# A Review on *Tinospora cordifolia* and its Immunomodulatory Activities

Submitted By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

Department of pharmacy
Brac University
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## Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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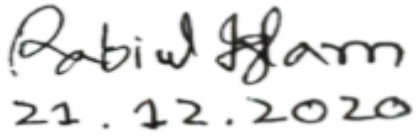


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# Approval

The thesis titled "A Review on *Tinospora cordifolia* and its Immunomodulatory Activities" submitted by Md. Rabiul Islam (ID-16346020) of Summer, 2016 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.) on 21st December, 2020.

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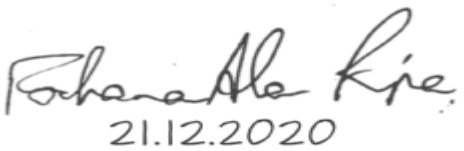


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## Ethics Statement

This study does not involve any kind of animal or human trial.

## Abstract:

Medicinal plants have always been considered as a valuable source of treatment since the ancient times. This review paper was done focusing on *Tinospora cordifolia* and its immunomodulatory properties. *T. cordifolia* is a large climbing shrub having many pharmacological properties and this medicinal plant has a great demand among the folk people of the Indian subcontinent. Different parts of *T. cordifolia* consist of many pharmacologically active compounds like alkaloids, phenols, glycosides, steroids, terpenoids, tannins, carbohydrates and other unique compounds having immunomodulatory, anti-diabetic, anti-inflammatory, hypotensive, scizonticidal, antibacterial, antifungal, antioxidant, antidiarrheal and many other properties. This plant can play a significant role in the modulation of the immune system as several components of this plant can increase the phagocytic activity of macrophage, antibody production, nitric oxide production, production of cytokines, lymphocyte proliferation, chemotactic index, plaque forming cell production and many other immune system modifying activities. The finding of this study is that this plant can increase the efficiency of the immune system.

**Keywords:** *Tinospora cordifolia*; Cordifolioside; Aerial roots; Immune system; Neutrophils; Boost.

# Dedication

*I want to dedicate this project to my family and my respectable supervisor, Dr. Farhana Alam Ripa (Assistant Professor, Department of Pharmacy, Brac University)*

## Acknowledgement

First of all, I am very thankful to Almighty Allah for blessing me with patience, strength and self-confidence which helped me most to get this project done properly.

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## List of Acronyms

| | |
|---|---|
| Tc | *T. cordifolia* |
| DC | Dendritic Cell |
| TCR | T-cell Receptor |
| CD | Differentiation Cluster |
| APC | Antigen Presenting Cells |
| LPT | Lymphocyte Proliferation Test |
| MTB | Mycobacterium Tuberculosis |
| LPS | Lipopolysaccharide |
| ANOVA | One-way Analysis of Variance |
| PBS | Phosphate Buffered Saline |
| DTH | Delayed Type Hypersensitivity |
| HBSS | Hank's Balanced Salt Solution |
| PMN | Polymorphonuclear |
| RIP | Radioimmunoprecipitation |
| SRBC | Sheep Red Blood Cells |
| NOS | Nitric Oxide Synthase |
| TNF | Tissue Necrosis Factor |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |

| | |
|---|---|
| CTL | Cytolytic T Lymphocytes |
| GFP | Green Florescence Protein |
| IL | Interleukin |
| PI | Phagocytic Index |
| NO | Nitric Oxide |
| IFN | Interferon |
| LTT | Lymphocyte Transformation Test |
| PPX | Propoxyphene |
| DAPI | 4′,6-diamidino-2-phenylindole |
| NK | Natural Killer cells |
| NETs | Neutrophil Extracellular Traps |
| WBCs | White Blood Cells |

# Chapter 1

# Introduction

## 1.1 Medicinal Plants

Life cycle of every living species directly depends on the nature. The main basis of the treatments of human diseases are plants, minerals and animals (Firenzuoli & Gori, 2007). Medicinal plants have always been preferred as a primary means of treatment since the ancient times. Every medicinal plant is a vast collection of medicinal components. The individuals initially used plants to Meet their dietary criteria. Natural flora has been a really beneficial thing. Sources for improving health and curing several diseases through different forms of diseases there are human communities and a range of species of plants available that are in several parts of the world, such as Asia, South America and Africa, treatments for many diseases are still in use. While the World Health Organization has estimated that conventional medicines constitute the primary health care system for 60 percent of the world 's population, a significant number of plant species with potential biological activities have not been explored. Due to their better compatibility with the human body, better cultural acceptability throughout the world and lower side effects, the efficacy of traditional medicines is now a putative act (Mustafa, Arif, Atta, Sharif, & Jamil, 2017). There are a large number of plants which have good therapeutic and prophylactic potential as they are compose of many valuable medicinal components like alkaloids, tannins, flavonoids, glycosides and coumarins (Mittal, Sharma, & Batra, 2014). If we analyze the history of medicinal plants we will notice that Approximately Indian people started the use of medicinal plants as medicine almost 5000 years ago and the people of central Asian people scripted about their use of medicinal plants 2500 year ago (Ang-Lee, Moss, & Yuan, 2001). In ancient times, Persians used plants for the treatment purposes and also used as aromatic agent and antiseptic agent (Hamilton, 2004). More than 35,000 plant species are used for medicinal purposes in different human cultures around the world and

almost 80 percent of the world 's populations rely on these traditional medicines for primary health care. For that reason, usage of plant extracts most of the time is included (Mustafa et al., 2017). Because of being a cluster of medicinal components traditional medicinal plants, its phytomedicines and metabolites have achieved a positive attention of the researchers all over the world (Dhama et al., 2017). Earlier, when the role of medicinal plants in narcotics was discovered, a number of drugs such as codeine, cocaine, quinine, digitoxin and morphine were isolated. Beginning with morphine isolated from opium in the early 19th century, active compounds are now isolated from medicinal plants. Any of these medicines are still being used by us (Mustafa et al., 2017). Medicinal plants have created a noticeable demand with an progressive acceptance in the world market of medicines (Jamshidi-Kia, Lorigooini, & Amini-Khoei, 2018). People who believe in Islam have diverse academic and analytical backgrounds. Date palm's meaning (*Phoenix dactylifera*) this verse of the Holy Quran is mentioned in it. In Verse no.61, Surah Baqarah, the importance of garlic (*Allium sativum*) is given. Verse no. 61, Surah Baqarah, describes the significance of garlic, cucumber, lentils, and onion. The importance of ginger is mentioned in Chapter No. 76, Verse No. 17 of Surah Ad-Dahr. The importance of many medicinal species of plants in Ahaidth has been described and more than 70 plant names are mentioned there (Aslam et al., 2016). Medicinal plants are most promising in providing a means of developing new efficient drugs (Abdul Rasool Hassan, 2012). In the developing world, much of the population is struggling to increase living standards and improve the delivery of health care because of the rising poverty and population. It is estimated that 70-80 per cent of the developing world relies on. As pharmaceuticals are highly priced, traditional plant-acquired remedies are available. The supposed "Green Wave "caused by Increased bionomical awareness has led to increased participation all over the globe in herbal formulations. Many novel compounds have been isolated from aquatic organisms over the past few years, and many of these substances have been shown to possess fascinating biological

activities. Research is currently based on the isolation of pharmacologically active compounds from natural sources in the field of diseases where medicines that are currently available are not so efficient (Aslam et al., 2016). Moreover, Some modern medicines like aspirin were developed from plant sources indirectly (R. Singh, 2015). In addition, some other valuable and modern medicines such as paracetamol, quinine, vinblastine, acetyl salicylic acid and digitalis are developed from compounds derived from some medicinal plants like *Digitalis purpurea*, black willow, calisaya bark, *Vinca rosea* etc. (Donald P. Briskin, 2000). Furthermore, medicinal plants has a very important contribution in the development of chemotherapeutic drugs (Spandana, Ali, Nirmala, Santhi, & Sipai Babu, 2013). Nowadays, the medicines derived from medicinal plants is termed as "Alternative Medicines" and it indicates the use of plants as medicines (Abdul Rasool Hassan, 2012). The basic basis of medicine is plants. Some significant medications that are still in use today are derived from ancient herbs of medicine. Ethnobotany and ethnopharmacology have been involved in the hunt for new medicines, a new route as a significant information source that has led to various sources and groups of compounds. If the planet had no plants on it, it would be difficult to foresee the future of the human race. Human beings' dependency on plants date back to the human race 's beginning. Popular sources of medicine include medicinal plants. There should be strong proof, cited in favor of herbs used in ancient systems for the treatment of diseases and the regeneration and fortification of body systems Medicine, such as traditional Ayurvedic, Unani, and Chinese medicine (Aslam et al., 2016). Medicinal plants usually produce and store some valuable substances in their different body parts which are termed as active substances (Phillipson, 2001). There are many ways of using a plant for medicinal purposes and one efficient way is administration of various type of extracts of the plant containing valuable medicinal constituents like vincristine, berberine, morphine, psilocin etc. (Balandrin, Klocke, Wurtele, &

Bollinger, 1985). These substances have many pharmacological properties for which it can also work efficiently like a synthetic drug (Mittal et al., 2014).

### 1.2 *Tinospora cordifolia*

*T. cordifolia* is also a medicinal plant with a good therapeutic potential (Figure-2). Raghu et al. (2006) defined this plant as a large climbing shrub which grows in all over India and it is also found in Bangladesh, Srilanka and China. The local name of this plant is "Guduchi" and it is a natural herbal shrub which belongs to the family Menispermaceae (Tiwari, Nayak, Prusty, & Sahu, 2018). This family contains 70 genera and 450 species in total which are generally found in tropical low land areas and the genera "Tinospora" includes 15 species (Spandana et al., 2013). These species are distributed throughout Madagascar, Africa, Australia and Asian regions (Natho G., 2008). Moreover, there are four species of this genus found in all over the India; Two species are *T. sinensis* and *T. cordifolia* which are generally seen in south India and the other two species are *T. glabra* and *T. crispa* (L.) which are seen to grow in North India (Arigela & Singh, 2018). The plants belonging to this family contains higher amount of terpenes and alkaloids (Abhimanyu et al., 2010).

The taxonomic classification of *T. cordifolia* is illustrated in Figure-1.

| | |
|---|---|
| Kingdom | : Plantae |
| Division | : Magnoliophyta |
| Class | : Magnoliopsida |
| Order | : Ranunculales |
| Family | : Menispermaceae |
| Genus | : Tinospora |
| Species | : *T. cordifolia* |

*Figure 1: Taxonomic classification of T. cordifolia (Sinha, Mishra, Singh, & Khanuja, 2004)*

*T. cordifolia* has created a great demand among the folk people and pharmaceutical companies because of its supremely good medicinal properties and this plant is added in the most 29 highly prioritized medicinal plants in agro climatic zone 8 in India (Mittal et al., 2014).

*Figure 2: T. cordifolia plant (Spandana et al., 2013).*

Many national and international pharmaceutical companies have also become very interested to manufacture and distribute the extract of this plant for its medicinal significance and commercial exploitability ( Pandey et al., 2012). They have also formulated various types of clinical and preclinical preparations with special properties like immunomodulatory, hepatoprotective, antidiabetic, antidepressant etc. properties (D. Singh & Chaudhuri, 2017). Moreover, this plant is also included in Indian Pharmacopoeia, British Pharmaceutical codex and Ayurvedic Pharmacopoeia for its importance in treatment of many diseases with greater level of safety (Choudhary, Muslim, & Pradesh, 2013). This plant is an indispensable plant which is very popular in the folk medicine system and it is proved to enhance our body defense system very effectively than other plants ( Pandey et al., 2012). Like other plants, this plants also show a greater number of genetic diversity in its physiological and morphological characteristics (Ghosh & Saha, 2012). These genetic diversities are determined by using its random amplified polymorphic DNA as a marker (Rout, 2006).

Abhimanyu et al. (2010) Collected some common names of this plant. The names are mentioned in Table-1.

*Table 1: Various names of T. cordifolia in different regions and languages* (Abhimanyu et al., 2010)

| **Languages** | **Names** |
|---|---|
| Latin | *Tinospora cordifolia* |
| English | Gulancha/ Indian tinospora |
| Sanskrit | Guduchi, Madhuparni, Amrita, Chinnaruha, Vatsadaani, Tantrika, Kundalini & Chakralakshanika. |
| Hindi | Giloya, Guduchi |
| Bengali | Gulancha |
| Telugu | Tippatiga |
| Tamil | Shindilakodi |
| Marethi | Shindilakodi |
| Gujarathi | Galo |
| Kannada | Amrita balli |

The medicine system of India shows an annual consumption of about 1000 tonnes of *T. cordifolia* (A. Sharma, Shanker, Tyagi, Singh, & Rao, 2008). In Ayurveda, this plant is considered as "Rasayana" and used in the treatment of hyperglycemic shock, cardiovascular, vitalizer, skin diseases, dysentery, diabetes, complexities in immune system etc. (Rege, Thatte, & Dahanukar, 1999). The tribal or folk groups of India also use this plant as medicine with a

greater importance (Upadhyay, Kumar, Kumar, & Mishra, 2010). Moreover, the 100 herbal active substances of this plant is called the constituents of "heavenly elixir" or "Soma" (Leonti & Casu, 2014). The plant not only has active medicinal substances but also the stem this plant is rich in minerals such as phosphorus, zinc, calcium, copper, manganese and iron (Mahima et al., 2014) Moreover, this plant is also considered as a versatile medicinal plant as it possesses a vast spectrum of therapeutic indications (Ghosh & Saha, 2012). Furthermore, it is proved to be safe while using with other drugs as an adjunct (Bahadur et al., 2016). Administration of this plant extracts in our body is safe because it does show mutagenic effect in the erythrocytes of bone marrow and also it does not alter the DNA sequence of peripheral white blood cells (Chandrasekaran, Mathuram, Daivasigamani, & Bhatnagar, 2009). However, Dhama et al. (2017) predict a good future of this medicinal plant and they see this plant constituents as a better substituent of the synthetic immunomodulatory drugs. This plant has an increasing demand in the dye industries as it contains two valuable pigments named Lycopene and berberine (Imtiyaj Khan, Sri Harsha, Giridhar, & Ravishankar, 2011).

Mittal et al. (2014) described about the growth requirements of this plant. They stated that although this plant has the ability to grow in almost all types of climates for having a very rigid structure, it grows very well in warm weather. They further stated that this plant grows very well in light back or red soil although it can be cultivated in various types of soil having sufficient moisture and organic substances. It is seen to be grown by itself in dry forest at an altitude of 1000ft (Choudhary et al., 2013). However, Mittal et al. (2014) also said that this plant can be cultivated from its seeds or vegetative cutting but for commercial purpose and large scale of production this traditional method is not applicable and its clonal propagation has also got some problems like less viable seeds, poor germination of seeds etc. Therefore, they mentioned a technique by which large scale production in short time is possible and the techniques is tissue culture. They also added that time of planting this tree is in between July

and August (Rainy season) and it needs a support to grow for being a shrub as well as climber. Moreover, they informed that Moringa (*Moringa oleifera*), Neem (*Azadirachta indica*), Jatropha (*Jatropha curcas*) and mango tree as these plants have a fast-growing property. Moreover, when the Neem (*Azadirachta indica*) is used as the support, they both are named as NEEM GILOY and also they both have almost similar composition showing a good therapeutic potential (Shefali & Nilofer, 2013). The starch derived from NEEM GILOY has good medicinal efficiency and the starch possesses bitter test (D. Singh & Chaudhuri, 2017).

### 1.3 Immune System and Immunomodulation

Immune system of our body is one of the important systems which protects our body from being infected by pathogenic microbes. The body 's chief defense against infection is the immune system. Its main purpose is to remove foreign substances that come into contact with the organism. These foreign substances are referred to as pathogens and include bacteria, viruses, fungi, parasites, and other organic matter. By creating responses to protein molecules that exist on their surface, the immune system removes pathogens from the body. These substances are referred as antigen. The immune system often removes body cells that have been transformed by infection or malignancy from their normal state and promotes the repair of tissues that have been weakened by Wound which was resulted from Accident. White blood cells are called the cells of the immune system. They are primarily located in the bone marrow, thymus, spleen, lymph nodes, and the skin, lung, and gastrointestinal tract tissues. Given that access to these compartments requires surgical procedures, most human studies examine the role of the immune system as it occurs in circulating blood. Immune system components are present in circulating blood because it transports them between immune compartments and sites of infection and injury, along with the lymphatic system (Miller & Cohen, 2001). The effectiveness of the infection immune response depends on its ability to sense and assess microbial threats, to organize the removal of the threat and to minimize the damage to the

targeted tissues. This delicate balance is accomplished by the coordinated operation of the immune system's innate and adaptive form (Figure-3) (Messaoudi, Estep, Robinson, & Wong, 2011).

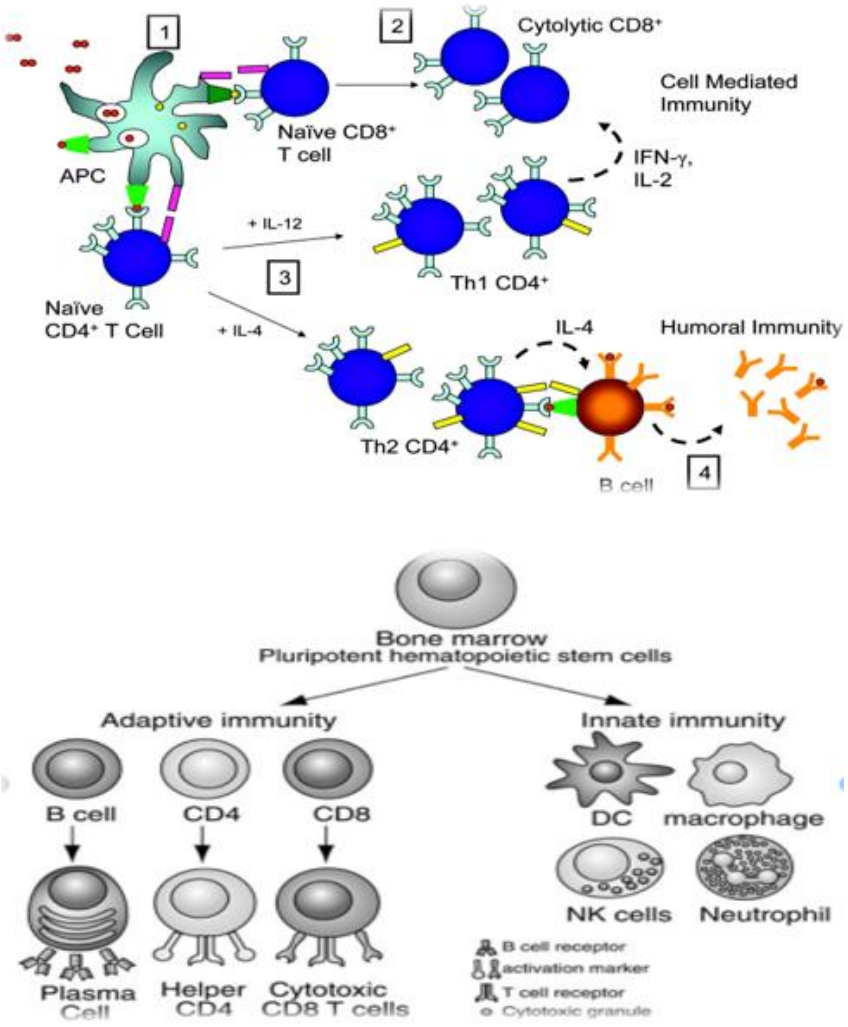


*Figure 3: Different types of immunity (Messaoudi et al., 2011; Randolph, 2005).*

The innate immune system is the first line of defense against pathogens and uses multiple pathogen detection mechanisms that can typically be grouped into three areas: a) microbial non-self-detection; b) missing self-recognition; and c) altered self-recognition. In mediating innate immune responses, multiple immune cell subsets play a critical role. Neutrophils, natural killer (NK) cells, dendritic cells (DC) and macrophages are among these (Messaoudi et al., 2011). The adaptive immune system consists of T cells, B cells, and their products. T cells are

lymphocytes characterized by their T-cell receptor (TCR) surface expression and are divided into subsets based on their surface expression of various proteins that have been allocated numbers of differentiation clusters (CD). T cells expressing CD8 are called cytolytic T lymphocytes (CTLs) or killer cells and are essential for killing virally infected cells (Randolph, 2005). Upon the identification of antigens by antigen-presenting cells (APCs), which stimulate T cells in the form of T helper cells, the humoral immune response is initiated. Via major histocompatibility complex class II (MHC II), these helper cells activate activated B cells which have already exhibited the same antigens on the cell surface. Activated B cells then proliferate and differentiate into plasma cells or long-lived memory B cells (for secondary responses to the same antigenic challenge) that secrete antibodies. Memory cells can further be activated in the presence of antigens and enter the clonal expansion and antibody synthesis cycle. Antibodies can bind to antigens to form immune complexes following secretion, which can be recognized and cleared by phagocytes (Konini, Kang, & Moghadas, 2016). Antibodies are involved in fish-specific humoral defense and are an important mechanism for bacterial disease prevention. Antibodies act as anti-toxins, anti-adhesins, and anti-invasins in fish. Antibodies also activate the classical complement system. When antibodies bind to them, toxins produced and secreted by bacteria are neutralized efficiently. By binding to the adhesins on the bacterial surface, antibodies serve as anti-adhesins to prevent bacterial adherence to fish epithelial cells. In the mucosal layers of the skin, gut, and gills, anti-adhesin antibodies are found. For the prevention of bacterial invasion of non-phagocytic cells, anti-invasins are used. To evade the immune response of the host, bacteria enter non-phagocytic cells. Antibodies that serve as anti-invasins avoid the invasion of host cells by bacteria, allowing bacteria to be eliminated by phagocytic cells. Moreover, the classical complement system can be activated by antibodies attached to bacterial surfaces (Lindell, 2012).

