1.1 INTRODUCTION

India has an ancient heritage of traditional medicine that the knowledge of Ayurveda is accepted as the oldest written medicinal system, and its prescriptions are that also supposed to be more effective in certain cases than modern therapies. Charaka Samhita is the first recorded treatise on Ayurveda. It consists of eight sections, divided into 150 chapters and describes 341 plants used in medicine. The second treatise on Ayurveda is Sushruta Samhita (1800BC) with special emphasis on surgery. It has six sections, covering 186 chapters and describes 395 medicinal plants, 57 drugs of animal origin, 64 minerals and metal as drugs. The next important authority on Ayurveda was Bagbahtta of Sind who practiced during about 17th century his manuscript entitled "Astanga Haridaya" is considered unrivaled for principles and practice of medicine. The manuscript is divided into six sections covering 120 Chapter and contains 7444 verses. Like Ayurveda, the Unani system of medicine was based on ancient principle and it origin to Greece ancient "Theory of four humour" of Hippocrates (460-3777 BC) refined and expounded centuries later by Galen (131-200 A.D), physician of Roman emperor Marcus Aurelis. The spread of Islamic across much of the ancient world from Andalusin to Persia between 8th and 11th centaury greatly facillated the revival of ancient Greek medicine and its further development. Johanna Ibn (777-857 A.D.) translated the Greek manuscript into Arabic and wrote a medicinal book.¹

The traditional medicine all over the world is nowadays revalued by extensive research on different plant species and their therapeutic principles. The World Health Organization has estimated that up to 80% of the world’s population depends on traditional medicine for primary health care because such drugs are easily available at low cost, comparatively safe due to their negligible side effects and, moreover, people have faith in such remedies. There has been a dramatic revival in recent years in the use of herbal preparations for the treatment of a wide range of ailments. Some products are being recommended for use as specifics to treat particular illnesses or conditions, much like conventional synthetic medications are prescribed in North America, Western Europe and the rest of the economically developed world.²

Nevertheless, there are a fair number of traditional medical systems that have incorporated routine use of adjuvant preparations or treatments into their health care delivery systems. All of these preparations are used to keep body in proper tune so to speak. In the traditional–Hindu medical (Ayurvedic) system of India they are called Jamu. In fungshui system of China, in which one seeks to balance between man and nature to create a harmonious
environment, they are called Zi bu or huifu (meaning a tonic and restorative, respectively in Mandarin) and in Russian Toniziruyuxhie sredstva (meaning tonic substances). Each of these connotes or means that which makes new again, or that which helps restore ones youth full state of physical and mental health as well as helps expand a state of happiness.

At least 25% of the prescription drugs issued in USA and Canada contain bioactive compounds that are derived from or modeled after plant natural products. Many of these drugs were discovered by following lead provided from indigenous knowledge systems. Historically ethno-botanicals leads have resulted in different types of drug discovery

<table>
<thead>
<tr>
<th>Drug</th>
<th>Medicinal use</th>
<th>Plant source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ajmaline</td>
<td>For heart arrhythmia</td>
<td>Rauwolfia spp.</td>
</tr>
<tr>
<td>Atropine</td>
<td>Pupil dilator</td>
<td>Atropa belladonna</td>
</tr>
<tr>
<td>Benzoin</td>
<td>Oral disinfectant</td>
<td>Styrax tonkinensis</td>
</tr>
<tr>
<td>Caffeine</td>
<td>CNS stimulant</td>
<td>Camellia sinensis</td>
</tr>
<tr>
<td>Camphor</td>
<td>For rheumatic pain</td>
<td>Cinamomum camphora</td>
</tr>
<tr>
<td>Cascara</td>
<td>Purgative</td>
<td>Rhamnus purshiana</td>
</tr>
<tr>
<td>Cocaine</td>
<td>Ophthalmic anesthetic</td>
<td>Erythroxylon coca</td>
</tr>
<tr>
<td>Codeine</td>
<td>Analgesic, antitussive</td>
<td>Papaver somniferum</td>
</tr>
<tr>
<td>Colchicine</td>
<td>For gout</td>
<td>Colchicum autumnale</td>
</tr>
<tr>
<td>Demecolcine</td>
<td>For leukemia, lymphomata</td>
<td>Colchicum autumnale</td>
</tr>
<tr>
<td>Deserpidine</td>
<td>Antihypertensive</td>
<td>Rauwolfia canescens</td>
</tr>
<tr>
<td>Dicoumarol</td>
<td>Anti thrombotic</td>
<td>Melilotus officinalis</td>
</tr>
<tr>
<td>Digoxin</td>
<td>For atrial fibrillation</td>
<td>Digitalis purpurea</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>For atrial fibrillation</td>
<td>Digitalis purpurea</td>
</tr>
<tr>
<td>Emetine</td>
<td>For amoebic dysentery</td>
<td>Psychotria ipecacuanha</td>
</tr>
<tr>
<td>Ephedrine</td>
<td>Bronchodilator</td>
<td>Ephedra sinica</td>
</tr>
<tr>
<td>Eugenol</td>
<td>For toothache</td>
<td>Syzygium aromaticum</td>
</tr>
<tr>
<td>Gallotanins</td>
<td>Haemorrhoid Suppository</td>
<td>Hamamelis virginia</td>
</tr>
<tr>
<td>Hyoscyamine</td>
<td>Anticholinergic</td>
<td>Hyoscyamus niger</td>
</tr>
<tr>
<td>Ipecac</td>
<td>Emetic</td>
<td>Psychotria ipecacuanha</td>
</tr>
<tr>
<td>Compound</td>
<td>Action</td>
<td>Plant</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>Ipratropium</td>
<td>Bronchodilator</td>
<td><em>H. niger</em></td>
</tr>
<tr>
<td>Morphine</td>
<td>Analgesic</td>
<td><em>Papaver somniferum</em></td>
</tr>
<tr>
<td>Noscapine</td>
<td>Antitussive</td>
<td><em>Papaver somniferum</em></td>
</tr>
<tr>
<td>Papain</td>
<td>Attenuator of mucus</td>
<td><em>Carica papaya</em></td>
</tr>
<tr>
<td>Papaverine</td>
<td>Antispasmodic</td>
<td><em>Papaver somniferum</em></td>
</tr>
<tr>
<td>Physostigmine</td>
<td>For glaucoma</td>
<td><em>Physostigma venenosum</em></td>
</tr>
<tr>
<td>Picrotoxin</td>
<td>Barbiturate antidote</td>
<td><em>Anamirta cocculus</em></td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>For glaucoma</td>
<td><em>Pilocarpus jaborandi</em></td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>For condyloma acuminatum</td>
<td><em>Podophyllum paltatum</em></td>
</tr>
<tr>
<td>Proscillaridin</td>
<td>For cardiac malfunction</td>
<td><em>Drimia maritima</em></td>
</tr>
<tr>
<td>Protoveratrine</td>
<td>Antihypertensive</td>
<td><em>Veratrum album</em></td>
</tr>
<tr>
<td>Pseudo ephedrine</td>
<td>For rhinitis</td>
<td><em>Ephedra sinica</em></td>
</tr>
<tr>
<td>Psoralen</td>
<td>For vitiligo</td>
<td><em>Psoralea corylifolia</em></td>
</tr>
<tr>
<td>Quinine</td>
<td>For malaria</td>
<td><em>Cinchona Pubescens</em></td>
</tr>
<tr>
<td>Quinidine</td>
<td>For cardiac arrhythmia</td>
<td><em>Cinchona Pubescens</em></td>
</tr>
<tr>
<td>Rescinnamine</td>
<td>Antihypertensive</td>
<td><em>Rauwolfia serpentina</em></td>
</tr>
<tr>
<td>Reserpine</td>
<td>Antihypertensive</td>
<td><em>Rauwolfia serpentina</em></td>
</tr>
<tr>
<td>Sennoside A, B</td>
<td>Laxative</td>
<td><em>Cassia angustifolia</em></td>
</tr>
<tr>
<td>Scopololamine</td>
<td>For motion sickness</td>
<td><em>Datura stramonium</em></td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>Steroidal precursor</td>
<td><em>Physostigma venenosum</em></td>
</tr>
<tr>
<td>Strophanthin</td>
<td>For congestive heart failure</td>
<td><em>Strophantus gratus</em></td>
</tr>
<tr>
<td>Tubocurarine</td>
<td>Muscle relaxant</td>
<td><em>Chondrodendron tomentosum</em></td>
</tr>
<tr>
<td>Teniposide</td>
<td>For bladder neoplasm</td>
<td><em>Podophyllum paltatum</em></td>
</tr>
<tr>
<td>Theophylline</td>
<td>Diuretic, antiasthematic</td>
<td><em>Camellia sinensis</em></td>
</tr>
<tr>
<td>Toxiferine</td>
<td>Relaxant in surgery</td>
<td><em>Strychnos guianensis</em></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>For Hodgkin’s disease</td>
<td><em>Catharanthus roseus</em></td>
</tr>
<tr>
<td>Vincristine</td>
<td>For Paediatric leukaemia</td>
<td><em>Catharanthus roseus</em></td>
</tr>
</tbody>
</table>
The search for new drugs is an important way to find a better treatment for many diseases such as cancer, heart disease, AIDS, infectious disease, etc. New drugs can be discovered in various ways, for instance, using the synthesis of combinatorial libraries of compounds, the rational synthesis of compounds based on a particular molecule target, and computer-based molecular modeling design. Standard medicinal chemistry approaches and the discovery of new bioactive compounds from nature are also used to find new drugs. The search for a new drug from nature is based on a biological and ecological rationale, since natural sources such as plants, microorganisms, and marine species produce bioactive compounds as their defense substances and for other purposes.

For instance, plants that must coexist with animals and microorganisms have developed defense strategies to assist in their survival in a competitive environment, and one of the common strategies is the production of toxic and other bioactive compounds.

Natural products have thus played an important role in drug discovery in the past and promise to provide still more drugs in the future. This significance is supported by a report that natural compounds, their derivatives, and their analogs represent over 50% of all drugs in clinical use, with higher plant-derived natural substances representing about 25% of the total. A recent review has listed 32 natural products or their analogs which are in clinical use or in clinical trials as antitumor agents over the last few years. Another survey indicates that 39% of new drugs approved between 1983 and 1994 are of natural origin, consisting of original natural compounds, semi synthetic and synthetic drugs based on natural product models. The discovery of novel drugs from nature is also important because many isolated molecules are quite complex, and would not be obtained by a simple synthetic approach. In some cases, the isolated lead compounds may not be potent enough to be drugs in their own right, but they can serve as pharmacophores for chemical modification and drug design, which often yield clinically useful drugs.

1.2 HISTORY OF IMMUNITY

The concept of immunity has intrigued mankind for thousands of years. Between the time of Hippocrates and the 19th century, when the foundations of the scientific method were laid, diseases were attributed to an alteration or imbalance in one of the four humors (blood, phlegm, yellow bile or black bile). Also popular during this time was the miasma theory, which held that diseases such as cholera or the Black Plague were caused by a miasma, a noxious form of "bad air". If someone was exposed to the miasma, he or she could get the disease.
The modern word “immunity” is derived from the Latin word *immunis*, meaning exemption from military service, tax payments or other public services. The first written descriptions of the concept of immunity may have been made by the Athenian Thucydides who, in 430 BC, described that when plague hit Athens “the sick and the dying were tended by the pitying care of those who had recovered, because they knew the course of the disease and were themselves free from apprehensions. For no one was ever attacked a second time, or not with a fatal result”. The term “immunes”, is also found in the epic poem “Pharsalia” written around 60 B.C. by the poet Marcus Annaeus Lucan to describe a North African tribe’s resistance to snake venom.

The first clinical description of immunity which arose from a specific disease causing organism is probably *Kitab fi al-jadari wa-al-hasbah* (A Treatise on Smallpox and Measles, translated 1848) written by the Islamic physician Al-Razi in the 9th century. In the treatise, Al-Razi describes the clinical presentation of smallpox and measles and goes on to indicate that that exposure to these specific agents confers lasting immunity (although he does not use this term). However, it was with Louis Pasteur’s Germ theory of disease that the fledgling science of immunology began to explain how bacteria caused disease and how, following infection, the human body gained the ability to resist further infections.

The birth of active immunotherapy may have begun with Mithridates VI of Pontus. To induce active immunity for snake venom, he recommended using a method similar to modern toxoid serum therapy-by drinking the blood of animals which fed on venomous snakes. According to Jean de Maleissye, Mithridates assumed that animals feeding on venomous snakes acquired some detoxifying property in their bodies, and their blood must contain attenuated or transformed components of the snake venom. The action of those components might be strengthening the body to resist against the venom instead of exerting toxic effect. Mithridates reasoned that, by drinking the blood of these animals, he could acquire similar resistance to snake venom as the animals feeding on the snakes. Similarly, he sought to harden himself against poison, and took daily sub-lethal doses to build tolerance. Mithridates is also said to have fashioned a ‘universal antidote’ to protect him from all earthly poisons. For nearly 2000 years, poisons were thought to be the proximate cause of disease, and a complicated mixture of ingredients, called Mithridate, was used to cure poisoning during the Renaissance. An updated version of this cure, Theriacum Andromachi, was used well into the 19th century. In 1888, Emile Roux and Alexandre Yersin isolated diphtheria toxin and, following the 1890 discovery by
Behring and Kitasato of antitoxin based immunity to diphtheria and tetanus, the antitoxin became the first major success of modern therapeutic immunology. In Europe, the induction of active immunity emerged in an attempt to contain smallpox. Immunization, however, had existed in various forms for at least a thousand years. The earliest use of immunization is unknown. However, around 1000 A.D. the Chinese began practicing a form of immunization by drying and inhaling powders derived from the crusts of smallpox lesions. Around the fifteenth century in India, the Ottoman Empire, and east Africa, the practice of variolation (poking the skin with powdered material derived from smallpox crusts) became quite common. Variolation was introduced to the west in the early 18th century by Lady Mary Wortley Montagu. In 1796, Edward Jenner introduced the far safer method of inoculation with the cowpox virus, a non-fatal virus that also induced immunity to smallpox. The success and general acceptance of Jenner's procedure would later drive the general nature of vaccination developed by Pasteur and others towards the end of the 19th century.

The immune system is primarily concerned with resistance against foreign invaders and protection against neoplastic cells. Leukocytes (B- and T-lymphocytes, natural killer (NK) cells, macrophages and granulocytes) are the major cellular players involved, arising during development from stem cells within the yolk sac and then the mammalian fetal liver. Immune cells in adult mammals are produced primarily by the bone marrow, which serves both hematopoetic and immunopoetic functions. This organ is of paramount importance, as it is the only lymphoid tissue capable of providing the complete restoration of primary lymphoid tissue (spleen, thymus, lymph nodes and liver), and therefore can alone prevent adult mammal death following lethal irradiation. In most mammalian species, the bone marrow itself acts also as a primary and secondary lymphoid organ, regulating the production, differentiation, and maturation of lymphocytes9,10.

The immune system can give rise to several disorders when it is weakened or over activated (Table-1.1). For instance, a defective adaptive immune response can lead to recurrent infection despite previous encounters with antigens, to lowered tolerance to self-organs and tissues leading to organ-specific auto- immunity, and to faulty recognition and elimination of transformed cells leading to cancer. However, an over activated innate immune response can cause chronic infection because of inefficient clearing of pathogen or chronic inflammation due to an inefficient regulation or resolution of the inflammatory response. Chronic inflammation can lead to extensive non-specific destruction of neighboring tissue. In fact, inflammation and the
immune system are intimately tied. In addition, inflammation is increasingly found to be involved in the development of several chronic diseases such as arteriosclerosis, diabetes, neurodegenerative diseases and even cancer. Immune dysfunction has also been linked to conditions such as chronic pain, anxiety and depression, albeit sometimes as a consequence rather than a cause, and may be involved in other disease processes in a manner that is currently not fully understood. Indeed, its diverse influence upon health may be in part due to its evolution as a defense mechanism and in part due to its origins, possibly as a sensory organ.  

Table 1.1: Health effects of imbalances of the immune system

<table>
<thead>
<tr>
<th>IMMUNE SYSTEM STATUS</th>
<th>ATTRIBUTABLE PATHOLOGIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak</td>
<td>Prone to opportunistic infectious diseases</td>
</tr>
<tr>
<td></td>
<td>Prone to cancer establishment and tumor escape</td>
</tr>
<tr>
<td></td>
<td>Chronic inflammation and autoimmunity</td>
</tr>
<tr>
<td></td>
<td>(e.g. Type 1 diabetes)</td>
</tr>
<tr>
<td>Over activated</td>
<td>Heart disease, cancer (e.g. lymphoid)</td>
</tr>
<tr>
<td></td>
<td>Skin disease, allergies and asthma</td>
</tr>
<tr>
<td></td>
<td>Joint and tissue destruction</td>
</tr>
</tbody>
</table>

1.3 NON-SPECIFIC / NATURAL / INNATE IMMUNITY

Nonspecific immunity or resistance comprises defense mechanisms that provide a general response against invasion by a wide range of pathogens.  

Nonspecific host defenses are those innate mechanisms with which a host is genetically endowed. Examples include physical and mechanical barriers: specifically, the skin, mucous membranes, the respiratory system, gastrointestinal tract, genitourinary tract and the eye. Other nonspecific defenses include chemical barriers such as bacteriocins, β-lycin, and other polypeptides.

Inflammation, the alternative complement pathway, phagocytes, cytokines, fever, and natural killer cells are other examples of nonspecific defenses that help protect the host against microorganisms and cancer. In mammals, an infectious disease process begins with penetrance of the first defense barriers by foreign invaders. The mammalian physical and chemical barriers include the skin, mucous membranes, saliva, and gastrointestinal and other secretions, consisting of symbiotic bacteria or lytic enzymes to destroy or halt the progress of the pathogen.
After invader penetration, mammals exert similar first-line defense immunity, which is non-specific in nature and therefore does not involve prior pathogen sensitization. These reactions primarily involve myeloid and mononuclear phagocytes, which respond to chemotactic factors (produced by the affected tissue cells or the invader itself) and destroy any foreign particles or microorganisms quickly and indiscriminately via phagocytosis.\(^\text{16}\)

Phagocytic destruction involves engulfment of the invader into vacuoles and lysosomes, followed by a lethal respiratory burst reaction. This process uses H\(_2\)O\(_2\), myeloperoxidase or catalase, and halide anions to produce hypohalides, especially -OCl, a very powerful reactive oxygen species that rapidly attacks and destroys most biological molecules.\(^\text{17,18}\)

Thus, phagocytes are immune cells that engulf, or eat, pathogens or particles. To phagocytose a particle or pathogen, a phagocyte extends portions of its plasma membrane, wrapping the membrane around the particle until the entire particle is enveloped (i.e. the particle is now inside the cell). Once inside the cell, the invading pathogen is contained inside a vacuole which merges with another type of vacuole called a lysosome. The lysosome contains enzymes and acids that kill and digest the particle or organism. Phagocytes generally patrol the body searching for pathogens, but are also able to react to a group of highly specialized molecular signals, called cytokines, produced by other cells. The phagocytic cells of the immune system include Macrophages, Neutrophils and Dendritic cells.\(^\text{19-20}\)
Components of the immune system communicate with one another by exchanging chemical messengers called cytokines. The term cytokine [Greek cyto, cell, and kinesis, movement] is a generic term for the soluble protein or glycoprotein released by one cell population that acts as an intercellular mediator or signaling molecule. When released from mononuclear phagocytes, these proteins are called monokines; when released from T-lymphocytes they are called lymphokines. When produced by a leukocyte and the action is on another leukocyte, they are interleukins; and if their effect is to stimulate the growth and differentiation of immature leukocyte in the bone marrow, they are called colony-stimulating factors (CSFs). Recently cytokines have been grouped into the following categories or families: chemokines, hematopoietins, interleukins and members of tumor necrosis factors (TNF) family.22 These proteins are secreted by cells and act on other cells to coordinate an appropriate immune response. Cytokines include a diverse assortment of interleukins, interferons, and growth factors. 23
Table 1.2: Some examples and functions of Cytokines

<table>
<thead>
<tr>
<th>CYTOKINES</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon ($\alpha$, $\beta$ &amp; $\gamma$)</td>
<td>-Antiviral &amp; antiproliferative actions, mostly stimulatory e.g. growth, proliferation, chemotaxis of B and T cells, bone marrow progenitors.</td>
</tr>
<tr>
<td>Interleukins</td>
<td>-Regulate the proliferation &amp; differentiation of bone marrow progenitor cells</td>
</tr>
<tr>
<td>Colony stimulating factors</td>
<td>-Antiparasitic action, mediates inflammatory reactions, antitumor actions, stimulates B cell proliferation</td>
</tr>
<tr>
<td>(GM-CSF, G-CSF, M-CSF)</td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td></td>
</tr>
<tr>
<td>(TNF$\alpha$ &amp; $\gamma$)</td>
<td></td>
</tr>
</tbody>
</table>

Natural killer cells, often referred to as NK cells, are similar to the killer T-cell subset (CD8+ T cells). They function as effector cells that directly kill certain tumors such as melanomas, lymphomas and viral-infected cells, most notably herpes and cytomegalovirus-infected cells. NK cells, unlike the CD8+ (killer) T-cells, kill their targets without a prior "conference" in the lymphoid organs. However, NK cells that have been activated by secretions from CD4+ T cells will kill their tumor or viral-infected targets more effectively.24

The complement system is a biochemical cascade of the immune system that helps clear pathogens or marks them for destruction by other cells. The cascade is composed of many small plasma proteins, synthesized in the liver, primarily by hepatocytes, which work together:

- To trigger the recruitment of inflammatory cells.
- To tag the pathogen for destruction by coating, or opsonizing, the surface of the pathogen.
- Or to disrupt the target cell's plasma membrane resulting in cytolysis, and death, of the pathogen.

Other specific proteins include Protease C3-convertase, which is synthesized in the liver and connects to other molecules that are commonly found in bacteria but non-existent in humans, stimulating the complement system and phagocytosis.25-27

The complement system is made up of about 25 proteins that work together to “complement” the action of antibodies in destroying bacteria. Complement also helps to rid the body of antibody - coated antigens (antigen-antibody complexes). Complement proteins, which cause blood vessels to become dilated and then leaky, contribute to the redness, warmth, swelling, pain, and loss of function that characterize an inflammatory response.
Complement proteins circulate in the blood in an inactive form. When the first protein in the complement series is activated—typically by antibody that has locked onto an antigen—it sets in motion a domino effect. Each component takes its turn in a precise chain of steps known as the complement cascade. The end product is a cylinder inserted into—and puncturing a hole in—the cell’s wall. With fluids and molecules flowing in and out, the cell swells and bursts. Other components of the complement system make bacteria more susceptible to phagocytosis or beckon other cells to the area.\textsuperscript{28}

Inflammation \{Latin, \textit{inflammatio}, to set on fire\} is an important non-specific defense reaction to tissue injury, such as that caused by pathogen or wound. Acute inflammation is the immediate response of the body to injury or cell death. The cardinal signs of inflammation include redness (\textit{rubor}), warmth (\textit{calor}), pain (\textit{dolor}), swelling (\textit{tumor}), and altered function (\textit{functio laesa}).\textsuperscript{29}

Inflammation is one of the first responses of the immune system to infection or irritation. Inflammation results in the recruitment of cells, called neutrophils, to the site of injury. Neutrophils then trigger the immune system by releasing factors that summon other innate immune cells and lymphocytes. Inflammation also serves to establish a physical barrier against the spread of infection, and to promote healing of any damaged tissue following the clearance of pathogens.

Inflammation is stimulated by chemical factors released by injured cells. These factors (histamine and bradykinin) sensitize pain receptors, cause vasodilatation of the blood vessels at the scene, and attract phagocytes, especially neutrophils.\textsuperscript{30}

Inflammation is manifested by:

- An increase in blood supply to the affected area.
- Increase in capillary permeability caused by retraction of endothelial cells.
- Influx of phagocytic cells.

