Dietary lectins induced immunomodulation targeted to tumour suppression

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For the award of the Degree of

Doctor of Philosophy

In

Biochemistry

by

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❁❁❁❁❁❁❁

Dedicated

To my Beloved Parents

Mr. Padiyappa D.R.
Smt. Gowramma
&
My lovely sister
Ms. Lavanya shree D .P

DECLARATION

I hereby declare that the Ph.D. thesis entitled **"DIETARY LECTINS INDUCE IMMUNOMODULATION TARGETED TO TUMOUR SUPRESSION"** submitted herewith to the Kuvempu University for the award of the Degree of "Doctor of Philosophy in Biochemistry", is the result of the research work carried out by me in the Molecular Biomedicine Laboratory, Post Graduate Department of Studies and Research in Biotechnology, Sahyadri Science College , Kuvempu University, Shivamogga, under the supervision of **Dr. S. N Pramod,** Associate Professor, Food Allergy and Immunology Laboratory, Department of Studies in Food Technology, Davangere University, Shivagangotri, Davangere and Co-supervision of **Dr. B. T Prabhakar,** Associate Professor, Molecular Biomedicine Laboratory, Post Graduate Department of Studies and Research in Biotechnology, Sahyadri Science College, Kuvempu University, Shivamogga. during the period 2019-20 to 2022-23.

I further declare that the results contained in this thesis have not been previously submitted for any other Degree or Fellowship.

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Introduction

1. General introduction

The use of medicinal plants is as old as human civilization. Since antiquity, plants have been used to treat many ailments and are the invaluable, incredible and traditional sources for the curability of various diseases in the form of medicines (Guerra *et al*., 2003). Plants are the main source of drugs that being used from the ancient times as a herbal remedies for the health care, prevention and cure of various diseases and ailments (Kalia, 2005). Plants secondary metabolites have been implicated for most plant therapeutic activities (Timothy *et al*., 2008). Drug discovery from plants involves a multidisciplinary approach combining botanical, ethnobotanical, phytochemical and biological techniques. They continue to provide us new chemical entities (lead molecules) for the development of drugs against various pharmacological targets. However, it was not until the 1800s that pure compounds were isolated from plants, paving the way for modern pharmaceuticals. In the beginning Morphine was isolated from the opium poppy (*Papaver somniferum*). The isolation of salicylic acid from the bark of the willow tree (*Salix alba*), aspirin was synthesized in 1897. Ephedrine was isolated from the Chinese herb Ma huang (Ephedra) and became popular with physicians for its bronchodilation and decongestant properties. Sodium cromoglyate, which is a khellin derivative, was isolated from Egyptian khella seeds (*Ammivisnaga*). The antimalarial drug artemisinin was developed from the Chinese herb qinghao (Sweet wormwood, *Artemisia annua L*.). These examples illustrate the rich history of plant-based medicines (Spainhour 2005).

According to WHO out of 252 drugs which are considered as basic and essential 11% are of plant origin, it says that about three quarters of world population depends upon traditional remedies (Ansari and Inamdar, 2010). Plants use as medicines have not been occurred recently, but its use as a traditional and folklore drug throughout the world for the health services is a matter of tremendous historical legacy and evidences (Hoareau and Dasilva, 1999). Apart from various therapeutic properties of plants today the most emphasis of researchers at present on a variety of immunomodulators which could enhance the immune system and eventually combat the disease or infection by modulating immune responses. Some of the plants with established immunomodulatory activity are *Viscum album, Panax ginseng, Asparagus racemos, Tinospora cordifolia* etc. (Satakopan, 1994). Many fruits like Avocado, are having beneficial effect on

immune system along with their minor cytotoxic property may be utilized for making natural immune modulators (Kulkarni *et al*., 2010).

1.1. Immunomodulation and human immune system

The immune system comprises of Innate (non-specific) and Adaptive immunity (specific) (Tan and Vanitha, 2004) and other cells like macrophages, natural killer cells. Antigen Presenting Cells (APCs) are involved in the fighting against antigens. These antigens could be (fungi, viruses, bacterial toxin) which are processed by APC's presented to T-cells for further processing. Phagocytic cells which are involved in immune system are neutrophils, basophils, eosinophils and monocytes, they engulf and destroy the antigens or foreign substances with their intra cellular mechanisms (Ranjith *et al*., 2008). The first thing done in the field of immunomodulation was the search of immunomodulatory agent for the treatment diseases (Agarwal and Singh, 1999). Cytokines, antibodies and interferon are used in the immunotherapy of cancer (Nadkarni, 2000). Cyclosporin is a potent immunosuppressant proved to be boon for prevention of graft rejection (Agarwal and Singh, 1999), it is also been in autoimmune diseases. Thus, it can be said that the term immunomodulation is a processing of suppressing or stimulating the immune system of the host in order to fight against various diseases and infections (Tan and Vanitha, 2004).