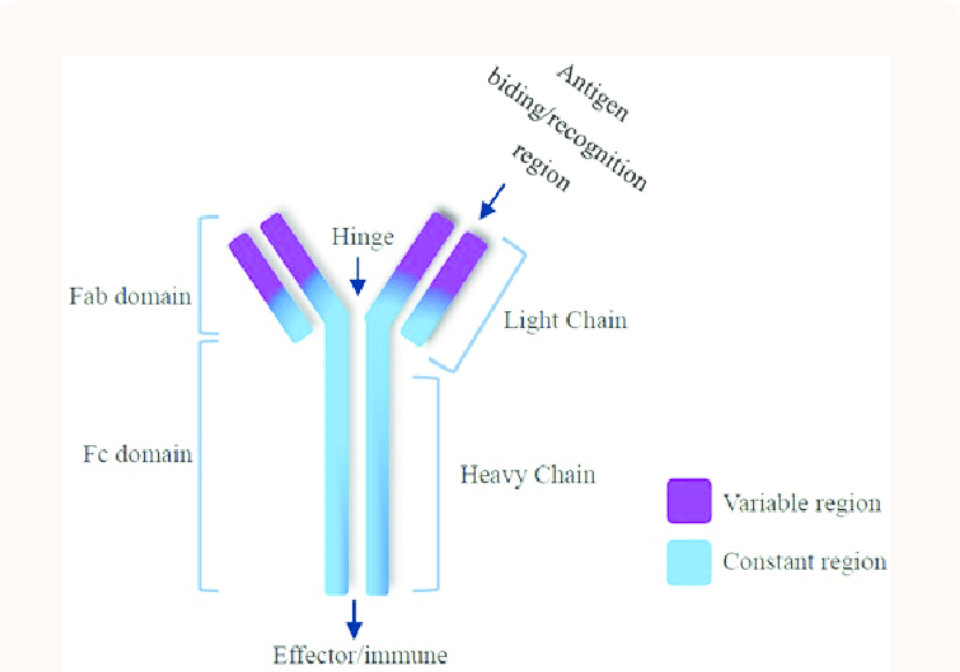


*Figure 4: Different parts and regions of an antibody (Haque Saeed, 2016).*

Antibodies are often referred to as immunoglobulins (Ig), which play a role. The essential role of a large number of invading microorganisms and other disease-causing agents or antigens in internal defenses or immunity (Figure-4). They are a significant group of glycoproteins that are part of the neutralizing immune response present in vertebrate blood or other body fluids. These antibodies have five common isotypes (IgG, IgE, IgA, IgM and IgD), including IgGs that are permeable to extravascular spaces, have a longer half-life and have the most therapeutic potential as they bind to the neonatal Fc receptor (FcRn) compared to other isotypes. In nonhuman primates and humans, FcRn binding affinity, cross-reactivity, and the elimination pathways are comparable. Because of the greater sequence homology, therapeutic mAbs typically bind to nonhuman primate antigens than rodent antigens (Haque Saeed, 2016).

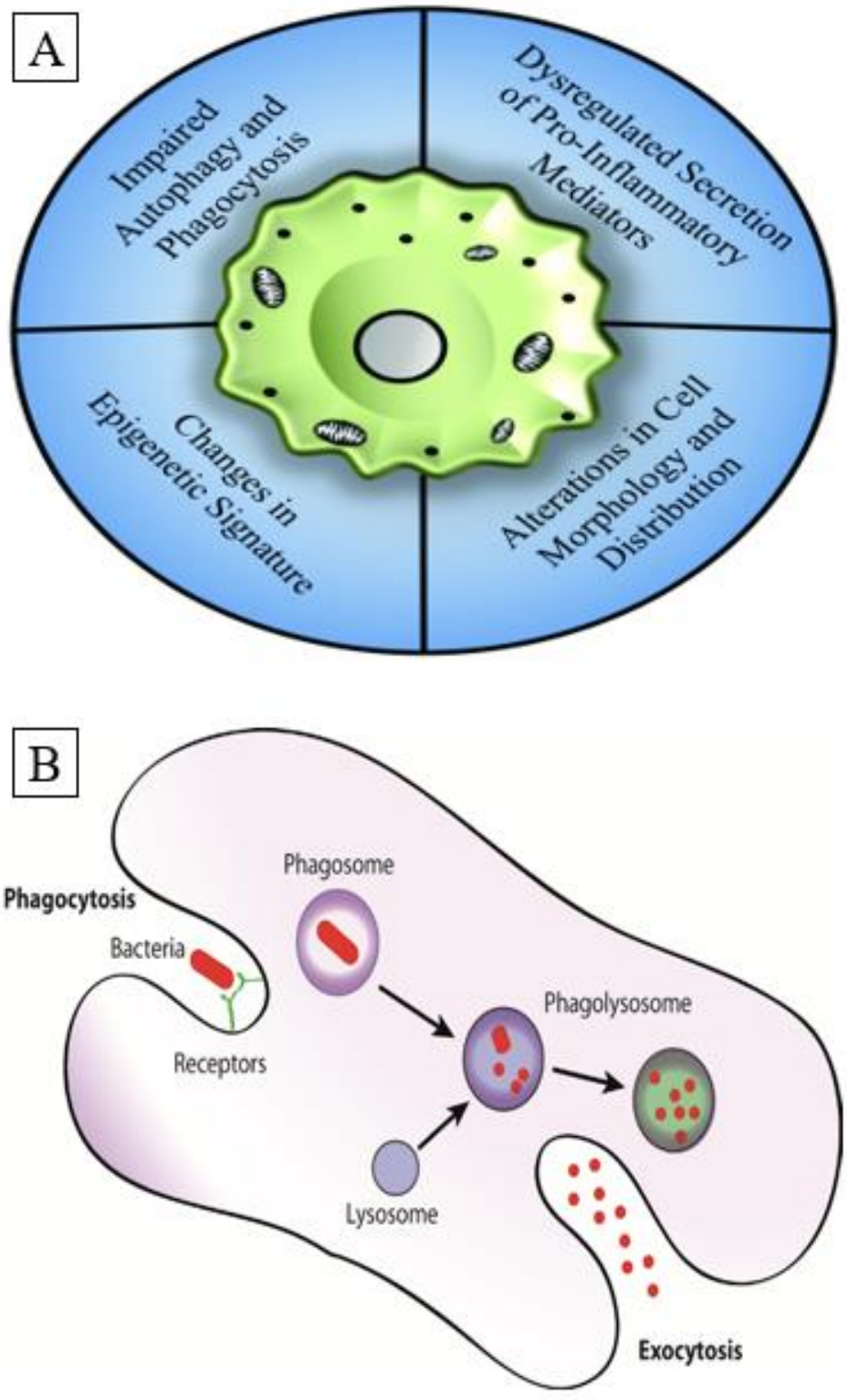


*Figure 5: A. Properties of macrophage (Stahl, Haschak, Popovic, & Brown, 2018). B. Phagocytosis (Lindell, 2012).*

The bacteria on a phagocyte are recognized by surface receptors. The bacteria are engulfed by the phagocyte and a phagosome is produced (Figure-5). During maturation, phagosome

formation needs several antimicrobial factors. The phagosome then fuses with a lysosome, resulting in a phagolysosome in which degradation or digestion of the engulfed particles takes place (Lindell, 2012). A number of surface receptors expressed on different types of cells make it possible to differentiate between self and non-self (or altered) material, allowing pathogens and apoptotic cells to phagocytose. The phase of phagocytosis can be divided into four main steps : 1) binding of the phagocyte to the target particle, 2) internalization of particles and phagosome formation by plasma membrane remodeling, 3) maturation of the phagosome, and 4) destruction of particles in the phagolysosome (Melcarne, Lemaitre, & Kurant, 2019).

Edward invented the notion of "immunomodulation" Jenner's discovery in 1796 of the small pox vaccine, which attenuated some infectious agents may use the same or closely associated infectious agents to strengthen the human immune system to fight against subsequent infections. The Ours the multi-tier immune system of the body appears to preserve a stable state of homeostasis, but it is continually exposed to invasive pathogens including viruses, bacteria, parasites from protozoa, allergens, etc. Furthermore, chronic changes in stress, disease, the environment and lifestyle adversely affect the immune system and the immune homeostasis of the host are compromised. The homeostasis may be due to the discovery of antibiotics and traditional chemotherapy. The use of chemical agents as medicines is further detrimental to the restoration, however the impacts on your immune system. Provided the enhanced knowledge of phytomedicines have been used for the side effects of chemotherapy in the last few decades, there has been a substantial rise (Tzianabos, 2000). Any substance having immunomodulatory effects means the substance can modify the response of the immune system (Bascones-Martinez, Mattila, Gomez-Font, & Meurman, 2014). Any natural and synthetic substances that can modulate immune compounds positive or negative responses are referred to as "Immunomodulators."

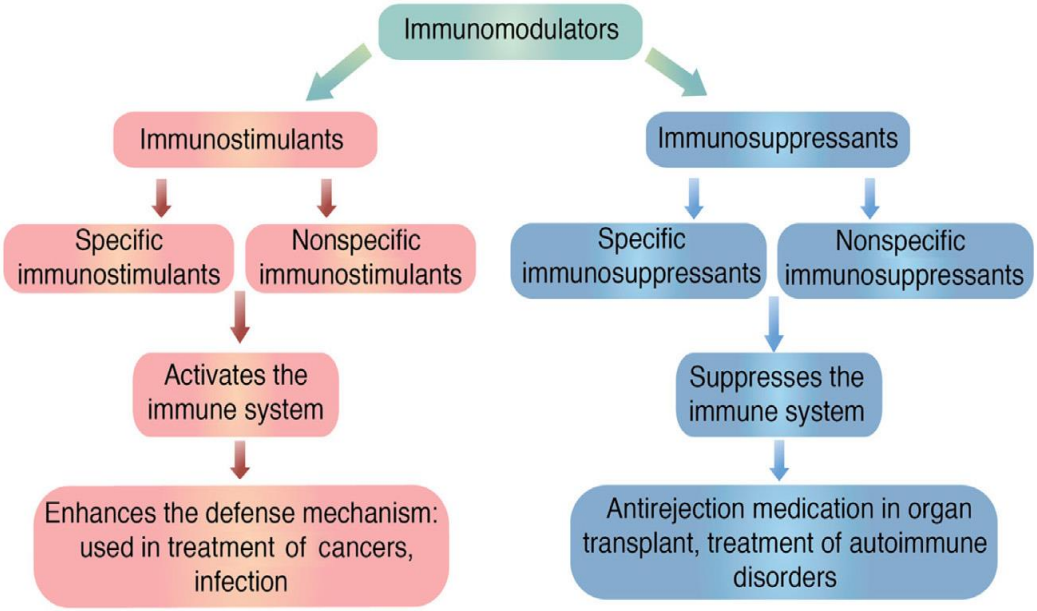


*Figure 6: Actions of immunomodulators (Nair, Chattopadhyay, & Saha, 2018).*

These are based on their effects on the immune system. As a "suppressor," or a "stimulant," or an "adjuvant," agents are classified (Figure-6). A hyperactive immune system fails to malfunction in autoimmune disorders understanding self from non-self and contributes to the dissolution of self-entities. Immunomodulators are not actual antigens, but are mitogens (imitating the action of actual antigens) or antigenomimetics. These may have modes of action that are unique as well as non-specific. Booster needed for antigenomimetics for proper working, doses. In the body, including drug metabolism, the efficacy of immunomodulators depends on different factors, such as the dose, period, mode and duration of administration, as well as the duration of administration, system Immune-Responsiveness. Here, to maintain normalcy, immunosuppressors play a key role in suppressing the immune system. Immunostimulators are used to treat the diseases resulted from immune system deficiency, like AIDS (Nair et al., 2018). These substances alter the immune response by altering the phagocytic activity, antibody production, nitric oxide production, the activity of cytokines etc. The demand of efficient immunomodulators is increasing day by day.

# Chapter 2

# Plant Description

In this segment the habitat, morphology and active constituents of *T. cordifolia* will be described.

## 2.1 Origin and Habitat

Every organism needs a suitable environment to survive with a higher reproduction success. For plants, they grow only in an environment which support its growth otherwise they will not be able to survive (Biggs, 1996). Plants can't choose their habitat as they are immobile and they only grow where they get their requirements for growth such as optimal pH, optimal temperature, nutrient composition of the soil etc. (Bazzaz, 1991). However, *T. cordifolia* also grows in some selective places. It can grow in wide range of pH (acidic to basic), soil (sands to fertile lands) and temperature (25ºC to 45ºC) although it requires a certain amount of soil moisture (Choudhary et al., 2013; Tiwari et al., 2018). This plant is seen to be grown in the tropical regions of India at 1000 feet of altitude and it also grows well in south Asia, Philippians, Myanmar, Srilanka, China, Thailand, Bangladesh and Indonesia (Figure-7) (Spandana et al., 2013). Moreover, this plant also grows by itself in dry forest at an altitude of 1000 feet (Choudhary et al., 2013). This plant also prefers those places which contain a rich amount of organic substances, valuable nutrients and minerals (Mittal et al., 2014). In India, the plant is seen from Kuman to Assam at an altitude of 1200 m from the sea level and this plant is distributed from the west Bengal to Kerala (Sinha et al., 2004). The regions where it grows are generally rich in the number of fast growing small plants like Moringa (*Moringa oleifera*), Neem (*Azadirachta indica*), Jatropha (*Jatropha curcas*) and mango tree because this plant is a climbing shrub (Mittal et al., 2014).

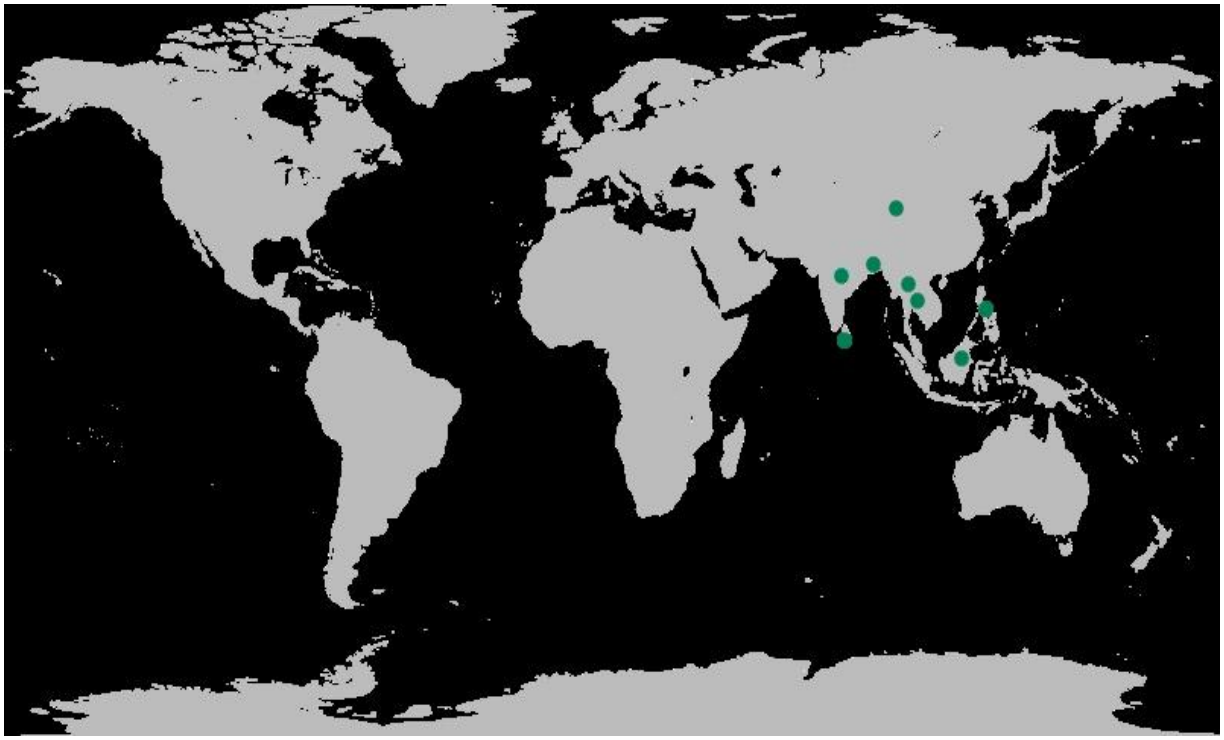

*Figure 7: Regions where T. cordifoila is seen to be grown and the green marked countries are India, Philippians, Myanmar, Srilanka, China, Thailand, Bangladesh and Indonesia (Spandana et al., 2013).*

## 2.2 Morphology

*T. cordifolia* is a climbing shrub with a extensively spreading nature and it also contains some coiling and elongated twining branches (Mittal et al., 2014; Sharma1 & Kumar, 2013; Sinha et al., 2004). The morphologies of different parts of this plant are described below:

Stem: *T. cordifolia* has a green succulent stem which is climbing in nature, long enough, fleshy, filiform and branched (Figure-8) and from the branches a type of roots arises named aerial roots (Mittal et al., 2014; Tiwari et al., 2018). The stem contains bicol lateral vascular bundles which are covered by pericycle fiber and there are 2-3 cork layers that arises from the sub-epidermal layers (Sinha et al., 2004). The fresh stem of this plant is covered by a thin layer of brown colored papery bark containing warty lenticels and the stem constricts resulting the separation of the bark from wood when the stem becomes dried (Choudhary et al., 2013). The lenticels look like large rosette and they contain deep clefts and Longitudinal fissures are seen

in the surface of the stem with 3 - 8 cm in diameter and 3 - 5 cm long (Tiwari et al., 2018). All over the region of parenchyma of stem is the store point of starch (Khosa & Med, 1971).

Aerial roots: The roots of *T. cordifolia* are slender pendulary fleshy, striate and terete types of roots that contain a pale or shining or glabrous and tubercled bark (Choudhary et al., 2013). The roots resemble like a long thread and the color is light grayish brown or dark white with bitter taste and odorless characteristics (Khosa & Med, 1971; Sharma1 & Kumar, 2013; Tiwari et al., 2018). The roots are usually called aerial roots which has two types of structure such as tetra and penta-arch primary structure (Mittal et al., 2014). Although, threadlike structures of the roots are seen in young roots, mature roots don't have the same structures as they don't contain nodal swellings (M. Sharma & Kumar, 2013). The cortex of roots has thick walled outer area and inner parenchymatous region which contain secretory canals (Sinha et al., 2004).

*Figure 8: Stems of T. cordifolia (Spandana et al., 2013)*

**Leaves:** The leaves of *T. cordifolia* have the following characteristics (Figure-9):

i) The leaves are very simple in its structural organization (Raghunathan & K, 1969).

ii) They don't have stipulate. So, the leaves are ex-stipulate (Raghunathan & K, 1969).

iii) The leaves are usually 5 to 10 cm long (Choudhary et al., 2013).

*Figure 9: Leaves of T. cordifolia (Venkateswarlu et al., 2019).*

iv) They have a 2.5 – 7.0 cm long petiole (Choudhary et al., 2013).

v) The shapes of the leaves are usually round, half way round, heart shaped or partially twisted (Choudhary et al., 2013; RAGHUNATHAN & K, 1969).

vi) It has an ovate lamina which is 10-20 cm long, 8-15 cm width, deeply cordate, 7-9 nerved and membranous (Choudhary et al., 2013; RAGHUNATHAN & K, 1969).

vii) The leaf of this plant don't contain trichomes (Sinha et al., 2004).