The increase in capillary permeability permits larger molecules to traverse across the endothelium and thus allows the soluble mediators to reach the site of infection. Similarly, the migration of leukocytes out of the capillaries into the surrounding tissues, makes them move towards the site of infection by the process of chemotaxis.\textsuperscript{31}

\textbf{1.4 SPECIFIC / ACQUIRED / ADAPTIVE IMMUNITY}

Specific resistance or immunity involves the production of specific antibodies or activation of \textit{T} cells against a particular pathogen or other foreign substance.\textsuperscript{32}
Mammals demonstrate specific, well-defined humoral and cell-mediated immune function, and possess major cellular and protein components.\textsuperscript{33-36}

Once the first-line defenders recognize a foreign entity, a specific immune response is initiated against the epitopes (cell-surface markers / receptors) of the invader by an interaction with major histocompatibility complexes (MHC I or II), interleukins, and either T-helper cells (Th) or cytotoxic T-cells (Tc). \textsuperscript{37, 38}

As mentioned previously, the macrophages can also retain a small peptide sequence of the invader to display on their cell surface. Therefore, macrophages (and also B-cells, dendritic/langerhans cells, and other IL-1 producing cells) are denoted as antigen presenting cells (APCs), capable of recognizing and presenting antigen to specific immune cells. Following phagocytosis of extracellular antigen, small peptide fragments of the invader (10-20 amino acids) are produced, which has high affinity for the MHC II transport protein within the cell. Transport of the antigen-MHC II complex to the surface of the cell allows the Th cell to recognize it as foreign, and a specific antibody mediated response could be initiated. Th cells cannot recognize antigen unless MHC II, which is termed MHC restriction, binds it. Alternatively, some APCs are poorly phagocytic, but can bind antigen to cell-surface MHC II, and present to Th cells in this manner.\textsuperscript{39}

If the antigen is endogenous (derived from inside the cell, such as viral proteins), the cell fragments the protein, but now carries high affinity for MHC I. Transport to the cell surface now allows Tc cells to recognize the foreigner, and a certain cell-mediated response ensues.\textsuperscript{40}

The immune system stores just a few of each kind of the different cells needed to recognize millions of possible enemies. When an antigen appears, those few matching cells multiply into a full-scale army. After their job is done, they fade away, leaving sentries behind to watch for future attacks.

All immune cells begin as immature stem cells in the bone marrow. They respond to different cytokines and other signals to grow into specific immune cell types, such as T cells, B cells, or phagocytes.

\textbf{1.4.1 Lymphocytes}

B-cells and T-cells are the main types of lymphocytes. B-cells work chiefly by secreting substances called \textit{antibodies} into the body’s fluids. Antibodies ambush antigens circulating the bloodstream. They are powerless, however, to penetrate cells. The job of attacking target cells—either cells that have been infected by viruses or cells that have been distorted by cancer—is left
to T-cells or other immune cells. Each B-cell is programmed to make one specific antibody. For example, one B-cell will make an antibody that blocks a virus that causes the common cold, while another produces an antibody that attacks a bacterium that causes pneumonia.

When a B-cell encounters its triggering antigen, it gives rise to many large cells known as plasma cells. Every plasma cell is essentially a factory for producing an antibody. Each of the plasma cells descended from a given B-cell manufactures millions of identical antibody molecules and pours them into the bloodstream.

An antigen matches an antibody such as key matches a lock. Some match exactly; others fit more like a skeleton key. But whenever antigen and antibody interlock, the antibody marks the antigen for destruction.

Antibodies belong to a family of large molecules known as immunoglobulins. Different types play different roles in the immune defense strategy.

- Immunoglobulin G, or IgG, works efficiently to coat microbes, speeding their uptake by other cells in the immune system.
- IgM is very effective at killing bacteria.
- IgA concentrates in body fluids—tears, saliva, the secretions of the respiratory tract and the digestive tract—guarding the entrances to the body.
- IgE, whose natural job probably is to protect against parasitic infections, is the villain responsible for the symptoms of allergy.
- IgD remains attached to B-cells and play a key role in initiating early B-cell response.

Unlike B-cells, T-cells do not recognize free-floating antigens. Rather, their surfaces contain specialized antibody-like receptors that see fragments of antigens on the surfaces of infected or cancerous cells. T-cells contribute to immune defenses in two major ways: some direct and regulate immune responses; others directly attack infected or cancerous cells.

Helper T cells or Th cells coordinate immune responses by communicating with other cells. Some stimulate nearby B cells to produce antibody, others call in microbe-gobbling cells called phagocytes, and still others activate other T cells.

Killer T cells are also called cytotoxic T lymphocytes or CTLs—perform a different function. These cells directly attack other cells carrying certain foreign or abnormal molecules on their surfaces. CTLs are especially useful for attacking viruses because viruses often hide from other parts of the immune system while they grow inside infected cells. CTLs recognize small
fragments of these viruses peeking out from the cell membrane and launch an attack to kill the cell.

In most cases, T-cells only recognize an antigen if it is carried on the surface of a cell by one of the body’s own MHC, or major histocompatibility complex, molecules. MHC molecules are proteins recognized by T-cells when distinguishing between self and nonself. A self-MHC molecule provides a recognizable scaffolding to present a foreign antigen to the T-cell.

Although MHC molecules are required for T-cell responses against foreign invaders, they also pose a difficulty during organ transplantations. Virtually every cell in the body is covered with MHC proteins, but each person has a different set of these proteins on his or her cells. If a T-cell recognizes a nonself MHC molecule on another cell, it will destroy the cell. Therefore, doctors must match organ recipients with donors who have the closest MHC makeup. Otherwise the recipient’s T-cells will attack the transplanted organ, leading to graft rejection.

The immune system is amazingly complex. It can recognize and remember millions of different enemies, and it can produce secretions and cells to match up with and wipe out each one of them. The secret to its success is an elaborate and dynamic communications network.
Millions and millions of cells, organized into sets and subsets, gather like clouds of bees swarming around a hive and pass information back and forth. Once immune cells receive the alarm, they undergo tactical changes and begin to produce powerful chemicals. These substances allow the cells to regulate their own growth and behavior, enlist their fellows, and direct new recruits to trouble spots.

1.4.2 Organs of the Immune System

The organs of the immune system are positioned throughout the body. They are called lymphoid organs because they are home to lymphocytes, small white blood cells that are the key players in the immune system.

*Bone marrow,* the soft tissue in the hollow center of bones, is the ultimate source of all blood cells, including white blood cells destined to become immune cells.

The *thymus* is an organ that lies behind the breast bone; lymphocytes known as *T lymphocytes,* or just "*T cells,"* mature in the thymus. Lymphocytes can travel throughout the body using the blood vessels. The cells can also travel through a system of lymphatic vessels that closely parallels the body’s veins and arteries.

Cells and fluids are exchanged between blood and lymphatic vessels, enabling the lymphatic system to monitor the body for invading microbes. The lymphatic vessels carry *lymph,* a clear fluid that bathes the body’s tissues. Small, bean-shaped lymph nodes are laced along the lymphatic vessels, with clusters in the neck, armpits, abdomen, and groin. Each lymph node contains specialized compartments where immune cells congregate, and where they can encounter antigens. Immune cells and foreign particles enter the lymph nodes via incoming lymphatic vessels or the lymph nodes’ tiny blood vessels. All lymphocytes exit lymph nodes through outgoing lymphatic vessels. Once in the bloodstream, they are transported to tissues throughout the body.

They patrol everywhere for foreign antigens, then gradually drift back into the lymphatic system, to begin the cycle all over again.

The *spleen* is a flattened organ at the upper left of the abdomen. Like the lymph nodes, the spleen contains specialized compartments where immune cells gather and work, and serves as a meeting ground where immune defenses confront antigens.41
1.4.3 Antigen

An antigen is defined as a substance that reacts with antibody molecules and antigen receptors on lymphocytes. An immunogen is an antigen that is recognized by the body as nonself and stimulates an adaptive immune response. Chemically, antigens are large molecular weight proteins (including conjugated proteins such as glycoproteins, lipoproteins, and nucleoproteins) and polysaccharides (including lipopolysaccharides). These protein and polysaccharide antigens are found on the surfaces of viruses and cells, including microbial cells (bacteria, fungi, and protozoan) and human cells. For simplicity, both antigens and immunogens are usually referred to as antigens. To be immunogenic, an antigen must possess three characteristics:

- A high molecular weight,
- Chemical complexity, and
- Foreignness (recognized as nonself by the body).
1.4.4 Antibodies

The immunoglobulins/antibodies are a group of glycoproteins present in the serum and tissue fluids of all animals. Immunoglobulins function as antibodies; the antigen binding proteins are present on the B cell membrane and are also secreted by plasma cells. The secreted antibodies serve as the effector molecules of humoral immunity and are said to circulate in the blood, searching for and neutralizing or eliminating antigens.

Their production is induced when host’s lymphoid system comes in contact with antigens and bind specifically to it, which induces their formation. It was 1939 when Tiselius and Kabat subjected the serum of ova albumin immunized rabbits to electrophoresis, and obtained four fractions; albumin, the alpha (α), beta (β), and the gamma (γ). While the same serum when reacted with an antigen formed a precipitate. The serum left over after separating the precipitate when subjected to electrophoresis showed a significant drop in the amount of γ-globulin fraction contained serum antibodies, which they named as antibodies.

Porter and Edelman in 1960s first separated the γ-globulin fraction of serum into a high molecular weight fraction with a sedimentation constant of 19S and a low molecular weight fraction with a sedimentation constant of 70S with a molecular weight of 1,50,000. They designated this 70S fraction of γ-globulins as immunoglobulin IgG for their studies. IgG antibodies are made up of four polypeptide chains, comprising two identical light chains and two identical heavy chains and can be thought of as forming a flexible Y-shaped structure. Each of the four chains has a variable region at its amino-terminus, which contributes to the antigen-binding site, and a constant region, which in the case of the heavy chain, determines isotype and hence the functional properties of the antibody.

The light chains are bound to the heavy chains with disulphide bridges and the variable regions of the heavy and light chains pair to generate two identical antigen-binding sites, which lie at the tips of the arms of the Y. This allows antibody molecules to crosslink antigens. The trunk of the Y, or Fc fragment, is composed of the two carboxy-terminal domains of the two heavy chains. Joining the arms of the Y to the trunk are the flexible hinge regions. The Fc fragment and hinge regions differ in antibodies of different isotypes, thus determining their functional properties. However, the overall plan of all isotypes is similar.
The three major effective functions that enable antibodies to remove antigens and kill pathogens are:

- **Opsonization**, which promotes antigen phagocytosis by macrophages and neutrophils
- **Complement activation**, which activates a pathway that leads to the generation of a collection of proteins that can perforate cell-membranes; and
- **Antibody-dependent cell-mediated cytotoxicity (ADCC)**, which can kill antibody-bound target cells.  

1.5 IMMUNOLOGICAL RESPONSES

1.5.1 Humoral immune response

B-lymphocytes, unlike T-Lymphocytes, which are free to circulate around the body, are fixed in lymphoid tissue (e.g. the spleen and lymph nodes). B-lymphocytes, unlike T-lymphocytes, recognize and bind antigen particles without having to be presented with them by an antigen-presenting cell. Once its antigen has been detected and bound, and with the help of a helper T-lymphocyte, the B-lymphocyte enlarges and begins to divide (clonal
expansion). It produces two functionally distinct types of cells, plasma cells and Memory B-cells.

**Plasma cells** secrete antibodies into the blood. Antibodies are carried throughout the tissues, while the B-lymphocytes themselves remain fixed in lymphoid tissue. Plasma cells live no longer than a day, and produce only one type of antibody, which targets the specific antigen that originally bound to the B-lymphocytes. Antibodies:

- Bind to antigens, labeling them as targets for other defense cells such as cytotoxic T-lymphocytes and macrophages
- Bind to bacterial toxins, neutralizing them
- Activate complement.

**Memory B-cells**, remain in the body long after the initial episode has been dealt with, and rapidly respond to another encounter with the same antigen by stimulating the production of antibody-secreting plasma cells.

The fact that the body does not normally develop immunity to its own cells is due to the fine balance that exists between the immune reaction and its suppression. Autoimmune diseases are due to the disturbance of this balance.

Humoral immunity targets exogenous foreign material via antibody binding, and is mediated primarily by the B1 lymphocytes-small round cells with a single large, round nucleus and very little cytoplasm. These lymphocytes make up only about 5% of the total lymphocyte population and are relatively short-lived, but are nonetheless very important. Most originate from the bone marrow in mammals, and mature in this organ before migrating to the other primary and secondary lymphoid tissues.
All lymphocytes carry antigenic surface molecules/receptors known as cluster of differentiation (CD) antigens, which denote function and identification. There are over 80 CD antigens described to date in mammals, and most are homologous across mammalian species. As well, most B-cells carry both MHC class I and II antigens, various interleukin receptors, and all B-cells carry a distinct B-cell receptor (BCR-capable of binding either APC processed or free antigen) and a complement receptor for recognition and response to foreign antigen.  

B-cells initiate body protection after recognition of antigen through the B-cell receptor with appropriate Th help. Remarkably, there is enough variability among the population of lymphocyte specific receptors that nearly any type of foreign invader can be recognized by the immune system. When the antigen is presented in conjunction with an MHC II molecule, a CD4+ Th cell can recognize it and bind. Strong binding alone, however, is not sufficient to promote an immune response, and indeed, may lead to tolerance to the antigen. Therefore, IL-1, a very important co-stimulator molecule released by APCs is necessary. Under these correct circumstances, the Th cell will then secrete IL-2 and IL-4, which will immediately promote the B-cells to begin a proliferation or clonal selection process, greatly elevating the proportion of B-
cells specific for the particular antigen. These cells in turn differentiate into plasma cells (mature B-cells capable of producing large quantities of antibody proteins) or memory cells (B-cells which can very quickly proliferate and produce an even greater amount of antibody upon a subsequent exposure to the same antigen).  

Certain antigens are capable of provoking antibody production in the absence of Th cells. These are T-independent antigens and usually are simple repeating polymers, such as *Escherichia coli* lipopolysaccharide (LPS). Because they are repeating polymers, they process multiple identical epitopes and so can cross-link several BCRs at one time. As a result, the effective dose of these epitopes must be relatively large to provide a sufficient stimulus for the proliferation of at least some B-cells. Characteristically, T-independent antigens trigger only IgM responses in mammalian B cells and fail to generate memory cells, due to their failure to induce the appropriate interleukins from Th cells, and so can not trigger the switch from IgM to other isotypes.

Antibodies (Ab) are proteins known as immunoglobulins, produced by immature and mature B-cells (plasma cells) in response to each recognized exogenous foreign invader. In mammals, there are five main isotypes of immunoglobulin: IgM (mostly stimulated by the primary response), IgG (for the memory response), IgA (in secretory fluids), IgE (cell-associated, often implicated in allergic reactions), and IgD (on membranes of B-lymphocytes, possibly important in recognition).

When antibodies bind to antigens, forming antigen-antibody (Ag-Ab) complexes, complement-mediated lysis can occur. Complement is composed of a set of protein enzymes whose activation results in a reaction cascade, ultimately leading to the disruption of cell membranes and the destruction of cells or invading microorganisms. There are two pathways of complement activation, the classical and the alternate. The classical pathway requires antibody bound to antigen for activation, while the alternate pathway can be activated against some Gram-negative bacteria containing endotoxin without involving specific antibody.

1.5.2 Cell-mediated immunity

Cell-mediated immunity involves the production of cytotoxic T-lymphocytes, activated macrophages, activated NK cells, and cytokines in response to an antigen and is mediated by T-lymphocytes.
Cell-mediated immunity (CMI) is an immune response that does not involve antibodies but rather involves the activation of macrophages and NK-cells, the production of antigen-specific cytotoxic T-lymphocyte, and the release of various cytokines in response to an antigen. Cellular immunity protects the body by:

- Activating antigen-specific cytotoxic T-lymphocytes (CTLs) that are able to lyse body cells displaying epitopes of foreign antigen on their surface, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumor antigens;
- Activating macrophages and NK cells, enabling them to destroy intracellular pathogens; and
- Stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses.

Cell-mediated immunity is directed primarily to the microbes that survive in phagocytes and microbes that infect non-phagocytic cells. It is most effective in removing virus-infected cells, but also participates in defending against fungi, protozoans, cancers, and intracellular bacteria. It also plays a major role in transplant rejection.
1.6 ANTIGEN-ANTIBODY REACTIONS

An antibody is bivalent, and an antigen is multivalent, so lattice formation can occur. The complex may be fibrous, particulate, matrix-like, soluble, or insoluble. These characteristics dictate the means of its disposal. Four fundamental reactions describe these processes: neutralization, precipitation, agglutination and bacteriolysis.

Neutralization is an immunological disposal reaction for bacteria and for toxins (which are small and soluble). Once they bind the antibody, they are no longer toxic because their active site structures are covered and they cannot bind their targets.

In the precipitation, a soluble antigen reacts with an antibody, and may form an insoluble particulate precipitate. Such a complex cannot remain in the blood stream in its insoluble state. These species must be removed by the spleen or through the reticuloendothelial system by phagocytosis.

In the agglutination, bacterial cells may be aggregated by binding to antibodies that mask negative ionic surface charges and crosslink cellular structures. The bacteria are thus immediately immobilized. This limits their ability to maintain an infection, but forms a particulate matrix. This type of complex must also undergo elimination through the reticuloendothelial system.

Bacteriolysis is a complement-mediated reaction. The last five proteins in the cascade self-assemble to produce a membrane attack complex that disrupts the cell membranes of bacteria, acting like bacitracin or amphotericin B. The cell membranes lose integrity, cell contents leak out, membrane transport systems fail, and the cell dies. This type of reaction yields products that require no special treatment. 

1.7 HYPERSENSITIVITY REACTIONS

Not all the immune responses against an antigen produce a desirable resistance, when the sensitivity is beyond limits it is termed as hypersensitivity. It can be defined as when an adaptive immune response occurs in an exaggerated or inappropriate form causing tissue damage, then the condition is termed as hypersensitivity. It depends on an individual. It usually occurs to people who have been previously ‘sensitized’ by an exposure to an antigen, i.e., upon second contact with a particular antigen. It may be broadly classified under two main headings:

✔ Immediate or humoral hypersensitivity
✔ Delayed or cell-mediated hypersensitivity
The hypersensitivity reactions given above are the basis of the clinically important group of autoimmune diseases. Some examples held to have a marked component of cell-mediated hypersensitivity are rheumatoid arthritis, multiple sclerosis and Type-I (insulin dependent) diabetes.

Immunosuppressive drugs and/or glucocorticoids are employed as part of the treatment of some autoimmune diseases, and the use of cytokines and of antibodies to T-cell surface antigens (e.g. the CD4 receptors) are being explored.\textsuperscript{53}

\textbf{1.8 IMMUNOMODULATORS}

Stimulation of non-specific defense mechanisms of human beings as one concept of therapy has a long tradition in medicine. Injection of body’s own blood, milk protein, suspensions of inactivated microorganisms, injection of animal or plant extracts as well as administration of inflammatory agents such as mustard oil, turpentine oil, etc. has been used for general reorientation of the being as a whole for chronic inflammatory, allergic and other diseases which are considered to be the consequence of impaired host defenses.\textsuperscript{54}

The impact of the immune system in human disease is enormous. Immune system mediated diseases are significant health care problems. Immunological diseases are growing at epidemic proportions that require aggressive and innovative approaches to the development of new treatments. These diseases include a broad spectrum of autoimmune diseases such as rheumatoid arthritis, diabetes mellitus, solid tumors and haematologic malignancies; infectious diseases; asthma and various allergic conditions.

Furthermore, one of the great therapeutic opportunities is for the treatment of many disorders in organ transplantation. However, immune system mediated graft rejection remains the single greatest barrier to widespread use of this technology.

An improved understanding of immune system has led to the development of new therapies to treat immune system-mediated diseases.

Development of these drugs are used to modulate the immune-response in 3-ways

(1) Immunosuppression (2) Tolerance (3) Immunostimulation.\textsuperscript{55}

Immunomodulators are natural and synthetic substances, which by altering the immune system affect a therapeutic benefit. They may have capacity to augment (immunostimulant and/or immunoenhancer), restore (immunosuppressants), or help to produce (adjuvant) the desired immune response.
Recently, interest in these agents has increased due to an expanding knowledge of immune function and a greater awareness that many human diseases are either immunologically mediated or are associated with immune abnormalities of pathological significance. This has spawned an explosion of new immunomodulatory agents as well as a steady rise in the number of potential therapeutic targets for diseases of immunomodulatory drugs. Immunomodulators are under development for the treatment of those diseases in which an abnormal immune response plays an important pathological role including cancer, autoimmune diseases and natural or acquired immune deficiency syndromes.56

The immunomodulator, cyclosporine, was originally isolated from a soil fungus, *Trichoderma polysporum*. This compound was a major breakthrough for organ transplantation, since it suppressed immunological rejection of the transplanted organ. Tacrolimus (FK – 506), a secondary metabolite of *Streptomyces tsukubaensis*, was approved in 1994 for use as an immunosuppressant in organ transplantation.57

Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders. There is a great potential for the discovery of more specific immunomodulators which mimic or antagonize the biological effects of cytokines and interleukins, and the refinement of assays for these mediators will create specific and sensitive screens. Natural remedies should be revisited as important sources of novel ligands capable of targeting specific cellular receptors.58 Medicinal plants and their active components have been shown to be an important source of immunomodulators. Thus, the development of drugs for immunomodulator activity from natural compounds has become an attractive area of research.59-60

1.8.1 Medicinal plants as immunomodulators

Fruits of *Emblica officinalis* (family: Euphorbiaceae) and whole plant of *Evolvulus alsinoides* (family: Convolvulaceae) have been extensively used in Indian Ayurvedic medicine for a variety of medical disorders. The immunomodulatory properties of *Emblica officinalis* and *Evolvulus alsinoides* were evaluated in adjuvant induced arthritic rat model. There was a significant reduction in swelling and redness of inflamed areas in the extracts treated animals than in untreated controls. The anti-inflammatory response of both extracts was determined by
lymphocyte proliferation activity and histopathological severity of synovial hyperplasia. Both extracts showed a marked reduction in inflammation and edema.\textsuperscript{61}

Ethanolic extract of \textit{A. calamus} inhibited proliferation of mitogen (phytohaemagglutinin) and antigen (purified protein derivative) stimulated human peripheral blood mononuclear cells (PBMCs). In addition, \textit{A. calamus} extract inhibited growth of several cell lines of mouse and human origin. It also inhibited production of nitric oxide (NO), interleukin-2 (IL-2) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)). Intracytoplasmic interferon-\(\gamma\) (IFN-\(\gamma\)) and expression of cell surface markers, CD16 and HLA-DR, on human PBMC, were not affected on treatment with \textit{A. calamus} extract but CD25 expression was down regulated.\textsuperscript{62}

Crude extract of \textit{Tinospora cordifolia} contained a polyclonal B cell mitogen which enhanced immune response in mice. An arabinogalactan polysaccharide from the stem of \textit{Tinospora cordifolia} was examined to modulate induced immunosuppression. Mice pre-treated with arabinogalactan polysaccharide exhibited protection against lipopolysaccharide (LPS) induced mortality.\textsuperscript{63} In further studies, intraperitoneal administration of alcoholic extract of \textit{Tinospora cordifolia} in Dalton's lymphoma bearing mice not only augmented the basic function of macrophages, such as phagocytosis, but also their antigen presenting ability and secretion of IL-1, TNF and RNI. It was also indicated that the extract slowed down the tumor growth and increased the life span of tumor bearing host, thus showing its anti-tumor effect through destabilizing the membrane integrity of Dalton's lymphoma cells directly or indirectly.\textsuperscript{64}

Ethanolic extract of \textit{Boerhaavia diffusa}, a plant used in Indian traditional system of medicine, significantly inhibited the cell proliferation.\textsuperscript{65} Extracts of \textit{B. diffusa} roots inhibited human NK cell cytotoxicity \textit{in vitro}, production of nitric oxide in mouse macrophage cells, interleukin-2 and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), in human PBMCs. However, intra-cytoplasmic interferon-\(\gamma\) (IFN-\(\gamma\)) and cell surface markers such as CD16, CD25, and HLA-DR did not get affected on treatment with \textit{B. diffusa} extract and demonstrates immunosuppressive potential of \textit{B. diffusa}.\textsuperscript{66}

Aqueous leaves extract of biopesticidal plant \textit{Nyctanthes arbor-tristis} has been found to be potent immunomodulator.\textsuperscript{67} The extract has been evaluated as immunorestorative or anti-immunosuppressive agent in the malathion exposed immunosuppressed mice by studying various immunological parameters (humoral, cell mediated immune, numerical values of immunocytes and functions of phagocytes) in treated or untreated mice. The results revealed that the immunological parameters which were suppressed with malathion reverted back to normal, when treated with aqueous leaves extract of \textit{Nyctanthes arbor-tristis}.\textsuperscript{68}
Methanolic extract of Eclipta alba and Centella asiatica increased phagocytic index and antibody titer significantly. The F ratios of the phagocytic index and WBC count were also significant, with linearity in the dose-response relationship. The ethanol extract of roots of the plant Cryptolepis buchanani caused significant stimulation of the delayed type hypersensitivity reaction and humoral antibody production in mice. An aqueous extract of Rhodiola imbricata rhizome stimulated production of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in human PBMCs as well as RAW 264.7 cell line. It also increased production of nitric oxide synergistically in combination with lipopolysaccharide in RAW 264.7. Furthermore, it increased the phosphorylated-IκB expression and activated the nuclear translocation of NF-κB in human PBMCs. Thus, Rhodiola most likely activated proinflammatory mediators via phosphorylated inhibitory κB and transcription factor NF-κB.