Commencing this we can say that the immune system is involved in the etiology as well as pathophysiologic mechanisms of many diseases (Muthaliar, 1998). Because of many unfavorable conditions, environment and frequent diseases attack, it becomes weak, enervated and needs an external source or factor which can augment the immune response in order to fight against various diseases. Modulation of the immune responses to alleviate the diseases has been of interest for many years (Nadkarni, 2000). Therefore, from a therapeutic point of view immunomodulation refers to a process and a course of action in which an immune response is altered to a desired level. The modulation of immune response by various agents in order to alleviate the disease has been of interest since many years and the concept of Indian Rasayana in Ayurveda has similarity with the concept (Vaghasiya *et al*., 2010). Modulators are classified as, immunosuppressant and immunostimulants.

- *Immunosuppressant:* These agents suppress the immune response and could be used for the control of pathological immune response in autoimmune diseases, graft rejections etc.
- *Immunostimulants:* These agents are envisaged to enhance body's resistance against infections, they enhance the basal levels of immune response and in individuals with impairment of immune response as immunotherapeutic agent.

1.2. Natural plant products as immunomodulators

There have been remarkable advances in the field of basic immunology during last three to four decades. Immunology is thus probably one of the most rapidly developing areas of biomedical research and has great promises with regard to the prevention and treatment of a wide range of disorders (Plaeger, 2003). The use of plant products as immunostimulants or immunosuppressants has a traditional history and the use of natural products with curative and remedial properties is as ancient as human civilization (Rates, 2001; De Pasquale, 1984). From the ancient times treatment of many diseases were done by modulating the immune system or function by using medicinal plants and their products, this was also the fundamental principle of therapeutic approach (Ismail and Asad, 2009). Plant based herbal medicines have been used from centuries for safety, efficacy, lesser side effect and cultural acceptability. Accordingly plant and its products are safe and as a result there is continuous use of plant product as a drug is found to be an alternative way to cure the patients and this approach is in practice from the ancient times (Subramanian *et al*., 2003). A large population of India is using plants for its healing, preventive, curative and many therapeutic properties together with immunomodulatory property (Ranjith *et al*., 2008). The present scenario also says that natural compounds can be lead compounds, allowing the design and rational planning of new drugs.

1.3. Plants as source of Immunomodulatory agents (Immunodrugs):

The main target of the immunomodulatory plant products is primarily there action on the macrophages which play a key role in the generation of an immune response. The therapeutic potential of immunomodulatory agents from plant products and the Ayurvedic concepts of preventive health care have been highlighted by many researchers (Dahanukar *et al.*, 2000). Scientists have described the immunomodulatory

properties of *Emblica offýcinalis* and *Evolvulus alsinoides* were evaluated in adjuvant induced arthritic rat model (Ganju *et al.,* 2003). Ethanolic extract of *A. calamus* inhibited proliferation of mitogen (phytohaemagglutinin) and antigen (purified protein derivative) stimulated human peripheral blood mononuclear cells (PBMCs). In addition, *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-α (TNF-α) (Mehrotra *et al.*,2003). Crude extract of *Tinospora cordifolia* contained a polyclonal B cell mitogen which enhanced immune response in mice.

An arabinogalactan polysaccharide, G1-4A from the stem of *Tinospora cordifolia* examined to modulate induced immunosuppression (Desai *et al.,* 2007). Ethanolic extract of *Boerhaavia diffusa*, a plant used in Indian traditional system of medicine, significantly inhibited the cell proliferation (Mungantiwar *et al.,* 1999). Methanolic extract of Avocado fruits enhances lymphocyte proliferation and reduces chromosomal abnormalities produced by cyclophosphamide treatment in cancer patients (Rajkumar *et al.,* 2010). Extracts of *B. diffusa* roots inhibited human NK cell cytotoxicity in vitro, production of nitric oxide in mouse macrophage cells, interleukin-2 and tumor necrosis factor-α (TNF-α), in human PBMCs. Whereas, intracytoplasmic interferon-γ (IFN-γ) and *B. diffusa* extract and demonstrates immunosuppressive potential. This describes about the clues which can lead to the development of immunostimulatory agents (Dahanukar *et al.,* 2000). In addition, purified bioactive compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicine and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies (Willianson *et al.,* 1996). This is a good sign of advancement and tremendous progress that in recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants.