Flowers: The flowers of this plant start to bloom in summer (March to June) when the plant is in leafless condition (Choudhary et al., 2013; Mittal et al., 2014; Tiwari et al., 2018). The characteristics of the flowers of *T. cordifolia* are:

*Figure 10: Flowers of T. cordifolia (Mittal et al., 2014).*

i) The flowers are unisexual and they have equal number of stalks that are uniformly distributed around the central stem and a rather lax raceme is seen which is about 5.0 cm long with elongated structure (Figure-10). The racemes are auxiliary and found in the

terminal position of the flower (Choudhary et al., 2013; Khosa & Med, 1971; Mittal et al., 2014).

ii) The color of the flowers is usually greenish yellow (Mittal et al., 2014)

iii) Clustered flowers are seen in the male flowers and female flowers has green sepals and are not a part of inflorescence and so they are called solitary flower (Choudhary et al., 2013; Mittal et al., 2014).

*Figure 11: Fruits of T. cordifolia (Tiwari et al., 2018)*

iv) There are two series of sepals containing total 6 sepals. Outer series of the sepals are small in size than the sepals of inner side (Mittal et al., 2014; M. Sharma & Kumar, 2013).

v) There can be 1-3 carpels that are separated from each other on a fleshy gynophore (Choudhary et al., 2013).

vi) There are total 6 petals which are membranous and free (Mittal et al., 2014; M. Sharma & Kumar, 2013).

Fruits: *T. cordifolia* has orange-red colored fruit (Figure-11) which is fleshy, smooth and has stalk containing droplets (Kirtikar & Basu, 1935). Fruits are structured as three sessile drupelets and fruits appear in the November (winter) (M. Sharma & Kumar, 2013; Tiwari et al., 2018).

Seeds: The seeds of *T. cordifolia* are usually curved (Figure-12) thus the family of the seeds is called moonseed family and the endocarps of the seeds consist various ornamental structures (Mittal et al., 2014).

*Figure 12: Seeds of T. cordifolia (Venkateswarlu et al., 2019)*

## 2.3 Active Constituents

There have been done extensive phytochemical studies on *T. cordifolia* to analyze its active constituents. The researchers have found this plant as a cluster of valuable and unique constituents. Many types of medicinal constituents are extracted from this plant (Figure-14). They are Alkaloids, quinones, carbohydrates, valuable oils, phenols and phenolic compounds, immune proteins, steroids and sterols, terpenoids, diterpinoids, triterpenoids, glycosides, tannins and many other unique compounds like Giloin, tinosporic acid, G1-4A arabigalactans etc. (Kapil & Sharma, 1997; U. Sharma et al., 2012; D. Singh & Chaudhuri, 2017; Sinha et al., 2004). Sinku (2018) did Millon's test and Nynhydrin test to check the presence of proteins and also got a positive result. He took Crude extract was mixed with 2ml of 2% solution of FeCl3.A blue- green or black colouration indicated the presence of phenols and tannins. By this test he also found the presence of phenols and tannins in this plant. The presence of steroids and sterols were found by performing Salkwosky's test (Patil, Kulkarni, & Pandey, 2017). Presence of lots

of alkaloids were observed by doing Wagner's test, Mayer's test and dragendorff test (Nazir & Chauhan, 2018). Pradhan et al. (2013) determined Total flavonoids by aluminium chloride colorimetric technique. 21. 0.5 gm sample was weighed and kept in 95% ethanol for 24 hours. It was than filtered and volume was made up to 25 ml with 80% ethanol. 0.5 ml of filtrate was then mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of potassium acetate and 2.8 ml water. The tubes were then incubated at room temperature for 30 min and absorbance was measured at 415 nm. The flavonoids content of the samples was calculated from the standard graph of quercetin. Glycosides were found by performing Legal test, Baljet test, Borntrager's test and Keller Kiliani test (Wani, Achur, & Nema, 2011). The alkaloids found from this plant are tembetarine, magnoflorine, berberine, jatrorrhizine, isocolumbin, tetrahydropalmitine, tinosporine, choline, palmatine, 1,2-Substituted pyrrolidine, magnoflorine and 18-norclerodane glycoside (Choudhary et al., 2013; Sinha et al., 2004). The compounds of carbohydrates or polysaccharide include glucose, galactose, xylose, rhamnose, mannos. arabinose, arabigalactan (G14A) and α-D-glucan (Raveendran Nair et al., 2004; D. Singh & Chaudhuri, 2017). Mishra et al. (2014) found some oils in this plant and they are Heptacosanol, nonacosan-15-one and octacosanol. Phenols and phenolic compounds include four phenyl propanoids, two benzenoid derivatives, three lignans (4-hydroxy-3-methyloxybenyl) and two flavonoids (D. Singh & Chaudhuri, 2017; Sinha et al., 2004; Van Kiem et al., 2010). There are several steroids and sterols present in *T. cordifolia* like hydroxy ecdysone, β-Sitosterol and giloinsterol, α-sitosterol and makisterone A (Mishra et al., 2014; Sinha et al., 2004). The structures of some important active constituents are illustrated in Figure-13.

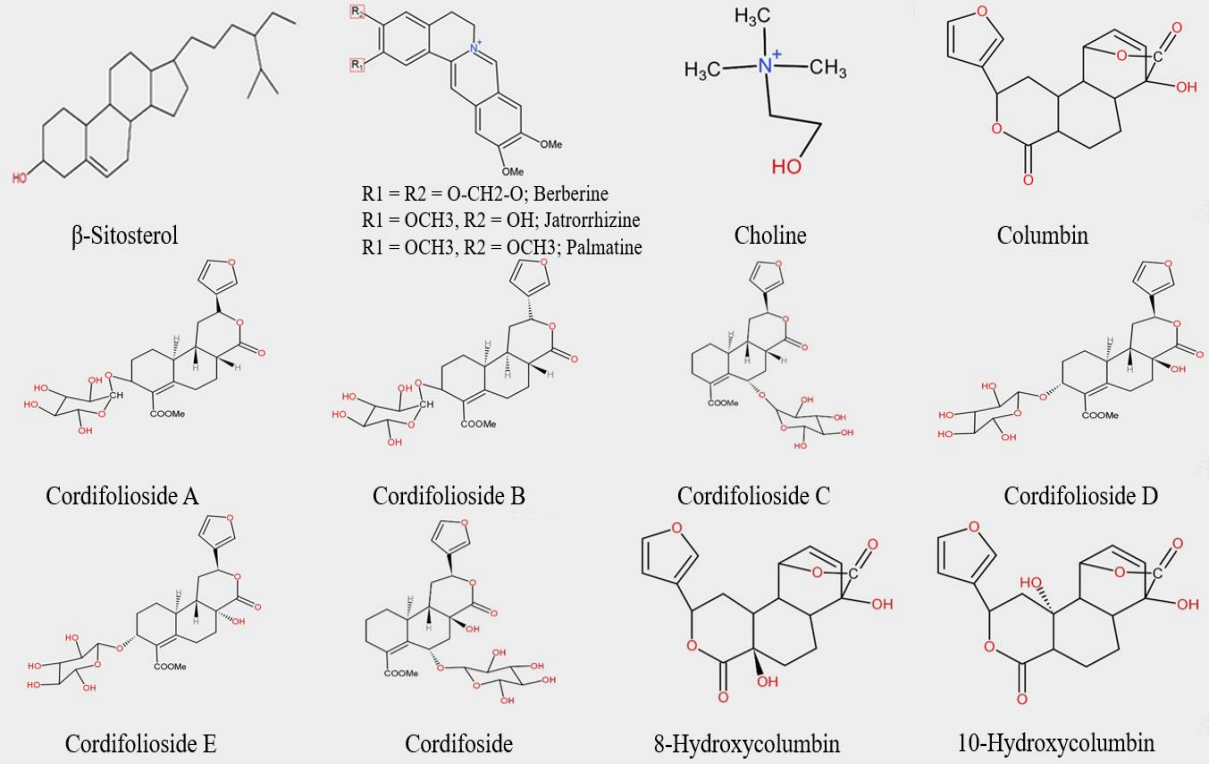


*Figure 13: Structures of various Tc derived active constituents drawn by KingDraw Chemical Structure Editor (D. Singh & Chaudhuri, 2017)*

Sesquiterpene tinocordifolin, cordioside, sesquiterpene glucoside tinocordifolia, furanoid diterpene, Tinosporide, Furanolactone clerodane diterpene, palmatosides C and F, Furanolactone diterpene, phenylpropene disaccharides cordifo- lioside, ecdysterone makisterone, cordifoliside D and E, Tinospora- side, Diepoxy-cleroda-13, Tinosporon, Furanolactone, Clerodane derivatives, Columbin and Jateorine are the various types of terpenoids, diterpenoids and triterpinoids which are present in *T. cordifolia* (Mishra et al., 2014; Mittal et al., 2014; Sinha et al., 2004). CordiofoliosideC, cordiofolioside E, furanoid diterpene glucoside, 18-norclerodane glucoside, palmatosides C, cordiofoliosideB, cordiofoliosideD, palmatosides P1 and cordiofoliosideA these glycosides are present in this plant (Gangan, Pradhan, Sipahimalani, & Banerji, 1994; Mishra et al., 2014) (Table-2). There are other valuable and unique constituents are present in this plant and they are Giloin, 4-di hydroxy-3 methoxy-benzyl tetrahydrofuran, Cordifol, Cordifelone, N transferuloyltyramineas diacetate,

giloinin, N-formylannonain, tinosporic acid, clerodane furano diterpene glycoside, syringin, cordioside etc. (Kapil & Sharma, 1997; Mittal et al., 2014; U. Sharma et al., 2012).

*Table 2: Various active constituents derived from different parts of T. cordifolia*

| **Class** | **Compounds** | **Part of the plant** (Sivakumar, 2014) | **Quantity** (Sivakumar, 2014) |
|---|---|---|---|
| Alkaloids (Choudhary et al., 2013; Sinha et al., 2004). | Tembetarine, Magnoflorine, Berberine, Jatrorrhizine, isocolumbin Tetrahydropalmitine, Tinosporine, Choline, Palmatine, 1,2-Substituted pyrrolidine, magnoflorine and 18-norclerodane glycoside. | Stem, leaf and root | Abundant |
| Quinones (D. Singh & Chaudhuri, 2017) | Hydroquinones | Leaf (Patil et al., 2017) | Low concentration (Patil et al., 2017) |
| Carbohydrates (Raveendran Nair et al., 2004; D. Singh & Chaudhuri, 2017) | Glucose, galactose, xylose, rhamnose, mannos. Arabinose, arabigalactan (G14A) and α-D-glucan. | Stem and leaf (Nazir & Chauhan, 2018) | Average |
| Fats/oils (Mishra et al., 2014) | Heptacosanol, nonacosan-15-one and octacosanol. | Stem | Average (3.1%)w/w (Nazir & Chauhan, 2018) |
| Phenolics (Choudhary et al., 2013; Sinha et al., 2004; Van Kiem et al., 2010). | Four phenyl propanoids, two benzenoid derivatives, three lignans (4-hydroxy-3-methyloxybenyl) and two flavonoids. | Stem and leaf | Abundant (7.2%) w/w |

(Table-2 Continued)

| | | | |
|---|---|---|---|
| Proteins(Aranha, Clement, & Venkatesh, 2012). | Guduchi Im p protein | Stem and leaf (Nazir & Chauhan, 2018) | Average (4.5-11.2%)w/w (Nazir & Chauhan, 2018) |
| Steroids and sterols (Mishra et al., 2014; Sinha et al., 2004). | Hydroxy ecdysone, β-Sitosterol and Giloinsterol, α-sitosterol and makisterone A. | Stem and leaf | Abundant |
| Terpenoids (Mishra et al., 2014; Sinha et al., 2004). | Sesquiterpene tinocordifolin, cordioside, sesquiterpene glucoside tinocordifolia, furanoid diterpene, Tinosporide, Furanolactone clerodane diterpene, palmatosides C and F, Furanolactone diterpene, phenylpropene disaccharides cordifo- lioside, ecdysterone makisterone, cordifoliside D and E and Tinospora- side. | Leaf (Nazir & Chauhan, 2018) | Average |
| Glycosides (Gangan et al., 1994; Mishra et al., 2014). | CordiofoliosideC, cordiofolioside E, furanoid diterpene glucoside, 18-norclerodane glucoside, palmatosides C, cordiofoliosideB, cordiofoliosideD, palmatosides P1 and cordiofoliosideA. | Stem and leaf | Abundant |
| Tannins (Van Kiem et al., 2010) | Gallic acid, gallotannin and ellagic acid. | Stem and leaf | Abundant (8.8%) w/w |

(Table-2 continued)

| | | | |
|---|---|---|---|
| Moisture content | $H_2O$ | Stem and leaf | Stem (0.98%) and leaf (0.85%) w/w (Sinku, 2018) |
| Diterpenoid Lactones (Mittal et al., 2014) | Diepoxy-cleroda-13, Tinosporon, Furanolactone, Clerodane derivatives, Columbin and Jateorine. | Whole plant | Average |
| Other compounds (Kapil & Sharma, 1997; Mittal et al., 2014; U. Sharma et al., 2012) | Giloin, 4-di hydroxy-3-methoxy-benzyl tetrahydrofuran, Cordifol, Cordifelone, N transferuloyltyramine as diacetate, Giloinin, N-formylannonain, Tinosporic acid, clerodane furano diterpene glycoside, syringin, cordioside etc. | Root and stem | Average |

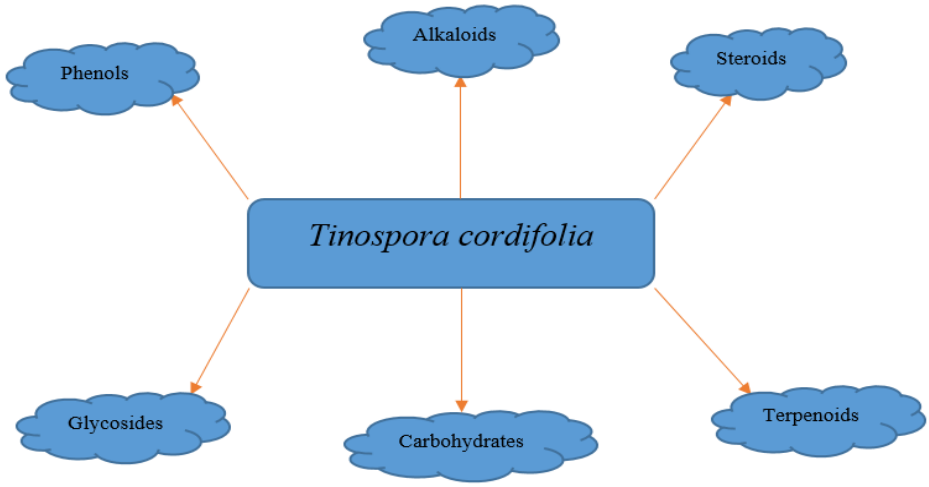


*Figure 14: Illustrations of the groups of the active constituents*

*T. cordifolia* is also very rich in the contents of minerals (Table-3). Potassium (0.845%), iron (0.28%) chromium (0.006%), calcium (0.131%), phosphorus (0.0024%), manganese (0.0012%), zinc (0.0007%) and copper (0.00037%) are present in this plant (Mutalik & Maitreyee, 2011); (Mahima et al., 2014).

*Table 3: Minerals and their amount (w/w) present in T. cordifolia*

| **Minerals** | **Amount (w/w)** |
|---|---|
| Potassium | 0.845% (Mutalik & Maitreyee, 2011) |
| Iron | 0.28% (Mutalik & Maitreyee, 2011) |
| Chromium | 0.006% (Mutalik & Maitreyee, 2011) |
| Calcium | 0.131% (Mutalik & Maitreyee, 2011) |
| Phosphorus | 0.0024% (Mahima et al., 2014) |
| Manganese | 0.0012% (Mahima et al., 2014) |
| Zinc | 0.0007% (Mahima et al., 2014) |
| Copper | 0.00037% (Mahima et al., 2014) |

Active compounds showing immunomodulatory activities:

Nearly every constituents of Tc are pharmacologically active and shows various types of activity. As this review is mainly on the immunomodulatory effects of Tc, the compounds responsible for immunomodulating effects are mentioned separately in Table-4. The compounds showing various types of immune system modifying activities are Cordifolioside A, Hydroxymustakone, N-methyl-2-pyrrolidone, Magnoflorine, tinocordiside, N formylannonain, G14A, Guduchi imp protein, Cordioside, Cordifolioside B, Ecdysteron and Syringin (Aranha et al., 2012; Chiang, Wang, & Wu, 1992; Kapil & Sharma, 1997; U. Sharma et al., 2012; D. Singh & Chaudhuri, 2017; Sudhakaran, Srirekha, Devasree, Premsingh, & Michael, 2006).

*Table 4: Compounds of Tc which have immunomodulatory activities.*

| Compounds | Immunomodulatory activities |
|---|---|
| Cordifolioside A (U. Sharma et al., 2012) | immune system stimulating properties |
| Hydroxymustakone, N-methyl-2-pyrrolidone (U. Sharma et al., 2012) | nitric oxide production enhancing properties |
| Magnoflorine, tinocordiside, N-formylannonain (U. Sharma et al., 2012) | phagocytic activity enhancing properties |
| G1-4A, Guduchi imp protein (Aranha et al., 2012; D. Singh & Chaudhuri, 2017) | Potential immunomodulator. |
| Cordioside (Sudhakaran et al., 2006) | An immunomodulator. |
| Cordifolioside B (Kapil & Sharma, 1997) | Stimulatory effects on immune system |
| Ecdysteron (Chiang et al., 1992) | immune system stimulating properties |
| Syringin (Kapil & Sharma, 1997) | Potential immunomodulator. |

Compounds with other properties:

The compounds derived from Tc have a broad spectrum of biological activities. Berberine has Antiphotooxidative properties, antibacterial properties, antifungal, anthelmintic and psychiatric activity (Cernakova & Košťálová, 2002; Choudhary et al., 2013; Kim et al., 2000; Mishra et al., 2014; Mittal et al., 2014). Cordioside, Columbin and Tinocordifolioside have got Scizonticidal properties (J. P. Patel et al., 2010). Palmatine shows antidiarrhoeal, antiplasmodial and antifungal properties (Choudhary et al., 2013; Xiao et al., 2015). Flavonoids, Cordifolioside A-D and β-Sitosterol have very effective anti-diarrhoeal properties (Choudhary et al., 2013). Other important biological activities of some active constituents have been mentioned in Table-5.

*Table 5: Other activities of important active constituents*

| Compounds | Biological activities |
|---|---|
| Cordioside | Scizonticidal properties (J. P. Patel, Gami, & Patel, 2010) |
| Berberine | Antiphotooxidative properties (Kim, Jung, Kim, & Kim, 2000), antibacterial(Cernakova & Košťálová, 2002), antifungal (Mishra et al., 2014), Anthelmintic (Choudhary et al., 2013), psychiatric activity (Mittal et al., 2014). |
| Columbin | Scizonticidal properties (J. P. Patel et al., 2010) |
| Ecdysteron | Anabolic activity (Mishra et al., 2014). |
| Jatorrhizin | Antibacterial activity (Mishra et al., 2014). |
| Magnoflorine | Antioxidant (Mishra et al., 2014), Cytotoxic (Došlov-Kokoruš, Ivanović, Simić, Vajs, & Kovačević, 2006). |
| Syringin | Hypotensive (Ahmad & Aftab, 1995), anti-inflammatory (Mishra et al., 2014). |
| Palmatine | Antifungal (Xiao, Ji, Wei, Liu, & Bao, 2015), Antiplasmodial effect (Mishra et al., 2014), Antimicrobial, antidiarrhoeal (Choudhary et al., 2013). |
| Tinocordifolioside | Scizonticidal properties (J. P. Patel et al., 2010), antihypertensive (Mittal et al., 2014). |
| Flavonoids. | Antimicrobial, Antidiarrhoeal and Anthelmintic (Choudhary et al., 2013) |
| Cordifolioside A-D | Antidiarrhoeal (Choudhary et al., 2013), anticancer activities (Mittal et al., 2014) |
| β-Sitosterol | Antidiarrhoeal (Choudhary et al., 2013) |

# Chapter 3

## Therapeutic use

*T. cordifolia* is mentioned specially for its use in tribal or folk medicine in different parts of the world. In ethnobotanical surveys conducted by ethnobotanists, almost all parts of the plant are recorded to be helpful. In folk and tribal medicine, whole herb, root and stem bark powder, root and stem decoction, root juice, leaf juice or paste, and stem of *T. Cordifolia* is used for the treatment of different ailments like jaundice, diarrhea, dysentery, general fatigue, cough, asthma, leucorrhea, skin diseases, fractures, insect bites, venomous snake, etc. (Choudhary et al., 2013). *T. cordifolia* is a very potential medicinal plant and has become very demandable for its various medicinal activities. Stem, bark, roots, leaf even the whole plant have many therapeutic uses (Table-6) and that's why Indian pharmaceutical industries are very much interested in formulating products containing Tc extracts (Table-7). The aerial roots of Tc plant are used in the treatment of HIV infection, immune system booster, anxiety, inflammations and cancer (Ly, Singh, & Shaw, 2007; Mittal et al., 2014; Mukherjee, De, & Ram, 2010; S. S. Patel, Shah, & Goyal, 2009). Leaf and bark are useful in the treatment of gout, ulcer, leprosy, allergic reactions and muscle spasm problem (Choudhary et al., 2013; Ikram, Gul Khattak, & Naeemuddin Gilani, 1987). The stem is mainly considered as the medicinally most important part of *T. cordifolia* plants. Stems are used to treat viral infections, Neurological and psychological disorders, diabetes, inflammation, misbalanced immune system, cancer, improper digestion, jaundice, malarial fever etc. (Choudhary et al., 2013; Kapil & Sharma, 1997; Ly et al., 2007; Mittal et al., 2014; S. S. Patel et al., 2009; Upadhyay et al., 2010). Other uses include as antidote against snake bite, skin infection, UTI infection, bronchitis etc. (Choudhary et al., 2013; Tradeway, 1998).

