity by scavenging the free radical generated by DPPH. The free radical scavenging activity of ethanolic extract is shown in Table 3. The extracts tested, the ethanolic extract of the stem of *T. cordifolia* displayed an excellent activity against the free radicals. The ethanolic extract showed the highest scavenging activity (71.76%) at 10 mg/ml and

lowest (43.80%) at 1 mg/ml (Table 4). The ethanolic stem extract showed the highest absorbance value (0.97700±0.001155) at 10 mg/ml and lowest (0.51700±0.001155) at 1 mg/ml (Table 4). It was found that generally when the concentration of extract was decreased the absorbance values also get decreased regularly.

Table 3: Percentage scavenging activity of ethanolic stem extract of *Tinospora cordifolia*.

Plant part used for pure extraction of phytochemical	**Solvent used**	**Concentration of extract (mg/ml)**	**DPPH Scavenging activity (%)**
Stem	Ethanol	10	71.76
		9	70.38
		8	66.03
		7	63.33
		6	59.65
		5	52.96
		4	50.99
		3	46.89
		2	45.32
		1	43.80

Table 4: Antioxidant profile of ethanolic stem extract of *Tinospora cordifolia*

Plant part used for pure extraction of phytochemical	Solvent used	Concentration of extract (mg/ml)	Absorbance at 518nm
Stem	Ethanol	10	0.97700±0.001155
		9	0.92133±0.000882
		8	0.91667±0.000882
		7	0.91300±0.000577
		6	0.86500±0.001155
		5	0.61533±0.001453
		4	0.56800±0.000577
		3	0.56267±0.001453
		2	0.55467±0.000333
		1	0.51700±0.001155

±Standard error

4.3 Evaluation of antibacterial activity

All the bacterial strain were treated with different concentration of ethanol stem extract of *T. cordifolia* , by taking a 100 µL of (100 mg/ mL, 50 mg /mL and 25 mg/ mL, 12.5 mg/mL,) and 5% DMSO and 10 microgram chloramphenicol as negative and positive control respectively. Antibacterial activity was determined by agar well diffusion method. Antibacterial activities of all the four different concentration against selected bacterial strains were recorded in the form of inhibition zone and measured in millimeter (mm) with Vernier Caliper. All four different concentration of plant extract was performed in triplicate against all selected strain of bacteria. The inhibition zones values of bacterial strains, against this different concentration, were shown in Table 4.

Results obtained in the present study relieved that the tested medicinal plant's extracts posses potential antibacterial activity against MRSA, VRE, *P. aeruginosa, K. pneumonia,*

and S. aureus as shown in Table 5. The highest zone of inhibition (19.3mm) was observed against MRSA at 100 mg/ml and lowest zone of inhibition (9.7mm) was observed against *K. pneumonia* at 12.5 mg/ml. The bacterial strain of *K. pneumoniae* was not inhibited at the concentration of 12.5 mg/ml which indicates *K. pneumoniae* were somewhat resistant to lower concentration of ethanolic stem extract of *T. cordifolia.*

Table 5: Zone of inhibition(mm) of a different strain of bacteria against the various concentration of ethanolic stem extract of *T. cordifolia.*

S.N	**Bacterial strain**	**Zone of inhibition(mm)**					
		Concentration(mg/mL)					
		100mg/ml	**50mg/ml**	**25mg/ml**	**12.5mg/ml**	**5%DMSO**	**C10µg**
1.	MRSA	19.3	16.3	12.7	10.3	-	25.3
2.	VRE	17	15	12.7	9.7	-	20
3.	*P. aeruginosa*	15.7	14.3	12	10.3	-	20.3
4.	*K.pneumoniE*	16	14.3	10	-	-	18.7
5.	*S. aureus*	19	17.3	14	12.3	-	22

MRSA: Methicillin resistant Staphylococcus aureus.

VRE: Vancomycin resistant enterococcus

C10 µg: Chloramphenicol 10 µg.

4.4 Cytotoxic effect of ethanol stem extract of *T. cordifolia*

4.4.1 Brine shrimp lethality assay(BSLA)

The ethanolic extracts of the *T. cordifolia* tested showed good brine shrimp larvicidal activity. The lethality concentration (LC50) of *T. cordifolia* was 150 µg/ml or 150 ppm.