1.4. Plant Lectins –potential biological molecules

Lectins are proteins or glycoproteins of non-immune origin, which bind specifically to the glycan part of glycoconjugates (e.g., glycoproteins, glycolipids), oligosaccharides and polysaccharides in a sugar-specific manner (Sharon and Lis, 2004). They are a diverse group of multivalent sugar-binding proteins and often have

two or more binding sites per molecule (Rini, 1995). They are abundant and are found to be ubiquitous in all forms of living matter, including bacteria and viruses (Wu *et al.,* 1988). Lectins have been defined by their ability to bind specifically to carbohydrate and by their characteristic property of agglutinating different cell types viz., erythrocytes, leukocytes, tumor cells and bacteria (Goldstein *et al.,* 1980; Green and Baenziger, 1987). The lectin-stimulated agglutination and effector functions suggest the possible involvement of the membrane glycoproteins or glycolipids containing specific carbohydrate residues in the interactions with the lectin (Wu *et al,* 1988). Overall, now plant lectins are defined as "plant proteins comprising at least one non-catalytic domain, which binds reversibly to a particular mono or oligosaccharide" (Peumans and Van Damme, 1995; 1998).

A redefinition was necessary because monovalent lectins have been identified which cannot precipitate glycoconjugates or agglutinate cells (Peumans and Van Damme, 1998). By binding to specific carbohydrates on cell surfaces, lectins can elicit multiple changes in cell and tissue metabolic processes. Lectins bind specifically to different terminal carbohydrates and thus elicit various effects on the cells.

Many plant species, including several important crop and food plants, contain carbohydrate-binding proteins better known as ‘lectins’, ‘agglutinins’, or ‘hemagglutinins’ (Van Damme *et al.,* 1998). By virtue of their specificity toward well-defined glycans, especially of animal origin, plant lectins are used intensively in glycoconjugate research as sensitive molecular probes. In addition, many plant lectins are widely exploited in biological and biomedical research because of their unique biological activities on various animal and human cells (Sharon and Lis, 1972; 2004). Plant lectins were primarily regarded as bioactive proteins by Stillmark in 1888. As a consequence, the majority of all currently known lectins have been tested extensively to reveal their possible effects on cells and organelles. Until the discovery of the carbohydrate-binding activity of lectins, the underlying mechanism(s) of the biological activities of plant lectins remained enigmatic. As soon as it became evident that the activity of lectins is based on the recognition and binding of specific sugar residues, the concept of lectin-receptor was introduced to explain the various activities of plant lectins in molecular terms (Sharon, 1993; Pusztai and Bardocz, 1996). Along with the discovery of many novel lectins, evidence accumulated that different plant lectins

exhibit obvious differences with respect to their biological activities toward animal and human cells (Van Damme *et al.,* 1998). The differences were intimately linked to the structure of cell surface glycan receptor(s) of the respective lectins.

Lectins are usually considered much extended group of plant proteins, because at first sight, the list of lectin-containing species is almost endless (Van Damme *et al.*, 1998). Plant lectins are not typical seed proteins. They have been found in virtually all types of vegetative tissues, and according to the recent compilations, the number of documented non-seed lectins surpasses that of the seed lectins (Rudiger and Gabius, 2001; Van Damme *et al.,* 1998). It is also worth noting that several of the most intensively used lectins are purified from vegetative tissues (e.g., the bulb lectin from snowdrop and the bark lectin from elderberry). The concentration of lectins in seeds and vegetative tissues varies strongly. Seed lectins usually account for 1 to 10% of the total seed protein (Liener, 1997).