*Table 6: Therapeutic indications of different parts of Tc*

| Parts of plant | Therapeutic indications | References |
|---|---|---|
| Stem | • Used to treat severe viral infections.<br>• Various neurological disorders like Parkinson disease, dementia, depression etc.<br>• Used as anti-hyperglycemic agent to treat both type-1 and type-2 diabetes.<br>• Treatment of cytokines induced inflammations.<br>• Used to boost immune system.<br>• Indicated as an adjunct therapy in chemotherapy.<br>• Also cure problems related with improper digestion.<br>• Active against intestinal worms and jaundice when the stem is used as infusion formulation.<br>• It is also used to reduce malarial fever by administering its decoction preparation. | (Choudhary et al., 2013; Kapil & Sharma, 1997; Ly et al., 2007; Mittal et al., 2014; S. S. Patel et al., 2009; Upadhyay et al., 2010). |
| Root | • Used to treat HIV infections as protease inhibitor.<br>• Indicated as an alternative of the resistant drugs in the treatment of HIV infection.<br>• Indicated for boosting immune system.<br>• Anxiety, depression<br>• Colorectal cancer.<br>• Indicated to treat inflammation. | (Ly et al., 2007; Mittal et al., 2014; Mukherjee et al., 2010; S. S. Patel et al., 2009) |
| Bark | • Bark is very useful in treating unexpected allergic reactions.<br>• It can also cure muscles spasm problem.<br>• Effective against leprosy. | (Choudhary et al., 2013; Tradeway, 1998) |
| Leaf | • Very effective in the treatment of gout.<br>• Leaves are also used to treat ulcer. | (Choudhary et al., 2013) |
| Stem + root | • Used as antidote against snake bite<br>• Skin infections, Urinary tract infection and bronchitis. | (Choudhary et al., 2013; Tradeway, 1998) |

Some market formulations (in India) and their indications:

*Table 7: Available(Indian) market formulation of Tc extracts and their indications* (Mittal et al., 2014)

| **Product name** | **Indications** |
|---|---|
| MadhuMehari | Fatigue, misbalanced blood sugar levels and genitourinary problems. |
| Brave Heart capsule | Diuretic and indicated for hyperlipidemia |
| Abhaibhubejhr | Anxiety, depression and stress. |
| Safe herb | Anemia |
| Cirrholiv capsules | Hepatoprotective |
| Mussaffen | Allergic reaction |
| Cirrholiv-ds syrup | Hepatoprotective |
| Guduchi | Boosting immune system |
| *Tinospora Cordifolia* pellets | Immune diseases |
| Rebuild | anxiety |
| Toplex | Decreased immunity. |

# Chapter 4

# Immunomodulatory properties of *T. cordifolia*

## 4.1 Effects on phagocytic activity

Aderem and Underhill (1999) stated that cells show different types of techniques to neutralize an invaded particle and the strategies include pinocytosis, receptor mediated endocytosis and phagocytosis. They also included that these mechanisms are responsible for our innate immune response followed by adaptive response. They informed that phagocytosis is a process of engulfing large particles into cells and cells use actin-dependent mechanism to perform this process. They also explained that macrophages and neutrophils are the main phagocyte of our immune system and by doing phagocytosis they also participate in tissue remodeling, immune response and inflammation. They mentioned some professional phagocytes named monocytes, macrophages and neutrophils. Phagocytosis is also important for the wound healing process and removal of invaded particles along with its immunological functions (Wan, Park, & Lau, 1993). Phagocytes perform micro biocidal functions by engulfing the pathogens (Figure-15) and this process includes membrane derived vacuole formation, formation of phagosome followed by the degeneration of the invaded pathogen (Lee, Harrison, & Grinstein, 2003). The cells involved in this process are called phagocytic cells and neutrophils, macrophages, monocytes, dendritic cells etc. are the examples of phagocytic cells and they keep role in our innate immunological response through releasing some products which are toxic to the invaded particles (Rosen, Pou, Ramos, Cohen, & Britigan, 1995). Wan et al. (1993) claimed phagocytosis assay as beneficial means to evaluate the immunotoxicological and immunopharmacological functions of macrophages.

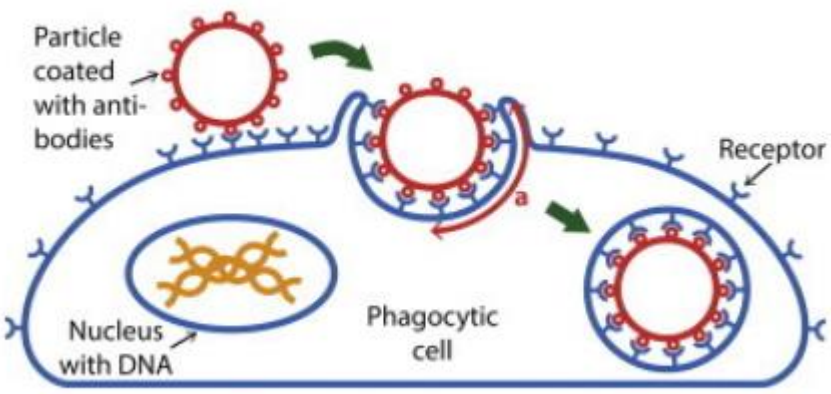


*Figure 15: A phagocytic cell performing phagocytosis. (Richards & Endres, 2014)*

They stated that many procedures are followed to conduct this assay and the conventional protocol is the examination of individual phagocytosis microscopically although some researchers modify this protocol to make the process more conventional. Various methods of phagocytosis involve the enumeration of the invaded particle, its microscopic observation and determination of the density of hemocyte lysates (Abdul-Salam & Michelson, 1980; Cheng & Sullivan, 1984)**.** Moreover, there is a technique which track the invasion organism by hemocyte doing luminescence measurements (Blaise, Trottier, Gagné, Lallement, & Hansen, 2002).

To evaluate the immunomodulatory effects of the medicinal plants, phagocytosis assay is a very significant assay as this assay indicates the efficiency of phagocyte cells to perform phagocytosis. Enhanced phagocytosis of our body phagocytes indicates a better immune system. Therefore, if a plant shows a positive result in phagocytosis assay, this plant will be considered as a plant with immunomodulatory activity. *T. cordifolia* is also considered as a plant with immune modulatory activity. So, this plant is supposed to increase phagocytic activity of our immune system. Many phagocytosis assays have been done (Table-8) on *T. cordifolia*. Let's have a look how promising the outcomes are:

Gupta et al., (2016) did a phagocytosis assay on a polysaccharide named G1-4A which is derived from *T. cordifolia.* In this assay, they used RAW 264.7 cell line of murine macrophage which was infected with MTB bacteria. At first, they cultured RAW 264.7 bacteria cells and treated the cell with g1-4A, LPS and one group left untreated. After 8 hours they infected those cells with GFP expressing MTB at MOI 10. Moreover, they incubated the culture at 37ºC for couple of hours. Furthermore, the culture was treated with 10µg/ml amikacin to make the culture clear of extracellular organisms. For staining purpose, DAPI was applied. Finally, cover slips were treated with Prolong Gold Ant-fade and Nikon TE 2000e laser microscope was used to analyze the culture. After getting the data the following equation was used to find out the phagocytic index.

Phagocytic index = (Percentage of phagocytes which engulfed at least one MTB × Average number of bacteria per positive cell)

The statistics the assay got were presented as mean ± SD and Significance of the data determined by student's t-test or one-way analysis of variance (ANOVA). However, they wanted to assess the effect of G1-4A on the phagocytic activity of macrophages. After finishing the assay, they found out that G1-4A and LPS treated macrophages showed an enhanced phagocytic activity. In addition, the phagocytic index of cells is comparable among the cells treated with G1-4A and LPS. Moreover, the enhanced phagocytosis index was noticeably higher than the control group (significance, $p<0.05$). Furthermore, a significant increase is seen in the percentage of GFP positive cells. 56.4±6.6 percent was FOR G1-4A ($p<0.01$) and 6.2±1.2 percentage for the control group. The key points of this assay is outlined in table-1. More and Pai (2017) experimented the phagocytosis assay on the aqueous extract of *T. cordifolia.* They used two types of organisms, one is heat killed yeast (non-infective microorganism) and another is *E. coli* (infective microorganisms). To assay the phagocytosis of dried and killed yeast cells, they used macrophages which were in late log phase of growth.

At first, they treated the macrophages with *T. cordifolia* (80µg/ml), LPS (10µg/ml) and some left untreated. Then they incubated the culture at 37ºC for 24 hours and 48 hours. Consequently, they removed the supernatant and treated the culture with 100µL of yeast cell suspension (1mg/ml) and incubated for 90 minutes. Moreover, PBS was used to wash out the extracellular organisms. Furthermore, the cells were treated with 100% methanol and stained by adding 0.1% of crystal violate and the slides were dipped in PPX. The slides were analyzed by electronic microscope at 40X magnification. Finally, they found some data after being finished with the analyzation and calculated the phagocytic index and the percentage of phagocytosis by using the following formulas.

$$\text{Percentage of phagocytosis (P\%)} = \frac{\text{Macrophages which engulfed yeast cells}}{\text{Total number of macrophages}} \times 100\%$$

$$\text{Phagocytosis index (PI)} = \frac{\text{Number of engulfed yeast cells}}{\text{Macrophages which engulfed yeast cells}} \times P\%$$

After finishing the assay, they found out that macrophages which were treated with *T. cordifolia*, exerted higher phagocytic activity compared to the control group. At 24 hours, the phagocytic index of *T. cordifolia,* LPS and control group were 4.5±0.12, 4.5±0.37 and 2.9±0.2. At 48 hours, the phagocytic index of *T. cordifolia*, LPS and control group were 5±0.75, 4.5±0.37 and 3.01±0.2. However, the phagocytic index was significantly higher in the *T. cordifolia* treated macrophages.

On the other hand, they also treated the macrophages with E. coli to compare the effect with the experiment carried on dead yeast cell. At first, they treated the macrophages with *T. cordifolia* (80µg/ml), LPS (10 µg/ml) and some left untreated. Then they incubated the culture at 37ºC for 24 hours. Moreover, 50 µg/ml of gentamycin was used to make a control group. After removing the supernatant, 100µL of E. coli was added. Furthermore, PBS was used to wash out the extracellular organisms and the cells were treated with 100% methanol and stained by adding 0.1% of crystal violate and the slides were dipped in PPX. After observation of all night, colonies of bacteria appeared. After finishing this experiment, there was no colony of bacteria in the plates which were treated with gentamycin, less colony was seen in the untreated plates and high number of colony were seen in the plates which were treated with plant extract which indicates high amount of phagocytized cells. Ranjith et al., (2008) used the aqueous, ethanolic, chloroform and ethyl acetate extract of *T. cordifolia* to experiment phagocytosis assay in vitro. At first, the prepared macrophage suspension from the exudates collected from the peritoneal cavity of mice. To prepare the suspension, they centrifuged the exudates at 500 rpm for 10 minutes. The concentration of the cell was made 105 cells per ml. Then they made the suspension of yeast cell (Candida albicans). Yeast was collected from the medium at its late log phase. The final concentration of yeast cells was 108 yeast cells per ml. 0.3 ml of macrophage suspension was mixed with 0.1 ml of C. albicans. Moreover, 0.3 ml of MEM and 0.1 ml of blood serum with AB blood group were added. Furthermore, 1,5,10,15 and 20 µg/ml of aqueous extract was added to the tube. Then they incubated the tubes at 37ºC for 90 minutes. However, the number of engulfed candida was counted as well as the phagocytic index and the percentage of phagocytosis.

At 5 µg/ml, the aqueous extract of *T. cordifolia* exerted enhanced phagocytic activity of the macrophages. At 1 µg/ml, no activity was seen. The effect was seen between 5 µg/ml and 20 µg/ml. Other extracts (ethanolic, chloroform and ethyl acetate) exerted zero activity in these

concentration. Bishayi et al., (2002) assessed the immunomodulatory activity of *T. cordifolia* on carbon tetrachloride (CCl4) intoxicated mature rats. They tested the phagocytic activity, chemotactic migration and cell adhesiveness to evaluate its immunosuppressive activity. They treated adult male albino rats of the Wister stain (weighing 150-160 g) with a polysaccharide preparation (satwa) derived from aqueous extract of *T. cordifolia*. They injected carbon tetrachloride to the rats to 9induce toxicity and the dose of the polysaccharide was 100 mg/kg and continued for 15 days. Consequently, they sacrificed the animals and made a preparation of the preparation of the peritoneal macrophages of the animals. Moreover, they incubated the preparation at 37ºC for 2 hours. They took 100 µL of HBSS-BSA (106 cells/ml) with macrophages and added 10% SRBC to examine the phagocytic activity of the macrophages. Moreover, they incubated the slides at 37ºC for 3 hours. To remove extracellular cells, they added 50% of methanol and stained the slides with Giemsa. Finally, they observed the data under an oil emersion microscope and calculated the phagocytic index. After finishing the assay, they observed that after the treatment of CCl4 phagocytic index of the control was decreased from 2700 ± 6130 to 1800 ± 572 and after the treatment with *T. cordifolia* extract the phagocytic index was reached at 30800 ± 653. Salkar et al., (2014) used human PMN cells and candida culture with different extracts of *T. cordifolia* to assay the phagocytic activity of this plant. After collecting the hydroalcoholic extract, they isolated human polymorphonuclear leukocytes (PMN) cells from 20 ml of blood collected from a human volunteer. Then they prepared a dilute suspension of the cells of Candida albicans and the final concentration of the suspension was 25×107 cells/ml and heated at 100ºC for 30 minutes. To conduct the assay, they took 0.2 ml of PMN cells (2×107 cells/ml) in 1ml of HBSS and added 0.05 ml of the test sample. Moreover, they incubated at 37ºC for 7 minutes. Furthermore, 0.1 ml of candida cell suspension were added and incubated again for 60 minutes. EDTA was used to stop the reaction. Finally, they counted the number of candida cells which was engulfed by PMN cells

*Table 8: Assays on the effect of Tc on phagocytic activity*

| **References** | ***In vivo/ in vitro*** | **Extract /compound used** | **Dose** | **Statistical analysis** | **Findings** |
|---|---|---|---|---|---|
| Test done by Gupta et al., (2016) | *In vitro* | G1-4A was isolated and purified from methanolic extract of *T. cordifolia.* | RAW 264.7 cells of macrophage were treated with sufficient amount of G1-4A. | • Experiments were presented as mean ±SD<br>• Significance of the data determined by student's t-test or one-way analysis of variance (ANOVA). | • Phagocytic activity enhanced for both G1-4A and LPS groups.<br>• The phagocytic index was significantly higher than the control group. (significance, $p<0.05$)<br>• A significant increase is seen in the percentage of GFP positive cells. |
| Test done by More and Pai (2017). | *In vitro* | Aqueous extract | Plant extract (80 µg/mL) and LPS (10 µg/mL) | • Experiments were presented as mean ±SD<br>• Significance of the data determined by student's t-test | • For Yeast cells, macrophages which were treated with *T. cordifolia,* exerted higher phagocytic activity compared to the control group. the phagocytic index was significantly higher in the *T. cordifolia* treated macrophages.<br>• For *E. coli,* high number of colonies were seen in the plates which were treated with plant extract which indicates high number of phagocytized cells. |

(Table-8 continued)

| | | | | | |
|---|---|---|---|---|---|
| Test done by Ranjith et al., (2008). | *In vitro* | Aqueous, ethanolic, ethyl acetate and chloroform extracts. | 1,5,10,15 and 20 µg/ml of plant extract. | • Experiments were presented as mean ±SD | • Enhanced phagocytic activity of macrophages shown from 5 µg/ml to 20 µg/ml.<br>• No significant change detected for the other extracts (Ethanolic, chloroform and ethyl acetate). |
| Test done by Bishayi et al., (2002). | In vitro | A polysaccharide preparation (satwa) was made from the aqueous extract of *T. cordifolia.* | 100 mg/kg of the polysaccharide preparation. | • Phagocytic index was presented as Mean ±SD.<br>• Significance was P<0.01 | • After the treatment of CCl4, phagocytic index was deceased from 2700 ± 6130 to 1800 ± 572.<br>• After treatment with *T. cordifolia*, phagocytic index reached at 30800 ± 653 (p<0.01) |
| Test done by Salkar et al., (2014). | *In vitro* | Aqueous and hydro-alcoholic extracts. | 0.5 and 1.0 mg/ml of the test sample was used. | • Significance of the data was determined by using student's t-test. | • The percentage of phagocytosis was increased in a dose dependent manner.<br>• Approximately, 30% of phagocytosis percentage was observed for 1.0 mg/ml.<br>• Both hydro-alcoholic extract and aqueous extract showed a percentage of 35 to 40%. |

and calculated the percentages of phagocytosis. They treated six samples with 0.5 and 1.0 mg/ml concentration of the extracts and observed that the percentages of phagocytosis of five samples were increased in a dose dependent manner and one sample didn't affect the PMN cells. Approximately 30% of phagocytosis percentage was observed for 1.0 mg/ml of concentration. However, both aqueous and hydro-alcoholic extracts showed a percentage of 35 to 40%.

## 4.2 Effects on the production of antibody

Antibody is also called immunoglobulin which is one type of glycoprotein and it can kill pathogens after being produced by the plasma cells of our immune system. By using its pathogenic antigen-binding region it can recognize the antigen for which it is considered specific (Litman et al., 1993). Moreover, humoral immune system works mainly by producing antibodies against the invaded pathogens (Pier et al. 2004). So, we can say that our immune system produces specific antibody when it finds any invaded antigen in our body. A number of assays, including radioimmunoprecipitation (RIP) assays, ELISA, and surface plasmon resonance procedures, have been used to detect anti-erythropoietin antibodies. Each of these tests can yield insightful outcomes and have unique benefits and limitations. ELISA, for instance, typically allows high throughput and is fast, relatively easy to use, and relatively inexpensive; thus, they are often used as screening tests for antibody detection. They can, however, generate non-specific matrix results, which typically manifest as false-positive outcomes. Antigen immobilization may alter the conformation of the native protein, and antibodies of "low affinity" may not be identified by ELISA; both of these problems may lead to false-negative results. In the solution process, RIP assays test antigen-antibody binding, which can be helpful and can be highly sensitive and specific, but they are typically low-throughput assays that are difficult to automate. They need a radiolabeled antigen source, which

has a shelf life that is relatively limited. RIP assays may be unique to the antibody isotype and can fail to detect antibodies that dissociate quickly. Procedures for surface plasmon resonance to measure anti-erythropoietin antibodies have been limited to methods of Biacore study. These procedures may be automatic and precise, but they may be less sensitive than other binding tests. Data on antibody kinetics, relative binding affinity, concentration and isotype can be given.