The degree of lethality was directly proportional to the concentration of the extract. Maximum mortalities (100%) were observed at a concentration of 1000 ppm. Based on the results, the brine shrimp lethality of the plant extracts was found to be concentration-dependent. The observed lethality of the plant extracts to brine shrimps indicated the presence of potent cytotoxic and probably antitumor components of these plants. According to (Meyer, Ferrigni et al. 1982), crude plant extract is toxic (active) if it has an LC50 value of fewer than 1000 µg/mL while non-toxic (inactive) if it is greater than 1000 µg/mL.The maximum larvicidal activity and minimum larvicidal activity was observed at 1000 µg/mL i.e 100% and at 1 µg/mL i.e 27%.

Table:6 The number of shrimp nauplii that survived after treating with the plant extracts and the percentage mortality

S.N.	**Plant extract**	**Concentration(µg/mL or ppm)**	**Number of surviving Nauplii After 24 hours**			**Total number of survivors**	**% Mortality**
			T1	T2	T3		
1.	Ethanolic stem extract of *T.cordifolia*	1	7	8	7	22	27%
2.		10	5	6	5	16	47%
3.		100	5	4	5	13	57%
4.		1000	0	0	0	0	100%

T1=Test number first

T2=Test number second % Mortality = (no.of dead nauplii/Total nauplii)×100%

T3=Test number thre

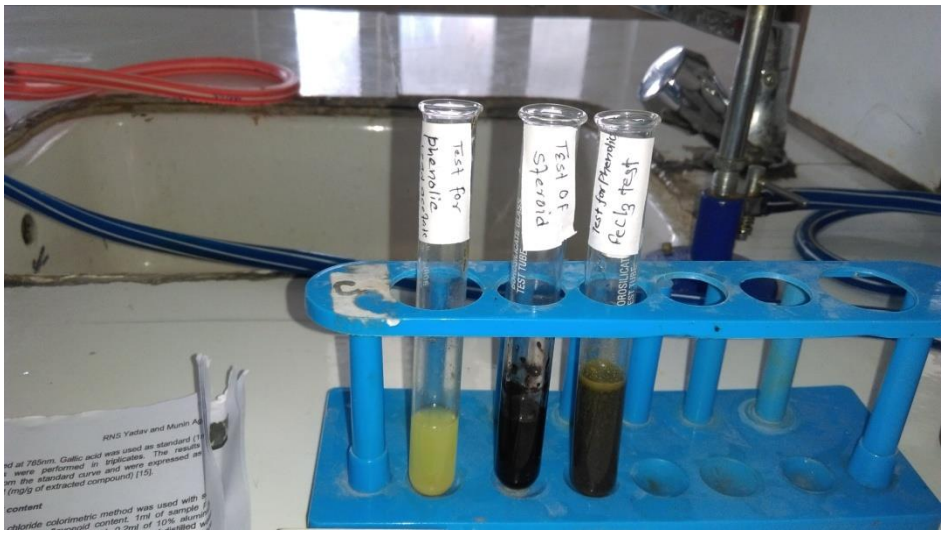
PHOTOGRAPHS

Photograph 1: Extraction Phytochemical by

Soxhlet apparatus

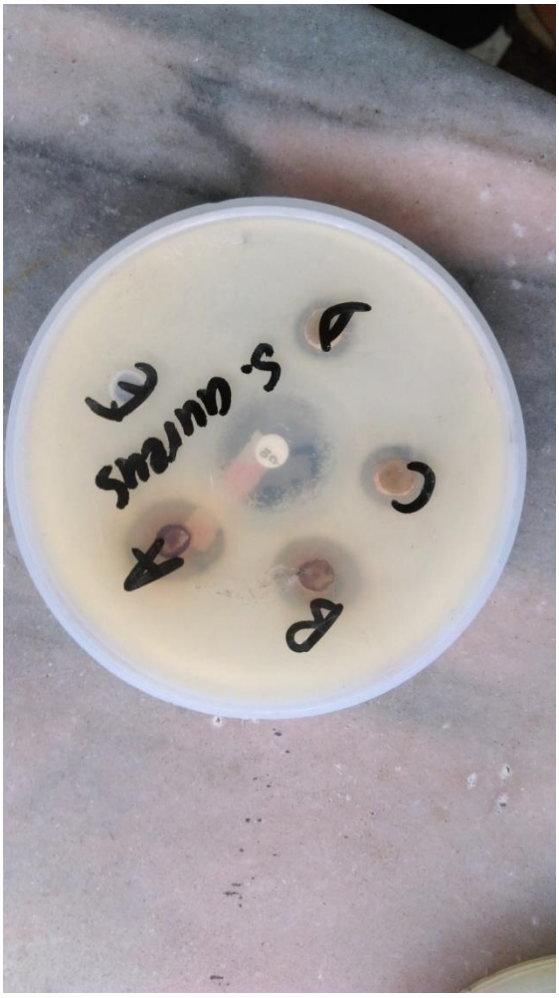
Photograph 2: Brine shrimp hatchery set up

PHOTOGRAPH



Photograph 3: Test tube after different biochemical tests

Photograph 4: Measuring antioxidant activity on UV-Visible double beam spectrophotometer in Molecular lab(STC)



Photograph 5*: S. aureus* growth

Inhibition seen on MHA

Photograph 6: Bacterial growth inhibition of different species.

CHAPTER 5
DISCUSSION

5.1 Antioxidant activity

It has been determined that the antioxidant effect of plant products is mainly due to radical scavenging activity of phenolic compounds such as flavonoids, polyphenols, tannins, and phenolic terpenes (Rahman, Aziz, & Moon, 2007). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Hasan et al., 2008). The oxidative injury now appears the fundamental mechanism underlying a number of human neurologic and other disorders such as inflammation, viral infections, autoimmune pathologies, and digestive system disorders including gastrointestinal inflammation and ulcer (Aruoma, 2003). For instance in diabetes, increased oxidative