In some species, even higher values have been reported (up to 50%), whereas in others the lectins are barely detectable with the techniques currently in practice. The same holds true for the lectin concentration in vegetative tissues. Many plants accumulate large quantities of lectins in their vegetative storage tissues. Lectins usually account for 1 to 20% of total protein content of bark, bulbs, tubers, rhizomes and corns (Rudiger and Gabius, 2001). In some cases, values up to 50% have been reported. Fruits, leaves, flowers, ovaries, roots, and other tissues usually contain low levels (<1% of the total protein) of lectin(s). It's been clear that expression of lectin and their abundancy is highly regulated both in seeds and vegetative tissues (Peumans and Van Damme, 1995).

The definition of lectins includes a wide range of proteins; plant lectins are divided into five major families of structurally or functionally related proteins based on their affinity towards specific monosaccharide ligands: Legume lectins, monocot mannose-binding lectins, lectins related to jacalin lectins that bind to chitin and type-4 ribosome-inactivating proteins (Van Damme *et al.,* 1998a). Another way of classifying lectins is based on their structure, and they fall into three classes: merolectins, hololectins, and chimerolectins (Sharon and Lis, 2003; 2004). Merolectins consist of only one carbohydrate-binding domain and hence, are incapable of agglutinating cells. Hololectins, the class in which the majority of plant lectins belong have at least two

domains, composed of only carbohydrate-binding domains as opposed to chimerolectins. The latter consist of one or more carbohydrate-binding domains and, in addition, an unrelated domain with well-defined biological activity such as enzymatic activity. Despite the obvious three-dimensional diversity observed in the different families of plant lectins, some common structural requirements are apparently necessary for specific sugar-binding activity. It is noteworthy that β-sheets, irrespective of their overall three-dimensional organization, occur predominantly. If not exclusively, in all proteins characterized as lectins. The overall folding of the monomers, which are typically built from β-sheets connected by turns and loops, creates very tight structural scaffolds. Carbohydrate-binding sites emerge from these scaffolds as depressions or pockets resulting from the convergence of flexible loops (Edelman *et al.*, 1972; Sharon and Lis 2004). In addition, the flattened shape of the monomers favors their subsequent oligomeric arrangement, which, in turn, confers a multivalent character to most lectins (Weis and Drickamer, 1994; Drickamer 1988). This quaternary arrangement strongly enhances the ability of lectins to bind to sugars and especially complex N-linked sugars, and is therefore very important for plant proteins involved in recognition processes.

Table 1. Occurrence, molecular structure and specificity of plant lectin families

Lectin Family	Occurrence		Nominal Specificity	References
	Taxonomic distribution	Approx. no. identified lectins		
Chitin-binding lectins	Ubiquitous Diverse Solanaceae	>50 <10	$(GlcNAc)_n$ $(GlcNAc)_n$	[Raikhel *et al.*,1993]
Jacalin related lectins	Monocots and dicots	<10	Gal/GalNAc Mannose/Maltose	[Sastry *et al.*,1986]
Legume lectins	Legumes	>100	Mannose/Glucose Fucose, $(GlcNAc)_n$	[Sharon *et al.*,1990]
Monocot mannose- binding lectins	Liliales, Arales Orachidales Bromeliales	>100	Gal/GalNAc Sialic acid, Complex mannose	[Van Damme *et al.*, 1998; Pusztai *et al.*,1996]
Type 4 RIP	Monocots and dicots	>20	Gal/GalNAc or Siaα2-6 Gal/GalNAc	[Van Damme *et al.*, 1998;]

Gal: Galactose, **GalNAc:** N-acetyl-D-galactosamine, **$(GlcNAc)_n$**: polymer of N-acetyl-D-glucosamine ; **RIP:** ribosome-inactivating protein.

1.5. Dietary Lectins as immunostimulators or immunomodulators

Mitogenic agents are capable of inducing mitosis and cell division. Lectins are able to induce cell division in different kinds of cells, and as mentioned earlier, some plant lectins are mitogenic towards enterocytes. The *in vitro* mitogenicity of lectins is typically measured as their ability to induce proliferation of lymphocytes from lymph organs or blood. Lectins are used to induce proliferation in experimental immunology; the best described and most used lectins are PHA and Con A. They are used as polyclonal activators irrespective of antigenic specificity, and the frequency of responding cells is very high. PZR is a major receptor identified for con A on various cells and has an important role in cell signaling, resulting various biological activity of con A (Zhao *et al.*, 2002). After prolonged contact with lectins, lymphocytes proliferate and become mature effector cells that secrete cytokines and may exert effector functions such as cellular toxicity and antibody production (Kilpatrick, 1999).