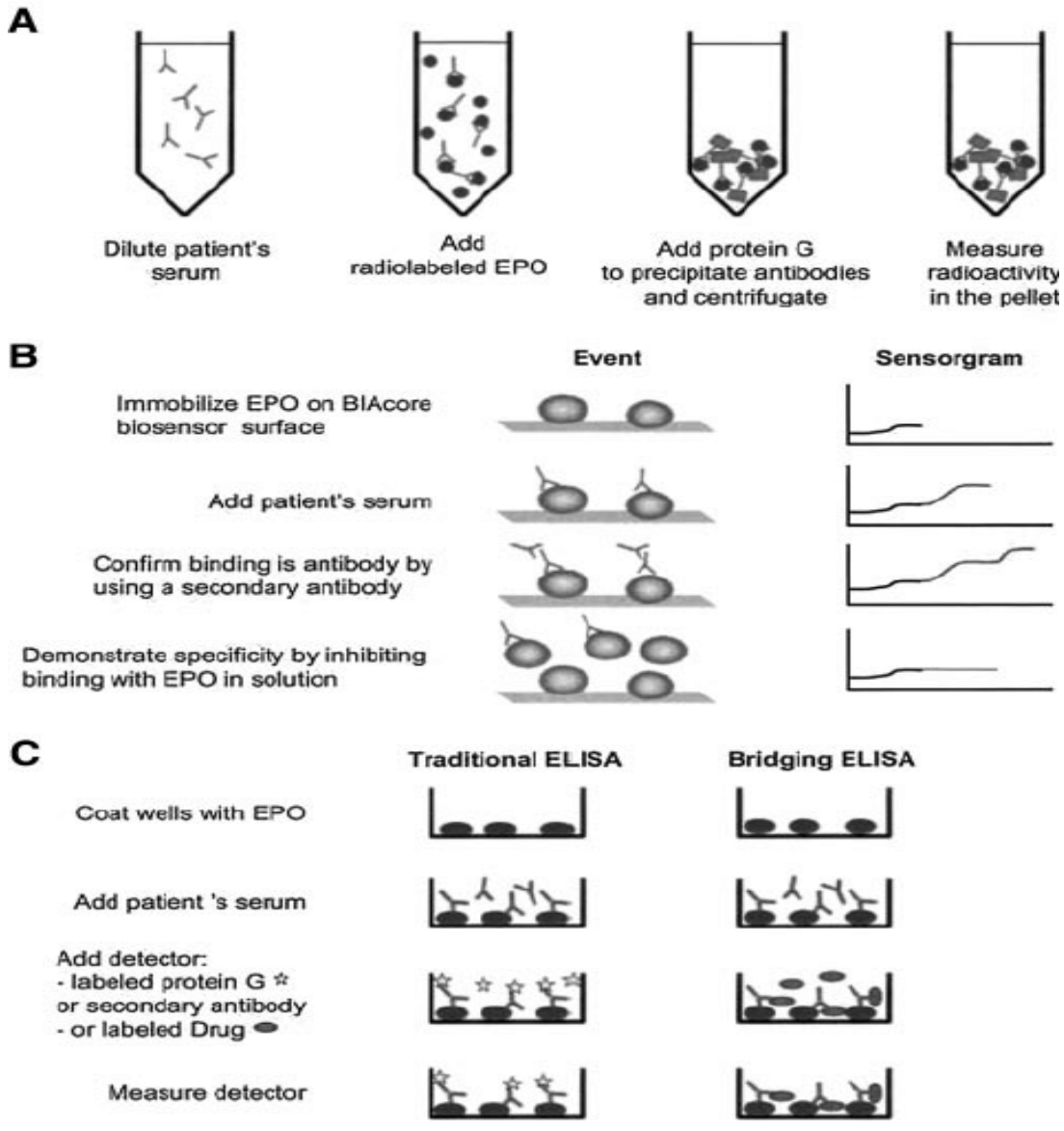


*Figure 16: Three types of antibody titer test developed to detect the presence of anti-erythropoietin antibodies (Pollock et al., 2008).*

Biacore analysis allows detection of rapidly dissociating, low-affinity antibodies not identified by ELISA, RIP assays, and other non-real - time procedures for anti-erythropoietin antibodies; however, they re-quire costly, dedicated equipment (Figure-16). In addition, antigen immobilization may alter the conformation of the native protein, and the antigen may degrade the regeneration stage (Pollock et al., 2008). If we can determine the production of antibody against a specific antigen, it will be easy to evaluate the immunomodulatory activity of a compound or drugs. Researchers use to determine antibody production to evaluate the immunomodulatory activities of medicinal plants. Many techniques are developed to determine antibody titre and hemagglutination inhibition assay is one of the popular techniques. An American virologist George Hrist developed hemaglutination inhibition assay in 1941-42 by which the concentration of antibodies could be determined (Hirst, 1942). Many types of organism like, bacteria, virus etc. can be used to perform agglutination of erythrocytes. The goal of this assay is to determine the concentration of antibodies in a sample and this assay is very popular in the laboratories all over the world and researchers perform this test for determination, comparison and standardization (Noah, Hill, Hines, White, & Wolff, 2009). I have collected and reviewed some of the experiments done by researchers about hemagglutination assay on *T. cordifolia* plant (Table-9). I found that most of the tests are *in vivo.* These assays are described and outlined below:

Siddiqui et al. (2008) evaluated the immunomodulatory effect of the ethanolic extracts of *T. cordifolia* and *C. asiatica* in mice against SRBC as an antigen. They examined delayed type hypersensitivity reaction, neutrophil count and hemagglutination titer to evaluate this effect of the selected plants. They used an immunosuppressant named cyclophosphamide to compare with the effects of the plant extracts. They prepared the plant extracts by using 95% ethanol. Moreover, hey distributed six groups of treated mice with six members in each. Then they induced sensitization with SRBC on day and started the collection of blood samples of

individual groups on day. Furthermore, on the day they collected blood samples from all groups. Finally, they determined antibody titer by following the method explained by Puri et al. (1994). In brief, the plate was treated with 25 µL saline and 25 µL of 0.1% (v/v) SRBC and incubated the plate at 37ºC for 1 hour. They expressed the antibody titer as a graded manner. They presented the data as mean ± S.E.M and they analyzed the data by ANOVA and Dunnett's test and considered $P<0.05$ as statically significant. They found no remarkable change in the hemagglutination (group II and III) after 10 days of the treatment but on day 14 all treated groups showed a remarkable humoral antibody response. The values were significantly higher than the control group. On the other hand, cyclophosphamide decreased HA titer compared to the control group. Ranjith et al. (2008) used the aqueous, ethanolic, ethyl acetate and chloroform extracts to assess the effects of this plant on the antibody production in vivo. They used Wister rats (150 -200 g) for the assay and distributed the animals into 4 groups and each group had six animals. Then they treated each group with the prepared plant extracts with a dose of 10 mg/kg and two animals was selected as control group. They fed extracts to the animals orally for 14 days. Consequently, they applied SRBC at a concentration of $0.5 \times 10^9$ cells/ml after 14 days. However, they did micro-agglutination test to estimate the antibody production. To titrate the 2-mercaptoethanol- resistant hemagglutithin, the same amount of 0.1M 2-ME in phosphate-buffered saline was added and incubated for 1 hour at 37°C. The results were presented as mean ± SD and analyzed by student's t-test. For treating the animals with *T. Cordifolia* aqueous and ethanolic extracts for 14 days at a dose of 10 mg/kg, the antibody production against the antigen used in rats was significantly higher than the control group but there was no increase of antibody production seen for other extracts of the plant. Mehta et al. (2011) investigated the effect of *T. Cordifolia* stem in vivo on the antibody production using its alcoholic extracts. At first, they made a suspension of the extracts with 5% dimethyl sulfoxide (DMSO) to make the extracts administrable to the animals. They used

Wister albino rats (age 6-8 weeks and weight 150-180 g) and SRBC as antigen to run the experiment. They did the experiment by following the method stated by Puri et al. (1993). They immunized the animals with 0.1 ml of SRBC at a concentration of $0.5 \times 10^9$ cells/ml on day zero. On 7th day, they collected blood samples from the animals and centrifuged the samples. However, they used hemagglutination technique to estimate the production of the antibodies and made a serial dilution series of the preparation. Consequently, they added 25µL of 1% suspension of SRBCs in saline and incubated the plate for 1 hour at 37°C. Finally, they examined the suspension under an electronic microscope observing the serum agglutination. The data were presented as mean ± SD and one-way ANOVA test was used to analyze the significance of the result. Moreover, $p<0.05$, $p<0.01$ and $p<0.001$ were considered as significant. They compared the values of antibody titer of both extracts of the plant with the control group to evaluate the actual effects of the extracts. The average values of the antibody titer were remarkably higher than the control group ($p<0.05$ and $p<0.001$) which confirms the effect of the plant on our humoral immunity. Mathew and Kuttan (1999) did an experiment on the immunomodulatory activities of the methanolic extract of *T. cordifolia*. They determined the antibody titer after the treatment with the extract to find out its effects on humoral immune response. They collected *T. cordifolia* stem to make its methanolic extract and re-suspended the extract in water with 1% acacia gum to make the extract administrable and the final concentration was 40 mg/ml. They used BALB/c and Swiss albino mice weighing 20 ± 2 g to run the experiment. At first, they immunized two groups of animals (each contained six animals) by 20% SRBC. They administered *T. Cordifolia* extract to the expected treated group at a dose of 200 mg/kg daily for 5 days before the group had been immunized. They collected the blood samples every third day after 5th dose for 1 month from the caudal vein of the animals. However, they followed hemagglutination technique to determine antibody titer and they gave only saline to the control group at a concentration of 5 ml/kg.

*Table 9: Assays on the effect of Tc in antibody production*

| **References** | ***In-vivo/in-vitro*** | **Extract /compound used** | **Dose** | **Statistical analysis** | **Findings** |
|---|---|---|---|---|---|
| Test done by Siddiqui et al. (2008). | *in vivo* (Swiss albino mice weighing 20 - 25 g,) | Ethanolic extract was used. | 100 mg/kg body weight of the plant extract. Administered from day1 to day 14. | • The statistics were presented as mean ± S.E.M.<br>• The data were analyzed by ANOVA and Dunnett's test.<br>• P<0.05 was considered as statistically significant. | • No remarkable change was seen in hemagglutination of group II and III after 10 days of the treatment.<br>• On 14$^{th}$ day all treated groups showed a remarkable humoral antibody response.<br>• The values were significantly higher than the co9ntrol group. (P>0.05) |
| Test done by Ranjith et al. (2008). | *In vivo* (Wister rats 150-200g) | Ethanolic, ethyl acetate, chloroform and aqueous extracts | 10 mg/kg for 14 days. | • The results were presented as mean ± SD.<br>• The data were analyzed by student's t-test. | • The antibody production against the antigen used in rats was significantly higher than the control group for aqueous sand ethanolic extracts.<br>• No antibody production seen for the other plant extracts. |

(Table-9 continued)

| | | | | | |
|---|---|---|---|---|---|
| Test done by Mehta et al. (2011). | *In vivo* (Wister albino rats age 6-8 weeks, body weight 150-180 g) | Ethanolic and methanolic extracts. | 50,100,200 and 300mg/kg | • The results were presented as mean ± SD.<br>• The data were analyzed by one-way ANOVA.<br>• Considered significant values are $p< 0.05$, $p<0.01$ and $p<0.001$ | • The average values of the antibody titer were remarkably higher than the control group.<br>• This outcome confirms its effect on humoral immunity. |
| Test done by Mathew and Kuttan (1999). | *In vivo* (BALB/c and Swiss albino mice weighing 20 ± 2 g) | Methanolic extract of the plant stem. | 200mg/kg of the plant extract preparation daily for 5 days. | • The results were presented as mean ± SD.<br>• The data were analyzed by student's t-test. | • On $18^{th}$ day, the value of antibody titer was maximum for the treated group. |

After being done with the estimation, they presented the data as mean ± SD and analyzed the data using student's t-test. They found that *T. cordifolia* extract increased the production of the

antibody significantly and on 18th day, 256 of maximum antibody titer was found out. However, the control animals exhibited only 32 of maximum antibody titer on the same day.

## 4.3 Effects on nitric oxide production

Lowenstein et al. (1994) complimented nitric oxide as a physiologic messenger. He also added that nitric oxide is an inorganic compound which has many cardiovascular, neurological and immunological functions (Figure-17). According to Assreuy et al. (1994), the macrophages of murine becomes activated after the entrance of a pathogen in blood stream and being activated the macrophages produce nitric oxide (NO) and superoxide. In addition, they also proved that these two free radicals react and produce peroxynitrite which was an antioxidant and responsible for killing the invaded pathogens. Macrophages use L-arginine with nitric oxide synthase (NOS) and arginine and trigger the production of nitric oxide (NO) and urea (Chang, Liao, & Kuo, 1998). Activated macrophages produce this nitric oxide by using the terminal guanidine nitrogen atom contained by L-arginine and it does not leave guanidine carbon atom while converting into L-citrulline (Hibbs, Taintor, Vavrin, & Rachlin, 1988). In macrophages, dendritic cells, and natural killer (NK) cells and in cell lines, clones, hybridomas and tumor cells of B or T cell origin, either iNOS or eNOS have been identified. Whether any of the nitric oxide synthase iso- types are expressed by primary T or B lymphocytes remains questionable. Expression of iNOS is regulated by cytokines and is primarily determined by de novo synthesis and iNOS mRNA and protein stability. NNOS and eNOS, on the other hand, exist as preformed proteins in the cell whose function is turned on by elevation of intracellular Ca2 + concentrations and calmodulin binding in response to neurotransmitters or vasoactive substances.

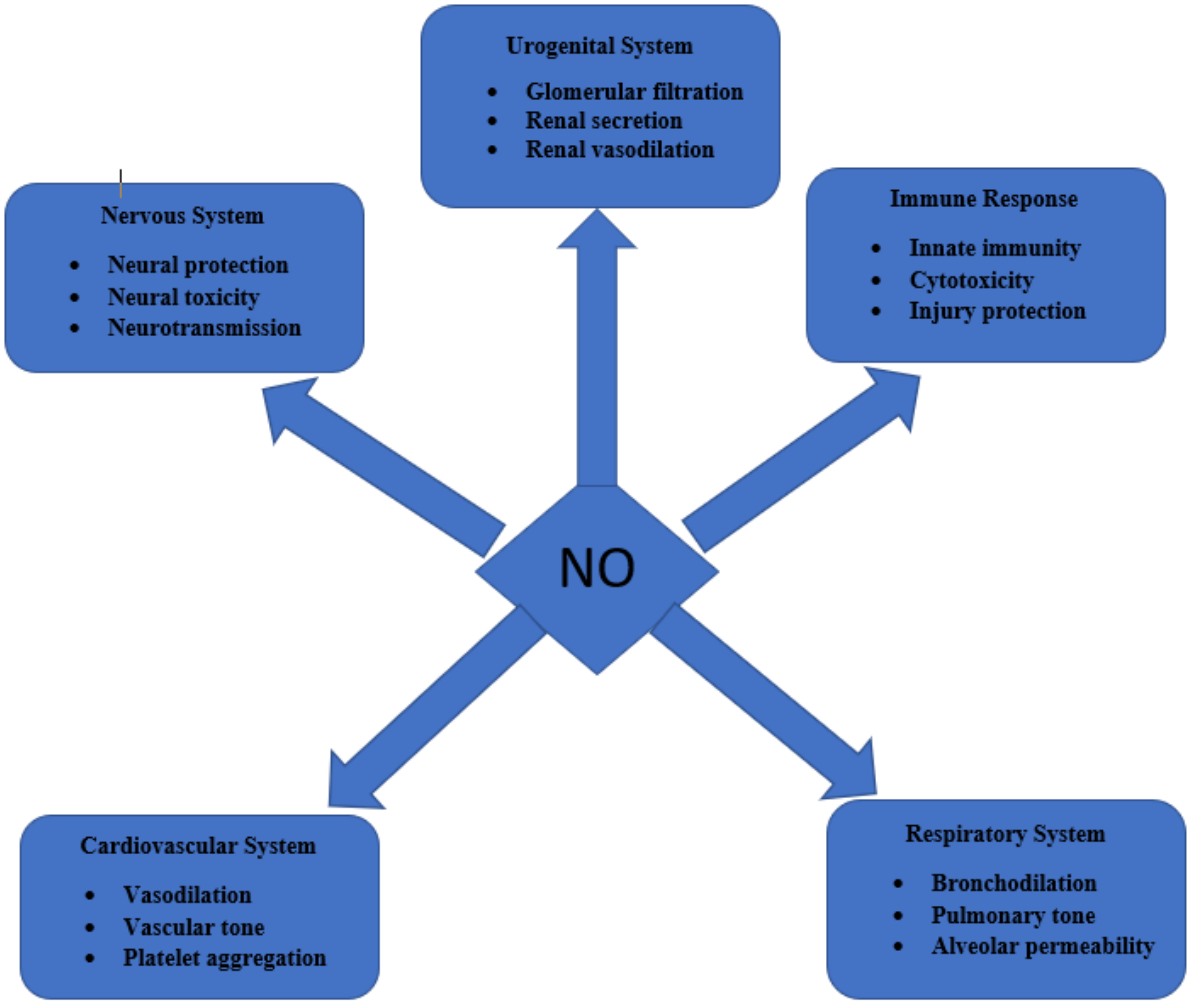


*Figure 17: Several functions of nitric oxide in our body* (Mian, Aranke, & Bryan, 2013)

For all three NOS isoforms that can operate during immune responses, additional levels of control exist beyond this specific paradigm. A significant iNOS mode of activation of the iNOS gene promoter is cytokine control, which was most extensively studied in mouse macrophages and lines of human hepatocytes and epithelial cells (Figure-18). Participating transcription factors include NF-nB, AP-1, transcription signal transducer and activator (STAT)-1af, interferon, regulatory factor-1 (IRF-1), nuclear factor interleukin-6 (NF-IL-6) and the high-mobility group-I(Y) protein (Bogdan, 2001).

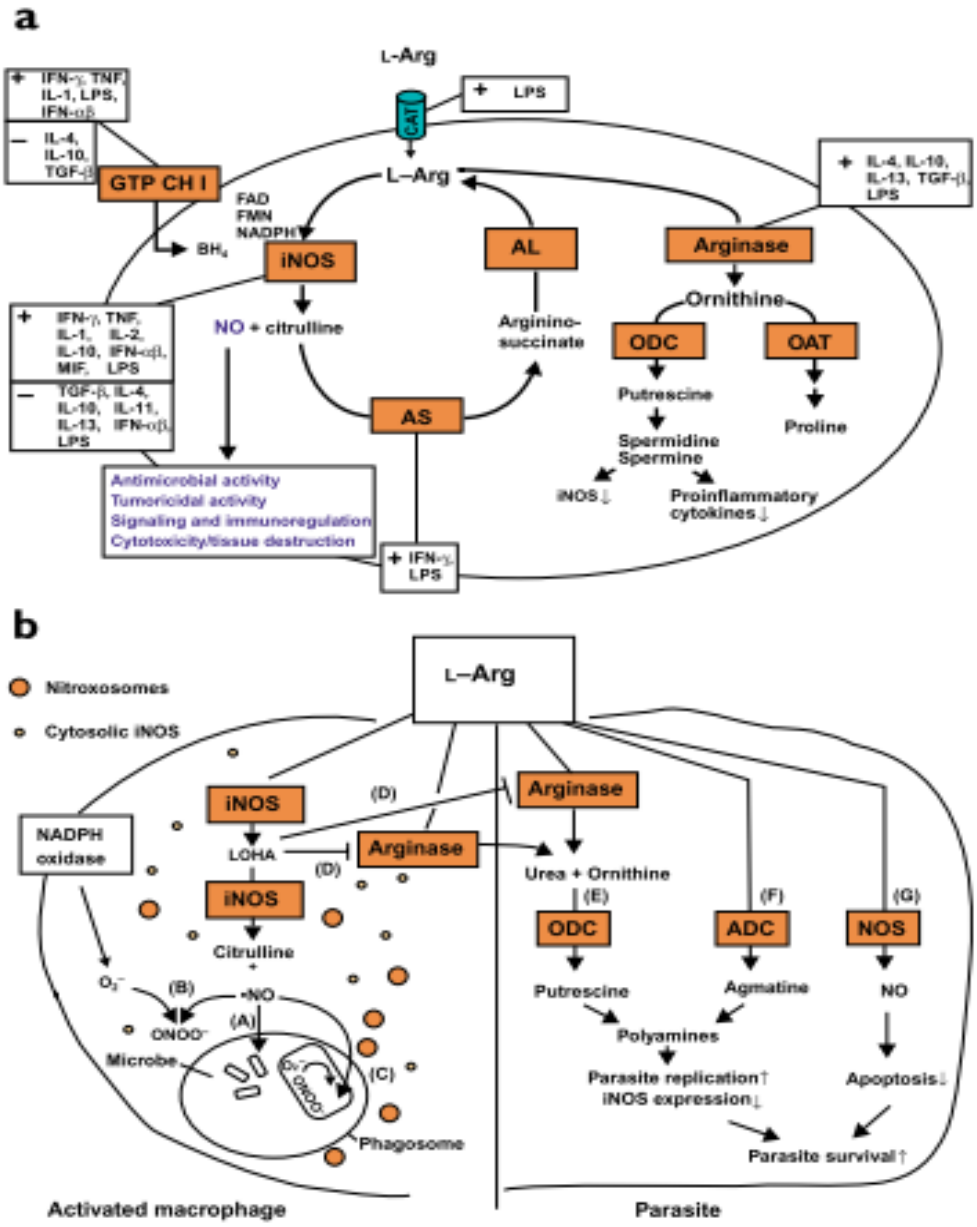


*Figure 18: Pathways of nitric oxide and its antimicrobial activity (Bogdan, 2001)*

However, Epstein et al. (1993) explained the L-arginine pathway of NO in terms of pathologic invasion. They stated that the phenomenon of macrophage activation was linked with the production of nitric oxide synthase and the resulted immunity was non-specific. They also stated that the system involved in this process was reticuloendothelial system. They reviewed the process briefly and suggested that nitric oxide also kept role in specific immunity but nobody could clear the actual role. They concluded by stating that NO is a good indicator of

the efficiency of macrophages to phagocyte a pathogen and any negative alteration in the production of NO indicates a pathological condition. To analyze the phagocytic activity or the activation properties of the macrophage researchers prefer the determination of NO production the macrophages. Therefore, they prioritized the estimation of NO production while analyzing a plant's immunological effects. There are various techniques by which NO production of NO can be determined. In this review, I will describe some of the methods. Moreover, maximum methods are *in-vitro.* However, there are some common procedures in these methods. Such as, collection of murine macrophages, making a culture of using cell lines, infecting the cell lines with an antigen and counting the concentration of NO by taking $NOS_2$ as a standard (Aranha et al., 2012; Desai, Ramkrishnan, Chintalwar, & Sainis, 2007; Gupta et al., 2016; Upadhyaya, Pandey, Sharma, & K, 2011)**.** The techniques they followed are described below and outlined in Table-10.