stress which co-exists with reduction in the antioxidant status has been postulated: Oxygen free-radical can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes, and play a role In the long-term complication of diabetes (Sabu & Kuttan, 2002). Similarly, in carcinogenesis, reactive oxygen species are responsible for initiating the multistage carcinogenesis process starting with DNA damage and accumulation of genetic events in one or few cell lines which leads to progressively dysplastic cellular appearance, deregulated cell growth, and finally carcinoma (Tsao, Kim, & Hong, 2004). Hence, therapy using free-radical scavenging antioxidants has the potential to prevent, delay or ameliorate many of these disorders (Dichter & Delanty, 2000). Over the past two decades, an expanding body of evidence from epidemiological and laboratory studies have demonstrated that some edible plants as a whole or their identified ingredients with antioxidant properties have substantial protective effects on human carcinogenesis (Greenwald, 2002; Kinghorn et al., 2004; Tsao et al., 2004).

It has been reported that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking

the free radical chain by donating a hydrogen atom (Kumar, Mishra, & Pandey, 2013). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The presence of reluctant (antioxidants) in the herbal extracts causes the reduction of Fe3+/ferric cyanide complex to ferrous form (Chung, Chang, Chao, Lin, & Chou, 2002). It is, therefore, possible that the activity of extracts might be due to the presence of higher amounts of reductions, which could react with free radicals to stabilize and block the radical chain reactions.

Polyphenolic contents of all the extracts appear to function as good electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and ROS to more stable products. Higher activity observed in *T. cordifolia* extracts could also be attributed to the total phenolic contents.

The transition metal ion, Fe2+, possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively nonreactive radicals(Kumar, Gupta, & Pandey, 2013; Kumar, Mishra, et al., 2013).The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions. Chelation therapy reduces iron-related complications and thereby improves quality of life and overall survival. Therefore, the continuing search for finding alternative sources of iron chelating activity with lower side effects from plant sources bears significance.