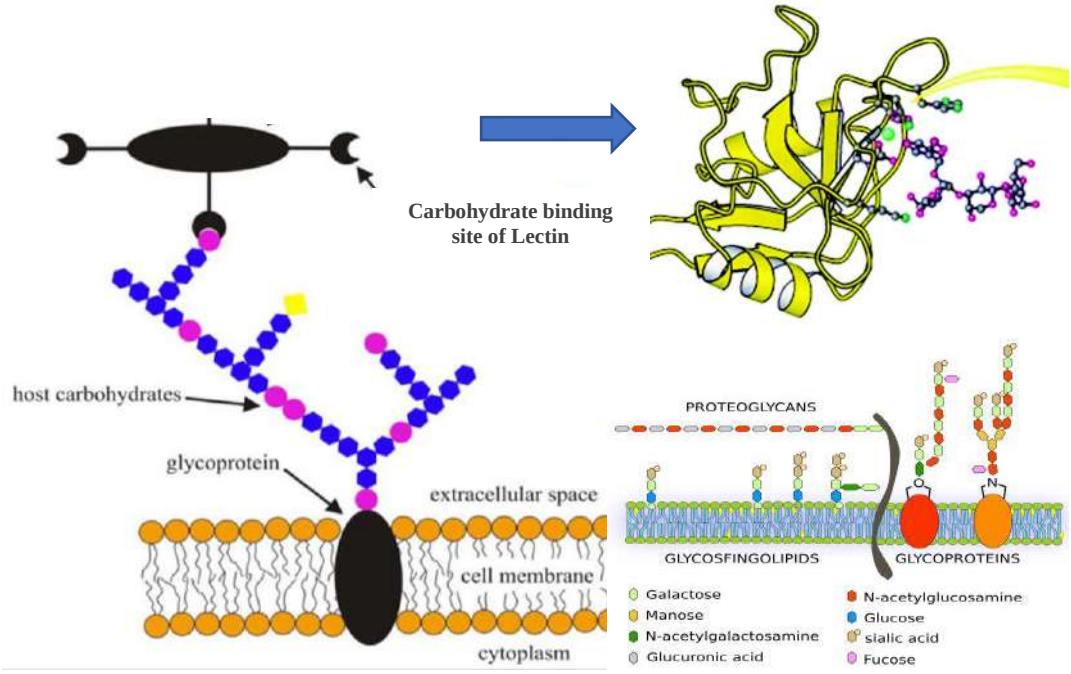


Fig.1: Plant lectins and their interaction: *The general structure of plant lectin and schematic illustration of interactions of plant lectins with the representative structure of cell surface glycoproteins and glycolipids.*

However, not all plant lectins are mitogenic; they can be grouped as mitogenic, non-mitogenic or anti-mitogenic. Actually, WGA has been found to be non-mitogenic (Muraille *et al.*, 1999), antimitogenic (Barret *et al.*, 1983), and mitogenic for either T

cells or B cells depending on the concentration of the lectin or the purity of the examined cells. Antimitogenic lectins inhibit the performance of mitogens in co-culture experiments. Whether a lectin is mitogenic or antimitogenic might also depend on the position of the sugar moiety to which the lectin binds. If the carbohydrate to which the lectin binds is located in close proximity to the binding site of the receptor, binding of lectin does not lead to activation, but the lectin may act as an antagonist due to steric hindrance of ligand binding. Some of the so-called antimitogenic lectins that fall in this category are potato and tomato lectins (McCurrach and Kilpatrick, 1988). Lectins shown to be non-mitogenic may also influence cells of the immune system. For example, WGA has been found to be non-mitogenic, but at the same time the lectin was able to induce secretion of IL-12 and IFNγ (Muraille *et al.*, 1999). The mitogenicity of a lectin can differ depending on the animal from which the cells originate, as well as the immune compartment (Fronkiaer *et al.*, 1997). The variation in cell responsiveness towards different lectins is not surprising, as cell surface saccharides change during development and differentiation and cells from different immune compartments are at different developmental stages. It has been shown that binding of some lectins to the T-cell receptor induces mitogenesis (Chilson and Kelly-Chilson, 1989). Reviews on the mitogenic action of lectins have appeared (Heegaard and Muller, 1988, Kilpatrick, 1999), however, knowledge of the mechanism by which lectins induce mitogenesis remains in its infancy.