1.8.2 Immunomodulatory activity of plant derived compounds

**Sterols and sterolins**

The phytosterols, β-sitosterol, and its glucoside enhanced the *in vitro* proliferative response of T-cells stimulated by sub-optimal concentrations of phytohaemagglutinin several fold at extremely low concentrations (femtogram level). A 100:1 (mass:mass) ratio of β-sitosterol: β-sitosterol glucoside showed higher stimulation than the individual sterols at the same concentration. The mixture of the sterols (β-sitosterol) and sterolins (β-sitosterol glucoside) has the ability to enhance the cellular response of T lymphocytes both *in vitro* and *in vivo*. The mixture enhances the cytotoxic ability of natural killer (NK) cells against the target cancer cell line NK562. It has also been postulated that the sterol-sterolin mixture in a specific ratio could reinstate a balance between the TH1–TH2 cells, a delicate balance that determines the final outcome of an immune response. The same mixture inhibited the release of pro-inflammatory cytokines from endotoxin activated monocytes: interleukin-6 and tumour necrosis factor-α secretion. In HIV-infected patients, analysis of the CD4 cell-type (TH1 vs TH2-type) showed that those receiving the sterol-sterolin mixture maintained a favorable TH1 response, which implies that their cell-mediated response was possibly responsible for the viral control and inhibition of CD4 cell loss. Immunomodulatory effect of a β-sitosterol glycoside, daucosterol was also observed against disseminated candidiasis caused by Candida albicans due to the CD4+ TH1 immune response.
**Cannabinoids**

Advances in understanding the physiology and pharmacology of the endogenous cannabinoid system have potentiated the interest of cannabinoid receptors as potential therapeutic targets. Cannabinoids have been shown to modulate a variety of immune cell functions and have therapeutic implications on central nervous system (CNS) inflammation, chronic inflammatory conditions such as arthritis, and may be therapeutically useful in treating autoimmune conditions such as multiple sclerosis. Many of these drug effects occur through cannabinoid receptor signalling mechanisms and the modulation of cytokines and other gene products.⁷⁶ Cannabidiol and cannabis-based medicines are potential therapeutic agents, because the immune system has been widely demonstrated to be affected by psychoactive cannabinoids, such as Δ9-tetrahydrocannabinol. Cannabidiol significantly attenuated the elevation of IL-2, IL-4, IL-5, and IL-13 steady-state mRNA expression elicited by Ova challenge in the lungs. Plant derived immunomodulatory cannabinoids exhibited potential therapeutic utility in the treatment of allergic airway disease by inhibiting the expression of critical T cell cytokines and the associated inflammatory response.⁷⁷

**Polysaccharides**

Polysaccharides exhibit a number of beneficial therapeutic properties, and it is thought that the mechanisms involved in these effects are due to the modulation of innate immunity and, more specifically, macrophage function. Furthermore, botanical and microbial polysaccharides bind to common surface receptors and induce similar immunomodulatory responses in macrophages, suggesting that evolutionarily conserved polysaccharide structural features are shared between these organisms. Thus, the evaluation of botanical polysaccharides provides a unique opportunity for the discovery of novel therapeutic agents and adjuvants that exhibit beneficial immunomodulatory properties.⁷⁸ The immunomodulatory effects of the polysaccharide of *Cistanche deserticola* have been evaluated by *in vitro* proliferation of murine
thymus lymphocytes by MTT method. The enhancing effect of polysaccharide on murine thymus lymphocyte proliferation was related to its promotion on thymus intracellular Ca+2 delivering. High molecular weight substances were isolated from *Salicornia herbacea*, which has been used to treat a variety of diseases including cancers in traditional oriental remedy. The active components of the extract have been described as polysaccharides, which not only activate monocytic cells strongly, but also induce differentiation of monocytic cells into macrophages.

**Alkaloids**

Plant bis-benzylisoquinoline alkaloid, tetrandrine, is an active purified compound from dried tuberous root of the creeper *Stephania tetrandra* and is a potent immunomodulator used to treat rheumatic disorders, silicosis and hypertension in mainland China.

Tetrandrine effectively suppressed cytokine production and proliferation of CD28-costimulated T cells. Recently, it has been it is reported that tetrandrine down regulated IκBα kinases- IκBα -NF-κB signaling pathway in human peripheral blood T cell. Four tetrandrine analogs (tetrandrine, dauricine, berbaméine and hemandezine) were compared; dauricine appeared to be the most potent inhibitor of CD28, but not of H2O2- induced NF-κB DNA-binding activities. Studies have shown that tetrandrine might modulate lipopolysaccharide induced microglial activation by inhibiting the NF-κB-mediated release of inflammatory factors.

Alcoholic extract of the fruits of well known spice *Piper longum* (black pepper) and its component alkaloid, piperine, exhibited immunomodulatory and antitumor activity to Dalton's lymphoma ascites cells and Ehrlich ascites carcinoma (EAC) cells. *Piper longum* extract and piperine increased the total WBC count, number of plaque forming cells, and bone marrow cellularity and α-esterase cells.

**Flavonoids**

Eupalitin-3-O-β-D-galactopyranoside, purified from the ethanolic extract of *Boerhaavia diffusa* root, inhibited phytohemagglutinin stimulated proliferation of peripheral blood mononuclear cells, two-way MLR and natural killer cell, as well as lipopolysaccharide induced NO production by RAW 264.7. It further inhibited production of phytohemagglutinin stimulated IL-2 at the protein and mRNA transcript levels; lipopolysaccharide stimulated TNF-α production in human peripheral blood mononuclear cells. It blocked the activation of DNA binding of nuclear factor-κB and AP-1, two major transcription factors centrally involved in expression of the IL-2 and IL-2R gene, which are necessary for T cell activation and proliferation. On the other hand, eupalitin showed little activity on the above experiments. *Bidens pilosa* is an ethnical
medicine for bacterial infection or immune modulation in Asia, America and Africa. Flavonoid, centaurein and its aglycone, centaureidin has been isolated from the n-butanol subfraction. The study suggested that centaurein regulated IFN-γ transcription as an immunomodulator, probably via NFAT and NFκB in T cells.  

![Chemical Structures](image)

**Lectins**

A plant lectin from *Viscum album* has been previously shown to increase the number and cytotoxic activity of natural killer cells and to induce antitumor activity in animal models. Lectin-sugar interactions on the cell surface of immunocompetent cells can induce cytokine gene expression and protein synthesis. Recently, plant lectin *Viscum album* agglutinin-I also demonstrated interesting potential therapeutic properties and immunomodulatory activities. Lectin alters mitochondrial transmembrane potential and increases intracellular levels of reactive oxygen species.

**Glycoprotein**

Immunomodulatory effect of a C3 binding glycoprotein, isolated from the parasitic plant *Cuscuta europea* has been reported. The glycoprotein showed a dose dependent in vivo immunostimulation against SRBC-immunized mice. The *in vitro* stimulation was assessed by an increase in the number of hemolytic plaque forming cells and hemagglutination titers. Later studies demonstrated that C3 binding glycoprotein induced proinflammatory and immunoregulatory cytokine production, in the highest degree IL-12, followed by IL-6 and in
lower degree TNF-α. IL-12 quantity was significantly increased in glycoprotein stimulated cultures in comparison with LPS, PHA and PWM stimulated PBMC. The authors suggested that a part of the mechanism of action of C3 binding glycoprotein is mediated through NF-κB signal transduction pathway.\textsuperscript{91}

1.8.3 Herbal formulations as immunomodulators

The immunomodulatory activities of Triphala, an Indian Ayurvedic formulation of three plants \textit{(Terminalia chebula, Terminalia belerica and Emblica officinalis)} were assessed by testing the various neutrophil functions like adherence and phagocytosis (phagocytic index and avidity index and nitro blue tetrazolium) reduction in albino rats. Oral administration of Triphala stimulated the neutrophil functions in the immunized rats. Stress induced suppression in the neutrophil functions were also significantly prevented by Triphala.\textsuperscript{92}

1.8.4 Patented immunomodulators

\textit{Polysaccharides}

Extracellular polysaccharides of \textit{Aphanothece halophytica} has been patented for regulating immunity and treating and/or preventing pulmonitis.\textsuperscript{93} A polysaccharide fraction (0.1-20%), extracted from callus plant tissue of \textit{Ungernia} species, was evaluated for its antimutagenic, immunomodulating, and antitumor activities. Per oral administration of the polysaccharide fraction at a daily dose during 20-30 days to mice enhanced effectiveness and safety of treatment.\textsuperscript{94} Gulvel is extensively used in Ayurveda as a single or polyherbal formulation. A process for the preparation of an immunomodulator from several plants of genus \textit{Tinospora}, such as \textit{T. cordifolia}, \textit{T. malabarica}, etc., are well known has been described. A branched polysaccharide, arabinogalactan, was selectively precipitated from the polar extracts in aqueous medium by methanol. The active polysaccharide was further purified by high-performance gel permeation chromatography. It is polyclonally mitogenic to β-cells, and augments antibody response as well as enhances T-cell responses to model antigens.\textsuperscript{95}

\textit{Coumarinolignoids}

A novel pharmaceutical composition consisting of a combination of three coumarinolignoids isolated from the seeds of the plant \textit{Cleome viscosa} has been described to modulate humoral and cell mediated immune response.\textsuperscript{96}
**Polyphenols**

Polyphenols are useful for prophylactic and therapeutic treatment of allergy. Apple polyphenol containing ~50% proanthocyanidin significantly promoted interferon-γ formation later and inhibited IL-5 and IL-10 in ovalbumin-immunized murine spleen cells.\(^{97}\)

**Stilbenoids**

Extraction of pharmaceutically active stilbene derivatives (e.g., resveratrols, ε-viniferin, viniferin derivatives, hopeaphenol, and Ampelopsis A) with immunomodulating activity from spermatophyte plants has been reported.\(^{98}\)

**Herbal formulation**

An immunomodulator for the prevention and the treatment of AIDS comprises of Cordyceps plants (such as *Cordyceps militaris* L., Brazil Cordyceps, and FENG Cordyceps), Radix glycyrrhizae, Rhizoma atractyloidis macrocephalae, Radix angelicae sinensis, Rhizoma coptidis, Radix aconiti lateralis preparata, Minor Decoction of Bupleurum, and AZT. This composition can maintain or improve the immunologic function of patients.\(^{99}\)

**Fve proteins and peptides**

The Fve protein (i.e., a protein from the golden needle mushroom *Flammulina velutipes*) upregulated expression of TH1/TC1 cytokines such as interferon-γ and tumor necrosis factor-α; whereas down-regulated expression of TH2/TC2 cytokines such as IL-4 and IL-13. In addition, it upregulated expression of T regulatory cell cytokines IL-10 and transforming growth factor-β. Furthermore, Fve proteins exhibited hemagglutination, lymphocyte aggregation, and lymphoproliferation. This peptide may be used as an immunomodulator, as an adjuvant, either alone or as a fusion protein with an antigen or allergen.\(^{100}\)
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17, 1140.
2.1 REVIEW OF LITERATURE

PLANT PROFILE (Rhododendron arboreum)

✓ Synonyms

- English: Rhododendron, Rose tree
- Hindi: Burans
- Nepali: Lali guras
- Punjabi: Ardawal
- Tamil: Alingi

✓ Classification

- Kingdom: Plant
- Division: Magnoliophyta
- Class: Magnoliatae
- Order: Sympetalae
- Suborder: Ericales
- Family: Ericaceae
- Subfamily: Rhododendroideae
- Genus: Rhododendron L.
- Species: Rhododendron arboreum Sm.

Figure 2.0 Flowers of Rhododendron arboreum.

Rhododendrons: A global purview

The term ‘Rhododendron’ comes from the Greek words ‘Rhodo’ meaning rose and ‘dendron’ meaning ‘tree’, in combination ‘Rose-tree’. Rhododendron, the largest genus of the Ericaceae family, includes 1200 species distributed throughout Northeast Asia and Eurasia,
Western Europe and North America.\textsuperscript{5,6} \textit{Rhododendrons} cover a vast section of South East Asia, comprising of Northwestern Himalaya through Nepal, Sikkim, Eastern Tibet, Bhutan, Arunachal Pradesh, and upper Myanmar, western and central China. More than 90\% of the world’s natural population of \textit{Rhododendrons} is from this region.\textsuperscript{7} They are widely distributed at higher elevation in the Sino-Himalayan regions, with maximum concentration in western China.\textsuperscript{8} The first of many \textit{Rhododendrons}, which were to come from southeastern Asia, was the tree species. \textit{Rhododendron} is a spectacular genus consisting of evergreen, semi deciduous and deciduous shrubs and trees. \textit{Rhododendrons} grow well in loose, open, and well aerated; lime free, cool and acidic soil with plenty of humus to retain moisture.\textsuperscript{9-11} They occur in the high altitudes from 1500m to 6000m and have ecological significance and economic importance in addition to its graceful flowers. Since \textit{R. arboreum} is a small tree with deep scarlet flowers, often associated with “banj” and “kharsu” plants.\textsuperscript{11} \textit{Rhododendron arboreum} is Nepal’s national flower, locally known as \textit{Lali Guras} or ‘rose tree’ in English. \textit{Rhododendrons} are usually clustered inflorescence (trusses) at the branch tips and occur in all colours except a true bright blue. They mostly blossom during summer months from March to July. There are two distinct flowering seasons, viz. March–May and May–July. However, a large number of species flower during April–June, except for a few species like \textit{R. edgeworthii}, \textit{R. griffithianum} and \textit{R. maddenii} that flower during May–July.\textsuperscript{12}

In 1753, Linnaeus, who devised binomial system of plant taxonomy, created two genera, \textit{Rhododendron} and \textit{Azalea}, into which the nine hitherto recognized species were placed. Over the next 240 years, many botanists have furthered the taxonomy of \textit{Rhododendrons}\textsuperscript{13} that range in size from tiny mat-like plants to trees up to 30 m height. \textit{Rhododendrons} are classified into four groups: the scaly \textit{Rhododendrons} (subgenus \textit{Rhododendron}); true \textit{Rhododendrons} (subgenus \textit{Hymenanthes}); the azaleas, which comprise the subgenera \textit{Azalea} and \textit{Tsuts sia}; and false azaleas (subgenus \textit{Azaleastrum}).\textsuperscript{14}

\textbf{Rhododendrons in India}

The history of Himalayan \textit{Rhododendron} commences with the visit of Captain Hardwick of the Siwalik Mountains of Srinagar, Kashmir in 1796, where he encountered \textit{R. arboreum}. Sir James Smith described it in 1805 in Exotic Botany. Sir Joseph Hooker’s journey to the Sikkim Himalayas between 1847–51 opened the doors to the \textit{Rhododendrons} of this area. Within a brief span of time that he travelled in Sikkim, Hooker gathered and described 34 new species and provided details of all the 45 species from the Indian region in his monograph entitled \textit{The
Rhododendrons of the Sikkim-Himalaya, published in 1849 and considered to be the standard text for the study of Himalayan Rhododendron. The monograph still remains unparalleled in its exhaustive details and elegant paintings.\textsuperscript{15, 16} Reportedly, 98\% of the Rhododendrons are confined to the Himalayan region of a total of 72 species, 20 subspecies and 19 varieties have been listed from India. The western Himalayan region has 8 species, whereas eastern Himalayan region harbours 71 species. The Rhododendrons are found between 1200 m (e.g. \textit{R. dalhousiae}, \textit{R. dendricola}) and 6000 m (e.g. \textit{R. nivale}, \textit{R. leptocarpum}) altitude, but majority of them occur between 2200 and 4000 m. Among all the Indian species, \textit{R. arboreum} is widely distributed from 1500 m elevation upto 4000 m,\textsuperscript{17} from western to eastern Himalayan region and other neighbouring countries. One subspecies (\textit{R. arboreum} ssp. nilagiricum) is found in the Nilgiri Hills of Western Ghats, Tamil Nadu. Some species (e.g. \textit{R. arboreum}, \textit{R. cinnabarinum}, \textit{R. dalhousiae}, \textit{R. setosum}, \textit{R. thomsonii}) are poisonous. Out of the 72 species known from India, Arunachal Pradesh has 61 species. However, a recent estimate shows that 12 species, 2 subspecies and 5 varieties are endemic to India. In terms of biological endemism; Rhododendrons contributed 7 species, 2 subspecies and 3 varieties in India.\textsuperscript{18, 19} In India, \textit{Rhododendron} is the state flower of Nagaland and state tree of Sikkim.\textsuperscript{20}

**Chemical constituents**\textsuperscript{21-28}

- **Flowers:**
  - Steroids, Terpenoids, Flavonoids, Anthocyanins, Saponins, Azaleatin, Flavonols, Alkaloids, Carbohydrates, Phytosterols, Tannins.

- **Leaves:**
  - Friedelin, Ursolic acid, Azaleatin, Caryatin, Flavonols, Gossypetin, Kaempferol, Glutinane, Epirfiedlanol, \(\beta\) and \(\alpha\)-amyrrins, Flavone glycosides, Terphthalic acid, \(\beta\)-Sitosterol and Ericolin.

- **Bark:**
  - Quercetin, Tarexerol, Ursolic acid, Betulinic acid

**Utilization prospects of \textit{R. arboretum}**

- **Aesthetic and sacred value:**
  - Flowers are considered to be sacred, offered in temples and monasteries.\textsuperscript{29} The grained wood is used for making khukri handles, pack-saddles, gift-boxes, gun stockes, posts, fuel wood and at high altitude trekking corridors for tourism.\textsuperscript{30}

- **Therapeutic action:**
Young leaves are poisonous in nature. 31, 32 R. arboreum is used therapeutically in some Ayurvedic preparations; its toxic potentialities should be taken into consideration.33

✓ **Traditional Uses:**

The fresh and dried corolla is acid sweet in nature and is given when fishbones get stuck in the gullet.34 Dried flowers fried with ghee are supposedly highly efficacious in checking diarrhoea and blood dysentery.35 Paste of young leaves is applied to the forehead for headache, Squash is made from the flowers and consumed once daily up to 2-3 months for the treatment of mental retardation.36, 37 Flowers are used for brewing a local wine to prevent high-altitude sickness in Darjeeling hills of eastern Himalayas.38 In Homeopathic Materia Medica, the dried leaves have been mentioned to be used in gout and rheumatism.39 Ayurvedic prepration “Ashoka Aristha” containing R. arboreum possesses oxytocic, oestrogenic and prostaglandin synthetase inhibiting activities.40 The beautiful red flowers of R. arboretum, in addition to being eaten raw as salad, are used for curing nasal bleeding.41

✓ **Biodynamic Notes:**

Leaf-effect on respiration. Leaf and flower CVS and CNS active. Flower-anti-cancer. Leaf and stem bark-spasmolytic.42

✓ **Modern Therapy:**

Water extract of leaf acts as a CNS depressant activity like chlorpromazine.43 Water, 50% ethanolic and methanolic extracts of flowers act as anti-inflammatory agents.44

**PREVIOUS STUDIES ON THE PLANT**

Uniyal et. al. reported that the flowers of R. arboreum were eaten raw as salad, used for curing nasal bleeding, and young leaves of the plant were considered to be poisonous, in the survey of the tribal communities of Chhota Bhangal, Western Himalaya.45

Paul et. al. documented biodiversity and conservation of Rhododendrons in Arunachal Pradesh in the Indo-Burma biodiversity hotspot.46 Singh et. al. reported the conservation of Rhododendron in the Sikkim Himalayas.47

Shaifulla et. al. isolated flavone glycoside 5,2’-dihydroxy-7-methoxy-4’-O-glucoside and dimethyl ester of terphthalic acid from the leaves of Rhododendron arboreum.48

It has been reported that water extract of mature dried leaves have CNS depressant effects in mice.49 In Homeopathic Materia Medica, the tincture of dried leaves has been mentioned to be used in gout and rheumatism.50 The Ayurvedic preparation “Ashoka Aristha”, containing R.
arboreum, possesses oxytocic, oestrogenic and prostaglandin synthetase inhibiting activities.\textsuperscript{51} Flowers of \textit{R. arboreum} have been claimed for cholinergic and anti-inflammatory activities.\textsuperscript{52, 53}

Water extract of leaves protected the mice against amphetamine induced group excitement, suggesting a CNS depressant activity like chlorpromazine. However, the extract did not produce analgesic effect with a sub hypnotic dose (10mg/kg.).\textsuperscript{54}

Agrawal \textit{et. al.} found significant anti-inflammatory activity of aqueous, 50\% ethanolic and methanolic extract of the flowers of \textit{R. arboreum} against rat’s hind paw oedema induced by carrageenan, PG (E\textsubscript{2}), histamine and 5HT.\textsuperscript{55}

Dixit \textit{et. al.} isolated \(\beta\)sitosterol, acetate, ursolic acid, quercetin and friedelin from the leaves and bark of \textit{R. arboreum}.\textsuperscript{56}

Harborne \textit{et. al.} identified uniform flavonoid pattern and quantitated various flavonoids such as Gossypetin, Kaempferol, Myricetin, Azaleatin, Caryatin, Dihydromyricetin, Dihydroquercetin, Dihydrokaempferol and Coumarins in the leaf survey of 206 \textit{Rhododendron} species, subspecies and varieties.\textsuperscript{57}

Harborne studied the natural distribution of flavonol 5-methyl ethers in the leaves and petals of 50 species of \textit{Rhododendron} and other higher plants.\textsuperscript{58}

Sharma \textit{et. al.} studied the anti-inflammatory activity of aqueous decoction and ethanolic extracts of the leaves. The inflammation was induced by carrageenan in Albino rats and mice. Aqueous extract showed significant activity (P < 0.001), comparable to the reference drug used.\textsuperscript{59}

Kunwar \textit{et. al.} reported various biological properties of the plant: bark of the plant is used in treatment of cough, diarrhea, dysentery, liver disorders, jaundice and diabetes. Flowers are used as an appetizer and in menstrual disorders and dysentery. Leaves were found to be effective in respiratory disorders.\textsuperscript{60}

Prakash \textit{et. al.} evaluated the hepatoprotective activity of the ethanolic extract of leaves against carbon tetrachloride-induced hepatotoxicity in Wistar rat model. Hepatoprotective effect was studied by assaying the activities of serum marker enzymes like SGPT, SGOT, ALP, direct and total bilirubin, triglycerides, cholesterol and estimation of ascorbic acid in urine.\textsuperscript{61}

Swaroop \textit{et. al.} performed the simultaneous determination of quercetin, rutin and coumaric acid in flowers of \textit{Rhododendron arboreum} by HPTLC. The separation was performed on TLC aluminium plates precoated with silica gel RP-18 F254S. Good separation was achieved
in the mobile phase of methanol-water-formic acid (40:57:3, v/v/v) and densitometric
determination of these compounds was carried out at 280 nm in reflectance/absorbance mode.\textsuperscript{62}

Kiruba \textit{et. al.} reported three biologically active phenolic compounds, i.e. quercetin, rutin
and coumaric acid in the flowers of \textit{Rhododendron arboreum} Sm. ssp. nilagiricum using
HPTLC.\textsuperscript{63}

Verma \textit{et. al.} evaluated the hepatoprotective potential of ethyl acetate fraction of the
flowers of \textit{Rhododendron arboreum} (Ericaceae) in Wistar rats against carbon tetrachloride
induced liver damage in preventive and curative models. The substantially elevated serum
enzymatic activities of SGOT, SGPT, SALP, $\gamma$-GT, and bilirubin due to CCl\textsubscript{4} treatment were
restored normal in a dose-dependent manner. The biochemical observations were supplemented
with histopathological examination of rat liver sections.\textsuperscript{64}

Verma \textit{et. al.} investigated and standardized ethyl acetate fraction of \textit{Rhododendron arboreum}
flowers (EFRA) for antidiarrheal activity in experimental animals. Quantitative
estimation of hyperin (0.148\%) in the above fraction was done by HPTLC. EFRA displayed a
significant potential in castor oil and magnesium sulfate-induced diarrhea. The antidiarrheal
activity was found to be dose dependent.\textsuperscript{65}

Giriraj \textit{et. al.} reported the climatic and ecological distribution of \textit{Rhododendron arboreum}
Sm. ssp. nilagiricum (Zenker) Tagg (Ericaceae), in the Nilgiri, Annamalai, Palni and
Meghamalai hills of the Western Ghats, using ecological niche modeling.\textsuperscript{66}

Mudgal \textit{et. al.} studied the preventive effect of the whole plant extract of \textit{Rhododendron arboreum}
on cardiac markers, lipid peroxides and oxidative stress in normal and isoproterenol-
induced myocardial necrosis in rats. Serum cardiac marker enzymes, like lactate dehydrogenase
(LDH), aspartate transaminase (AST) and alanine transaminase (ALT) and lipid peroxidative
product, like lipid hydroperoxidase, were elevated in tissue and serum and a decline in enzymatic
antioxidant status (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic
reduced glutathione antioxidants.\textsuperscript{67}
PLANT PROFILE (*Carum copticum*)

*Carum copticum* (Ajwain) or commonly called Bishop's weed is widely cultivated in gardens and commercially for its value as a spice and medicine. Its essential oil, thymol, was in great demand in past due to its antiseptic properties, before the synthesis of a substitute. It is commonly used as household medicine for bowel disorders.