Upadhyaya et al. (2011) evaluated the immunomodulatory and cytotoxicity activities of *T. cordifolia* by following in vitro and ex vivo models. They did nitric oxide assay to check how the plant extracts affect the phagocytic activity of macrophages. At first, they prepared aqueous extract of *T. cordifolia* and purified the proteins derived from the extract. They injected 4% sodium thioglycolate solution to the mice and collected the peritoneal exudates and centrifuged at 1100 rpm for 10 minutes. Moreover, they re-suspended the pellet in RPMI and incubated at 37°C for 3 hours. Then they added the different concentrations of the extract preparation. Furthermore, they incubated the pellet for 48 hours to produce NO. Finally, the amount of NO was estimated using $NaNO_2$ as a standard. They presented the collected data as mean ± SD analyzed by student's t-test.

They found that the production of nitric oxide (NO) was enhanced by the macrophages because of the treatment of the *T. cordifolia* extract. Among all the doses of *T. cordifolia* 1 mg/kg exerted highest enhancement in the production of NO. However, they noticed that the

enhancement was done in a dose-dependent manner. As higher NO production indicates higher number of activated macrophages, they decided that *T. cordifolia* enhances the phagocytic activity of macrophages. They also noticed the increasing pattern followed a cascade of the signing pathway.

Desai et al. (2007) experimented the immunomodulatory effects of *T. cordifolia* by using its polysaccharide named G1-4A. They stated that G1-4A showed 100% efficiency against LPS induced mortality in mice and this compound worked via getting attached to the macrophages. At first, they isolated and purified G1-4A from the methanolic extract of *T. cordifolia* and acetone was used to facilitate the isolation. They chose C3H mice to collect spleens which is required for the test. They isolated the spleens and made a single cell suspension by sacrificing the animal and the final concentration was 5 × 106 cells/ml. They stimulated the cells by administering LPS and G1-4A in vitro for 48 hours and added Griess reagent. Then they took the absorbance of the suspension and determined the concentration of NO2 considering sodium nitrite as a standard. They used student's t-test to analyze the data and considered $p<0.05$ as statistical significance.

They claimed that G1-4A stimulated the splenic adherent cells to release nitric oxide and the process was in vitro. After 48 hours, they noticed that at 0.5 mg/ml concentration, G1-4A stimulated NO production but the rate was lower than the LPS of 0.05 mg/ml concentration. They also noticed that by increasing the production of NO G1-4A also influenced the endotoxin tolerance. Gupta et al. (2016) tested the effects of *T. cordifolia* on the production of NO in macrophage. To conduct the test, they used MTB infected RAW 264.7 macrophages and treated the cell lines with purified G1-4A. They stated that G1-4A increased NO production following TLR4-MyD88 dependent manner. At first, they prepared a cell line plate at a concentration of 2.5 × 105 cells per well and made total 24 well plates and incubated for overnight at 37°C. Then they isolated G1-4A from the methanolic extract of *T. cordifolia* with

the help of acetone. Moreover, they treated RAW 264.7 cells with G1-4A and they infected the cells with MTB after 8 hours. Furthermore, they treated the cells again with G1-4A for 48 hours. They stated that nitrite is an end product of NO and they determined its concentration by Griess's reaction. In addition, they used sodium nitrite concentrations to make a standard curve which indicated the production of NO. However, they presented the statistics as mean ± SD and analyzed the data by using student's t-test or one-way ANOVA. They used sigma stat software version 3.5 to analyze these tests and they considered $p<0.05$ as a significant value.

After being done with the assay, they found that MTB stain-2 produced maximum NO than stain-1 without the treatment of G1-4A and the NO production increased noticeably after the treatment of G1-4A. They also stated that G1-4A also upregulated NOS2 in the infected cells. Aranha et al. (2012) isolated immunomodulatory protein from the extracts collected from the stem of *T. cordifolia*. At first, they collected and purified the immunomodulatory protein from the aqueous extract of *T. cordifolia*. Then they isolated peritoneal exudates from BALB/c mice and centrifuged the exudates at 1000 rpm for 10 minutes. Moreover, the cells were suspended using 10% (v/v) FCS, 1 µg/ml of LPS, 100U/ml of streptomycin and made a suspension with a density of 2 × 105 cells/ml. They seeded the suspensions in some well plates which contained different concentration of the immunomodulatory protein (1,5,10 µg /ml) and incubated at 37°C for 48 hours. They performed Trypan blue exclusion test to determine the viability of the cells. Moreover, they used Griess reagent to determine nitrite concentration which is an indicator of the production of NO. For this purpose, they incubated the wells with this reagent at 37°C for 10 minutes and determined the absorbance at 540 nm. They stated that NO production indicates the activation of macrophages. They found a noticeable enhancement in the production of NO in comparison with the control. Moreover, the protein exhibited 2 to 3-fold increase in the production of NO at different doses (1,5 and 10 µg/ml).

*Table 10: Assays on the effects of Tc on nitric oxide production*

| **References** | ***In-vivo/in-vitro*** | **Extract /compound used** | **Dose** | **Statistical analysis** | **Findings** |
|---|---|---|---|---|---|
| Test done by Upadhyaya et al. (2011). | *In vitro* | Aqueous Extract | 1 mg/kg**,** 5 mg/kg, 10 mg/kg Of the plant extract. | • Experiments were presented as mean ± SD<br>• Significance of the data determined by student's t-test. | • After the treatment with *T. cordifolia* extract, a significant increase in the production of nitric oxide was noticed.<br>• 1 mg/kg exerted highest enhancement in the production of nitric oxide.<br>• The production of nitric oxide increased in a dose-dependent manner. |
| Test done by Desai et al. (2007). | *In-vitro* | Purified G1-4A from methanolic extract of *T. cordifolia* | 0.5 mg/ml of purified G1-4A. | • Student's t-test was used to analyze the data.<br>• $p<0.05$ considered as the value of statistical significance. | • After 48 hours, G1-4A of 0.5 mg/ml enhanced NO production but lower than the effect of LPS of 0.05 mg/ml.<br>• Endotoxin tolerance enhanced according to the increase of the production of NO. |

(Table-10 continued)

| | | | | | |
|---|---|---|---|---|---|
| Test done by Gupta et al. (2016). | *In vitro* | G1-4A was isolated and purified from the methanolic extract of *T. cordifolia*. | Sufficient amount of G1-4A was used to treat the MTB infected cell lines. | • The statistics were presented as mean ± SD.<br>• Analyzed by Student's t-test or one-way ANOVA.<br>• Significance value was $p<0.05$. | • Significant increase of NO production was seen in the MTB infected cell lines after the treatment of G1-4A.<br>• $NOS_2$ concentration was also upregulated in the cell lines after the treatment. |
| Test done by Aranha et al. (2012). | *In vitro* | Immunomodulatory protein was isolated and purified from the aqueous extract. | 1,5 and 10 µg/mL of the purified immunomodulatory protein was used. | • The statistics were presented as mean ± SD.<br>• Analyzed by Student's t-test or one-way ANOVA.<br>• Significance value was $p<0.05$. | • Noticeable enhancement in the production of NO was seen compared to the control group.<br>• All concentrations of the protein exhibited 2-3-fold increase in the production of NO. |

## 4.4 Effects on the production of cytokines

Cytokines are SSPs which are usually secreted by defensive cells of our immune system (Figure-19) showing some roles in the interactions between the cells and cytokines are also called lymphokine, monokine, chemokine and interleukin depending on the source of the release (Zhang & An, 2007). Cytokines have very significant roles in the development of bone, immunity, haemopoiesis and other biological functions (Thorpe, Wadhwa, Bird, & Mire-Sluis, 1992). Paul and Seder (1994) subdivided cytokines into the groups of hematopoeteins, interferons, tumor necrosis factor and chemokines. They prioritized hematopoeitins over other groups of cytokines and they included IFN- γ, IL-4, IL-13, IL-5, GMCSF, and IL-3 in this group.

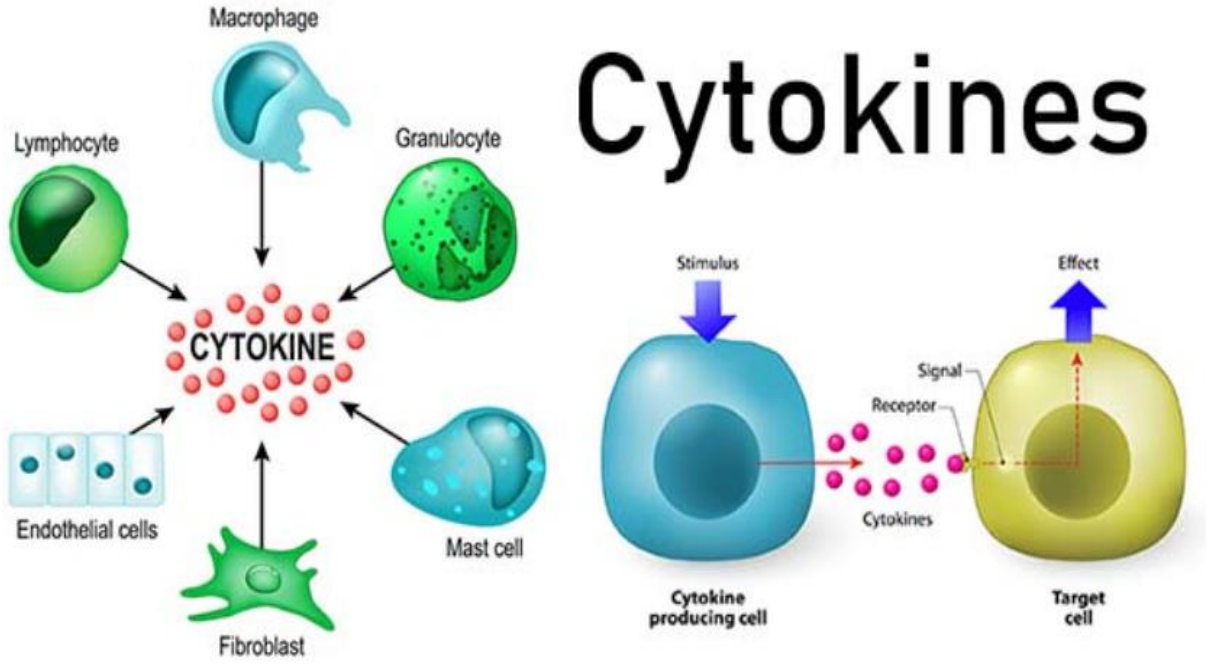


*Figure 19: An illustration of cytokines (Aryal, 2018)*

Different cytokines may have same functions and some cytokines provoke other cytokines by making them stimulated or inhibited which can result in a synergistic or antagonistic effect

(Zhang & An, 2007). IL-1β, IL-6 and TNF-α are usually released in response of the entrance of an infectious virus and IFN-α/β is produced when inactivated or killed virus are injected into the blood stream (Paul & Seder, 1994). IL-1β, IL-6, IL-8 and TNF-α are released from monocytes in response to gram positive and negative bacteria (Hessle, Andersson, & Wold, 2005).

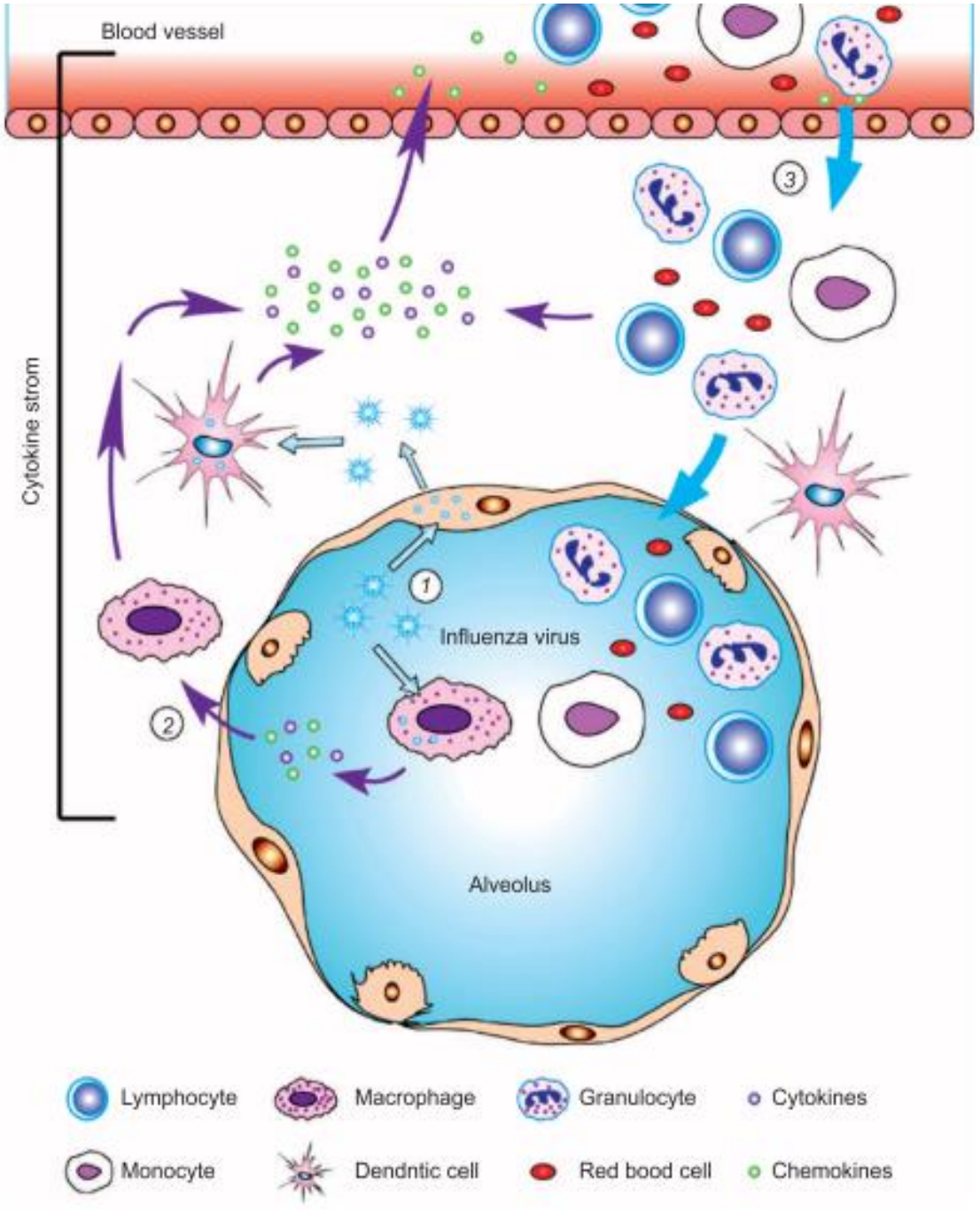


*Figure 20: Cytokines storm after the infection of influenza* (Liu, Zhou, & Yang, 2016)

Figure-20 describes about lung cytokine storm after a serious influenza infection. (1) Pulmonary epithelial cells and alveolar macrophages are infected with viruses to produce progeny viruses and release cytokines / chemokines (mainly interferons). (2) Macrophages triggered by cytokine / chemokine and virally infected dendritic cells contribute to a more extensive immune response and cytokine storm initiation. (3) Chemokines released attract more inflammatory cells to migrate to the site of inflammation from the blood vessels and these cells release additional chemokines / cytokines to amplify the cytokine storm. Unbalanced release of cytokines can result in many serious diseases (Thorpe et al., 1992). So, the measurement of cytokines can indicate the working efficiency of an immune system. Thorpe et al. (1992) stated that to determine the correct levels of cytokines the assay methodology must be accurate. They also mentioned some available assays named bioassay, immunoassay, immunochemical assay and receptor binding assay. They informed that usually researchers prefer bioassay and immunoassay for acceptable accuracy. A new microscopic technique has been developed to determine the levels of intracellular cytokines named indirect immune fluorescent staining method (Schauer, Jung, Krug, & Frew, 1996). There are some factors which give raise of some complexities in the assay like bioassay needs for standardization (Bienvenu, Monneret, Fabien, & Revillard, 2000)**.** I have collected some of the assays (Table-11) which was performed on *T. cordifolia.* The assays are:

Desai et al. (2007) examined the effect of a *T. cordifolia* derived polysaccharide on the cytokines production in mice. The polysaccharide they used was G1-4A and they isolated and purified the polysaccharide from the methanolic extract of *T. cordifolia* plant using acetone to facilitate the isolation. They measured the level of TNF-α, IL-1β, IL-6 and IFN-γ and saw some positive results. They performed the assay in vivo and used C3H mice of 8 to 10 weeks old. They prepared various dose of G1-4A polysaccharide to treat the mice. The concentrations were 2.5, 5., 12.5, 25 and 50 mg/kg body weight. At first, they treated the mice with various

prepared doses and also induced endotoxic shock by an injection of 16 mg/kg of LPS. Consequently, they treated the mice of one other group with D (+) galactosamine and little amount of LPS. However, they treated the mice with the G1-4A before 24 of the treatment of LPS. Finally, they assessed the effect of the polysaccharide on the cytokines production after 24 hours of the toxicity induction by collecting the blood and they mainly estimated the production monocyte derived cytokines, TNF-α and IL-1β of three groups of mice. One group was treated with G1-4A, another group was treated with LPS and the other group was treated with saline only. They collected the blood sample at different points of time (1h, 14h and 24h). They estimated the cytokines level of different group mice by specific ELISA kits. They presented the statistics of the data as mean ± S.E.M. and they analyzed the data by student's t-test. In addition, they considered $p<0.05$ as statistically Significant value.

They found that the levels of TNF-α and IL-1β increased significantly depending the concentrations of the polysaccharide at 5 mg/kg and 12.5mg/kg. Surprisingly, they found that other two high doses (25 mg/kg and 50 mg/kg) did not show any increase and these concentrations caused decrease in the production of cytokines. However, they stated that 12.5 mg/kg showed highest increase in the production of TNF-α and IL-1β. Gupta et al. (2016) used mycobacterium tuberculosis to assess the effect of *T. Cordifolia* derived polysaccharide (G1-4A) on the production of cytokines. The assay they performed was in vivo and the animals they used were female BALB/c (6 weeks old). Moreover, they isolated and purified the polysaccharide from the methanolic extract of *T. cordifolia* by using acetone.