1.6. Plant lectins on inflammation and cancer:

Inflammation is a protective pattern for higher organisms, in response to harmful insults like microbial infections, tissue injury and other toxic conditions. It is very essential for immune response by the host to eliminate the harmful stimuli and also to heal the tissue damage. Several studies have revealed that plant-derived lectins have the ability induce modulation in the immune system, by targeting inflammation cascade. For example, *Caulerpa cupressoides* lectin has anti-inflammatory activity by modulating NF-kB expression, thereby preventing leukocyte migration in an experimental asthma murine model (Vanderlei *et al*., 2010; Rogerio *et al.*, 2007). Cancer is enduring to be a major worldwide health problem, with environmental and genetic factors annexing to the risk of developing cancer. There is an eternal demand for a new cancer drug as nearly all attainable cancer drugs have some drawbacks (Amri

2014). Malignant transformation is mainly associated with alterations in glycosylation, which suggests that such molecules play an important role in malignant transformation. Cell surface glycosylation plays a vital role on cell development, signaling, interaction, proliferation, differentiation and migration (Christiansen *et al.,* 2014; Hakomori 1996). Carbohydrate residues of the membrane glycoproteins can be detected using lectins due to their binding specificity to carbohydrates (Hakomori S. 1996). The specificity of lectins provides an adjunct advantage of choosing peptides which are differently glycosylated and aberrantly expressed in cancer patients. Several studies have suggested a strong relationship between lectin binding pattern and their biological behavior with varied tumors (Nagata 2000). Lectins have been established to possess anti-cancer effects, that inducing proliferation of T lymphocytes, which in turn activating the immune system to inducing apoptosis of malignant cells (Udey *et al.,* 1980). Initial reports focused on cytotoxic effects of lectins like ricin and abrin as potential therapies for human cancer treatments (Lin *et al.,* 1970).

1.7. Plant lectins – possible biologicals for cancer therapy

In many biological studies, including cancer research, lectin has proven to be a promising candidate due to its inherent ability to bind specific sugar residues in glycoproteins and glycolipid complexes (Hamid *et al.,* 2013). Various plant lectins have been employed in the past with success as anti-tumor agents or anti-neoplastic drugs against different cancer types, such as leukemia, sarcoma, hepatoma and breast cancer (Li *et al.,* 2008). The anticancer effect of lectins is based on its capacity to limit the growth of tumors by triggering apoptosis and autophagy, which results in the downregulation of telomerase activity, suppression of angiogenesis, and cytotoxicity towards cancer cells (Hamid *et al.,* 2013; Yau *et al.,* 2015). Additionally, lectins are employed to differentiate between benign and malignant cells by recognizing a changed glycan structure that is primarily expressed in tumor cells (Haseenabeevi *et al.,* 1991). In addition to this, lectins work as a carrier to precisely target the tumor cells after being conjugated with chemotherapy drugs. For instance, the studies showed that functionalizing urothelial cells with WGA improved the nano carrier's binding affinity to those cells. Additionally, this micro particle had improved anti-neoplastic efficacy (Neutsch *et al.,* 2013). Similar to this, wistar rat intestinal mucosa showed increased drug delivery and absorption when exposed to WGA-tagged nanoparticles containing

thymopentin (Yin *et al.,* 2006). When encapsulated in alginate microbeads, lectins obtained from *Pisum sativum* seeds demonstrated improved drug delivery to hepatocellular cancer (El-Aassar *et al.,* 2014). ConA related microsphere also revealed a rise in the drug's adhesion to release from stimulated gastrointestinal fluids (Bakowsky *et al.,* 2008; Jain *et al.,* 2014). Targeting human prostate cancer, Bauhinia purpurea agglutinin-bound liposomes were demonstrated to inhibit cell proliferation after specifically attaching to DU145 cells in mice (Ikemoto *et al.,* 2016). In spite of all the differences in the properties of these lectins, most of them exhibit a common biological function *i.e.* they have antiviral, anti-insect, antifungal, anti-parasitic, immunomodulatory and antitumor activities.