✓ **Synonyms**: 68

- Sanskrit : Yavanika, Ajmada and Deepayaka
- Hindi : Ajowan and Omum
- English : Bishop’s weed
- Punjabi : Ajawain
- Telgu : Omamu

✓ **Classification:**

- Kingdom : Plantae
- Division : Magnoliophyta
- Class : Magnoliopsida
- Order : Apiales
- Family : Apiaceae
- Genus : *Trachyspermum*
- Species : *C. copticum*

![Figure 2.1 Seeds of *Carum copticum*.](image)

*Carum copticum* is a smooth or slightly hairy branched annual (or perennial) herb reaching a height of 90 cm. It is an aromatic spice, resembling thyme in flavor. The plant is indigenous to
India, Iran, and Egypt, but has also been introduced into the United States. Bishop's weed has small white flowers and leaves that are pinnately divided in twos or threes. The fruit is harvested from February to March and is separated when dried. The oval fruits are one-seeded. The aromatic seeds are grayish-brown in color.

**Chemical constituents**

An analysis of the ajwain seeds shows them to consist of moisture 7.4 per cent, proteins 17.1 per cent, fat 21.8 per cent, minerals 7.9 per cent, fibres 21.2 per cent and carbohydrates 24.6 per cent per 100 grams. Calcium, phosphorus, iron, carotene, thiamine, riboflavin and niacin are amongst the vitamins and minerals in it. Volatile oil is rich in cymene, terpene, thyme and steroptin. The oil of ajwain is an almost colourless to brownish liquid with characteristic odour and a sharp hot taste. If the liquid is allowed to remain undisturbed, a part of the thymol may separate from the crystals, which is sold in Indian markets under the name of ajwain ka phul or sat ajwain. It is much valued in medicine as it has nearly all the properties ascribed to the ajwain seeds.

**Traditional uses**

Seeds (fruits) are used as anti-spasmodic, stomachic, carminative, stimulant, tonic, in antidiarrhoeal, in atonic dyspepsia, colic, flatulence, indigestion and cholera. It acts as an excellent nervine tonic. Ajwain is popular mainly for stimulating digestion and correcting the digestive disorders. It may be taken as a powder after food for digestive troubles such as dyspepsia, heartburn, indigestion flatulent colic, diarrhoea due to indigestion, and gastrointestinal infection. Its carminative properties are enhanced when combined with black salt or rock salt. Administration it with each feed helps in easy digestion and prevents vomiting. Eating ajwain seeds with jaggery alleviates urticaria that results due to indigestion. Ajwain seeds are used in treatment of inflammation and umbilical hernia (children/infant). Powder of the seeds mixed with alum and common salt is used as a dentifrice. As it has kapha and vaata pacifying nature, it is often used in respiratory troubles and a variety of allergies. The infusion of ajwain with common salt is used in the treatment of throat troubles like acute pharyngitis, sore throat, horseness of the voice and cough. While infusion mixed with two grains of soda-bicarb (baking soda) is given as a medicine for indigestion and flatulence in children.

Seeds contain 4-6% essential oil, containing 45-55% thymol. Thymol is used as a condiment; ajwain oil is used as an antiseptic and to aid digestion. Omum water is distilled from the seeds, and the omum water and omum seeds are house hold substances is India. Roots are
used as diuretic and carminative. Tender leaves and leaf juice are used as vermicide. Seeds of *Carum copticum* increase virility and cure premature ejaculation.\(^72-74\)

**PREVIOUS STUDIES ON THE PLANT**

Boskabady *et. al.* studied the bronchodilatory effect of boiled extract of the seeds of *Carum copticum* in the airways asthamatic patients. They found significant increase in all pulmonary function tests (PFT) on the treatment with extract when compared with the standard drug, theophylline.\(^75\)

Gilani *et. al.* prepared aqueous-methanolic extract of *Carum copticum* seeds and found significant antihypertensive, antispasmodic, bronchodilator and hepatoprotective activities in rats. *Carum copticum* seed extract (CSE) caused a dose dependent fall in arterial blood pressure in anaesthetized rats, inhibitory effect on K+ induced contractions, Ca channel blocking effects and inhibition of carbachol and K+ induced bronchoconstriction.\(^76\)

Boskabady *et. al.* screened aqueous extract of *Carum copticum* seeds for antitussive effect in guinea pigs. The antitussive effects of aerosols of two different concentrations of aqueous and macerated extracts, carvacrol, codeine and saline were tested by counting the number of cough produced. The cough number obtained in the presence of higher concentration of the extracts was significantly less than the cough numbers produced by codeine.\(^77\)

Rani *et. al.* studied methanolic and aqueous extracts of fifty four important plants of Ayurveda against multi-drug resistant *Salmonella typhi*. Seeds of *Carum copticum* showed strong antibacterial activity.\(^78\)

Singh *et. al.* extracted essential oil from the seeds of seven spices, *Carum copticum* being one of them. It was found that the oil of *C. capticum* was very effective against all tested bacteria, when compared with standard antibiotics.\(^79\)

Biswas *et. al.* tested a herbal eye drop, containing *Carum copticum* as an ingredient, for the treatment of conjunctivitis, conjunctival xerosis (dry eye), acute dacryocystitis, degenerative conditions (pterygium or pinguecula) and postoperative cataract in patients.\(^80\)

Ishikawa *et. al.* isolated twenty five compounds, including five new monoterpenoid glucosides, a new monoterpenoid, two new aromatic glucosides, and two new glucides from the water-soluble portion of the methanol extract of the fruit of *Carum copticum*.\(^81\)

Boskabady *et. al.* studied Inhibitory effect of *Carum copticum* on histamine (H\(_1\)) receptors of isolated guinea-pig tracheal chains. The results of this study indicated a competitive antagonistic effect of *C. copticum* at histamine \(H_1\) receptors.\(^82\)
Dashti et al. investigated analgesic activity of the ethanolic extract of *Carum copticum* fruits and morphine (standard) by tail-flick induced analgesia in mice. Results indicated that the extract produced a significant increase in tail-flick latency (TFL) during two hours post-drug administration.\(^8^3\)

The headspace solid phase microextraction coupled with gas chromatography-mass spectrometry was used to identify the volatile oil components of *Carum copticum* cultivated in India by Dehghan et al. cultivated in Iran. Ten components were identified according to their retention indices and mass spectra (EI, 70 eV). The major components of the seed were found to be thymol, gamma-terpinine, p-cymene, myrcene and β-pinene.\(^8^4\)

The bronchodilatory effect of different fractions of essential oil from *Carum copticum* and theophylline in comparison with ethanol was examined by their relaxant effects on precontracted tracheal chains of guinea pig by KI and methacholine. Fraction 2 and 3 of the essential oil from *Carum copticum* showed potent and concentration dependent relaxant effects comparable to theophylline.\(^8^5\)

Magnetic resonance imaging was used to localize the site of essential oil accumulation in the fruit of *Carum copticum*. The presence of essential oil secretory structure in the fruit and an essential oil containing a high proportion of thymol were confirmed with optical microscopy and gas chromatography-mass spectrometry, respectively.\(^8^6\)

Vasudevan et al. studied the influence of intra-gastric perfusion of aqueous spice extracts on acid secretion in anesthetized Albino rats. All the spices tested increased gastric acid secretion, in some by cholinergic mechanism and some by other mechanism. Red pepper produced maximum increased acid secretion, but this was significantly reduced in injured stomachs. Cumin and coriander increased acid secretion in injured stomachs.\(^8^7\)

Nutrient composition of eight commonly consumed spices of South India was analyzed. *Carum copticum* was one of them. The nutrients analyzed were proximate principles, minerals, starch, sugars, dietary fibers, tannins, phytic acid, enzyme inhibitors and amino acids. Tannin contents of ajowan were found to be high. Spices had appreciable amount of essential amino acids like lysine and threonine.\(^8^8\)

An aqueous extract from roasted seeds of *Carum copticum* has cholinomimetic effects. It showed muscarinic effects on rabbit duodenum, guinea pig ileum and rat jejunum, and on the blood pressure of rat and cat. These effects were blocked by atropine.\(^8^9\)
Carvacrol is a monoterpene phenol, isolated from the volatile oil of some plants, including *Carum copticum*. The study showed carvacrol inhibited HepG2 cell growth by inducing apoptosis, as evidenced by Hoechst 33258 stain and flow cytometric analysis. Carvacrol may induce apoptosis by direct activation of the mitochondrial pathway, and the mitogen-activated protein kinase pathway.\(^{90}\)

Rezvani et al. studied the effect of aqueous extract of *Carum copticum* seeds (CCS) on kindling models of epilepsy. The sedative and anxiolytic effects of the extract were also assessed in an open-field apparatus and elevated plus maze, respectively on male Sprague-Dawley rats. Different doses of extract significantly delayed the incidence of every seizure stage in the pentylenetetrazol model of kindling.\(^{91}\)

Deb et al. investigated modulatory effects of methanolic extract of *C. copticum* seeds (MCE) against hexavalent chromium induced cytotoxicity, genotoxicity, apoptosis and oxidative stress on human bronchial epithelial cells (BEAS-2B) and isolated human peripheral blood lymphocyte (PBL) *in vitro*. MCE showed beneficial effects in preventing Cr (VI) induced toxicity in BEAS-2B and PBL cells.\(^{92}\)

Khan et al. isolated a new compound from the seeds of *Carum copticum*. The isolated compound was characterized as (4aS, 5R, 8aS) 5, 8a-di-1-propyl-octahydronaphthalen-1-(2H)-one, with a great potential to be used as a therapeutic agent against dental caries.\(^{93}\)

Bekhechi et al. demonstrated that isothymol is the major component of ajowan essential oil, when the plant was collected near the sea at low altitude and at the beginning of the flowering stage, while in other cases, thymol is the predominant constituent.\(^{94}\)

Zahin et al. reported that the methanol fraction showed highest antioxidant activity in the phosphomolybdenum and DPPH assay, followed by other fractions comparable to ascorbic acid and BHT (Butylated hydroxytoluene). Based on the antioxidant activity, methanol fraction was evaluated for antimutagenic potential against direct acting mutagens, sodium azide (NaN\(_3\)) and methyl methane sulphonate (MMS), and indirect acting mutagens, 2-aminofluorene (2-AF) and benzo(a)pyrene (B(a)P), using Salmonella typhimurium (TA97a, TA98, TA100, and TA102) tester strains. The methanolic fraction showed no sign of mutagenicity at tested concentrations (25-100 microg/plate).\(^{95}\)

Alizadeh et al. evaluated parameters of antifungal activity of the essential oils of *Zataria multiflora, Thymus migricus, Satureja hortensis, Foeniculum vulgare, Carum capticum* and
thiabendazol fungicide on survival and growth of different species of Aspergillus. The results showed that all essential oils could inhibit the growth of Aspergillus species.  

2.2 RESEARCH ENVISAGED

Seeds of Carum copticum have been prescribed for colic, diarrhea, other bowel disorders, and in the treatment of asthma. Ajowan seeds made hot are used as a dry fomentation to the chest in asthma, hands and feet in cholera, fainting, syncope, and in rheumatism. In Homeopathic Materia Medica, the nature of dried leaves of Rhododendron arboreum (Sm.) has been mentioned to be useful in gout and rheumatism.

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disorder that causes the immune system to attack the joints, where it causes inflammation (arthritis) and destruction. It is usually described as Type III hypersensitivity. Asthma is an immune disorder resulting from an immune response to inhaled allergens. The humoral immune system produces antibodies against the inhaled allergens.

Naturally occurring triterpenoids such as glycyrrhizic acid, ursolic acid, oleanolic acid and nomilin showed immunomodulatory activity by remarkable inhibition of delayed type hypersensitivity reaction (DTH). β-Sitosterol and its glucoside (steroline) enhanced the cellular response of the T-lymphocytes in both, in-vitro and in-vivo, studies. They also showed immunomodulatory effect against disseminated candidiasis from Candida albicans caused by the CD4+ and TH1 immune responses. Eupalitin-o-β-D-galactopyranoside is a flavonoid isolated from Boerhaavia diffusa root. It showed immunomodulatory activity through the inhibition of phytohemagglutinin-stimulated proliferation of peripheral blood mononuclear cells (PBMC) and two-way MLR and natural killer cells.

Mixtures of essential oils, containing thymol and carvacrol, displayed potential immunomodulatory activity by increasing the proportion of CD4+, CD8+ and double positive T cells in peripheral blood and mesenteric lymph nodes. Thymol, when used alone, enhances total IgA and IgM serum levels and exhibited some local anti-inflammatory properties, as indicated by a reduction in TNF-α mRNA in the stomach of post-weaned pigs.

Flavonoids (flavone glycoside 5, 2’-dihydroxy-7-methoxy-4’-O-glucoside, kaempferol, dihydroquercetin, dihydrokaempferol, flavonol 5-methyl ethers and quercetin), triterpenoids and phytosterol (ursolic acid and β-sitosterol) were reported in the leaves of R. arboreum.

Seeds of Carum copticum contain 4-6% essential oil; containing 45-55% thymol as one of the major constituents of the plant.
On the basis of above findings, regarding the traditional uses and chemical constituents of the selected plants, it was decided to explore the immunomodulatory principles from leaves of *Rhododendron arboreum* and seeds of *Carum copticum*.

### 2.3 PLAN OF WORK

1. Collection and authentication of the plant materials.
2. Preparation of the plant extracts by cold percolation method with 95% ethanol.
3. Immunomodulatory activity of the crude extracts: The parameters to be used in this model are:
   - **Humoral Immunity** - Haemagglutination assay would be used for the evaluation of humoral immune responses.
     - *Primary humoral immune response*
     - *Secondary humoral immune responses*
   - **Cell-mediated Immunity** - Cellular immune response would be investigated by-
     - *Footpad reaction method / Delayed type hypersensitivity reaction*
     - *Phagocytosis*
4. Fractionation of the active crude extracts.
5. Immunomodulatory activity of the above fractions.
6. Isolation of pure compounds from the active fraction by using column chromatography and other feasible techniques.
7. Characterization of the active compounds by using various group tests, chromatographic and spectroscopic methods.
8. Immunomodulatory activity of the pure isolated compounds, if obtained in sufficient quantity.
9. Evaluation of any other pharmacological activity as the case may be.
REFERENCES


3.1 PHYTOCHEMICAL SCREENING

Phytochemistry is, in the strict sense of the word, the study of phytochemicals. These are chemicals derived from plants. In a narrower sense, the term is often used to describe the large number of secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant diseases. They also exhibit a number of protective functions for human consumers. Techniques commonly used in the field of phytochemistry are extraction, isolation and structural elucidation (MS, 1D and 2D NMR) of natural products, as well as various chromatographic techniques (MPLC, HPLC, LC-MS).}

3.1.1 MATERIALS AND METHODS

The authentic markers of quercetin, rutin and thymol were procured from S.D. Fine Chemicals, India. All the chemicals and solvents used were of analytical and HPLC grade. Melting ranges were determined by open capillary method using a melting point apparatus, Edutek instrumentation. UV-Visible spectra were recorded on UV-1700 pharmaspec-UV-Visible spectrophotometer, Shimadzu. IR spectra were recorded on Shimadzu FTIR-8400S in the range of 400-4000 cm\(^{-1}\) with KBr pellets. NMR spectra were obtained on a Bruker ADVANCE DRX 300 MHz spectrometer, with TMS as an internal standard, at room temperature (\(\delta\) in ppm, \(J\) in Hz). ESIMS and DART-MS were carried out on a JEOL SX 102/DA-600 mass spectrometer. Camag Linomat IV applicator was used for sample application. Camag Twin trough glass chamber (20 x10 cm) was used for the development of plates and Camag TLC scanner-3, equipped with win CATS 1.4.4.6337 version software, was used for interpretation of data. Optical and specific rotation were carried out on Rudolph research analytical Autopol-III, automatic polarimeter. Gas chromatograms were recorded on Perkin Elmer Autosystem-XL chromatograph with flame ionization detector (FID). HPLC chromatograms were recorded on D-7000 HPLC system with PDA detector, MERK. Fractions were monitored by TLC (silica gel 60F\(_{254}\), E. Merck, Darmstadt, Germany) and spots were visualized using iodine vapors and UV light. Solvents were distilled prior to use.

3.1.1.1 Phytochemical screening of the plant R. arboreum

Collection and authentication of the plant and plant parts of R. arboreum

Leaves and flowers of *Rhododendron arboreum* were collected from the forests of “Tala” village of Ukhi Math hills, situated in Rudraprayag (Uttarakhand), India. The plant was identified and authenticated by the Pharmacognosy and Ethnopharmacology Division, National
Botanical Research Institute, Lucknow (India). Plant herbarium was submitted to NBRI bearing Ref. No. NBRI/CIF/72/2009.

**Alcoholic extract of the whole flowers of R. arboreum (ERAF)**

Flowers of *R. arboreum* were shade-dried and powdered. The powder (100 g) was packed in a soxhlet apparatus and subjected to hot continuous percolation for about 8 hr using 350 ml of ethanol as the solvent. The extract was concentrated to a semi-solid mass under vacuum and completely dried in a desiccator (yield 38.25% w/w).

**Aqueous extract of the whole flowers of R. arboreum (ARAF)**

Approximately 100 g of shade-dried powder of *R. arboreum* was taken in a 1 L beaker and chloroform: water (1:99) was added up to a sufficient level to immerse the drug completely. Chloroform was added as a preservative to prevent microbial infection. This set up was placed aside for 72 h, with stirring at alternate intervals. Finally, the extract from the beaker was vacuum filtered to get a clear watery brown coloured extract. The extract was concentrated under high vacuum and completely dried in a desiccator (yield 6.9% w/w).

**Alcoholic extract of the leaves of R. arboreum (ERAL)**

Powdered leaves of *R. arboreum* (400g) were packed in a soxhlet apparatus and subjected to hot percolation for four days, using 800 ml of ethanol (95%v/v) as the solvent. The solvent was evaporated under vacuum using rotary evaporator (IKA-RV 10 basic), and was further dried in a desiccator under reduced pressure for complete drying.

**Table 3.0 Preliminary phytochemical investigation of the flower and leaf extracts of R. arboreum.**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Chemical test</th>
<th>Observation</th>
<th>Inference</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><strong>Alkaloids:</strong> To the extract, dil. HCl was added, shaken well filtered. To the filtration perform the following tests, were performed</td>
<td>Cream coloured ppt.</td>
<td>Presence of alkaloids</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td><strong>Mayer’s Test:</strong> Few drops of potassiomercuric iodide solution.+ Filtrate.</td>
<td>Yellow colour</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td><strong>Hager’s Test:</strong> Few drops of saturated solutionary of Picric acid + Filtrate.</td>
<td>Reddish-brown colour</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Wagner’s test:</strong> iodine solution in KI + filtrate.</td>
<td></td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

59
<table>
<thead>
<tr>
<th>2</th>
<th><strong>Carbohydrates:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molisch Test</strong> - (general test)</td>
<td>2 ml of 10% etanolic solution of 1-naphthol. + Extract + Few drops of concentrated H$_2$SO$_4$ from the sides of test tube.</td>
</tr>
<tr>
<td><strong>Fehling’s Test</strong></td>
<td>1 ml Fehling’s solution-A + 1 ml Fehling’s solution-B + equal vol. of extract and heated on a boiling water bath.</td>
</tr>
<tr>
<td><strong>Benedict’s Test</strong></td>
<td>Benedict’s soln + equal vol. of ext. and heat at boiling water bath</td>
</tr>
</tbody>
</table>

**Red ring appears at interface.**

**Red ppt. formed.**

**Red ppt. formed.**

| Presence of carbohydrates | + | + | + |
| Presence of steroids | + | - | + |

| 3- | **Steroids:** |

**Salkowski Test** - To 2 ml of the extract, 2 ml chloroform and 2 ml conc. H$_2$SO$_4$ were added, through the sides of the test tube. |

**Red colour appeared in chloroform layer and green fluorescence in acid layer.**

**Presence of steroids.**

| Presence of amino acids | – | – | – |

| 4- | **Amino acids:** |

**Ninhydrin Test** - 0.25% of n-butanolic soln. of ninhydrin + Extract, placed on a boiling water bath for 10 min. |

**Blue-violet colour.**

**Presence of amino acids.**

| Presence of phenolic compounds | + | + | + |

| 5- | **Phenolic compounds:** |

1% ‘neutral’ ferric chloride solution + Extract. |

**Transient or permanent purple/blue or green colour.**

**Presence of phenolic compounds.**

| Presence of saponins | + | + | + |

| 6 | **Flavonoids:** |

**Pew’s Test** - Zinc powder + 1 drop of 5N HCl + Extract |

**Deep purple-red/cherry red colour.**

**Presence of flavonoids.**

| Presence of flavonoids | + | + | + |

**Shinoda Test** - Mg powder + 1 drop of 5N HCl + Ext. |

**Deep-red or magenta colour.**

| Presence of flavonoids | + | + | + |

| 7- | **Saponins:** |

The extract was shaken in a conical flask. |

**Foaming occurred.**

**Presence of saponins.**

| Presence of flavonoids | + | + | + |

1= ERAF, 2=ARAF, 3=ERAL

**Fractionation and Isolation of ERAF**

About 20 gm of ERAF was suspended in 200 ml of distilled water and was extracted several times, by taking 10 ml each with solvents of decreasing polarity (such as n-hexane,
chloroform, n-butanol), in a separating funnel. All the fractions were concentrated under vacuum, using rotary evaporator, and placed in a desiccator at reduced pressure for complete drying.

**Table 3.1:** Percentage yields of ERAF fractions.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fractions</th>
<th>Weight (gm)</th>
<th>% Yield (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-Hexane</td>
<td>0.09</td>
<td>0.45</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform</td>
<td>0.152</td>
<td>0.76</td>
</tr>
<tr>
<td>3</td>
<td>n-Butanol</td>
<td>8.73</td>
<td>42.2</td>
</tr>
</tbody>
</table>

Approximately 6 gm of completely dried n-butanol fraction (dark brown tarry mass) was dissolved in 200 ml of distilled water. The insoluble material was filtered out and the filtrate was set aside at 4°C for 48 hr. No precipitation or crystallization was observed. The filtrate was then kept at room temperature for 1 hr and further extracted several times with diethyl ether. The ethereal fractions were pooled in a petri-dish and the solvent evaporated in air. A brownish-yellow amorphous powder was obtained, which was dissolved in 20 ml of ice-cold water and centrifuged at 2000 rpm for 5 min to remove the brown coloured pigment. The supernatant was discarded and the remaining residue was extracted with ethanol. This was repeated two times. A yellowish-green amorphous powder was obtained (7.0 mg, 0.0167% w/w).

**Characterization of the isolated compound**

The isolated compound was characterized by melting point, chemical tests, TLC, paper chromatography, UV-Visible spectroscopy, FT-IR Spectroscopy, $^1$H-NMR, $^{13}$C-NMR and mass spectrometry. All the above spectra of isolated compound were then compared with the obtained spectra of authentic standard (quercetin). It was confirmed that the compound thus isolated was quercetin (IQ).

**M.P. >308°C**

**Identification tests**

Shinoda, and Pew’s tests of flavonoids were found to be positive with IQ.

**TLC and paper chromatography**

TLC and paper chromatography of IQ was performed using various solvent systems, viz. n-Butanol: Acetic acid: Water (BAW- 4:1:5), conc. HCl: Acetic acid: Water (Forestal, 3:30:10), 50% HOAc $^5$, Chloroform: Acetic acid: Water (CAW- 10:9:1), and Chloroform: Methanol: Water (CMW- 35:7:2)$^9$, on aluminium foil precoated silica gel 60 F$_{254}$ and Whatman number-
filter paper respectively. Chromatograms were evaluated under UV light at short and long wavelengths to detect the flavonoids. Finally, these were developed with NH₃ solution to confirm the presence of flavonoids. Co-chromatography of IQ was performed with standard (Quercetin) and Rf x 100 was measured for all chromatograms given in Table 3.3. Well-defined spots were obtained in two solvent systems including BAW and CAW.