The present results suggest that all the tested plant extracts have moderate to potent antioxidant activity. Since a variety of constituents are known from the extracts studied, it becomes difficult to ascribe the antioxidant properties selectively to any one group of constituents without further studies which are beyond the scope of this paper. Thus, further extensive investigations are necessary to find out the active antioxidative principles present in these plants.

5.2 Antibacterial activity

From the results, it dictates that the greater activity resides in ethanolic stem extracts of the plant. This may due to the chemical constituents responsible for the antibacterial

activity are more soluble in ethanol extracts. It can be interpreted that the antibacterial activity against microorganisms is due to any one or more alkaloids of the plants (Nayak & Singhai, 2003). Present findings support the applicability of Tinospora cordifolia in traditional systems for it's claimed uses like fever inflammations, urinary and skin diseases. Further work is necessary to isolate and purification compounds in Tinospora cordifolia stem extracts, which will allow the scientific community to recommend their utilization as an accessible alternative to synthetic antibiotics.

Phytochemical screening of the *T. cordifolia* revealed the presence of some of the phytoconstituents in the extracts such as phenols, glycosides, and terpenoids (Table 1). Chemical basis of their presence in different fractions may be correlated with small structural differences in the compounds belonging to the same group that is critical to their activity as well as solubility. Occasionally tannins and terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents (Cowan, 1999). Since phenols have been attributed with antimicrobial and free radical scavenging activities.

Available reports tend to show that secondary metabolites such as alkaloids, flavonoids, tannins, and other compounds of phenolic nature are responsible for the antimicrobial activities in higher plants (Mishra, Kumar, Bhargava, Sharma, & Pandey, 2011). Monoterpenes, sesquiterpenes, alcohols, and aldehydes have been reported to exhibit antibacterial activity in spices against respiratory tract infections. Cyclic terpene compounds have been reported to cause loss of membrane integrity and dissipation of proton motive force (Mishra et al., 2011). Therefore, the presence of some of these phytochemicals along with phenolic compounds could to some extent justify the observed antibacterial activities in the present study. Many *T. cordifolia* extracts exhibited inhibition of pathogenic test bacteria (Table 4).

The antimicrobial activities of phenolic compounds may involve multiple modes of action. Essential oils degrade the cell wall, interact with the composition and disrupt cytoplasmic membrane, damage membrane protein, interfere with membrane integrated enzymes, cause leakage of cellular components, coagulate cytoplasm, deplete the proton motive force, change fatty acid and phospholipid constituents, impair

enzymatic mechanisms for energy production and metabolism, alter nutrient uptake and electron transport, influence the synthesis of DNA and RNA, and destroy protein translocation and the function of the mitochondrion in eukaryotes (Lambert, Skandamis, Coote, & Nychas, 2001; Pandey, Mishra, & Mishra, 2012; RACCACH, 1984). All of these mechanisms are not separate targets since some are affected as a consequence of another mechanism being targeted.

5.3 Cytotoxic effect of stem extract of *T. Cordifolia*

The evaluation of the toxic action of plant extracts is indispensable in order to consider a treatment safe; it enables the definition of the intrinsic toxicity of the plant and the effects of acute overdose (Cáceres, 1996).

Because there is currently a tendency to limit the use of laboratory animals in toxicological tests (Van Zutphen & Balls, 1997), and the brine shrimp is a crustacean whose larvae are sensitive to a variety of substances, the brine shrimp bioassay can be useful as a quick and simple test for predicting the toxicity of plant extracts and guiding their phytochemical fractionation (Cáceres, 1996).

This test is used particularly in developing countries, where 85 % of the population use medicinal plants in traditional therapy (Feroze, 1969). It has also been considered a bioindicator of environmental contamination by traces such as arsenic, lead, copper, zinc, cadmium, mercury, and selenium. Due to its commercial availability, Artemia salina L. is widely used in toxicological applications and research. Some authors say that there is no correlation between this bioassay and the toxicological effects in a whole animal (Sánchez, De Norieda, Gupta, Montenegro, & Vasquez, 1993); however, 20 plant extracts were toxicologically tested in a study using “in vivo” and “in vitro” methods, whose results showed a good correlation ($r = 0.85$ $p < 0.05$), suggesting that the brine shrimp bioassay is a useful alternative model(Parra, Yhebra, Sardiñas, & Buela, 2001).