Review of Literature

2. Review of literature

2.1 Lectins in the human diet (Dietary lectins)

Plant and animal materials used as foodstuffs contain lectins, some of which are denatured by cooking. There are, however, still active lectins in the diet as when foodstuffs are eaten raw (uncooked) and some lectins are still active after cooking or processing (Peumans and Van Damme, 1998). In general, most lectins are inactivated by heat treatment such as those involved in commercial processing or household cooking. This, however, still leaves foodstuffs like juices, tomatoes, raspberries, garden peas, salad ingredients, spices, dry cereals, and roasted nuts in which lectins are consumed in an agglutinative active form. Nachbar and Oppenheim (1980) found, by survey of the literature in combination with their own work, that 82 different edible plants contained agglutinative-active lectin.

In general, lectins appear to be refractory to hydrolysis by digestive enzymes. There is evidence that lectins, as phylogenetically distinct as those from wheat (Brady *et al.,* 1978) and tomato fruit (Kilpatrick *et al.*, 1985), can migrate from the human mouth to the gut intact without losing its functional attribute. Similar resistance to the digestive process has been found for various lectins introduced into the stomach of rodents (Pusztai, 1991). Therefore, dietary lectins appear to be bioactive in human gastrointestinal tract, that have the ability to substantially alter gut immune function (Wang *et al.,* 1998). Lectins may also be neutralized by provoking an IgA response; certainly, salivary IgA could interact with dietary peanut agglutinin, pea lectin and wheat germ agglutinin (Gibbsons and Dankers, 1983; 1986). The human diet emphasizing the degree to which the human population is exposed to dietary sources of lectins is accountable. However, the absence of any study involving the oral intake of lectins purified from these sources, and their possible physiological significance is still unclear.

2.2. Interactions and survival of dietary lectins in the digestive tract

As part of the normal turnover of the gut epithelium, cells are shed from the villus tips into the lumen and most cellular material is then digested and recycled. The presence of lectins attached to these cells does not interfere with the breakdown of cell contents, but the liberated lectins can move further down the gut and bind to the next receptor with an

appropriate carbohydrate moiety (Pusztai, 1991; 1993). Although lectin binding is most frequently studied in the small intestine, similar binding can occur throughout the entire digestive tract, from the stomach to the distal colon (Pusztai *et al.,* 1990). However, as surface glycosylation varies in the different functional parts of the gut, lectin binding is not uniform in the digestive tract.

Dietary lectins interact with buccal epithelial cells and/or bacteria or viruses. Certainly, for two lectins occurring in normal diets (peanut agglutinin and wheat germ agglutinin), binding to gut-epithelial cells has been noted (Gibbson and Dankers, 1983). These lectins also bind to oral bacteria and it is evident that they have the potential to alter oral bacterial ecology and may be of relevance to periodontal diseases. The influence of sugars on lectins in the diet could influence the nutritional significance of these lectins in the gut. Dietary lectins, in general, bind surface glycans of brush-border epithelial cells causing damage to the villi, which includes disarrangement of the cytoskeleton, increased endocytosis, and shortening of the microvilli (Liener, 1986; Sjolander *et al.,* 1986; Pusztai, 1993).

The structural changes induced by dietary lectins on gut and intestinal epithelial cells elicit functional changes including increased permeability (Sjolander *et al.,* 1984), which may facilitate the passage of undegraded dietary lectins into systemic circulation (Pusztai, 1993). The interaction of numerous lectins, particularly PHA, with the rat small intestine has been extensively studied (Pusztai, 1991). These studies led to the following generalization: (a) to a significant extent, lectins reach the gut in a structurally intact and biologically active form, (b) lectins may bind to the gut wall and act as local growth promoters, or also as systemic toxins, and (c) the toxic effects are not observed in germ-free animals, suggesting bacterial overgrowth and toxin production is crucial (Brady *et al.,* 1978). In rats, dietary wheat germ agglutinin is rapidly transported across the intestinal wall into the systemic circulation where it is deposited in blood and lymphatic vessel walls (Pusztai, 1993).