![Figure 3.0 TLC and Paper chromatography of IQ and Standard (Quercetin)](image)

**Table 3.2** TLC and Paper chromatographic observations of IQ and Standard (Quercetin)

<table>
<thead>
<tr>
<th>SOLVENT SYSTEMS</th>
<th>BAW</th>
<th>CAW</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TLC Analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rf (x100 cm.)</td>
<td>2.7/5.4 x100 =50</td>
<td>3.9/4.4 x 100 =88.7</td>
</tr>
<tr>
<td><strong>Colour of spots</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unaided</td>
<td>Dull brownish-yellow</td>
<td>Dull brownish-yellow</td>
</tr>
<tr>
<td>Short UV wavelength</td>
<td>Dull-brown</td>
<td>Dull-brown</td>
</tr>
<tr>
<td>Long UV wavelength</td>
<td>Black</td>
<td>Black</td>
</tr>
<tr>
<td>NH₃ Soln.</td>
<td>Intense-yellow colour</td>
<td>Intense-yellow colour</td>
</tr>
<tr>
<td><strong>Paper chromatography</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rf (x100 cm.):</td>
<td>6.1/7.1 x 100 =85.9</td>
<td>Tailing</td>
</tr>
<tr>
<td><strong>Colour of spots:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unaided</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>Short UV wavelength</td>
<td>Dull-brown</td>
<td>Dull-brown</td>
</tr>
<tr>
<td>Long UV wavelength</td>
<td>Black</td>
<td>Black</td>
</tr>
<tr>
<td>NH₃ Solution</td>
<td>Intense-yellow colour.</td>
<td>Intense-yellow colour</td>
</tr>
</tbody>
</table>
UV-Visible Spectroscopy

The UV-Visible spectroscopy of IQ and standard quercetin (SQ) was performed using ethanol as solvent and by adding, aluminium chloride, hydrochloric acid, sodium acetate and boric acid as diagnostic and shifting reagents.

Table 2.3 Comparison of the UV spectral effects of IQ with the standard (Quercetin)

<table>
<thead>
<tr>
<th>Shifting reagents</th>
<th>Spectral maxima (nm)</th>
<th>Spectral effect (Bathochromic shift)</th>
<th>Functional groups Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Band (I)</td>
<td>Band (II)</td>
<td></td>
</tr>
<tr>
<td>Unaided</td>
<td>370*</td>
<td>255*</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>372#</td>
<td>254#</td>
<td>Flavonoids</td>
</tr>
<tr>
<td>AlCl₃ (5% in EtOH)</td>
<td>437*</td>
<td>267*</td>
<td>67 nm band (I) and 12 nm in (II)</td>
</tr>
<tr>
<td></td>
<td>443#</td>
<td>268#</td>
<td>71 nm band (I) and 13 nm in (II)</td>
</tr>
<tr>
<td>AlCl₃+HCl</td>
<td>431*</td>
<td>266*</td>
<td>No significant shift in band (I) and (II) in comparison to AlCl₃</td>
</tr>
<tr>
<td></td>
<td>431#</td>
<td>264#</td>
<td></td>
</tr>
<tr>
<td>NaOAc</td>
<td>456*</td>
<td>270*</td>
<td>86 nm band (I) and 15 nm in (II)</td>
</tr>
<tr>
<td></td>
<td>448#</td>
<td>269#</td>
<td>76 nm band (I) and 15 nm in (II) in comparison to Unaided.</td>
</tr>
<tr>
<td>NaOAc+H₃</td>
<td>440*</td>
<td>269*</td>
<td>70 nm band (I) and 14 nm in (II)</td>
</tr>
<tr>
<td>BO₃</td>
<td>452#</td>
<td>270#</td>
<td>82 nm band (I) and 16 nm in (II) in comparison to Unaided.</td>
</tr>
</tbody>
</table>

* = IQ    # = standard quercetin

Table 3.4 Spectroscopic data of isolated quercetin (IQ)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spectrum</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IR (KBr) v₉max</td>
<td>3284, 1664, 1610, 1560, 1521 and 1448 cm⁻¹</td>
</tr>
<tr>
<td>2</td>
<td>EIMS (70eV) m/z</td>
<td>303.2 (M+1).</td>
</tr>
<tr>
<td>3</td>
<td>¹H-NMR (300MHz) DMSO, δ (ppm)</td>
<td>6.187 (s, H), 6.408 (s, H), 7.524-7.673 (d, 2H), 9.322-9.378 (d, 2H), 9.616 (s, H), 10.812 (s, H), 12.496 (s, H).</td>
</tr>
<tr>
<td>4</td>
<td>¹³C-NMR (300MHz) DMSO, δ (ppm)</td>
<td>93.71 (s), 98.54 (s), 103.36 (s), 115.41 (s), 115.95 (s), 120.34 (s), 122.31 (s), 136.07 (s), 145.40 (s), 147.16 (s), 148.04 (s), 156.49 (s), 161.07 (s), 164.22 (s), 176.18 (s),</td>
</tr>
</tbody>
</table>
Figure 3.1 FT-IR spectrum of compound IQ.

Figure 3.2 Mass spectrum of compound IQ.
Figure 3.3 $^1$H-NMR spectrum of compound IQ.

Figure 3.4 $^{13}$C-NMR spectrum of compound IQ
On the basis of above spectral studies, the isolated compound was identified as quercetin.

![Structure of Quercetin](image)

C₁₅H₁₀O₇
Exact Mass: 302.04
Mol. Wt.: 302.24
2-(3,4-Dihydroxy-phenyl)-3,5,7-trihydroxy-chromen-4-one
(Quercetin)

**Fractionation of the alcoholic extract of the leaves of R. arboreum (ERAL)**

Ethanolic extract (100 g) suspended in distilled water (200ml) was and then extracted in a separatory funnel with n-hexane (25ml x 10), chloroform (25ml x 8) and n-butanol (25ml x 4). All the fractions were concentrated under vacuum using rotary evaporator and then placed in a desiccator at reduced pressure for complete drying.

**Table 3.5** Percentage yields of the fractions of alcoholic extract.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fractions</th>
<th>Weight (gm)</th>
<th>% Yield (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-Hexane</td>
<td>27.7</td>
<td>27.7</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform</td>
<td>14.8</td>
<td>14.8</td>
</tr>
<tr>
<td>3</td>
<td>n-Butanol</td>
<td>2.3</td>
<td>2.30</td>
</tr>
</tbody>
</table>

**Column chromatography of n-hexane fraction of ERAL**

The fraction (25 g) was subjected to chromatography over silica gel (60-120 mesh) column using n-hexane with increasing polarity by ethyl acetate as eluent. Fractions of 10 ml of each were collected and similar fractions were pooled after monitoring their TLC. It afforded four pure compounds-

**Compound P-1a:**

A mixture of three compounds was obtained from the eluent of n-hexane: ethyl acetate (95:05) as light yellow colored semisolid. This mixture was recrystallised with petroleum ether and filtered. The residue was washed with chilled petroleum ether to get a white colored gummy amorphous powder with single spot on TLC.

Yield 3.7 mg, mp 283°C.
Table 3.6 Spectroscopic data of compound P-1a

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spectrum</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IR (KBr) $\nu_{\text{max}}$</td>
<td>3448, 3425, 2920, 2850, 1708, 1618, 1461, 1251 cm$^{-1}$</td>
</tr>
<tr>
<td>2</td>
<td>EIMS (70eV)</td>
<td>m/z 473.9 (M+1)</td>
</tr>
<tr>
<td>3</td>
<td>$^1$H-NMR (300MHz) DMSO, $\delta$ (ppm)</td>
<td>0.922-1.975 (m), 2.25-2.39 (s), 3.76-3.78 (m), 5.30-5.40 (m).</td>
</tr>
</tbody>
</table>

Figure 3.5 FT-IR spectrum of compound P-1a.
Figure 3.6 Mass spectrum of compound P-1a.

Figure 3.7 $^1$H-NMR spectrum of compound P-1a.
These spectral data were similar to those of the constituent isolated previously and identified as ursolic acid\textsuperscript{11}.

\[
\text{C}_{31}\text{H}_{53}\text{O}_3 \\
\text{Exact Mass: 472.39} \\
\text{Mol. Wt.: 472.74}
\]

10-Hydroxy-1,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-octadecahydro-2H-picene-4a-carboxylic acid; compound with methane

\textbf{(Ursolic acid)}

\textbf{Compound P-2a:}

A single compound was obtained from the eluent of n-hexane: ethyl acetate (95:05) as colorless fine crystals in elute after standing it overnight. These crystals were filter and washed with chilled n-hexane.

Yield 2.6 mg, mp 143°C.

\textbf{Table 3.7} Spectroscopic data of compound P-2a

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spectrum</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IR (KBr) $\nu_{\text{max}}$</td>
<td>3552, 3477, 3415, 2920, 2850, 1685, 1618 and 1461.cm\textsuperscript{-1}</td>
</tr>
<tr>
<td>2</td>
<td>EIMS (70eV)</td>
<td>m/z 708.2, 665.3, 664.3, 663.2, 409.2, 391.</td>
</tr>
<tr>
<td>3</td>
<td>$^1$H-NMR (300MHz) DMSO, $\delta$ (ppm)</td>
<td>0.598-2.196 (m), 3.09-3.18 (m), 4.49-4.61 (d, $J$=35.25), 5.058-5.113 (s).</td>
</tr>
</tbody>
</table>
Figure 3.8 FT-IR spectrum of compound 2a.

Figure 3.9 Mass spectrum of compound 2a
Compound P-3:
A single compound was obtained from the eluent of n-hexane: ethyl acetate (95:05) as light green amorphous powder. This was purified by washing with chilled n-hexane, Yield- 16 mg, mp 199 ºC.

Table 3.8 Spectroscopic data of compound P-3

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spectrum</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IR (KBr) νₘₐₓ</td>
<td>2921, 1639, 1620 1461, 1454, 1384 and 1190 cm⁻¹</td>
</tr>
<tr>
<td>2</td>
<td>EIMS (70eV)</td>
<td>m/z 426 (M⁺ peak), 425, 410, 409, 218, 205, 203 and 191</td>
</tr>
<tr>
<td>3</td>
<td>¹H-NMR (300MHz) CDCl₃, δ (ppm)</td>
<td>0.740-2.016 (m), 3.66 (s), 4.161-4.186 (d, J=7.5), 4.587-4.708 (d, J=36.3), 5.148-5.203 (s), 7.282 (s)</td>
</tr>
</tbody>
</table>
Figure 3.11 FT-IR spectrum of compound P-3.

Figure 3.12 Mass spectrum of compound P-3.
On the basis of above spectral studies, the data were found to be similar as reported in literature.\textsuperscript{12} Thus the isolated compound was identified as 3, 10-epoxy glutinane.

\textbf{Compound P-3C:}

A mixture of two compounds was obtained from the eluent of n-hexane: ethyl acetate (90:10), as a light yellow colored gummy amorphous powder. This mixture was further subjected to chromatography over silica gel (60-120 mesh) column, using chloroform with increasing polarity by methanol as eluent. A single compound was obtained from the eluent of chloroform: methanol (99:01) as a white-gummy amorphous powder.

Yield- 5.4 mg, Melting range: 146-150 °C.
Table 3.9 Spectroscopic data of the compound P-3C.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spectrum</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IR (KBr) ν&lt;sub&gt;max&lt;/sub&gt;</td>
<td>3406, 2920, 2852, 1618, 1381, 1033 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>EIMS (70eV) m/z</td>
<td>416.1 (M+2), 415 (M+1), 414 (M&lt;sup&gt;+&lt;/sup&gt; peak), 412, 354, 298 and 253</td>
</tr>
<tr>
<td>3</td>
<td>&lt;sup&gt;1&lt;/sup&gt;H-NMR (300MHz) CDCl&lt;sub&gt;3&lt;/sub&gt;, δ (ppm)</td>
<td>0.650-2.032 (m), 2.228-2.283 (m), 3.485-3.556 (m), 5.343-5.357 (s).</td>
</tr>
<tr>
<td>4</td>
<td>&lt;sup&gt;13&lt;/sup&gt;C-NMR (300MHz) CDCl&lt;sub&gt;3&lt;/sub&gt;, δ (ppm)</td>
<td>12.09-12.22 (d, J=39), 19.02 (s), 19.29-19.62 (m), 20.04 (s), 21.34 (s), 23.34 (s), 24.54 (s), 26.40 (s), 28.48 (s), 29.45 (s), 31.93 (s), 32.17 (s), 34.22 (s), 36.39-36.76 (s), 37.52 (s), 40.04 (s), 42.58 (s), 46.12 (s), 50.41 (s), 72.06 (s), 121.94 (s), 141.02 (s).</td>
</tr>
</tbody>
</table>

Figure 3.14 FT-IR spectrum of compound P-3C.
Figure 3.15 Mass spectrum of compound P-3C.

Figure 3.16 $^1$H-NMR spectrum of compound P-3C.
On the basis of above spectral studies, the data were found to be similar as reported in literature. Thus the isolated compound was identified as β-Sitosterol.
Column chromatography of chloroform fraction of ERAL

The fraction (13 g) was subjected to chromatography over silica gel (60-120 mesh) column, using chloroform with increasing polarity by methanol as eluent. Fractions of 10 ml each were collected and similar fractions were pooled after monitoring their TLC. It afforded a pure compound.

Compound P-4:

A pure compound was obtained from the eluent of chloroform: methanol (85:15) as a light greenish yellow amorphous powder. Yield 3.2 mg, mp >300 ºC.

Table 3.10 Spectroscopic data of the compound P-4.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spectrum</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IR (KBr) $\nu_{\text{max}}$</td>
<td>3417, 1662, 1612, 1560, 1521, 1458 cm$^{-1}$.</td>
</tr>
<tr>
<td>2</td>
<td>EIMS (70eV) m/z 304.09 (M+2),</td>
<td>303.08 (M+1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>$^1$H-NMR (300MHz) DMSO, $\delta$ (ppm)</td>
<td>3.346 (s), 6.180-6.186 (s), 6.400-6.406 (s), 6.86-6.89 (d, $J$=8.4), 7.519-7.553 (s), 9.30-9.58 (s), 12.489 (s).</td>
</tr>
<tr>
<td>4</td>
<td>$^{13}$C-NMR (300MHz) DMSO, $\delta$ (ppm)</td>
<td>93.80 (s), 98.62 (s), 103.46 (s), 115.51 (s), 116.05 (s), 120.42 (s), 122.40 (s), 136.19 (s), 145.51 (s), 147.25 (s), 148.15 (s), 156.58 (s), 161.17 (s), 164.33 (s), 176.29 (s).</td>
</tr>
</tbody>
</table>
Figure 3.19 FT-IR spectrum of compound P-4

Figure 3.20 $^1$H-NMR spectrum of compound P-4
Figure 3.21 Expended $^1$H-NMR spectrum of compound P-4

Figure 3.22 $^{13}$C-NMR spectrum of compound P-4.
On the basis of above spectral studies, the isolated compound was identified as quercetin.

\[
\begin{align*}
\text{C}_{15}\text{H}_{10}\text{O}_7 \\
\text{Exact Mass: 302.04} \\
\text{Mol. Wt.: 302.24}
\end{align*}
\]

\text{2-(3,4-Dihydroxy-phenyl)-3,5,7-trihydroxy-chromen-4-one (Quercetin)}

Simultaneous identification of quercetin and rutin in the flowers and leaves of \textit{R. arboreum} by HPTLC

Simultaneous identification of two biologically active flavonoidal compounds, quercetin and rutin, from the flowers and leaves of \textit{R. arboreum} was performed using high-performance thin-layer chromatography (HPTLC). TLC aluminum plates, precoated with silica-gel RP-18 F 254 S, were used with a mobile phase of methanol: water: formic acid (55: 42: 03 v/v/v) and densitometric determination of these compounds was carried out at 254 nm in the absorbance mode.

Standard solutions and samples preparation

Standard stock solutions of quercetin (10 mg/ml) and rutin (1 mg/ml) were prepared by dilution in ethanol. Alcoholic extracts (100mg) of the leaves and flowers of \textit{R. arboreum} were taken and dissolved in ethanol (10 ml) and then 1 ml of each was diluted to 10 ml separately and filtered. Working solutions of quercetin (1000, 500, 250, 125 and 62.5μg/ml) and rutin (100, 10, 1, 0.1 and 0.01 μg/ml) were prepared separately by serial dilution, using ethanol as the solvent.

Chromatographic conditions

1μl of standards and samples were applied on 10x10 cm TLC plate at 20 mm from X and 10 mm from Y axis and 5 mm spaces were left between adjacent spots. TLC plates were developed in a glass tank, which was pre-saturated with developing solvent system up to the height of 7.5cm. The solvent system was selected from the method previously developed. The composition of the solvent system was optimized on the basis of the resolution of the spots. After development, the plate was removed, air dried and scanned in a TLC scanner (CAMAG HPTLC) at 254 nm under the following conditions: slit width (6x0.4mm) and scanning speed (20 mm/s). Quantitative evaluation of the plate was performed in the absorbance mode.
**Figure 3.23** UV spectra comparison of extracts of flowers and leaves with standard quercetin and rutin.

**Figure 3.24** Chromatogram of standard quercetin.
Figure 3.25 Chromatogram of standard rutin.

Figure 3.26 Chromatogram of the extract of the leaves.
Isolation of rutin from aqueous extract of the leaves of R. arboreum

After fractionation of the alcoholic extract with n-hexane, chloroform and n-butanol, the remaining portion was filtered out and the insoluble portion discarded. 50 ml of the filtrate (aqueous extract), was concentrated under reduced pressure up to 20 ml and then refrigerated. A greenish-yellow amorphous powder settled down in the beaker, which was filtered and washed with chilled water. It was further purified with hot water.

Yield- 14.7 mg, melting range: 192-195 °C.

Table 3.11 Spectroscopic data of compound rutin.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spectrum</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IR (KBr) $\nu_{\text{max}}$</td>
<td>3434, 2902, 1677, 1585, 1498, 1282 cm$^{-1}$</td>
</tr>
<tr>
<td>2</td>
<td>EIMS (70eV)</td>
<td>m/z 611.4 (M$^+$), 610.4, 609.5, 300.7.</td>
</tr>
<tr>
<td>3</td>
<td>$^1$H-NMR (300MHz) DMSO, $\delta$ (ppm)</td>
<td>0.984 (s), 3.248 (s), 3.387 (s), 6.183 (s), 4.368-4.384 (s), 5.332 (s), 6.373 (s), 6.834-6.849 (s), 7.513-7.535 (s), 8.340 (s), 12.595 (s).</td>
</tr>
<tr>
<td>4</td>
<td>$^{13}$C-NMR</td>
<td>17.81 (s), 67.06, 67.15, 68.31 (s), 70.02, 70.42, 70.59,</td>
</tr>
</tbody>
</table>
(300MHz) DMSO, δ (ppm)  

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>71.88, 74.12, 75.93, 76.48, 79.24, 100.81, 101.27 (s), 93.70 (s), 98.84 (s), 103.85 (s), 115.28 (s), 116.28 (s), 121.18 (s), 121.64 (s), 133.31 (s), 144.83 (s), 148.52 (s), 156.50 (s), 156.61 (s), 161.24 (s), 164.56 (s), 177.35 (s).</td>
<td></td>
</tr>
</tbody>
</table>

Melting range and spectroscopic data were found similar as reported earlier.\textsuperscript{14} On the basis of above spectral studies, the isolated compound was identified as rutin.

![Chemical Structure of Rutin](image)

\[ \text{C}_{27}\text{H}_{30}\text{O}_{16} \]

Exact Mass: 414.39
Mol. Wt.: 414.71

Quercetin-3-rutinoside (Rutin)

Figure 3.28 FT-IR spectrum of isolated rutin.
Figure 3.29 Mass spectrum of isolated rutin.

Figure 3.30 $^1$H-NMR spectrum of isolated rutin.
3.1.1.2 Phytochemical screening of the plant *Carum copticum*

*Collection and authentication of the seeds of Carum copticum*

Seeds of *Carum copticum* were procured from the local market of Lucknow. The seeds were identified and authenticated by the Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute, Lucknow (India). Plant herbarium was submitted to NBRI bearing Ref. No. NBRI/CIF/88/2009.

*Preparation of the alcoholic extract of the seeds of Carum copticum*

Powdered *C. copticum* seeds (400 g) were packed in a soxhlet apparatus and subjected to hot percolation for about 4 days using 800 ml of ethanol (95% v/v) as the solvent. The solvent was evaporated under vacuum using rotary evaporator (IKA-RV 10 basic), and was further dried in a desiccator under reduced pressure for complete drying (10.90% w/w).
Table 3.12 Preliminary phytochemical investigation of the seeds extract of *C. copticum*

<table>
<thead>
<tr>
<th>S.No</th>
<th>Chemical test</th>
<th>Observation</th>
<th>Inference</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Alkaloids:</strong> To the extract, dil. HCl was added, shaken well and filtered. On the filtrate the following tests were performed.</td>
<td>Cream coloured ppt.</td>
<td>Presence of alkaloids</td>
<td>–</td>
</tr>
<tr>
<td>A</td>
<td><em>Mayer’s Test</em> - Few drops of potassiomercuric iodide solution + Filtrate.</td>
<td>Yellow colour</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td><em>Hager’s Test</em> - Few drops of saturated solution of Picric acid + Filtrate.</td>
<td>Reddish-brown colour</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td><em>Wagner’s test</em> - Iodine solution in KI + Filtrate.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><strong>Carbohydrates:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td><em>Molisch Test</em> - (general test) 2 ml of 10% ethanolic solution of 1-naphthol. + Extract + Few drops of concentrated H₂SO₄ from the sides of test tube.</td>
<td>Red ring appears at interface.</td>
<td>Presence of carbohydrates</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td><em>Fehling’s Test</em> - 1 ml Fehling’s soln. A + 1 ml Fehling’s soln. B + equal vol. of ext. and heat at boiling water bath.</td>
<td>Red ppt. formed.</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td><em>Benedict’s Test</em> - Benedict’s soln + equal volume of extract and heated on a boiling water bath.</td>
<td>Red ppt. formed.</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><strong>Steroids:</strong></td>
<td>Red colour appeared in the chloroform layer and green fluorescence in the acid layer.</td>
<td>Presence of steroids.</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>Salkowski Test</em> - To 2 ml of the extract, 2 ml of chloroform and 2 ml concentrated H₂SO₄ were added through the sides of the test tube.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><strong>Amino acids:</strong></td>
<td>Blue-violet colour.</td>
<td>Presence of amino acids.</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Ninhydrin Test</em> - 0.25% of n-butanolic soln. of ninhydrin + Extract, placed on a boiling water bath for 10 min.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><strong>Phenolic compounds:</strong></td>
<td>Transient or permanent purple/blue or green colour.</td>
<td>Presence of phenolic compounds</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1% ‘neutral’ ferric chloride solution + Extract</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Flavonoids:**

- **Pew’s Test** - Zinc powder + 1 drop of 5N HCl + Extract
  - Deep purple-red/cherry red colour.
  - Presence of flavonoids

- **Shinoda Test** - Mg powder + 1 drop of 5N HCl + Extract
  - Deep-red or magenta colour.
  - Presence of flavonoids

**Saponins:** Shake the extract in conical flask.

- Foaming occurred.
- Presence of saponins.

CC = alcoholic extract of the seeds of *C. copticum*

**Fractionation of the alcoholic extract of the seeds of Carum copticum**

Ethanolic extract (100 g) was suspended in distilled water (200ml) and then extracted in a separatory funnel with n-hexane (25ml x 15), chloroform (25ml x 10) and n-butanol (25ml x 4). All the fractions were concentrated under vacuum, using rotary evaporator, and then placed in a desiccator at reduced pressure for complete drying.

**Table 3.13** Percentage yield of the fractions of alcoholic extract of *Carum copticum*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fractions</th>
<th>Weight (gm)</th>
<th>% Yield (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-Hexane</td>
<td>33.7</td>
<td>33.7</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform</td>
<td>29.4</td>
<td>29.4</td>
</tr>
<tr>
<td>3</td>
<td>n-Butanol</td>
<td>7.30</td>
<td>7.30</td>
</tr>
</tbody>
</table>

**Column chromatography of n-hexane fraction**

The fraction (20g) was subjected to chromatography over silica gel (60-120 mesh) column, using n-hexane by increasing polarity with ethyl acetate as eluent. Fractions of 10 ml each were collected and similar fractions were pooled after monitoring their TLC. This afforded four sub-fractions

**Compound PC-b:**

A mixture of two compounds was obtained from the eluent of n-hexane: ethyl acetate (85:15) as yellow colored oily liquid. This mixture was further subjected to chromatography over silica gel (60-120 mesh) column using chloroform with increasing polarity by methanol as eluent. A single compound was obtained from the eluent of chloroform: methanol (99:01) as a light-yellow coloured oily-liquid.