*Table 11: Assays on the effect of Tc on the expression and release of cytokines*

| **References** | ***In-vivo/in-vitro*** | **Extract /compound used** | **Dose** | **Statistical analysis** | **Findings** |
|---|---|---|---|---|---|
| Test done by Desai et al. (2007) | *In-vivo* (C3H mice of 8 to 10 weeks old) | G1-4A was isolated and purified from the methanolic extracts. | 2.5, 5, 12.5, 25 and 50 mg/kg | • The statistics were presented as mean ± S.E.M.<br>• Data were analyzed by student's t-test.<br>• Value of significance was $p<0.05$. | • Production of cytokines increased in a concentration dependent manner for TNF-α and IL-1 cytokines.<br>• But decreased after the concentration of 25 mg/kg.<br>• Highest level of TNF-α and IL-1 were seen at a concentration of 12.5 mg/kg. |
| Test done by Gupta et al. (2016) | *In vivo* (femal e BALB /c mice of six weeks old) | G1-4A was isolated and purified from the methanolic extract of *T. cordifolia* | Sufficient amount of G1-4A polysaccharide to treat the animal. | • The statistics were presented as mean ± SD.<br>• Data were analyzed by student's t-test or one-way ANOVA.<br>• Value of significance was $p<0.05$. | • The level of IFN-γ was noticeably higher than the control group.<br>• The level of IL-4 had no change after the treatment of G1-4A.<br>• Higher ratio of IFN- γ and IL-4 indicates that the polysaccharide derived from *T. cordifolia* affected Th1 response more than the Th2 response. |
| Test done by Gupta et al. (2017) | *In vitro* | G1-4A was isolated and purified | Sufficient amount of G1-4A was added. | • Sigma State software was used to analyze the data.<br>• The statistics were presented as mean ± SD.<br>• | • the releases of IL-1β, IL-6, IL-12, IL-10 and TNF- α increased remarkably after 24 hours of the treatment.<br>• TNF- α reached a maximum level at 6 hours and after that the production did not show any increase. |

They mainly estimated the release of TNF-α, IL-12, IL-6, IL-4, IL-10, IL-1β and IFN-γ. They collected the serum of MTB infected BALB/c mice and analyzed the serum using ELIZA technique. The ELIZA plates they used had 96 wells and they kept the plates overnight at 4°C. Consequently, they added chromogenic substrate and streptavidin POD to finish the procedure. Finally, EDTA was added to stop the reaction and the absorbance was determined. They presented their statistical data as mean ± SD and they analyzed the data by using student's t-test or one-way ANOVA. However, they considered $p<0.05$ as a value of significance. They found that the level of IFN- γ in serum was noticeably higher than the control group although there was no change in the level of IL-4 after the treatment of G1-4A. As the ratio of IFN-gamma and IL-4 was higher than the control group, it was clear that the polysaccharide induced mainly Th-1 response. Gupta et al. (2017) measured the effect of *T. Cordifolia* derived polysaccharide on the production of various cytokines. They followed an in vitro technique to conduct the measurement. At first, they collected a cell line containing murine macrophages and organized them in 24 well plates with an amount of macrophage cells per well was 5×105. Then they treated the cell lines with G1-4A at different time points (6, 12, 24 and 48 hours) and stored the preparation at -80°C. They mainly counted the levels of IL-1β, IL-6, TNF- α, IL-12, IL-10, IL-4and IFN-γ. They stated that these cytokines were secreted into the supernatant of the cell lines. However, they used BD OptEIA mouse ELISA sets to determine the cytokine levels. To conduct ELISA technique, they added specific antibody in the wells and did incubation at 4°C. Moreover, they used blocking buffer to remove nonspecific sites of the cells and they added a standard with known concentration. Finally, they added substrate solution and stop solution after 30 minutes. For IL-1β, IL-6 and TNF- α, the concentrations were exhibited as ng/ml and pg/ml units was used for the other cytokine concentrations. They used Sigma Stat software to analyze the statistics and they presented the results as mean ± SD where $p< 0.05$ was the significance value. They found that the releases of IL-1β, IL-6, IL-12,

IL-10 and TNF- α increased remarkably in the cell lines than the untreated cell lines after 24 hours of the G1-4A treatment. They noticed a further increase in the release of IL-6 and IL-12 after 48 hours of the treatment of G1-4A. On the other hand, IL-1β, IFN-γ levels were reduced after 48 hours. Moreover, TNF- α reached a maximum level at 6 hours and after that the production did not show any increase.

## 4.5 Effects on the number of neutrophils

In recent years, our understanding of the role of neutrophils in inflammation has profoundly changed. An appreciation that activated neutrophils can perform most (if not all) of the macrophage functions has now replaced the initial view of the neutrophil playing a passive role and merely responding to external signals. Properly activated neutrophils are now recognized to secrete a range of pro-inflammatory cytokines and express MHC Class II (MHCII) in a way that enables T cells to present antigen and activate it. It is also known that neutrophils lead to the pathogenesis of a variety of human diseases such as chronic obstructive pulmonary disease, Behcet 's disease and inflammatory arthritis. Neutrophils form the main line of protection of the body against invading pathogens such as bacteria and make up 40 percent-60 percent of the population of white blood cells. Neutrophils live in a resting state in the bloodstream of healthy adults, which means that their toxic intracellular contents are not inadvertently released to damage the host tissue (Figure-21). Via a two-stage phase, neutrophils become activated. Agents that include bacterial products and cytokines or chemokines, e.g., may become primed by resting neutrophils. TNF-a, GM-CSF, IL-8 and IFN-g and primed neutrophils are then mobilized to the infection or inflammation site, where bacterial killing is caused by triggering signals (Wright et al., 2010).

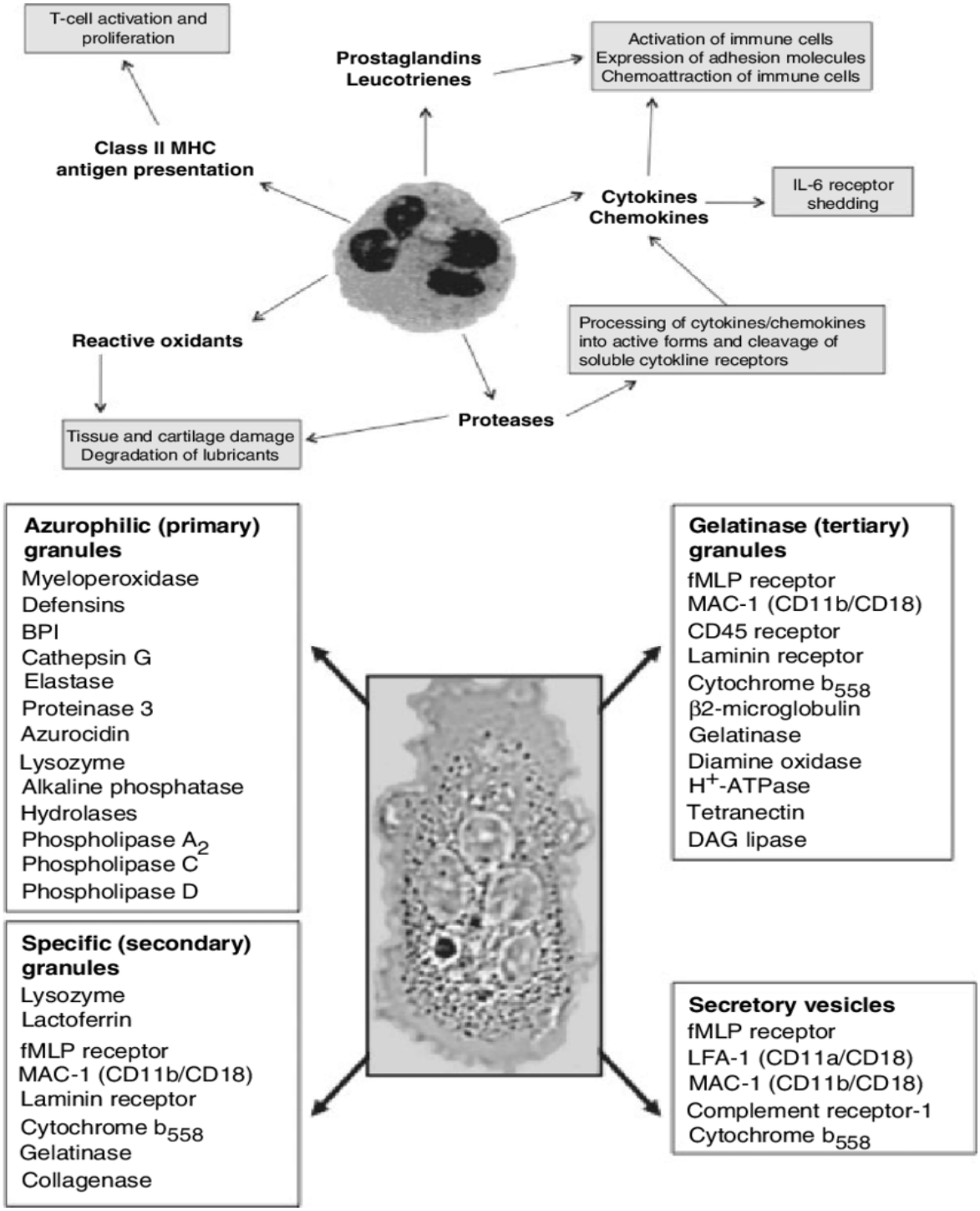


*Figure 21: Properties of Neutrophils* (Wright, Moots, Bucknall, & Edwards, 2010)

Various types of extract of *T. cordifolia* speed up the production of WBCs as well as increase the number of neutrophils (Table-12). Neutrophils are the main cells which are the base components of our body defense systems.

*Table 12: Assays on the effect of Tc on the number of WBCs.*

| **References** | ***In-vivo/in-vitro*** | **Extract /compound used** | **Dose** | **Statistical analysis** | **Findings** |
|---|---|---|---|---|---|
| (Siddiqui et al., 2008) | *In vivo* (Swiss albino mice) | Ethanolic extract. | 100 mg/kg | • The Data were presented as mean ± SD.<br>• Data were analyzed by student's t-test or one-way ANOVA. | • Every group showed slightly more percentage of neutrophil count on day 10 compared to day 1.<br>• After the treatment of Tc extract a significant increase ($P<0.01$) in the percentage of neutrophil was seen on day 14. |
| (Mathew & Kuttan, 1999) | *In vivo* (BALB/c mice) | Methanolic extract | 200mg /kg daily for 5 days. | • Data were Expressed as mean± SD and analyzed by student's t-test. | • After the treatment of Tc extract the percentage of neutrophil count was increased significantly. |

Some valuable assays are performed to check whether the extracts of this plant can increase the number of WBCs or not. Siddiqui et al. (2008) performed an assay *in vivo* (Animal) by using ethanolic extract of *T. cordifolia*. They found that on the $10^{th}$ day of administration the Number of neutrophils were increased slightly and a significant increase in the number of neutrophils was seen on the $14^{th}$ day. Moreover, Mathew and Kuttan (1999) used methanolic extract of this plant and found that there were a noticeable ($p<0.001$) increase in the number of leukocytes on the $15^{th}$ day.

## 4.6 Effects on Lymphocyte proliferation

Lymphocyte proliferation indicates rapid synthesis of DNA of lymphocytic cells to increase the number of new leukocyte cells. Higher Lymphocyte proliferation means rapid increase in the number of defense cells (Thome & Tschopp, 2001). During the late 1950s, the in vitro lymphocyte transformation phenomenon was first described. In short, in short-term primary cultures, human peripheral blood leukocytes (PBLs) differentiate, forming plasts. This result was subsequently due to the presence of a constituent (phytohemagglutinin [PHA]) of red kidney bean plant extract (Phaseolus vulgaris) used to separate peripheral leukocytes in the blood. PHA induces the accumulation and sediment of erythrocytes, allowing leukocytes to differentiate from whole blood preparations. Nowell showed in a later study that in cultured human leukocytes, PHA also initiates mitotic activity(transformation). To explain this procedure, the words' lymphocyte transformation test (LTT),' lymphocyte stimulation test '(LST), and 'lymphocyte proliferation test '(LPT) are interchangeably used. The treatment requires the incubation at non-toxic concentrations of PBMCs isolated from drug-hypersensitive patients with the incriminating agent and the detection of any rise in the rate of cell proliferation calculated by the in-corporation of thymidine. As a ratio between the proliferation of cells incubated with and without the drug, the increase in cell proliferation is expressed. A recent technique focused on staining of carboxyfluorescein succinimidyl ester (CFSE) intracellular proteins has been successfully used to measure in vitro T-cell proliferation. To non-specifically mark intracellular proteins, this fluorescent dye is used. In cell proliferation, as the stained proteins are separated during mitosis, the strength of the fluorescent signal is gradually reduced (Fiure-22). Cell proliferation that can be assessed by flow cytometry is demonstrated by an increase in the number of low-fluorescence cells (Elzagallaai et al., 2009).

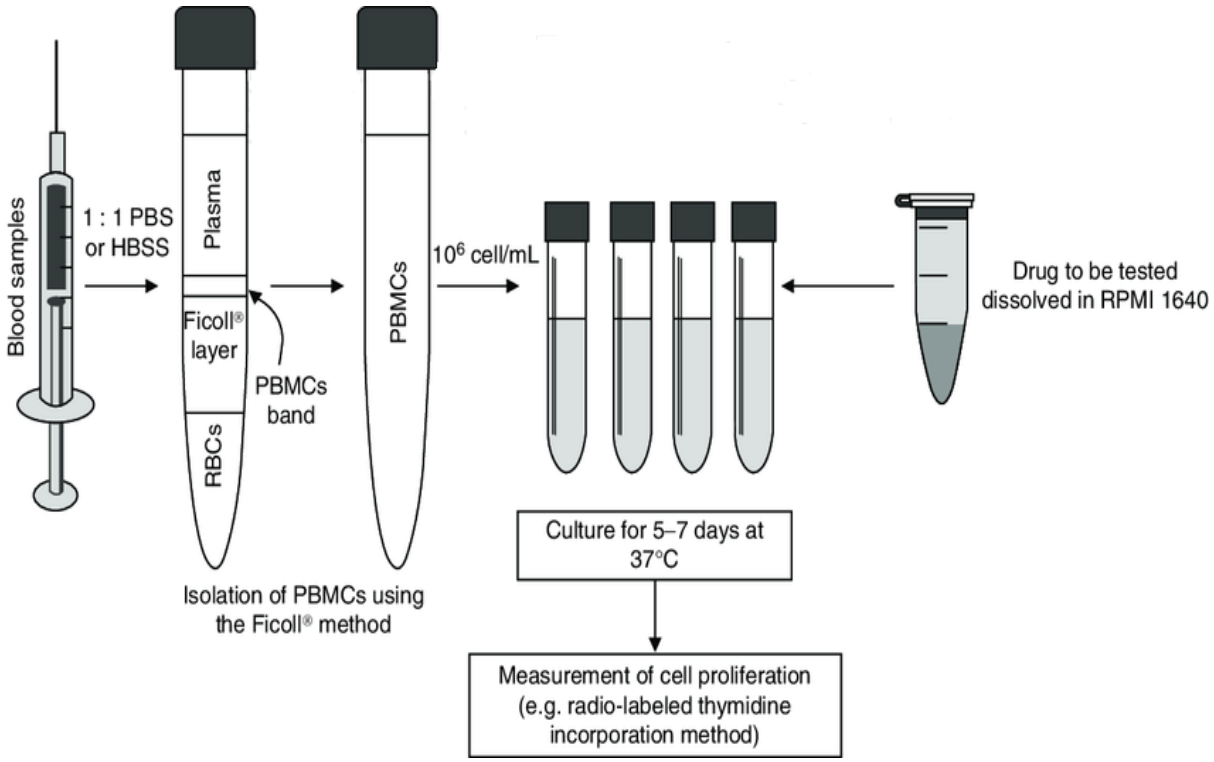


*Figure 22: Lymphocyte proliferation technique (Elzagallaai et al., 2009)*

Extracts of *T. cordifolia* have also the ability of speeding up the proliferation of lymphocytes (Table-13). Aranha et al. (2012) experimented whether this plant has any effect on the proliferation of lymphocytes and they found that a protein derived from the aqueous extract of this plant shows an increased (almost three-fold) proliferation rate of leukocytes. According to Aher and Wahi (2012), the ethanolic extract of this plant is also able to boost the proliferation rate in male Wister rats.

*Table 13: Assays on the effect of Tc on lymphocyte proliferation*

| **References** | ***In-vivo/in-vitro*** | **Extract /compound used** | **Dose** | **Statistical analysis** | **Findings** |
|---|---|---|---|---|---|
| (Aranha et al., 2012) | *In vivo* (BALB/c mice) | Purified protein from the aqueous extract of Tc. | 1,5 and 10 µg/ml | • Statistics were presented as Mean ± SD.<br>• One-way analysis of variance (ANOVA) was used to analysis the results. | • Nearly 3-fold increase in the lymphocyte proliferation after the treatment of the sample at 10 µg/ml of concentration. |
| (Aher & Wahi, 2012) | *In-vivo* (Male Wister rats weighing 150-180 g) | Ethanolic extract. | 100 mg/Kg | • Sigma State software was used to analyze the data.<br>• The statistics were presented as mean ± SD.<br>• Value of significance was $p<0.05$. | • The administration of Tc-ethanolic extract increased the spleen lymphocytes proliferation as compared to the vehicle-treated group. |

## 4.7 Effects on the expression and secretion of TNF-α

TNF-α is an inflammatory cytokine which is usually produced by monocytes and macrophages. This cytokine is responsible for the apoptosis or necrosis of a damaged cells and in plays a very important role to prevent infection and cancer (Cawthorn & Sethi, 2008). Optimal expression of this cytokine can assist in the defense system very effectively. One of the most important regulatory proteins in the immune system of animal cells is tumor necrosis factor-a TNF-α. Although previous observations in the 19th century had already shown that heat-killed bacteria could be used to induce tumor regression in cancer patients, it was discovered in 1975 by Lloyd

Old and his colleagues because of its anti-tumor activity. It is also currently understood that TNF-α mediates tumor initiation, metastasis and inflammation. A broad variety of inflammatory disorders, including asthma, dermatitis, cystic fibrosis, rheumatoid arthritis, inflammatory bowel/Crohn's disease, multiple sclerosis, psoriasis, systemic lupus erythematosus, type II diabetes, atherosclerosis, Alzheimer's disease, osteoporosis and autoimmune deficiency, have been shown by comprehensive studies of cell signaling cascades. Figure-21 shows the cascade of TNF-a and signaling. The binding of TNF-a to its TNFR receptor results in TRADD, TRAF2 and RIP recruitment (Figure-23). This is accompanied by IKK activation, IjBa binding to NFjB, IjBa phosphorylation and NFjB release and transport into the nucleus, which binds to many (inflammatory) targets genes. The binding of LPS to LBP and subsequently to CD14 and TLR4 activates JNK, p38MAPK and ERK1/2 signaling pathways for upregulation of TNF-α through activation of NFjB or certain other pathways. TRAF2 recruitment can also activate the JNK, p-38MAPK, ERK1/2 and AKT pathways for TNF-α regulation (Iqbal, Verpoorte, Korthout, & Mustafa, 2013).

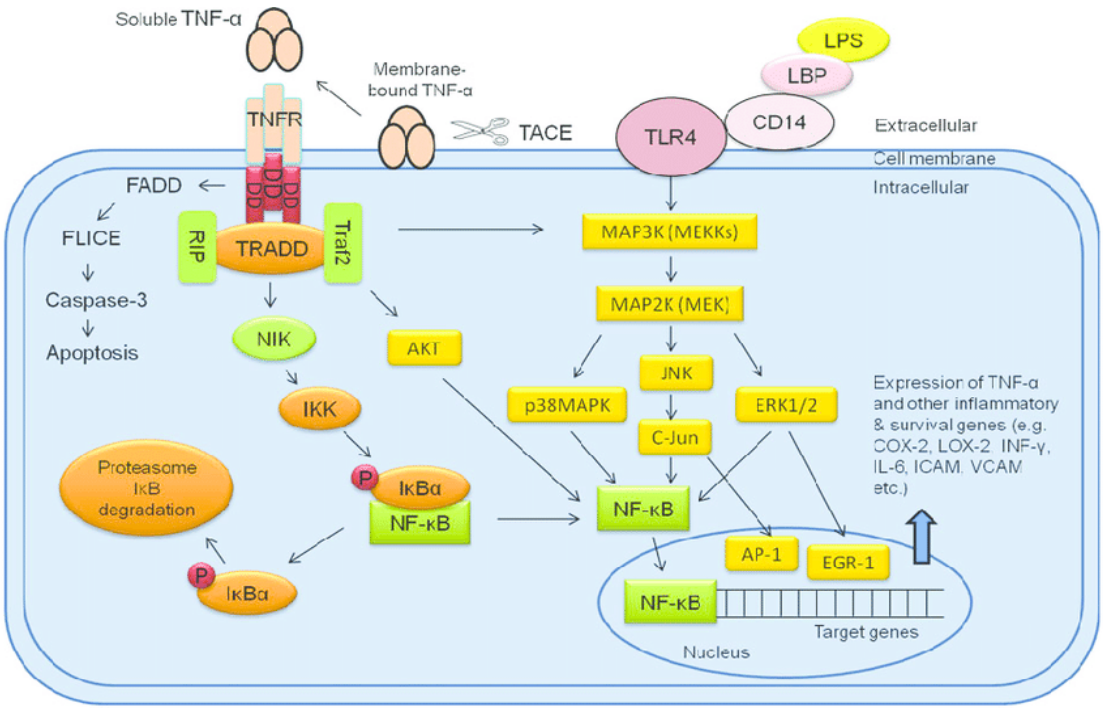


*Figure 23: Signaling cascade of TNF-α (Iqbal et al., 2013)*

While doing an assay on the effects of *T. cordifolia* on the expression and secretion of TNF-α Desai et al. (2007) found that the expression of TNF-α was increased in a concentration dependent manner. Moreover, Gupta et al. (2016) did same type of experiment and they found an upregulated expression rate of TNF-α after the treatment of Tc extract (Table-14).