In toxicity evaluation of plant extracts by brine shrimp bioassay, an LC50 value lower than 1000 µg/ml is considered bioactive (Meyer et al., 1982). In our study the extract has

LC50 values < 1000 µg/ml; therefore, they can have biological activity. Pharmacological properties of these plants have been demonstrated in preclinical and clinical studies.

This test is a quick, simple, practical, and low-cost method (it does not require aseptic techniques) and allows a great number of samples to be tested and processed adequately (Ohno, 1996) .

In general, the Artemia salina L. test is useful for the screening of plant extracts in order to predict their toxicity. However, although the method offers advantages such as quickness, simplicity, lack of animal use, and therefore low cost, it should be subjected to an adequate interlaboratory validation. That is the objective of the Group on Alternative Toxicological Studies in Cuba [Grupo de Estudios Toxicológicos Alternatives en Cuba (ETAC)], which is developing this test to be used in toxicological evaluations of medicinal plants so that they may be validated for popular use.

In the present study, DMSO was used as the solvent and as a negative control. This is in accordance with the previous report that brine shrimp nauplii can tolerate up to 11% of DMSO (Sam, 1993). Further studies are being conducted to isolate and purify the bioactive constituents for further evaluation in human cell line cultures for cytotoxic effects.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The study demonstrated the presence of various groups of phytochemicals like phenolics, alkaloids, tannins, glycosides, carbohydrates, and proteins etc in ethanolic stem extract of *T. cordifolia* which are responsible for showing considerable antibacterial, antioxidant, and cytotoxic activities.

The imbalance between reactive oxygen species and antioxidant defense system may increase the oxidative burden and lead to the damage of biomolecules such as DNA, carbohydrates, and protein. Such a process is thought to play a role in pathological processes of various diseases. Plants having vitamins (C, E, carotenoids , etc), flavonoids (flavones, isoflavones, flavanones, anthocyanins, and catechins), polyphenols (ellagic acid, gallic acid and tannins) possess remarkable antioxidant activity.

From the results, it could be concluded that the greater activity resides in ethanolic stem extracts of the plant since it inhibits the growth of the bacteria which are resistant to the standard antibiotics . This may due to the chemical constituents responsible for the antibacterial activity are more soluble in ethanol extracts. It can also be concluded that antibacterial activity against microorganisms is due to any one or more alkaloids of the plants (Nayak & Singhai, 2003). Present findings support the utility of *Tinospora cordifolia* in traditional systems for it's claimed uses like fever inflammations, urinary and skin diseases. Further investigation is necessary to isolate and purification of compounds in *Tinospora cordifolia* stem extracts, which will allow the scientific community to recommend their utilization as an accessible alternative to artificial synthetic antibiotics.

The leaf extracts of *Tinospora cordifolia* exhibited cytotoxic activity against the brine shrimp and considered as containing active or potent components. This is because their LC50 values are less than 1000 ppm or µg/mL. The ethnopharmacological activities of this plant species are due to the different bioactive compounds present in the plants.

Although BSLA is inadequate in determining the mechanism of action of the bioactive substances in the plant, it is very useful by providing a preliminary screen that can be supported by a more specific bioassay, once the active compound has been isolated. Thus, some useful drugs of therapeutic importance may develop out of the research work.

6.2 RECOMMENDATIONS

1. The present study was focused to determine the antioxidant, antibacterial, cytotoxic activities and presence of phytochemical constituents in ethanolic stem extract of *Tinospora cordifolia* . The next step will be a purification of compounds and to find out the molecular mechanisms of its antioxidants, antibacterial and cytotoxic.

2. The present study gives the result in only one solvent i.e ethanol, all the phytochemical compound cannot dissolve in it hence the comparison of phytochemical activities in different solvent seems necessary.

3. Determination of cytotoxic effect by brine shrimp lethality assay (BSLA) is only applicable for preliminary screening of phytochemical compound hence to know the exact cytotoxic or anticancer property it should be checked on human cancer cell lines.