Yield - 0.55 ml
Table 3.14 Optical rotation and spectral data of the compound PC-b

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Analysis</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Optical rotation</td>
<td>(+) 0.001 (dextrorotatory)</td>
</tr>
<tr>
<td>2</td>
<td>Specific rotation</td>
<td>0.8613</td>
</tr>
<tr>
<td>3</td>
<td>IR (KBr) $\nu_{\text{max}}$</td>
<td>3500, 2923, 2858, 1745, 1641, 1541, 1458, and 1147 cm$^{-1}$</td>
</tr>
<tr>
<td>4</td>
<td>EIMS (70eV)</td>
<td>m/z 135.27 (M+1), 136.20 (M+2)</td>
</tr>
<tr>
<td>5</td>
<td>$^1$H-NMR (300MHz) CDCl$_3$, $\delta$ (ppm)</td>
<td>0.858-0.890 (s), 1.115-1.137 (d, $J$=6.6), 1.262-1.304 (s), 1.551-1.682 (m), 2.034-2.054 (s), 2.294-2.343 (m), 2.750-2.788 (t, $J$=11.4), 4.113-4.172 (m), 4.27-4.323 (m), 5.247-5.359 (m) and 7.26 (s)</td>
</tr>
<tr>
<td>6</td>
<td>$^{13}$C-NMR (300MHz) CDCl$_3$, $\delta$ (ppm)</td>
<td>14.08 (s), 22.67 (s), 24.48 (s), 24.87 (s), 25.64 (s), 26.80 (s), 27.26 (s), 29.16-29.65 (m), 31.52 (s), 33.94-34.19 (s), 62.12 (s), 68.92 (s), 127.90-128.12 (d, $J$=66), 128.94 (s), 129.98 (s), 130.22 (s), 130.55 (s) and 172.80 (s), 173.09 (s).</td>
</tr>
</tbody>
</table>

Figure 3.32 Optical and specific rotation data of compound PC-b.
Figure 3.33 FT-IR spectrum of compound PC-b.

Figure 3.34 Mass spectrum of compound PC-b.
Figure 3.35 $^1$H-NMR spectrum of compound PC-b.

Figure 3.36 Expended $^1$H-NMR spectrum of compound PC-b.
Figure 3.37 Expended $^1$H-NMR spectrum of compound PC-b.

Figure 3.38 $^{13}$C-NMR spectrum of compound PC-b.
Figure 3.39 13C-NMR spectrum of compound PC-b.

Figure 3.40 13C-NMR spectrum of compound PC-b.
On the basis of above spectral studies, the isolated compound was identified as \( p \)-cymene.

\[
\text{C}_{10}\text{H}_{14} \\
\text{Exact Mass: 134.11} \\
\text{Mol. Wt.: 134.22}
\]

\( p \)-Cymene

**Compound PC-d:**

A pure compound was obtained from the eluent of chloroform: methanol (98:02) as a light brown coloured oily liquid.

Yield- 0.35 ml

**Table 3.15** Optical rotation and spectral data of the compound PC-d.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Analysis</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Optical rotation</td>
<td>(+) 0.004 (dextrorotatory)</td>
</tr>
<tr>
<td>2</td>
<td>Specific rotation( [\alpha]^{546\text{nm}} )</td>
<td>0.2648</td>
</tr>
<tr>
<td>3</td>
<td>IR (KBr) ( \nu_{\text{max}} )</td>
<td>3433, 2964, 2923, 2864, 1726, 1620, 1515, 1454, 1417, 1290, 1222, 1153, 1087, 945 and 808 cm(^{-1} )</td>
</tr>
<tr>
<td>4</td>
<td>EIMS (70eV)</td>
<td>m/z 151.12 (M+1)</td>
</tr>
<tr>
<td>5</td>
<td>(^1\text{H-NMR (300MHz)} ) CDCl(_3), ( \delta ) (ppm)</td>
<td>1.286-1.419 (m), 2.245-2.251 (s), 2.32 (s), 2.43 (s), 3.186-3.339 (m), 4.971 (s), 5.080 (s), 6.621 (s), 6.729-6.904 (m), 7.123-7.149 (s), 7.234-7.278 (s).</td>
</tr>
<tr>
<td>6</td>
<td>(^{13}\text{C-NMR (300MHz)} ) CDCl(_3), ( \delta ) (ppm)</td>
<td>14.05 (s), 20.08 (s), 22.65 (s), 29.67 (s), 30.87-31.90 (d), 76.58-77.43 (t), 116.05 (s), 121.61 (s), 126.20 (s), 128.91 (s), 130.58 (s), 131.41 (s), 136.52 (s) and 152.56 (s).</td>
</tr>
</tbody>
</table>
Figure 3.41 Optical and specific rotation data of compound PC-d.

Figure 3.42 FT-IR spectrum of compound PC-d.
Figure 3.43 Mass spectrum of compound PC-d.

Figure 3.44 $^1$H-NMR spectrum of compound PC-d.
Figure 3.45 Expended $^1$H-NMR spectrum of compound PC-d.

Figure 3.46 Expended $^1$H-NMR spectrum of compound PC-d.
On the basis of above spectral studies, the isolated compound was identified as carvacrol.

\[
\text{C}_{10}\text{H}_{14}\text{O}
\]

Exact Mass: 150.10
Mol. Wt.: 150.22
(Carvacrol)

**Compound PC-g:**
A pure compound was obtained from the eluent of chloroform: methanol (98:02) as a brown coloured oily liquid.

Yield- 0.45 ml
Table 3.16 Optical rotation and spectral data of the compound PC-g

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Analysis</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Optical rotation</td>
<td>(+) 0.001 (dextrorotatory)</td>
</tr>
<tr>
<td>2</td>
<td>Specific rotation [α]_{546nm}</td>
<td>0.0690</td>
</tr>
<tr>
<td>3</td>
<td>IR (KBr) ν_{max}</td>
<td>3461, 2923, 2854, 1743, 1458, and 1166 cm(^{-1})</td>
</tr>
<tr>
<td>4</td>
<td>EIMS (70eV)</td>
<td>m/z 136.03 (M+)</td>
</tr>
<tr>
<td>5</td>
<td>(^1)H-NMR (300MHz) CDCl(_3), (\delta) (ppm)</td>
<td>0.858-0.901 (m), 1.225-1.302 (m), 1.609-1.683 (m), 2.032-2.051 (s), 2.262-2.346 (m), 2.751-2.789 (m), 3.117-3.209 (m), 4.119-4.178 (m), 4.277-4.330 (m), 4.766 (s), 5.271-5.393 (m), 6.567 (s), 6.706-6.731 (s), 7.057-7.083 (d, (J=7.8)) and 7.244 (s).</td>
</tr>
<tr>
<td>6</td>
<td>(^{13})C-NMR (300MHz) CDCl(_3), (\delta) (ppm)</td>
<td>14.08 (s), 20.82 (s), 22.65 (s), 24.48-24.87 (s), 25.63 (s), 26.74-27.25 (s), 29.16-29.65 (m), 31.52 (s), 31.91 (s), 33.95-34.20 (s), 62.16 (s), 68.95 (s), 116.03 (s), 121.61 (s), 126.21 (s), 127.90-128.10 (s), 128.92 (s), 129.99-130.56 (m), 131.36 (s), 136.53 (s), 152.60 (s) and 172.94-173.25 (s).</td>
</tr>
</tbody>
</table>
Figure 3.48 Optical and specific rotation data of compound PC-g.

<table>
<thead>
<tr>
<th>No</th>
<th>OpRot</th>
<th>Result</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.001</td>
<td>0.069</td>
<td>29.9</td>
<td>15:37:23</td>
</tr>
<tr>
<td>2</td>
<td>0.001</td>
<td>0.069</td>
<td>29.8</td>
<td>15:37:31</td>
</tr>
<tr>
<td>3</td>
<td>0.001</td>
<td>0.069</td>
<td>29.8</td>
<td>15:37:40</td>
</tr>
<tr>
<td>4</td>
<td>0.001</td>
<td>0.069</td>
<td>29.8</td>
<td>15:37:48</td>
</tr>
<tr>
<td>5</td>
<td>0.001</td>
<td>0.069</td>
<td>29.8</td>
<td>15:37:57</td>
</tr>
<tr>
<td>6</td>
<td>0.001</td>
<td>0.069</td>
<td>29.9</td>
<td>15:38:05</td>
</tr>
<tr>
<td>7</td>
<td>0.001</td>
<td>0.069</td>
<td>29.8</td>
<td>15:38:14</td>
</tr>
<tr>
<td>8</td>
<td>0.001</td>
<td>0.069</td>
<td>29.8</td>
<td>15:38:22</td>
</tr>
<tr>
<td>9</td>
<td>0.001</td>
<td>0.069</td>
<td>29.9</td>
<td>15:38:31</td>
</tr>
<tr>
<td>10</td>
<td>0.001</td>
<td>0.069</td>
<td>29.9</td>
<td>15:38:39</td>
</tr>
</tbody>
</table>

Counts: 10
Average: 0.0690
Std.Dev.: 0.0069
Minimum: 0.069
Maximum: 0.069

Figure 3.49 FT-IR spectrum of compound PC-g.
Figure 3.50 Mass spectrum of compound PC-g.

Figure 3.51 $^1$H-NMR spectrum of compound PC-g.
Figure 3.52 ¹H-NMR spectrum of compound PC-g.

Figure 3.53 ¹H-NMR spectrum of compound PC-g.
Figure 3.54 $^{13}$C-NMR spectrum of compound PC-g.

Figure 3.55 Expended $^{13}$C-NMR spectrum of compound PC-g.
Figure 3.56 Expended $^{13}$C-NMR spectrum of compound PC-g.

On the basis of above spectral studies, the isolated compound was identified as α-pinene.

\[
\text{C}_10\text{H}_{16}
\]

Exact Mass: 136.13  
Mol. Wt.: 136.23  
(Alfa Pinene)

**Compound PC-i:**

This was obtained from the eluent of chloroform: methanol (98:02) as a dark brown coloured oily liquid.

Yield- 0.15 ml
Table 3.17 Optical rotation and spectral data of the compound PC-i

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Analysis</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Optical rotation</td>
<td>(+) 0.001 (dextrorotatory)</td>
</tr>
<tr>
<td>2</td>
<td>Specific rotation [α]_{546nm}</td>
<td>0.1294</td>
</tr>
<tr>
<td>3</td>
<td>IR (KBr) ν_{max}</td>
<td>2923, 1743, 1718, 1458, 1164 and1033 cm^{-1}</td>
</tr>
<tr>
<td>4</td>
<td>EIMS (70eV)</td>
<td>m/z 337.30, 339.31 (base peak), 340.31</td>
</tr>
<tr>
<td>5</td>
<td>$^1$H-NMR (300MHz) CDCl$_3$, δ (ppm)</td>
<td>0.681-1.606 (m), 1.820 (s), 2.172-2.310 (m), 2.768 (s), 3.13-3.15 (m), 3.72 (d), 4.153-4.267 (s), 5.34 (s), 6.504-6.571 (s), 6.709-6.73 (s), 6.952 (s), 7.059-7.085 (s) and 7.258 (s).</td>
</tr>
<tr>
<td>6</td>
<td>$^{13}$C-NMR (300MHz) CDCl$_3$, δ (ppm)</td>
<td>14.09 (s), 22.68 (s), 24.49-24.86 (m), 25.67 (s), 26.82-27.26 (s), 29.17-29.69 (m), 31.53 (s), 31.92 (s), 33.95 (s), 62.11 (s), 68.93 (s), 116.04 (s), 127.91-130.58 (m).</td>
</tr>
</tbody>
</table>

Figure 3.57 Optical and specific rotation data of compound PC-i.
Figure 3.58 FT-IR spectrum of compound PC-i.

Figure 3.59 Mass spectrum of compound PC-i.
Figure 3.60 $^1$H-NMR spectrum of compound PC-i.

Figure 3.61 Expanded $^1$H-NMR spectrum of compound PC-i.
Figure 3.62: Expanded $^1$H-NMR spectrum of compound PC-i.

Figure 3.63: $^{13}$C-NMR spectrum of compound PC-i.
Figure 3.64  
Expended $^{13}\text{C}$-NMR spectrum of compound PC-i.

Figure 3.65  
Expended $^{13}\text{C}$-NMR spectrum of compound PC-i.
Extraction of volatile oil from the seeds of Carum copticum

Volatile oil was extracted from the powder (50g) of the seeds of Carum copticum by steam distillation, using Clevenger apparatus. Volatile oil obtained was a transparent liquid with a pungent smell.

GLC and HPLC analysis of volatile oil (C-1)

20 µg/ml concentrations of sample (C-1) and standard (Thymol) were prepared in the chloroform. Co-chromatography of sample and standard was performed in HPLC.

Chromatographic conditions for GLC-
Column: OV-1, packed column 10 ft long
Carrier gas: Nitrogen
Injection volume: 2µl
Flow rate: 20ml/min.
Detector: FID (Flame Ionization Detector),
Run time: 45 min.
Temperature: Injection temp.-245ºC
Oven- 80ºC for 2 min., 4ºC-120ºC for 3min., 6ºC-180ºC for 5 min., 8ºC-220ºC for 30 min.
Detector temp. - 250ºC

Chromatographic conditions for HPLC-
Column: C-18
Mobile phase: Acetonitrile: Water (70:30)
Injection volume: 4µl
Flow rate: 0.8 ml/min.
Run time: 30 min.
Pump type: L-7100, pressure- 0-420 kgf/cm²
Detector: PDA (Photo diode array)
Calculation method: Area%
Peak identification window: % Time
Table 3.18 Physicochemical data of volatile oil (C-1)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Analysis</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yield</td>
<td>1.4 ml</td>
</tr>
<tr>
<td>2</td>
<td>Percentage yield</td>
<td>3% w/v</td>
</tr>
<tr>
<td>3</td>
<td>Refractive index</td>
<td>1.493</td>
</tr>
<tr>
<td>4</td>
<td>Optical rotation</td>
<td>0.029 (dextrorotatory)</td>
</tr>
<tr>
<td>5</td>
<td>Specific rotation $[\alpha]_{546nm}$</td>
<td>0.315</td>
</tr>
<tr>
<td>6</td>
<td>IR (KBr) $\nu_{\text{max}}$</td>
<td>3500, 3419, 2958, 2867, 2356, 1610, 1563, 1514, 1460, 1380, 1290, 1224, 1153, 1087, 1060, and 945 cm$^{-1}$</td>
</tr>
<tr>
<td>7</td>
<td>EIMS (70eV)</td>
<td>m/z 153.13, 151.12 (M+1, thymol), 137.14 (M+1, $\alpha$-pinene), 135.12 (M+1, $p$-cymene)</td>
</tr>
<tr>
<td>8</td>
<td>GLC</td>
<td>27.78% of thymol is present in the volatile oil and its peak is obtained at RT (retention time) 27.90 min., The RT of thymol in the volatile oil was compared with the obtained chromatogram of the standard thymol.</td>
</tr>
<tr>
<td>9</td>
<td>HPLC</td>
<td>Three peaks were obtained in the chromatogram. As per the chromatogram, thymol is the major component. The RT of thymol peak is 3.72 min.</td>
</tr>
</tbody>
</table>

Values of refractive index and optical rotation of volatile oil were similar to the reported values.\textsuperscript{15}
Figure 3.66 Optical and specific rotation data of compound C-1 (Volatile oil).

Figure 3.67 FT-IR spectrum of compound C-1 (Volatile oil).
Figure 3.68 FT-IR spectrum of compound C-1 (Volatile oil).

Figure 3.69 HPLC chromatogram of Volatile oil (C-1)
Figure 3.70 HPLC chromatogram of thymol (standard)

Figure 3.71 HPLC chromatogram of co-injection of C-1 and thymol (standard)
Figure 3.72 Gas liquid chromatogram of compound C-1.
Figure 3.73 Gas liquid chromatogram of thymol (standard)
3.2 CONCLUSION

Alcoholic (ERAF) and aqueous (ARAF) extracts of the flowers and alcoholic extract of the leaves (ERAL) were prepared using standard method of extraction. ERAF was fractionated with n-hexane, chloroform and n-butanol using a separatory funnel. One pure compound was isolated from the n-butanol fraction. It was identified and characterized as quercetin by co-chromatography (TLC, paper chromatography) with an authentic marker and various spectroscopic methods (UV-Visible, IR, PMR, CMR and mass spectrometry). ERAL was fractionated with n-hexane, chloroform and n-butanol using a separatory funnel. Four pure compounds were isolated from n-hexane fraction by column chromatography. Three (ursolic acid, 3, 10-epoxy glutinane and β-sitosterol) of them were characterized and one is unidentified. A pure compound (quercetin) was isolated from its chloroform fraction by column chromatography and one compound (rutin) was isolated from the aqueous fraction by solvent-solvent extraction method. Simultaneous RP-HPTLC method was developed for the identification of quercetin and rutin in the alcoholic extracts of the flower and leaves. Alcoholic extract of the seeds of Carum copticum was prepared and fractionated with n-hexane, chloroform and n-butanol. Four sub-fractions, containing essential oils, were isolated from the n-hexane fraction by column chromatography. Compositions of these sub-fractions were determined by various spectroscopic methods. One compound was the volatile oil of ajowan, isolated through Clevenger apparatus. The major component of volatile oil was thymol; which was identified and quantified by Gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC) with authentic marker and by mass spectrometry.
REFERENCES


15. The Pharmacopeia of India. The government of India: Delhi, 1966; Vol. 1, p 32.
4.1 PHARMACOLOGICAL SCREENING

The definition of immunomodulation refers to the action undertaken by the medication on autoregulating processes that steer the immunological defense system.

The present study was conducted to investigate the immunomodulatory potential of the plants *R. arboreum* and *Carum copticum* by SRBC antigenic challenge model in mice. Three parameters were used to evaluate the activity:

a) **Humoral immunity** - primary and secondary humoral immune responses were checked by haemagglutination technique.

b) **Cell-mediated immunity (CMI)** - Checked by Delayed type hypersensitivity (DTH) reaction and phagocytosis.

c) **Total leukocyte counts**

Screening of some other activities like antimicrobial, anti-tumor and anti-cancer were also performed on the plant *R. arboreum*.

4.1.1 IMMUNOMODULATORY ACTIVITY OF ERAL (ALCOHOLIC EXTRACT OF THE LEAVES OF *R. ARBOREUM*)

**Animals**

Swiss albino mice (25-30g) were procured from the Laboratory Animal Service Division of Central Drugs Research Institute, Lucknow. The experimental protocol was duly approved by the Institutional Animal Ethics Committee (IAEC, No. BBDNITM/IAEC/05/2010) and the experiments were performed as per the guidelines. All the animals were housed under standard conditions of temperature (23 ± 2 °C), 12 h light/dark cycles and fed with standard pellet diet and water *ad libitum*.

**Preparation of Sheep RBC (SRBC)**

Blood was withdrawn from the jugular vein of a healthy sheep with the help of a 10ml syringe fitted with a 22gauge needle. RBCs were obtained by centrifugation of blood at 4000 rpm using BL-120-Refrigerated centrifuge (Biolab). The RBCs were preserved in Alsevier’s solution. They were then suspended in phosphate-buffered saline for further use.

The animals were divided into five groups consisting of six animals each (n=6).

- **Group-1** = Control, received only vehicle (3% gum acacia suspension).
- **Group-2** = Positive control, received levamisole (2.5mg/kg body weight of mice).
- **Group-3** = ERAL treated group, received 10mg/kg body weight of mice.
- **Group-4** = ERAL treated group, received 30mg/kg body weight of mice.
Group-5= ERAL treated group, received 100mg/kg body weight of mice.

The above were administered once daily (orally) from day 1 to day 25. Mice from all groups received an antigenic challenge with intraperitoneal (i.p.) injection of sheep RBC (SRBC) in phosphate buffer saline (10% approx., 0.1ml) on 7th and 14th day of the treatment.

**Humoral immune response**

Humoral immune response was assessed by haemagglutination on day 14 and 21 of the treatment. The blood was withdrawn by puncturing the retro-orbital plexus of all antigenically challenged mice and the antibody levels were determined by haemagglutination technique. For this, 20µl of serum was mixed with 180µl of phosphate-buffered saline in 96-well microtitre plates, to get ten-fold dilution of the antibodies present in the serum. Further, two-fold dilutions of this diluted serum were similarly carried out so that the antibody concentration of any of the dilutions was half of the previous dilution. SRBC (1%, 100µl) were added to each of these dilutions. After mixing, the plates were incubated at room temperature for 4h and examined for haemagglutination. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titer or HA titer, which is defined as the “reciprocal” of the last dilution with positive reaction. The level of the antibody titer on day 14 of the experiment was considered as the primary humoral immune response, and the one on day 21 of the experiment was considered as the secondary humoral immune response.¹

**Cell-mediated immune response**

*Delayed Type Hypersensitivity reaction*

This was assessed by foot pad reaction method in mice. The increase in paw thickness, induced by an injection of SRBC (20 µl, 1% v/v) in the sub plantar region of right hind paw of each animal on day 22 was assessed after 48 h, i.e. on day 24th. The increase in paw thickness was considered as delayed type hypersensitivity (DTH) reaction and as an index of cell-mediated immunity. The thickness of left hind paw, injected similarly with phosphate buffer saline, served as the control.²

**Phagocytosis**

On day 25 of the experiment, cervical dislocation euthanasia was done on mildly anaesthetized mice. The peritoneal macrophages of treated mice were harvested by flushing the peritoneal cavity with 3.0ml of Hank balanced salt solution (HBSS), containing 1% bovine serum albumin. The macrophages present in the aliquots were incubated on glass slides at 37°C for 30 minutes in a humidified chamber. The glass slides were washed thoroughly to remove
non-adherent cells. The adhered cells were incubated with 50µl of heat killed (boiled for 30 min in normal saline) *Candida albicans* cells, at 37 ºC for 30 min. Finally, the cells on the slides were stained with Wright’s dye after thorough washing with HBSS to microscopically determine the adherent cells containing yeast cells. Three hundred cells were counted and are expressed as percent phagocytosis.³

*Total leukocyte count (TLC) and liver function tests*

On day 25 of the experiment, the blood was withdrawn by puncturing the retro-orbital plexus of all antigenically challenged mice and TLC and liver function tests were performed.
Primary humoral immune response

Table 4.1 Influence of ERAL on the primary humoral immune response in mice

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>HA-Titer (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>512 ± 78.38</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>896 ± 156.7*</td>
</tr>
<tr>
<td>3</td>
<td>ERAL</td>
<td>10</td>
<td>352 ± 78.38**</td>
</tr>
<tr>
<td>4</td>
<td>ERAL</td>
<td>30</td>
<td>320 ± 0.00***</td>
</tr>
<tr>
<td>5</td>
<td>ERAL</td>
<td>100</td>
<td>293 ± 26.66***</td>
</tr>
</tbody>
</table>

Figure 4.1 Effect of ERAL and levamisole on primary humoral immune response in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of Levamisole Vs. Treated Group was performed by one-way ANOVA (Turkey’s Multiple comparison test). * p < 0.05, ** p < 0.01 and *** p < 0.001.
Secondary humoral immune response

Table 4.2 Influence of ERAL on the secondary humoral immune responses in mice

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>HA-Titer (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>448 ± 78.38</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>768.0 ± 128.0**</td>
</tr>
<tr>
<td>3</td>
<td>ERAL</td>
<td>10</td>
<td>400.0 ± 80.0**</td>
</tr>
<tr>
<td>4</td>
<td>ERAL</td>
<td>30</td>
<td>386.67 ± 88.64**</td>
</tr>
<tr>
<td>5</td>
<td>ERAL</td>
<td>100</td>
<td>320 ± 87.63***</td>
</tr>
</tbody>
</table>

Figure 4.2 Effect of ERAL and levamisole on secondary humoral immune response in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of Levamisole Vs. treated group was performed by one-way ANOVA (Turkey’s Multiple comparison test). * p < 0.05, ** p < 0.01 and *** p < 0.001.
Delayed Type Hypersensitivity (DTH) reaction

Table 4.3 Influence of ERAL on the delayed type hypersensitivity reaction after 48 hrs in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>Paw edema (mm) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>0.400 ± 0.031</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>0.640 ± 0.024*</td>
</tr>
<tr>
<td>3</td>
<td>ERAL</td>
<td>10</td>
<td>0.375 ± 0.11*</td>
</tr>
<tr>
<td>4</td>
<td>ERAL</td>
<td>30</td>
<td>0.300 ± 0.031**</td>
</tr>
<tr>
<td>5</td>
<td>ERAL</td>
<td>100</td>
<td>0.200 ± 0.024***</td>
</tr>
</tbody>
</table>

Figure 4.3 Effect of ERAL and levamisole on delayed type hypersensitivity reaction in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of Levamisole Vs. treated group was performed by one-way ANOVA (Turkey’s Multiple comparison test). * P < 0.05, ** P < 0.01 and *** P < 0.001.
**Phagocytosis**

Table 4.4 Influence of ERAL on the phagocytosis in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>Per cent phagocytosis (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>61.20 ± 3.81</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>77.40 ± 1.50*</td>
</tr>
<tr>
<td>3</td>
<td>ERAL</td>
<td>10</td>
<td>57.25 ± 3.70**</td>
</tr>
<tr>
<td>4</td>
<td>ERAL</td>
<td>30</td>
<td>49.66 ± 3.23***</td>
</tr>
<tr>
<td>5</td>
<td>ERAL</td>
<td>100</td>
<td>35.80 ± 2.92***</td>
</tr>
</tbody>
</table>

Figure 4.4 Effect of ERAL and levamisole on Phagocytosis in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of Levamisole Vs. treated group was performed by one-way ANOVA (Turkey’s Multiple comparison test). * $P < 0.05$, ** $P < 0.01$ and ***$P < 0.001$. 