*Table 14: Assays on the effect of Tc on the expression of TNF-α*

| **References** | ***In-vivo/in-vitro*** | **Extract /compound used** | **Dose** | **Statistical analysis** | **Findings** |
|---|---|---|---|---|---|
| (Desai et al., 2007) | *In-vitro* | G1-4A polysaccharide was collected and purified from the methanolic extract of Tc. | 2.5, 5.0, 12.5, 25.0 mg/kg | • Mean ± SD<br>• One-way analysis of variance (ANOVA)<br>• P<0.05 was considered as statistically significant. | • The secretion of TNF-α was increased in a concentration dependent manner. |
| (Gupta et al., 2016) | *In-vitro* | G1-4A polysaccharide was collected and purified from the methanolic extract of Tc. | Sufficient amount of G1-4A. | • The statistics were presented as mean ± S.E.M.<br>• Data were analyzed by student's t-test. | • TNF-α, IFN-γ and NOS2 expression and secretion were upregulated after the administration of the polysaccharide in the MTB infected lung cell. |

## 4.8 Effects on the adhesion of neutrophils

The emigration of neutrophils at sites of inflammation apparently requires intercellular adhesion. Initially, leukocyte adherence is observed in post capillary venules where neutrophils

roll along the luminal surface of the endothelial cells before stopping, changing shape, and migrating into the perivascular tissue (Abbassi, Kishimoto, Mclntire, & Smith, 1993). The most abundant type of circulating white blood cells is neutrophilic granulocytes. They release neutrophil extracellular traps (NETs), web-like structures comprised of decondensed chromatin decorated with antimicrobial proteins, in a process called NETosis. Until both the nuclear envelope and the outer cell membrane rupture, the nuclear chromatin swells during NETosis. NETosis, as neutrophils can bind and destroy bacteria and other pathogens through NETs, is considered an essential immune defense mechanism. There are some additional NET-inducers such as phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharides (LPS), which induce NETosis in vitro, in addition to physiological stimuli such as pathogens, chemokine activated platelets or urea crystals (Figure-24). Although NETosis was originally identified as part of the inherent immune defense mechanism, today we know that dysregulated NETosis is also involved in a number of chronic inflammatory and autoimmune diseases Such as atherosclerosis, preeclampsia, systemic lupus erythematosus, as well as malignancies. Therefore, it is highly important to question which environmental factors play a role in this process and may affect the course of diseases (Erpenbeck et al., 2019).

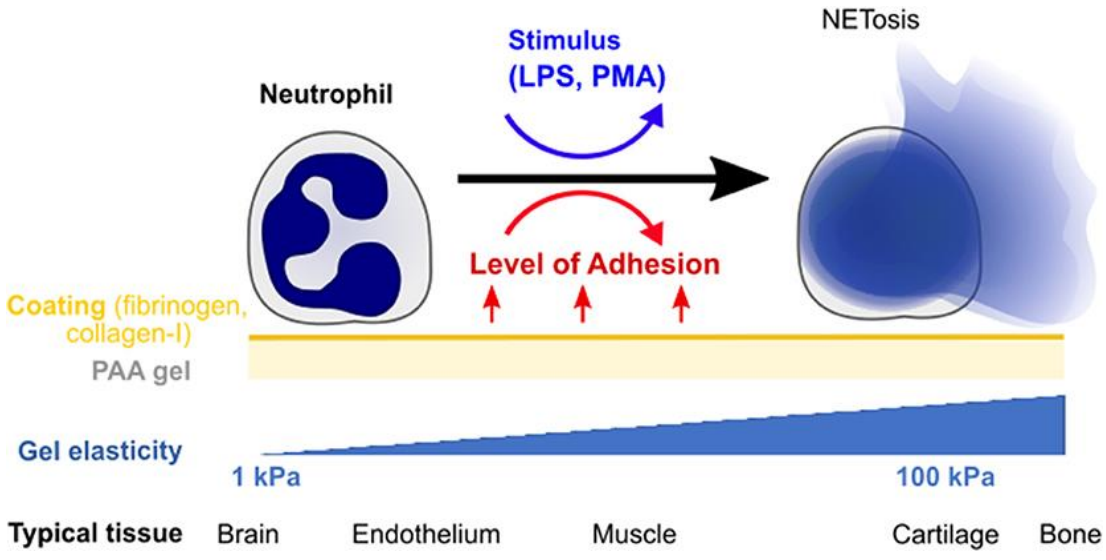


*Figure 24: Adhesion, stimulation and elasticity of neutrophils (Erpenbeck et al., 2019)*

So, higher adhesion of neutrophils indicates quicker and increased action of our defense system. Bishayi et al. (2002) did an *in vitro* experiment and found that the adhesion of neutrophils was started to increase after the treatment of a T. cordifolia derived polysaccharide (G1-4A). On the other hand, Mehta et al. (2011) experimented neutrophil adhesion test *in vivo* using Wister albino rats and found a significant ($p<0.05$) increase in the adhesion of neutrophils after treating the rats with *T. cordifolia* methanolic and ethanolic extracts (Table-15).

*Table 15: Assays on the effect of Tc on the adhesion of neutrophil*

| **References** | ***In-vivo/in-vitro*** | **Extract /compound used** | **Dose** | **Statistical analysis** | **Findings** |
|---|---|---|---|---|---|
| (Bishayi et al., 2002) | *In vitro* | Polysaccharide isolated from Tc extract | 100mg/kg | • P<0.05 was considered as statistically significant. | • A gradual increase in the adhesion of neutrophil was seen after the administration of the polysaccharide. |
| (Mehta et al., 2011) | *In vivo* (Wistar albino rats) | Ethanolic extract and methanolic extract. | 100, 200, 300 mg/kg. | • Data were expressed as mean ± SD<br>• Results were analyzed by ANOVA. | • After the administration of Tc extract the percentage of neutrophil adhesion increased in a concentration dependent manner compared to the control group. |

## 4.9 Effects on delayed type hypersensitivity reaction

Delayed type hypersensitivity (DTH) is an important *in vivo* manifestation of cell-mediated immunity. The development of DTH elicited by immunization or infection with intracellular parasites is mediated by antigen-specific T cells and involves the synthesis of activating and

chemotactic cytokines, increase in vascular permeability and recruitment of antigen non-specific effector cells to the site of the reaction. The reaction can be subdivided into tuberculin-type, Jones–Mote type and contact sensitivity. Tuberculin-type hypersensitivity is normally indurated, being characterized by a prominent mononuclear infiltration and substantial fibrin deposition. A sustained response that peaks at 48 hours is maintained for several days (Jacysyn, Abrahamsohn, & Macedo, 2001). Type IV HS is immunopathological damage that occurs approximately 24-72 hours after an antigen is presented to a sensitized person (hence, "delayed-type" hypersensitivity or DTH).

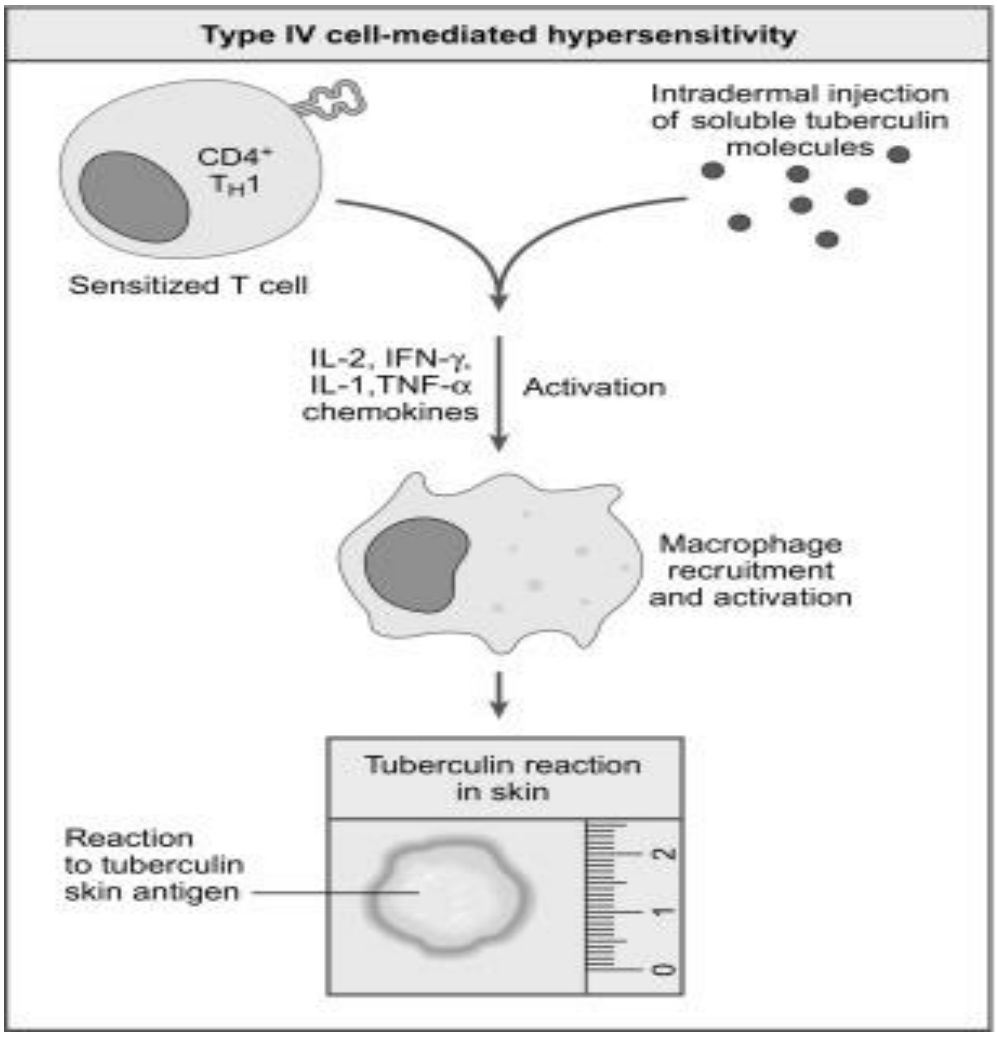


*Figure 25: Macrophage and antigen interaction in Type-IV Hypersensitivity reaction (Jayapal & Jayapal, 2007)*

Histologically, type IV HS reactions are differentiated by the penetration of basophils and mononuclear cells at the exposure site from other HS reactions. Indeed, unlike (all antibody-mediated) Type I, II, and III HS, Type IV HS mainly results from the activities of effector T cells and macrophages. Among the first to show that Type IV HS reactions were cell-mediated rather than antibody-mediated were Landsteiner and colleagues (Figure-25). These scientists transferred peritoneal exudate cells to a recipient from a host sensitized with antigen A. The animal displayed signs of a type IV HS reaction when the recipient was then challenged with antigen A. However, if serum from the sensitized animal was transferred instead, no anti-A DTH response occurred (Jayapal & Jayapal, 2007).

*Table 16: Assays on the effect of Tc on DTH reaction*

| **References** | ***In-vivo/in-vitro*** | **Extract /compound used** | **Dose** | **Statistical analysis** | **Findings** |
|---|---|---|---|---|---|
| (Mehta et al., 2011) | *In vivo* (Wistar albino rats) | Ethanolic extract and methanolic extract. | 50, 100, 200 and 300 mg/kg | • Data were expressed as mean ± SD<br>• Results were analyzed by ANOVA. | • Significant and dose-dependent increase in DTH activity was observed after the treatment of ethanolic and methanolic extract of *T. cordifolia.* |
| (Siddiqui et al., 2008) | *in vivo* (Swiss albino mice) | Ethanolic extract. | 100 mg/kg | • Data expressed as mean ± S.E.M.<br>• The results were analyzed by ANOVA and followed by Dunnett's test. | • The foot pad thickness was increased significantly after 24 h of treatment and normalized within 48 h. |

So, higher DTH reaction indicates high efficiency of cell mediated immunity. Mehta et al. (2011) said that during doing an experiment they found the alcoholic extracts of *T. cordifolia* to increase DTH in a dose dependent manner. Siddiqui et al. (2008) also did the same experiment and found that the thickness of mice foot pad enhanced after the treatment of *T. cordifolia* extracts (Table-16).

## 4.10 Other immune system modifying effects of Tc

Lots of assays have been performed on this plant to check its immunomodulatory effects (Table-17). Upadhyaya et al. (2011) found that this plant also has cytotoxic effect by which it can assist the cell mediated immunity response in a mouse. The alcoholic extract of this plant can also increase the number of mitochondrial antioxidant marker enzymes like glutathione, superoxide dismutase, catalase etc. and administration of *T. cordifolia* extracts in male Wister rats causes an increased expression and secretion of melatonin which confirms its immunomodulatory activities (Aher & Wahi, 2012)**.** Moreover, this plants extracts can increase the macrophage infection rate toward the bacterial burden in lung of mice and increase the level of IFN-γ (Gupta et al., 2016)**.** Furthermore, this plant can also increase pinocytosis rate of macrophages alongside increasing the phagocytosis (More & Pai, 2017). Bishayi et al. (2002) proved that T. cordifolia extracts are able to increase the chemotactic index in CCl4 intoxicated rats. Mehta et al. (2011) showed Tc extracts also gives promising results in carbon clearance test showing increased phagocytic index. Increased bone marrow cellularity indicates increased production of leukocyte cells. Mathew and Kuttan (1999) found out that *T. cordifolia* extracts also increse bon marrow cellularity in BALB/c rats.

*Table 17: Other assays which may prove Tc as an immunomodulator*

| **Assays** | ***In-vivo/in-vitro*** | **Extract /compound used** | **Dose** | **Statistical analysis** | **Findings** |
|---|---|---|---|---|---|
| In-vitro Cytotoxicity Assay (Upadhyaya et al., 2011). | *In vitro* | Aqueous Extract | 100 µg/ml, 50 µg/ml, 1 µg/ml, 0.1 µg/ml | • The results were presented as mean ± SD.<br>• The statistics were analyzed by student's t-test. | • The cytotoxic activity was increased in a dose dependent manner. |
| Estimation of antioxidant marker enzymes (Aher & Wahi, 2012). | *In-vivo* (Male Wister Rat) | Ethanolic extract. | 100 mg/Kg | • The results were presented as mean ± SD.<br>• The statistics were analyzed by student's t-test. | • After the sensation with SRBC the levels of glutathione, superoxide, dimusate and catalase were decreased.<br>• The levels were increased after the treatment of Tc extract. |
| SDS-PAGE analysis (Aher & Wahi, 2012). | *In-vivo* (Male Wister rats) | Ethanolic extract. | 100 mg/Kg | • The results were presented as mean ± SD.<br>• The data were analyzed by Student's t-test. | • At 248 kDa a thick band was observed after the treatment of Tc extract which indicates an enhanced secretion of melatonin and its immunomodulatory activity gets confirmed. |
| Macrophage infection and CFU | *In vivo* (femal | G1-4A was isolated | Sufficient amount | • The results were | • The bacterial burden in the lung of the mouse |

(Table-17 continued)

| | | | | | |
|---|---|---|---|---|---|
| assay (Gupta et al., 2016) | e BALB /c mice.) | from Tc extracts. | of G1-4A | presented as mean ± SD.<br>• The data were analyzed by student's t-test. | reduced day by day and the burden was minimum on day 60. |
| In vitro restimulation Assay (Gupta et al., 2016) | *In vitro* | G1-4A was isolated from the methanolic extract of Tc. | Sufficient amount of G1-4A. | • The results were presented as mean ± SD.<br>• The data were analyzed by student's t-test. | • The level of IFN-γ was significantly higher in the G1-4A treated group than the untreated group. |
| Pinocytosis assay (Measurement of pinocytic rate) (More & Pai, 2017). | *In vitro* | Aqueous extract | 80 µg/mL | • Experiments were presented as mean ±SD<br>• Significance of the data determined by student's t-test | • Tc extract treated macrophages showed an increased pinocytic rate.<br>• A significant increase was seen after 48 h of the treatment. |
| determination of chemo-tactic index (Bishayi et al., 2002) | *In vitro* | Polysaccharides were isolated from the methanolic extract of Tc. | 100 mg/kg | • P<0.05 was considered as the value of significance.<br>• Results were presented as Mean ± SD | • A significant increase in the chemotactic index of the CCl4 intoxicated rats was seen after the treatment with the extract. |
| Carbon clearance test (Mehta et al., 2011) | *In vivo* (Wistar albino rats) | Ethanolic and methanolic extract. | 100, 200, 300 mg/kg | • P<0.05 was considered as the value of significance.<br>• Results were presented as Mean ± SD | • All the treated group with various extracts showed significantly high phagocytic index in a dose dependent manner. |

(Table-17 continued)

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|---|---|---|---|---|---|
| Effect on plaque-forming cells (Mathew & Kuttan, 1999) | *In vivo* (BALB/c mice) | Methanolic extract | 200 mg/kg daily for 5 days. | • results are expressed as mean ± SD after repeating the experiment for two times.<br>• Data were analyzed by Student's t-test. | • The number of antibodies producing cells was increased significantly (P > 0.001) after the treatment. |
| Effect on bone marrow cellularity and esterase activity (Mathew & Kuttan, 1999) | *In vivo* (BALB/c mice) | Methanolic extract | 200 mg/kg daily for 5 days. | • results are expressed as mean ± SD after repeating the experiment for two times.<br>• Data were analyzed by Student's t-test. | • Bone marrow cellularity of the treated group were increased by 10 cells/ femur than the control group. |

# Chapter 5

## Discussion

No nation or country can neglect the potential of medicinal plants even if they are very rich in the production of alternative and synthetic medicines. Medicinal plants have been used since the ancient times to support our immune system. Although the present time has much more medical technologies and other advanced facilities, we are still not safe from the antibiotic resistance tragedies or the serious immunological side effects of alternative immunity stimulating medicines. Here comes the importance of immunomodulatory plants. There are a lot of plant having immunomodulatory properties but most of the time people are not acknowledged about that. However, *T. cordifolia* is one of those plants which has effective immunomodulatory properties but mainly used for its anti-diabetic, anti-inflammatory, hyperlipidemia, gout, peptic ulcer, rheumatoid arthritis etc. This plant can boost our immune system very effectively. I think the 'chapter-4' part of this review has a lot of evidences to support this statement. This plant contains some components like cordifolioside A, cordifolioside B, magnoflorine, tinocordiside, G1-4A, cordioside, ecdysteron etc. which have stimulatory effects on our immune system (Table-4). The various types of extracts of this plant are proved to enhance the phagocytic activity of macrophages (Table-8). Moreover, it also increases the activation rate of macrophages. Therefore, this plant is able to make our body defense system in a way that the invaded pathogens will be rapidly phagocytized. This plant has also the ability of boosting antibody production against the invaded pathogens (Table-9). So, this plant is also effective to improvise our body defense system to be more specific attack against specific antigens. The extracts of this plants are also proved to increase the production of nitric oxide in phagocytic cells which indicates increased phagocytic ability of the phagocytic cells (Table-10). Moreover, this plant also increases the production of cytokines (Table-11) and that means after administering this plant extracts our body defense system will

give the humoral response more rapidly and effectively. Furthermore, it boosts the production of WBCs which are the base component of our defense system (Table-12). However, this plant is also proved to increase lymphocyte proliferation, TNF-α expression, neutrophil adhesion, cytotoxic t cell activity, mitochondrial antioxidant marker enzyme production, expression of melatonin, macrophage infection rate, pinocytosis, chemotaxis of neutrophils, plaque forming cells and bone marrow cellularity (Table-13 to Table-17). It is now very clear that *T. cordifolia* can improvise our humoral immune response very effectively. This plant doesn't kill the invaded pathogens directly rather it improvises or modifies our immunity in a way that the body can fight against invaded pathogens or other immunological conditions very effectively. Therefore, this plant is a good immunomodulator and its use as immunomodulator should be increased.

# Chapter 6

# Conclusion

Immunological disorders, viral infections, bacterial infections and the infections of other pathogens are increasing nowadays. A good example is Covid-19 infection. To fight against these infections, we must have a good body defense system. If we take antibiotics extensively, we will be fallen in the trap of super-bug. Moreover, the adverse effects of the long-term intake of synthetic antiviral or antifungal drugs are not ignorable. To be prepared with a good body defense system, we must get the help of medicinal plants with immunomodulatory properties. There are many plants having immunomodulatory properties are being used in purpose of boosting immune system. Immunomodulatory activity is not the main activity of *T. cordifolia* plant but if we use this as an immune system modulator for its effectiveness, we will find one more option to boost our immunity. There are many assays performed on the immunomodulatory activities of this plant which showed very positive results (Chapter-4). Unfortunately, almost all *in-vivo* assays were performed in animals and I rarely found any trial in human. The possible reason behind this is people are using this medicinal plant with importance but not that much as an immunomodulatory purpose. Therefore, the main purpose of this review is to make people acknowledge about the importance of this plant as an immunomodulator. As the assays (which were performed in animals) looked very promising, I am very optimistic about a positive result in the future assays in human. I want to direct the future researchers to do more study on the immunosuppressive activity of this plant and also more comparative study with synthetic immunomodulator should be performed.

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