CHAPTER 7
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APPENDICES

Appendix A: Preparation of media and reagents

A. Composition and preparation of Different Culture Media

The culture media used were from

a. Hi-Media Laboratories Pvt. Limited, Bombay, India.

(All compositions are given in grams per liter and at 25^{o}C temperature)

1.Nutrient Agar(NA) M001

Ingredients	Gms / Litre
Peptone	5.000
Sodium chloride	5.000
HM peptone B#	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

**Formula adjusted, standardized to suit performance parameters

Preparation: 28 grams of NA was suspended in 1000 ml distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooled to 45-50°C. Mixed well and poured into sterile Petri plates.

2. MacConkey Agar(MA) (Hi-Media,M081B)

Ingredients	Gms / Litre
Peptones (meat and casein)	3.000
Pancreatic digest of gelatin	17.000

Lactose monohydrate	10.000
Bile salts	1.500
Sodium chloride	5.000
Crystal violet	0.001
Neutral red	0.030
Agar	13.500
pH after sterilization(at 25°C)	7.1±0.2

**Formula adjusted, standardized to suit performance parameters

Preparation:

49.53 grams of the dehydrated medium was suspended in 1000 ml purified/distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes i.e. validated cycle. Cooled to 45-50°C. Mixed well before pouring into sterile Petri plates.

3. Mueller-Hinton Agar (MHA) (M173,Hi-media)

Ingredients	Gms / Litre
HM infusion B from #	300.000
Acicase ##	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.4±0.1

**Formula adjusted, standardized to suit performance parameters

- Equivalent to a Beef infusion from

- Equivalent to Casein acid hydrolysate

Preparation:

38.0 grams of MHA was suspended in 1000 ml distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooled to 45-50°C. Mix well and poured into sterile Petri plates .

4. McFarland Standard

0.5 ml of 0.048 M BaCl2 (1.17% w/v $BaCl_2$ H_2O) was added to 99.5 ml of 0.18M H_2SO_4(1% w/v) with constant stirring. The MacFarland standard was thoroughly mixed to ensure that it is evenly suspended.

5. Preparation of Brine shrimp hatchery set up

Brine shrimp eggs were obtained from the Marine aquatics Nepal(Kathmandu) sample for the research work. Filtered, artificial seawater was prepared by dissolving 38 g of sea salt in 1 liter of distilled water for hatching the shrimp eggs. The seawater was put in a small plastic container (hatching chamber) with a partition for dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber while the lamp above the other side (light) will attract the hatched shrimp. Two days were allowed for the shrimp to hatch and mature as nauplii (larva).

Appendix B: Equipment, Materials, and Supplies

A. Equipment

1. Autoclave-SHIVA
2. Electric balance-K. Roy
3. Incubator- Digilab
4. Hot air oven-Digilab
5. Microscope- Olympus
6. Refrigerator-Samsung
7. Deep freeze-Haier
8. Micropipette-HumaPette
9. Spectrophotometer-Labtronics
10. Sieve of different pore size
11. Electric bulb (40 W)
12. Borer (6 mm)
13. Mortar and pestle

B. Microbiological / Biochemical Media

1. Nutrient Agar
2. MacConkey Agar
3. Nutrient Broth
4. Mueller Hinton Agar

C. Chemicals and reagents

1. Ethanol
2. Distilled water
3. DPPH
5. Ninhydrin
6. Fehling solution A&B
7. Benedict reagent
8. Molisch reagent
9. Sulphuric acid
10. Nitric acid
11. Iodine solution
12. Ferric chloride
13. Hydrochloric acid
14. Mg-ribbon
15. Sodium hydroxide
16. Acetic acid
17. Chloroform
18. DMSO
18. Ascorbic acid
19. Sea salt (Iodine free)

D. Glassware (Borosil)

1. Pipette
2. Funnels
3. Beakers
4. Test tubes
5. Petri plates
6. Conical Flasks
7. Soxhlet apparatus
8. Glass rods
9. Microscopic slides
10. Measuring Flasks
11. Reagent Bottles
12. Graduated cylinder
13. Screw-capped cylind

E. Antibiotic Discs

1. Chloramphenicol (10 micro gm)