125
Total Leukocyte Count (TLC)

Table 4.5 Influence of ERAL on the TLC in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>Leukocyte count (cells/mm³) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>7808.0 ± 417.28</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>11476 ± 596.56***</td>
</tr>
<tr>
<td>3</td>
<td>ERAL</td>
<td>10</td>
<td>6695 ± 438.90***</td>
</tr>
<tr>
<td>4</td>
<td>ERAL</td>
<td>30</td>
<td>6173.3 ± 338.62***</td>
</tr>
<tr>
<td>5</td>
<td>ERAL</td>
<td>100</td>
<td>3856 ± 604.71***</td>
</tr>
</tbody>
</table>

Figure 4.5 Effect of ERAL and levamisole on Total leukocyte count in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of Levamisole Vs. treated group was performed by one-way ANOVA (Turkey’s Multiple comparison test). * P < 0.05, ** P < 0.01 and ***P < 0.001.
4.1.2 IMMUNOMODULATORY ACTIVITY OF THE FRACTIONS OF ERAL

Preliminary immunomodulatory effectiveness of n-hexane, chloroform and n-butanolic fractions of ERAL were evaluated by haemagglutination, to obtain active fractions for further isolation.

The animals were divided into four groups consisting of six animals each (n=6).
Group-1= Control, received only vehicle (3% gum acacia suspension).
Group-2= n- Hexane fraction (HEF) treated group (50mg/kg body weight of mice).
Group-3= Chloroform fraction (CF) treated group (50mg/kg body weight of mice)
Group-4= n- Butanol fraction (BUF) treated group (50mg/kg body weight of mice)

The above were administered once daily (orally) from day 1 to day 14. Mice from all groups received an antigenic challenge with intraperitoneal (i.p.) injection of sheep RBC (SRBC) in phosphate buffer saline (10% approx., 0.1ml) on 7th day of the treatment.
Table 4.6 Influence of different fractions of ERAL on primary humoral immune response in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>HA-Titer (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>448 ± 78.38</td>
</tr>
<tr>
<td>2</td>
<td>HEF</td>
<td>50</td>
<td>144 ± 16*</td>
</tr>
<tr>
<td>3</td>
<td>CF</td>
<td>50</td>
<td>306.7 ± 78.43</td>
</tr>
<tr>
<td>4</td>
<td>BUF</td>
<td>50</td>
<td>384 ± 64</td>
</tr>
</tbody>
</table>

Figure 4.6 Effect of the ERAL-fractions on primary humoral immune response in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of control Vs. treated group was performed by one-way ANOVA (Turkey’s Multiple comparison test). *p < 0.05.
4.1.3 ASSESMENT OF LIVER AND LIVER-FUNCTION TESTS OF ERAL TREATED MICE

Physical condition of liver

Table 4.7 Influence of ERAL on the physical condition of liver in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>Weight of the liver (gm) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>1.61 ± 0.31</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>1.53 ± 0.39</td>
</tr>
<tr>
<td>3</td>
<td>ERAL</td>
<td>10</td>
<td>1.52 ± 0.80</td>
</tr>
<tr>
<td>4</td>
<td>ERAL</td>
<td>30</td>
<td>1.30 ± 0.19</td>
</tr>
<tr>
<td>5</td>
<td>ERAL</td>
<td>100</td>
<td>1.06 ± 0.20</td>
</tr>
</tbody>
</table>

Six mice were used in each group. Physical appearance of liver in the mice of all groups was found normal. There was no significant difference in the weights of liver of mice in each group.

Total bilirubin estimation

Serum total bilirubin was estimated spectrophotometrically using semi-autoanalyser (Stat Fax-3300). Blank (B) was prepared in a test tube by the addition of total bilirubin reagent (1.0 mL) and serum sample (50 µl). Test sample (T) was prepared in another test tube by the addition of total bilirubin reagent (1.0 mL), total diazo reagent (25 µl) and serum sample (50 µl). Mixed well both the tubes and incubated at room temperature for 10 minutes and read the absorbance of blank and test at 546 nm.

Total bilirubin in mg/dl = (Abs of T – Abs of B) x 26.31(Factor)

Table 4.8 Influence of ERAL on the total bilirubin in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>Total bilirubin (mg/dl) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>0.37 ± 0.094</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>0.29 ± 0.095</td>
</tr>
<tr>
<td>3</td>
<td>ERAL</td>
<td>10</td>
<td>0.27 ± 0.147</td>
</tr>
<tr>
<td>4</td>
<td>ERAL</td>
<td>30</td>
<td>0.27 ± 0.055</td>
</tr>
<tr>
<td>5</td>
<td>ERAL</td>
<td>100</td>
<td>0.29 ± 0.079</td>
</tr>
<tr>
<td>6</td>
<td>ERAL</td>
<td>400</td>
<td>0.30 ± 0.091</td>
</tr>
</tbody>
</table>

Six mice were used in each group. There was no significant difference in the amount of total bilirubin in mice of each group.
**Serum Glutamate Pyruvate Transaminase (SGPT) estimation**

Serum SGPT was estimated by UV kinetic method using semi autoanalyser. Enzyme reagent (1.0 ml) and serum (100μl) were taken in a test tube, mixed well and absorbance was measured against distilled water at 340 nm at 30 seconds time interval for 1 minute 30 seconds. The average change in absorbance per minute (ΔA/min) was calculated.

\[
\text{SGPT in IU/L} = \Delta A/\text{min} \times 1746 \text{ (Factor)}
\]

**Table 4.9 Influence of ERAL on the SGPT in mice.**

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>SGPT (IU/L) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>21.99 ± 7.57</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>21.99 ± 11.08</td>
</tr>
<tr>
<td>3</td>
<td>ERAL</td>
<td>10</td>
<td>22.69 ± 7.94</td>
</tr>
<tr>
<td>4</td>
<td>ERAL</td>
<td>30</td>
<td>20.59 ± 9.02</td>
</tr>
<tr>
<td>5</td>
<td>ERAL</td>
<td>100</td>
<td>25.83 ± 13.60</td>
</tr>
<tr>
<td>6</td>
<td>ERAL</td>
<td>400</td>
<td>26.53 ± 8.93</td>
</tr>
</tbody>
</table>

Six mice were used in each group. There was no significant difference in the amount of SGPT in mice of each group.

**Serum Glutamate Oxaloacetate Transaminase (SGOT) estimation**

Procedure was similar to that of SGPT determination.

\[
\text{SGOT in IU/L} = \Delta A/\text{min} \times 1746 \text{ (Factor)}
\]

**Table 4.10 Influence of ERAL on the SGOT in mice.**

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>SGOT (IU/L) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>29.67 ± 10.25</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>31.42 ± 6.98</td>
</tr>
<tr>
<td>3</td>
<td>ERAL</td>
<td>10</td>
<td>24.87 ± 8.83</td>
</tr>
<tr>
<td>4</td>
<td>ERAL</td>
<td>30</td>
<td>29.32 ± 11.20</td>
</tr>
<tr>
<td>5</td>
<td>ERAL</td>
<td>100</td>
<td>35.26 ± 6.22</td>
</tr>
</tbody>
</table>

Six mice were used in each group. There was no significant difference in the amount of SGOT in mice of each group.
4.1.4 IMMUNOMODULATORY ACTIVITY OF THE SEEDS OF *CARUM COPTICUM*

Immunomodulatory activity of the ethanolic extract of the seeds of *Carum copticum* (ECCS) was performed in the same way as in the case of *R. arboreum*. The parameters of activity were taken as primary and secondary humoral immune responses, Total leukocyte counts, DTH and phagocytosis.

The animals were divided into five groups consisting of six animals each (n=6).

Group-1= Control, received only vehicle (3% gum acacia suspension).
Group-2= Positive control, received levamisole (2.5mg/kg body weight of mice).
Group-3= ECCS treated group, received 100mg/kg body weight of mice.
Group-4= ECCS treated group, received 300mg/kg body weight of mice.
Group-5= ECCS treated group, received 500mg/kg body weight of mice.

The above were administered once daily (orally) from day 1 to day 25. Mice from all groups received an antigenic challenge with intraperitonial (i.p.) injection of sheep RBC (SRBC) in phosphate buffer saline (10% approx., 0.1ml) on 7th and 14th day of the treatment.
Humoral immune responses

Primary humoral immune response

Table 4.11 Influence of ECCS on the primary humoral immune response in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>HA-Titer (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>240 ± 50.60</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>853 ± 134.9*</td>
</tr>
<tr>
<td>3</td>
<td>ECCS</td>
<td>100</td>
<td>426 ± 67.46</td>
</tr>
<tr>
<td>4</td>
<td>ECCS</td>
<td>300</td>
<td>576 ± 186</td>
</tr>
<tr>
<td>5</td>
<td>ECCS</td>
<td>500</td>
<td>832 ± 192*</td>
</tr>
</tbody>
</table>

Figure 4.7 Effect of ECCS on primary humoral immune response in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of Levamisole Vs. Treated Group was performed by one-way ANOVA (Turkey’s Multiple comparison test). * p < 0.05
Secondary humoral immune response

Table 4.12 Influence of ECCS on the secondary humoral immune response in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>HA-Titer (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>288 ± 32</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>960 ± 143.1*</td>
</tr>
<tr>
<td>3</td>
<td>ECCS</td>
<td>100</td>
<td>384 ± 64</td>
</tr>
<tr>
<td>4</td>
<td>ECCS</td>
<td>300</td>
<td>768± 128</td>
</tr>
<tr>
<td>5</td>
<td>ECCS</td>
<td>500</td>
<td>853 ± 134.9*</td>
</tr>
</tbody>
</table>

Figure 4.8 Effect of ECCS on secondary humoral immune response in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of Levamisole Vs. Treated Group was performed by one-way ANOVA (Turkey’s Multiple comparison test). * p < 0.05.
Cell-mediated immune responses

*Delayed Type Hypersensitivity (DTH) reaction*

**Table 4.13** Influence of ECCS on the delayed type hypersensitivity reaction after 48 hrs in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>Paw edema (mm) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>0.220 ± 0.037</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>0.660 ± 0.024***</td>
</tr>
<tr>
<td>3</td>
<td>ECCS</td>
<td>100</td>
<td>0.240 ± 0.40</td>
</tr>
<tr>
<td>4</td>
<td>ECCS</td>
<td>300</td>
<td>0.360 ± 0.024*</td>
</tr>
<tr>
<td>5</td>
<td>ECCS</td>
<td>500</td>
<td>0.580 ± 0.037***</td>
</tr>
</tbody>
</table>

**Figure 4.9** Effect of ECCS on DTH in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of Levamisole Vs. Treated Group was performed by one-way ANOVA (Turkey’s Multiple comparison test). *p < 0.05, **p < 0.01 and ***p < 0.001.
**Phagocytosis**

**Table 4.14** Influence of ECCS on the phagocytosis in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>Per cent phagocytosis (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>37.50 ± 1.33</td>
</tr>
<tr>
<td>2</td>
<td>Levamisole</td>
<td>2.5</td>
<td>78.40 ± 1.74***</td>
</tr>
<tr>
<td>3</td>
<td>ECCS</td>
<td>100</td>
<td>40.33 ± 1.022</td>
</tr>
<tr>
<td>4</td>
<td>ECCS</td>
<td>300</td>
<td>47.60 ± 3.076*</td>
</tr>
<tr>
<td>5</td>
<td>ECCS</td>
<td>500</td>
<td>74.80 ± 3.680***</td>
</tr>
</tbody>
</table>

**Figure 4.10** Effect of ECCS on phagocytosis in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of Levamisole Vs. Treated Group was performed by one-way ANOVA (Turkey’s Multiple comparison test). * p < 0.05, ** p < 0.01 and *** p < 0.001.
4.1.5 IMMUNOMODULATORY ACTIVITY OF FRACTIONS OF CARUM COPTICUM SEEDS

Preliminary immunomodulatory effectiveness of n-Hexane, chloroform and n-butanol fractions of ECCS were evaluated by haemagglutination, to obtained active fractions for further isolation.

The animals were divided into four groups consisting of six animals each (n=6).
Group-1= Control, received only vehicle (3% gum acacia suspension).
Group-2= n- Hexane fraction (HEF) treated group (150mg/kg body weight of mice).
Group-3= Chloroform fraction (CF) treated group (150mg/kg body weight of mice)
Group-4= n- Butanol fraction (BUF) treated group (150mg/kg body weight of mice)

The above were administered once daily (orally) from day 1 to day 14. Mice from all groups received an antigenic challenge with intraperitonial (i.p.) injection of sheep RBC (SRBC) in phosphate buffer saline (10% approx., 0.1ml) on 7th day of the treatment.

Table 4.15 Influence of different fractions of ECCS on primary humoral immune response in mice.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Drugs</th>
<th>Dose (mg/kg, body wt.)</th>
<th>HA-Titer (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3% acacia suspension</td>
<td>288 ± 32</td>
</tr>
<tr>
<td>2</td>
<td>HEF</td>
<td>150</td>
<td>576 ± 64**</td>
</tr>
<tr>
<td>3</td>
<td>CF</td>
<td>150</td>
<td>373.3 ± 53</td>
</tr>
<tr>
<td>4</td>
<td>BUF</td>
<td>150</td>
<td>176 ± 39.19</td>
</tr>
</tbody>
</table>
Figure 4.11 Effect of ECCS-fractions on primary humoral immune response in mice. Values are Mean ± SEM (n = 6 in each group). The comparison of Levamisole Vs. Treated Group was performed by one-way ANOVA (Turkey’s Multiple comparison test) * $p < 0.05$, ** $p < 0.01$.

4.2 SCREENING OF OTHER PHARMACOLOGICAL ACTIVITIES OF R. ARBOREUM

4.2.1 ANTI MICROBIAL ACTIVITY

A large number of human, animal and plant disease are caused by pathogenic microbes (fungi, bacteria and algae). Infections due to fungi and bacteria have been a major cause of death in higher organisms. The discovery of antibiotic penicillin by Fleming is therefore considered to be one of the most important discoveries in the world. Historically many of the new antibiotics were isolated from natural sources (soil microbes, plants, etc.). Many more were later synthesized and introduced in clinical practices.

There are three major methods for anti microbial testing: (a) agar diffusion method (b) agar dilution method and (c) bioautographic method.

The agar diffusion method was adopted for the testing antimicrobial activity of ERAF, ARAF and isolated quercetin by Cup-Plate method due to certain advantages.

The advantages of the cup plate method are:

1. Handling of cylinders is eliminated.
2. It is more sensitive to low dilutions than the paper disc assay.
(3) Suspended material does not interfere with diffusion of the solution.
(4) Variation in the size of the cylinder is avoided.  
(5) It can be used as a screening test for substances elaborated by microorganisms.  
(6) It is adaptable to rapid assay.

**Microorganisms**

The microorganisms (*B. subtilis* MTCC 441, *E. coli* MTCC 1573, *S. aureus* MTCC 1430, *P. aeruginosa* MTCC 424, *A. tumifaciens* MTCC 3329 and *C. albicans* MTCC 183) were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

**Table 4.16** Growth media, incubation temperature and pH range for the microbial strains:

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Microorganisms</th>
<th>Growth Medium (Composition)</th>
<th>Incubation Temperature (°C)</th>
<th>pH Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Bacillus subtilis</em></td>
<td>Beef extract, Yeast extract, Peptone</td>
<td>30</td>
<td>6.8 – 7.0</td>
</tr>
<tr>
<td>2</td>
<td><em>Staphylococcus aureus</em></td>
<td>antal extract, Peptone, Agar</td>
<td>37</td>
<td>6.8 – 7.0</td>
</tr>
<tr>
<td>3</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Yeast extract, Peptone, Agar, Distilled water</td>
<td>37</td>
<td>6.8 – 7.0</td>
</tr>
<tr>
<td>4</td>
<td><em>Escherichia coli</em></td>
<td>Yeast extract, Tryptone, Sodium Chloride, Distilled water</td>
<td>30</td>
<td>6.8 – 7.0</td>
</tr>
<tr>
<td>5</td>
<td><em>Agrobacterium tumifaciens</em></td>
<td>Mannitol, KH2PO4, MgSO4.7H2O, NaCl, Yeast extract, CaCO3, Agar, Distilled water</td>
<td>28°C</td>
<td>7.2–7.4</td>
</tr>
<tr>
<td>6</td>
<td><em>Candida albicans</em></td>
<td>Yeast extract, Peptone, Dextrose, Agar, Distilled water</td>
<td>30°C</td>
<td>5.6</td>
</tr>
</tbody>
</table>
**Inoculums**

A loop full of microorganisms were inoculated into nutrient broth and incubated at 35 ± 2°C for 24 h. The turbidity of the resulting suspensions (except *C.albicans*) was diluted with nutrient broth to obtain a transmittance of 25.0% at 580 nm. That percentage was found spectrophotometrically comparable to one McFarland turbidity standard. This level of turbidity is equivalent to approximately 3.0 x 10^8 CFU/ml. A double beam UV-Visible Spectrophotometer: 2202, SYSTRONICS was used to adjust the transmittance of working suspensions.10

**Samples preparation**

The stock solutions of ERAF and ARAF were prepared (100 mg/ml) in distilled autoclaved water; further five concentrations (50, 25, 12.5, 6.25 and 3.125 mg/ml) were prepared from stock solutions. The stock solution (100 mg/ml) of isolated quercetin was prepared in DMSO; further other five concentrations (25, 12.5, 6.25, 3.125, and 1.5625 mg/ml) were prepared by serial dilution method. Norfloxacin (10µg/ml) in DMSO was used as the standard and DMSO as the blank/control.

**Method**

About 0.6 ml of nutrient broth suspensions of the test organisms (specific broth for *C.albicans* according to MTCC protocol) were added to 60 ml of sterile molten nutrient agar (specific agar media for *C.albicans* according to MTCC protocol), which had been cooled to 45°C, mixed well and poured into sterile plates. The agar was allowed to solidify; six well (cups) /plate were punched using a six millimeters diameter sterile cork borer (separate borer for each organism) to insure proper distribution of wells in the periphery with one in the center. Agar plugs were removed and 50 µl test samples of each concentration were poured in corresponding marked well by micropipettes after marking at the back of the wells. Triplicate plates of each organism were prepared. The plates were left at room temperature for 2 h. to allow diffusion of the samples and incubated face upwards at corresponding temperature of each microorganism for 24 h. The diameter of the zones of inhibition was measured to the nearest millimeter (the cup size also being included).11-12
(a) & (b) Zone of inhibition by ERAF & ARAF against *S. aureus* respectively. 
(c) & (d) Zone of inhibition by ERAF & ARAF against *E. coli* respectively.

**Figure 4.12** Zone of inhibition by ERAF and ARAF at various concentrations against *S. aureus* and *E. coli*
Table 4.17 Mean zone diameters of ERAF and ARAF against various microorganisms.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentrations (mg/ml)</th>
<th>Mean Zone Diameter (mm)</th>
<th>SA</th>
<th>EC</th>
<th>BS</th>
<th>PA</th>
<th>AT</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERAF</td>
<td>100</td>
<td>13</td>
<td>15</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>13</td>
<td>12</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>11</td>
<td>8</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>-ve</td>
<td>8</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
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<td>-ve</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>3.125</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>ARAF</td>
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<td>13</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
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</tr>
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<td>50</td>
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<td>9</td>
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<td>-ve</td>
<td>9</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>3.125</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>IQ</td>
<td>50</td>
<td>18</td>
<td>14</td>
<td>-ve</td>
<td>-ve</td>
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<td>11</td>
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<td>-ve</td>
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<td>-ve</td>
</tr>
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<td></td>
<td>12.5</td>
<td>11</td>
<td>8</td>
<td>-ve</td>
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<td>-ve</td>
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<td>-ve</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
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<td>-ve</td>
</tr>
<tr>
<td></td>
<td>3.125</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>1.5625</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>NOR</td>
<td>10 µg/ml</td>
<td>27</td>
<td>28</td>
<td>26</td>
<td>23</td>
<td>27</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>-----</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

SA= *S. aureus*; EC= *E. coli*; BS= *B. subtilis*; PA= *P. aerugenosa*; AT= *A. tumifaciens*; CA= *C. albicans*

NOR= Norfloxacin, IQ = Isolated quercetin

4.2.2 ANTI-TUMOR ACTIVITY OF *R. ARBOREUM*

Crown gall tumor inhibition assay (potato disc anti-tumor assay)

Crown gall is a neoplastic disease of plants, which is induced by a gram-negative bacterium, Agrobacterium tumefaciens. The bacteria possess large Ti (tumor inducing) plasmids which carry genetic information (T-DNA) that transform normal, wounded, plant cells into autonomous tumor cells. Since, the mechanism of tumor induction is similar to that in animals, this test has been used to evaluate and pre-screen the antitumor/cytotoxic properties of natural
products. The results suggest that the potato disc assay is safe, simple, rapid and inexpensive in-house screen of natural products for antitumor activity.

**Culture media**

Yeast mannitol agar medium was used for the activation of *Agrobacterium tumefaciens*, according to the MTCC protocol. Composition of media is below.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>10.0g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.5g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.2g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.4g</td>
</tr>
<tr>
<td>CaCO$_3$</td>
<td>4.0g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 L</td>
</tr>
</tbody>
</table>

**Broth culture of *Agrobacterium tumefaciens***

A loop ful of *Agrobacterium tumefaciens* was inoculated into sterile autoclaved yeast mannitol broth, in a screw-cap test tube and incubated at 30°C for 48 hours.

**Test samples preparation**

About 16 mg of ERAF and ERAL was dissolved in 2 ml of sterile water for injection, in a sterile test tube separately, and filtered each through a millipore filter into another sterile tube. Now, 0.5 ml of these solutions was added to 1.5 ml of autoclaved distilled water. Thus, final conc. of 2 mg/ml of ERAF and ERAL were prepared. Further, 1 mg/ml of ERAF and ERAL were prepared by double dilution with sterile water. Sterile water for injection was used as control.

**Method**

Fresh potato tubers of moderate size were surface sterilized by immersion in liquid bleach (Clorox) for 20 minutes; a core cylinder of tissue was removed from the potato by means of a surface sterilized (ethanol and flame) cork borer (6 mm diameter). The 2 cm ends of each potato cylinder were discarded and the remainder of the cylinder was cut into discs of uniform thickness with surface-sterilized knife. The discs were then transferred to 1.5% agar plate (1.5 g of agar was dissolved in 100 ml of distilled water, autoclaved and 20 ml poured into each sterile petri dish). Each plate contained 4 discs and 3 petri plates were prepared for each experimental sample. About 2 ml of broth culture of *Agrobacterium tumefaciens* (a 48 hours culture
containing 5x 10⁹ cells/ml) was added aseptically in 2 ml of each test sample. Control was prepared in the same way but sterile water for injection was used in the place of test sample. One drop (0.05ml) from each sample as well as control tubes was used to inoculate the respective potato discs and it was spread over the disc surface with the help of disposable micro tips fitted with micropipette.

The plates were incubated at room temperature for twelve days, the lids was sealed to minimize the moisture loss. After twelve days of inoculation, the tumors were counted with the aid of a dissecting microscope after staining with Lugol’s solution. The tumor cells lacked starch. The number of tumors in the control was used as a reference for determining the activity.

**Evaluation**

\[
\text{% Tumor} = \frac{\text{Mean No. of tumor (Test sample)}}{\text{Mean No. of tumor (Control)}} \times 100
\]

\[
\text{% Tumor Inhibition} = 100 - \text{% Tumor}
\]

**Table 4.18** Inhibition of Crown Gall tumor development by ERAF and ERAL

<table>
<thead>
<tr>
<th>Samples (mg/ml)/disc</th>
<th>Mean no. of tumor cells ± SD</th>
<th>% Tumors</th>
<th>% Inhibition of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>84.83 ± 17.42</td>
<td>---</td>
<td>--------</td>
</tr>
<tr>
<td><strong>ERAF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>42.33 ± 15.1</td>
<td>50.49</td>
<td>49.51***</td>
</tr>
<tr>
<td>1</td>
<td>54 ± 14.72</td>
<td>64.41</td>
<td>35.59***</td>
</tr>
<tr>
<td><strong>ERAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>26 ± 9.3</td>
<td>31.01</td>
<td>68.99***</td>
</tr>
<tr>
<td>1</td>
<td>33.33 ± 4.8</td>
<td>39.75</td>
<td>60.25***</td>
</tr>
</tbody>
</table>

Crown gall tumor inhibition effect of the ERAF and ERAL. Values are Mean ± SD. The comparison of control Vs. extracts treated discs. ***p < 0.001. Each sample tested on 3 plates with 4 discs. n = 12
Figure 4.13 Crown gall tumor inhibition by ERAF and ERAL in potato discs.
4.2.3 ANTI-CANCER ACTIVITY OF *R. ARBOREUM*

**Sulforhodamine B (SRB) assay**

Sulforhodamine B (SRB) is a bright pink aminoxanthene dye. Under mildly acidic conditions, SRB binds to basic amino acid residues of TCA fixed proteins. It provides a stable end-point that does not have to be measured within any fixed period of time. Once stained and air dried, plates can be kept for months before solubilization and reading. This assay has proven particularly useful in large scale anti cancer drug screening.\(^\text{13}\)

**Cell culture**

MCF-7 (Human breast cancer cell line) cells obtained from National Center for Cell Science, Pune, India and being maintained in tissue culture department of Central Drug Research Institute, Lucknow, (India) were cultured in DMEM pH-7.4, containing penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamicin (60 μg/ml), supplemented with 10% FCS and 10 mM HEPES. The cells were cultured and sub cultured in T-25 tissue culture flasks at appropriate intervals in a humidified atmosphere of 5% CO\(_2\) at 37°C in a CO\(_2\) incubator.\(^\text{14}\)

**Method**

The test procedure was based on the following methods: new colorimetric assay for anticancer drug screening, Skehan *et al.* \(^\text{15}\) and feasibility of high flux anticancer drug screen using a diverse panel of cultured human tumor cell lines Saxena *et al.* \(^\text{16}\)

A fully confluent flask of MCF-7 cells was trypsinized and \(10^4\) cells/well were taken in a 96-well flat bottom plate in 200μl DMEM, pH 7.4, and allowed to attach for 24 hours at 37°C in a humidified CO\(_2\) incubator. Subsequently, sample A and sample B dissolved in sterile PBS (pH-7.4) and tamoxifen (Standard) in ethanol were added at specified concentration and further incubated for 48 h as before. The cells were then fixed in 50 μl cold 50% TCA and incubated for 1 h at 4°C. The supernatant was discarded and the plate was washed five times with deionized water and air-dried. Hundred microliters of 0.4% (w/v) Sulforhodamine B (SRB) in 1% acetic acid was added to each well and incubated at room temperature for 30 min. Unbound SRB was removed by five washes with chilled 1% acetic acid and the plate was air-dried. Two hundred microliters of unbuffered 10 mM Tris base was added to solubilize the bound stain for 5 min at room temperature and OD (Optical Density) was read at 560 nm in a plate reader. The graph was plotted between OD and concentration. Best-fit lines were found by performing linear regression correlation analysis and the results interpreted.
Table 4.19 Anti-cancer effect of ERAF and ERAL on MCF-7 cells using SRB assay.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Concentration of Sample</th>
<th>Mean OD ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>TAM (µM/ml)</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1.66 ± 0.1129</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>1.67 ± 0.0401</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>1.53 ± 0.0660</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>1.21 ± 0.0387</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>0.92 ± 0.0712</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>0.19 ± 0.0040</td>
</tr>
<tr>
<td></td>
<td><strong>ERAF (µg/ml)</strong></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1.72 ± 0.0714</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>1.70 ± 0.0788</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>1.56 ± 0.0354</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>1.63 ± 0.0958</td>
</tr>
<tr>
<td>11</td>
<td>75</td>
<td>1.45 ± 0.1020</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>2.16 ± 0.0394</td>
</tr>
<tr>
<td></td>
<td><strong>ERAL (µg/ml)</strong></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>1.97 ± 0.1142</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>1.75 ± 0.0814</td>
</tr>
<tr>
<td>15</td>
<td>25</td>
<td>1.46 ± 0.1141</td>
</tr>
<tr>
<td>16</td>
<td>50</td>
<td>1.78 ± 0.0841</td>
</tr>
<tr>
<td>17</td>
<td>75</td>
<td>1.50 ± 0.0693</td>
</tr>
<tr>
<td>18</td>
<td>100</td>
<td>1.41 ± 0.0165</td>
</tr>
</tbody>
</table>

All data (TAM, ERAF and ERAL) in the table at various concentrations are represented as Mean O.D.± SEM.
Figure 4.14 Anti-cancer effect of ERAF and ERAL on MCF-7 cells using SRB assay

Table 3.20 Linear regression analysis of SRB assay.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Regression Eq.</th>
<th>Correlation</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAM</td>
<td>Y = 1.960 – 0.060 C</td>
<td>-0.916</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ERAF</td>
<td>Y = 1.60 – 0.0028 C</td>
<td>-0.424</td>
<td>NS</td>
</tr>
<tr>
<td>ERAL</td>
<td>Y = 1.832 – 0.0041C</td>
<td>-0.618</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The conc. of TAM, ERAF and ERAL was subjected to the best-fit regression line eq. Y=a+bc where a= intercept and b= the rate of change in O.D. w.r.t. the concentrations. In the table, TAM and ERAL have significant fit indicating that the O.D. and concentrations are linearly related. The –ve slope indicates the O.D. decrease with increase in concentration. This was determined by comparing the slope of the regression lines with the slopes of TAM and ERAL, that were significantly diff. with p<0.01.

4.3 STATISTICAL ANALYSIS

Data are expressed as mean ± SEM. All treated-groups were compared with control and positive control (Levamisole) by one way-Analysis of Variance (ANOVA) with Turkey’s multiple comparison tests. The significance level was set at p < 0.05 with the help of Graph Pad Prism ® software.
**4.4 CONCLUSION**

In the present study, ERAL exhibited significant reduction in humoral and cell mediated immune responses against control and levamisole treated mice. As per the results obtained, the alcoholic extract of the leaves of *R. arboreum* acts as an immunosuppressant. Most of the classical immunosuppressant agents cause hepatotoxicity, therefore liver function tests and physiology of the liver has been assessed in this study. There was no significant difference in the serum total bilirubin, SGPT and SGOT level in extract-treated mice compared with control. Therefore, this extract is a safe immunosuppressive agent. The alcoholic extract of the seeds of *Carum copticum* (ECCS) showed induction of humoral and cell mediated immunity. Therefore, this extract is an immunostimulant. Only n-hexane fractions of ERAL and ECCS were found to be significantly active immunomodulatory agents; therefore isolation was performed from n-hexane fractions. ERAF and ARAF showed dose dependent antimicrobial activity against two microorganisms (*S. aureus* and *E. coli*) out of six strains. Quercetin, a polyphenolic compound isolated from n-butanol fraction of ERAF, was also found effective against the above two microbes. ERAF and ERAL, demonstrated concentration-dependent anti-tumor activity against *A. tumefaciens* induced tumors on potato disc. ERAL has greater antitumor activity at each concentration than ERAF. The anti-cancer effect of both the extracts against MCF-7 breast cancer cell lines was found to be dose-dependent. ERAL showed significant anti-cancer activity in comparison with TAM, while ERAF showed some decline in the O.D. but the effect was found to be statistically insignificant.
REFERENCES

5.1 RESULTS AND DISCUSSION

In the present study, phytochemical and immunomodulatory screening of two plants (leaves of *Rhododendron arboreum* and seeds of *Carum copticum*) was done. Alcoholic extracts of the flowers (ERAF) and leaves (ERAL) of *R. arboreum* and seeds of *C. copticum* (ECCS) were prepared by continuous hot percolation method using soxhlet apparatus. Aqueous (ARAF) extract of the flowers of *R. arboreum* was prepared by cold maceration method. Carbohydrates, steroids, phenolic compounds, flavonoids and saponins were identified in ERAF and ERAL. ARAF gave the positive tests for the above compounds, except steroids. Chemical tests of ECCS represented the presence of alkaloids, carbohydrates, steroids and phenolic compounds.

ERAL and ECCS were fractioned with n-hexane, chloroform and n-butanol by solvent-solvent extraction method. Preliminary immunomodulatory activity of the above fractions was evaluated by haemagglutination technique. n-Hexane fraction of both the extracts were found to be most active. Therefore, this fraction of both the extracts was selected for further isolation.

Four pure compounds (P-1a, P-2a, P-3 and P-3C) were isolated from n-hexane fraction of ERAL. Three of these were identified as terpenoids and the fourth one was identified as a phytosterol. One flavonoid was isolated from the chloroform fraction and one flavonoid from the aqueous fraction of the alcoholic extract of the leaves of *R. arboreum*. All the compounds, except P-3C, from n-hexane fraction showed positive Liebermann-Burchard test, indicating their terpenoidal nature. Compounds isolated from chloroform and aqueous fractions showed positive tests for flavonoids (Pew’s and Shinoda tests).

Compound P-1a was a white amorphous powder. The IR spectrum of P-1a indicated the presence of OH (3425 cm\(^{-1}\)), C=C and C-O str. of COOH. \(^1\)HNMR data showed the presence of carboxylic, hydroxyl and cycloalkane protons. Mass spectrum showed the M+1 peak.

Compound P-2a was obtained as colourless crystals. This compound could not be identified due to low yields to further spectral studies. It showed a positive test for terpenoids (Liebermann-Burchard test).

Compound P-3 was a greenish-white amorphous powder. IR spectrum showed peaks at 2921 and 1190 cm\(^{-1}\) for C-H and C-O-C str. respectively. \(^1\)HNMR data showed the presence of cycloalkane protons and the evidence for the oxide bridge between carbon atom 3 and 10 was provided by the chemical shift of the proton α to the cyclic ether. In the five membered ring systems, these protons gave a signal at δ 3.66. Since in the compound P-3, the signal for the lone
proton appeared at δ 3.66, the oxygen bridge should be between C-3 and C-10 and thus the compound was identified as 3, 10-epoxy glutinane.

Compound **P-3C**, a white gummy amorphous powder, showed a positive test for sterol (Salkowski test). IR spectrum of **P-3C** indicated the presence of OH (3406 cm\(^{-1}\)), CH (2920, 2852 cm\(^{-1}\)) and C=C (1618 cm\(^{-1}\)) \(^1\)HNMR showed the signals for the protons of cycloalkane, hydroxyl and the proton α to the hydroxyl group. \(^{13}\)CNMR gave the signals of 29 carbons. Mass spectrum showed the M\(^+\), M+1 and M+2 peaks at m/z 414, 415 and 416 respectively.

Compound **P-4** was a light greenish-yellow amorphous powder. IR spectrum of **P-4** indicated the presence of bonded OH (3417 cm\(^{-1}\)), C=O (1662 cm\(^{-1}\)), aromatic ring (1612 cm\(^{-1}\)) and C=C of cycloalkene at 1560, 1521and 1458 cm\(^{-1}\). \(^1\)HNMR spectrum showed the signals for the protons of phenolic and enolic hydroxyl groups and for the protons of aromatic rings. \(^{13}\)CNMR gave the signals of 15 carbons. Mass spectrum gave the M+1 and M+2 peaks at m/z 303 and 304 respectively.

Rutin (**PR**) and quercetin were simultaneously identified from the alcoholic extracts of the flowers and leaves of *R. arboreum* using HPTLC. The method was a RP-HPTLC method. Rutin is being reported for the first time in the leaves *R. arboreum*. It was isolated from the aqueous fraction and characterized. IR spectrum of rutin indicated the presence of bonded hydroxy group (3434 cm\(^{-1}\)), C=O (1677 cm\(^{-1}\)), aromatic ring (1585 cm\(^{-1}\)) and C=C of aromatic ring at 1498 cm\(^{-1}\). \(^1\)HNMR spectrum showed the signals for the protons of phenolic and enolic hydroxyl groups and for the protons of aromatic rings. \(^{13}\)C-NMR gave the signals of the carbons of aglycone and sugar moiety. Mass spectrum gave the M+, M-1 and M-2 peaks at m/z 611, 610 and 609 respectively.

Quercetin was isolated as a yellowish-green amorphous powder from n-butanol fraction of the alcoholic extract of the flowers of *R. arboreum* by solvent–solvent extraction. Its flavonoidal nature was identified by Shinoda and Pew’s tests. Further, TLC, paper chromatography and co-chromatography with authentic standard confirmed its flavonoid nature. Structure was characterized as 3, 3’, 4’, 5, 7- penta hydroxy flavone (quercetin) by UV-Visible spectroscopy using various shifting reagents. The IR spectrum of quercetin gave signals of hydroxyl (3284 cm\(^{-1}\)), carbonyl (1664 cm\(^{-1}\)) and aromatic rings (1610). NMR spectra gave the signals of aromatic rings, ring fusions and hydroxyl groups. In the mass spectrum of quercetin, (M+H) peak was obtained at m/z 303 corresponding to the molecular formula C\(_{15}\)H\(_{10}\)O\(_7\).
Immunomodulatory activity of alcoholic extract of the leaves of *R. arboreum* was evaluated by SRBC antigenic challenge model in mice using three parameters i.e. humoral, cell-mediated immune responses and total leukocyte counts. Humoral immune response is a specific immune response against a specific antigen. In the present study, reduction in the primary and secondary humoral antibody titers, in ERAL treated mice (ETM), in comparison with control and levamisole treated mice (LTM), are the evidences of the suppression of the antibody production. Per oral administration of ERAL (10, 30 and 100 mg/kg body weight) resulted in the reduction of primary and secondary antibody syntheses in a dose dependent manner (*Figure-4.1 and Figure-4.2*). Primary as well as secondary antibody titers were found to be significantly reduced ($p<0.001$) at 100 mg/kg body weight in ETM, when compared with corresponding values for LTM. A significant difference was also observed in control vs. levamisole treated groups in primary ($p<0.05$) and secondary ($p<0.01$) HA titers.

The Delayed Type Hypersensitivity (DTH) response is directly correlated with the cell-mediated immunity (CMI). ETM showed a significant reduction in footpad thickness to SRBC challenge in a dose dependent manner. Footpad thickness in ETM was significantly different from LTM after 48 hrs. Maximum reduction in the paw edema was found at the highest dose (100mg/kg body weight) of the extract of *R. arboreum* ($p<0.001$). However, the delayed type response at the doses of 10mg/kg and 30mg/kg body weight was slightly less significant ($p<0.05$, $p<0.01$) when compared with the LTM (*Figure-4.3*).

ERAL indicated the overall immunosuppressant effect on cellular immunity when compared with levamisole. The results of phagocytosis demonstrated a significant reduction in the per cent phagocytosis in a dose dependent manner (*Figure-4.4*). ERAL exhibited almost similar effect in the reduction of phagocytic function at the doses of 30mg/kg and 100mg/kg body weight when compared with levamisole ($p<0.001$). The per cent phagocytosis was also significant at the lowest dose (10mg/kg body weight) in comparison with levamisole ($p<0.05$).

The results indicate that ERAL suppressed the cell mediated immunity of mice by causing lesser engulfment of the added *Candida* cells in the phagocytosis, in comparison with levamisole, thereby suppressing a non-specific immune response. Significant changes were observed in the white blood cell (WBC) profile of the treated mice. The total leukocyte count (TLC) was significantly lower at the doses of 30mg/kg and 100mg/kg ($p<0.001$) than the control and levamisole (*Figure-4.5*). The value of TLC at the lowest dose (10mg/kg body weight,
was also significantly different when compared with control, but equally significant 
\( p<0.001 \) at the higher doses when compared with levamisole.

As per the results obtained, ERAL acts as an immunosuppressive agent. 
Immunosuppressants are amongst the pharmacological agents with the greatest potential to cause 
induction of hepatotoxicity, which is paradoxical from the pathogenic point of view, since the 
response of the innate and the acquired immune system is a key element in the chain of events 
leading to chemical liver damage. Immunosuppressant therapy can favor the development of 
infections, which by themselves can cause liver damage, or reactivate latent chronic viral 
hepatitis. Methotrexate at high doses, and in patients with risk factors, can induce advanced 
fibrosis and cirrhosis. Thiopurine agents can cause a spectrum of hepatic lesions, including 
hepatocellular cholestatic lesions and hepatic vascular alterations.\(^1,2\)

Most of the classical immunosuppressive agents cause hepatotoxicity, therefore, liver 
function tests and physiology of the liver has been assessed in this study. There was no 
significant difference in the weight of liver of the ETM compared with the control and LTM 
(\textit{Table-4.7}). Serum total bilirubin, SGPT and SGOT levels were also not significantly different 
in ETM compared with control and LTM (\textit{Table-4.8, Table-4.9 and Table-4.10}).

On the basis of results, it has been concluded that ERAL is a safe immunosuppressive agent 
and would show potential in combating autoimmune diseases such as systemic lupus 
erythematosus, myasthenia gravis as well as hypersensitivity reaction, graft rejection etc.

The other plant studied was \textit{Carum copticum}. Ethanolic extract of the seeds of \textit{Carum copticum} (ECCS) was prepared by continuous hot percolation method using soxhlet apparatus. Chemical tests on ECCS represented the presence of alkaloids, carbohydrates, steroids and 
phenolic compounds. Three fractions of ECCS (n-hexane, chloroform and n-butanol) were 
prepared by solvent-solvent extraction method. Four oily sub-fractions (\textbf{PC-b}, \textbf{PC-d}, \textbf{PC-g} and \textbf{PC-i}) were isolated from n-hexane fraction of ECCS by column chromatography and one 
volatile oil (\textbf{C-1}) was extracted by steam distillation. Sub-fraction \textbf{PC-b} was a light-yellow 
coloured dextrorotatory oily liquid and \( p \)-cymene was identified as its major component. IR 
spectrum of \textbf{PC-b} indicated the presence of aromatic rings (2923 cm\(^{-1}\) and 1458 cm\(^{-1}\)). \(^1\)H-NMR 
spectrum showed the signals for the protons of aromatic rings and alkyl groups. \(^13\)C-NMR gave 
the signals of the carbons of aromatic rings and aliphatic substituent. Mass spectrum gave the 
M+1 and M+2 peaks at m/z 135.27 and 136.20 respectively.

153
Sub-fraction **PC-d** was a light-brown coloured oily liquid and was characterized by spectroscopic methods, indicating carvacrol to be its major component. IR spectrum of **PC-d** indicated the presence of aromatic rings (2964 cm\(^{-1}\) and 1454 cm\(^{-1}\)) and phenolic hydroxyl group (3433 cm\(^{-1}\)). \(^1\)H-NMR spectrum showed the signals for the protons of aromatic rings, hydroxyl and alkyl groups. \(^1\)H-NMR spectrum showed the signals for the protons of aromatic rings, hydroxyl and alkyl groups. \(^1\)C-NMR gave the signals of the carbons of aromatic rings and aliphatic substituent. Mass spectrum gave the M+1 peak at m/z 151.12.

Sub-fraction **PC-g** was a brown coloured oily liquid which was characterized by spectroscopic methods indicating terpinene to be its main component. Its IR spectrum indicated the presence of cycloalkene (1654 cm\(^{-1}\)) and alkyl (2923cm\(^{-1}\)) groups. \(^1\)H-NMR spectrum showed the signals for the protons of cycloalkene and alkyl groups. \(^1\)C-NMR gave the signals of the carbons of cycloalkene and aliphatic substituent. Mass spectrum gave the peak at m/z 136.03.

Sub-fraction **PC-i** was a dark-brown coloured oily liquid. This oil contained unidentified components. IR spectrum of **PC-i** indicated the presence of aromatic rings (2964 cm\(^{-1}\) and 1454 cm\(^{-1}\)) and alkyl groups (3433 cm\(^{-1}\)). \(^1\)H-NMR spectrum showed the signals for the protons of aromatic rings, alkenes and alkyl groups. \(^1\)C-NMR gave the signals of the carbons of aromatic rings and aliphatic substituent. Mass spectrum gave the peaks at m/z 337.30, 339.31 (base peak), 340.31 etc.

Volatile oil (**C-1**) was extracted by steam distillation from the seeds of *Carum copticum* using Clevenger apparatus. **C-1** was a very light-yellow colored transparent liquid. It contained three major components, which were characterized by mass-spectrometry. Mass spectrum gave the peaks at m/z 135.12 (M+1 for \(\rho\)-cymene), 137.14 (M+1 for \(\alpha\)-pinene) and 151.12 (M+1 for thymol). These three components were further confirmed by HPLC (Figure-3.69-3.71). Out of the three, thymol was found to be the major component. Therefore, the quantitative estimation of thymol was carried out in **C-1** by GLC, with an authentic marker (Figure-3.72-3.73).

In the immunomodulatory studies, animals treated with different doses of the ECCS showed an increase in the HA titers, DTH-response and phagocytosis in a dose dependent manner. Per oral administration of ECCS (100, 300 and 500 mg/kg body weight) resulted in significant (P<0.05) increase in the primary and secondary antibody syntheses at the highest dose (Figure-4.7-4.8). The agglutination of the humoral responses, as evidenced by an enhancement of antibody responsiveness to SRBC, indicate enhanced responsiveness of macrophages and B-Lymphocytes that are closely associated with antibody production. Per oral administration of ECCS created delayed type hypersensitivity (DTH) reaction in mice. The DTH-response of
ECCS is a direct correlate of cell-mediated immunity (CMI) and showed a significant increase in footpad thickness in 48h, as compared with the control and Levamisole group. DTH responses were significant at the doses of 300 and 500 mg/kg body weight. DTH-response for the highest dose was found to be better than the medium dose (Figure-4.9). This indicated the overall stimulatory effect of ECCS on cellular immunity when compared with the levamisole treated group. The results of the in-vitro polymorphonuclear (PMN) function test (phagocytosis) showed a significant increase in the per cent phagocytosis. ECCS exhibited significant phagocytosis at the doses 300 and 500 mg/kg body weight. Higher dose showed better phagocytosis then lower (Figure-4.10). This may be due to the ECCS enhanced phagocytic efficacy of the macrophages, causing greater engulfment of the added Candida cells in comparison with the control, thereby stimulating a non-specific immune response.

Immunomodulatory effect of n-hexane, chloroform and n-butanol fractions of ECCS was checked by haemaglutination, to get active fraction for further isolation. n-Hexane fraction (HEF) of the ECCS was found to be significantly active against humoral immunity. The other two fractions (CF and BUF) of ECCS were also active but they did not show any significant activity (Figure-4.11). Therefore, it was decided to further isolation from HEF of C. copticum.

In the additional studies, ERAF, ARAF and isolated quercetin (IQ) showed dose dependent antimicrobial activity against two microorganisms (S. aureus and E. coli) out of a total of six strains (Figure-4.12). ERAF and ERAL, demonstrated concentration-dependent anti-tumor activity against A. tumefaciens induced tumors on potato discs. ERAL has greater antitumor activity at each concentration than ERAF (Figure-4.13 and Table-4.18). The anti-cancer effect of both the extracts against MCF-7 breast cancer cell lines was found to be in dose-dependent. ERAL showed significant anti-cancer activity in comparison with TAM, while ERAF showed some decline in the O.D (optical density) but the effect was found to be statistically insignificant (Figure-4.14 and Table-4.19, 4.20).

5.2 Conclusion

On the basis of the results obtained in this study, it can be concluded that alcoholic extract of the leaves of R. arboreum has a potential for immunosuppressive activity. Unlike the hepatotoxicity observed with classical immunsuppressive agents, it showed insignificant changes in total bilirubin, SGPT and SGOT in the blood serum. Therefore, ERAL is a safe immunosuppressive agent and may show potential in combating autoimmune diseases such as
systemic lupus erythmatosus, myasthenia gravis as well as hypersensitivity reaction, graft rejection etc.

Alcoholic extract of the seeds of *C. copticum* act as an immunostimulant. ECCS expressed good prospect of being developed into a drug entity that may be used to stimulate the immunity, as an accessory to therapy in immune disorders. Additionally, the leaves of *R. arboreum* exhibit anti-tumor and anti-cancer activities. Also, significant anti-microbial, anti-tumor and anti-cancer activities were observed with flowers of *R. arboretum*. 
REFERENCES