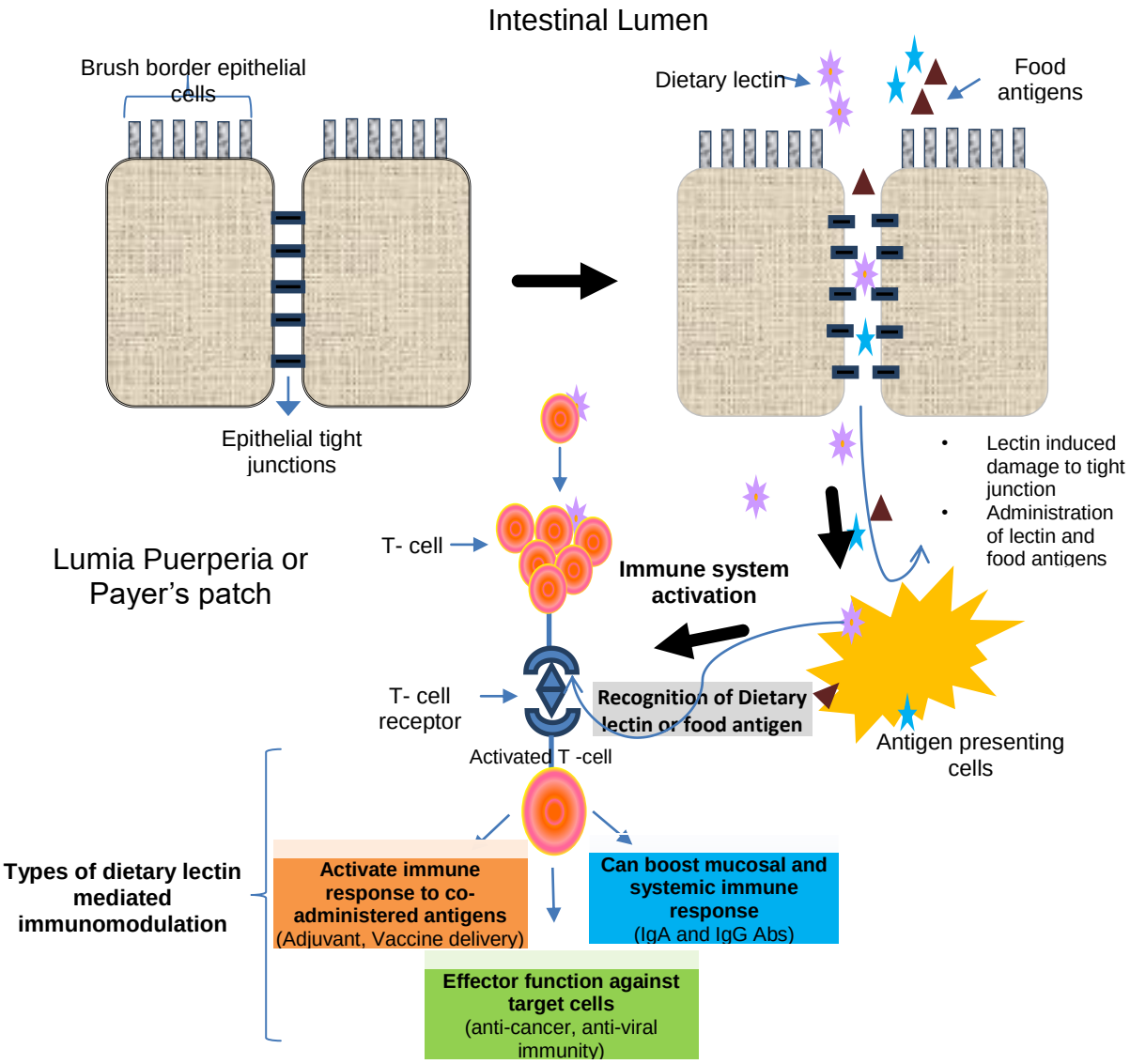


Fig.2: Plant lectins as immunomodulators: *Schematic representation of lectins contributing to the evolvement of the intestinal immune system.*

2.3. Toxicity and biological effects of lectins in foods

A very important *in-vivo* biological activity is the striking effects that some dietary lectins have on gut function. Such lectins react with the surface epithelium of the digestive tract, and are in some cases mitogenic for enterocytes (Banwell *et al.*, 1993; Otte *et al.*, 2001). Phytohemagglutinin (PHA) is a powerful growth factor for the gut cells and by interacting with the brush border epithelial receptors induce extensive proliferation of epithelial cells (Banwell *et al.*, 1993). It has been shown that PHA reversibly induces hyperplasic and hypertrophic growth of the small bowel (Bardocz, 1996). In addition to its role as a growth factor for the gut, PHA induces enlargement of the pancreas (Pusztai *et al.*, 1995). Many lectins are very potent exogenous growth signals; some can even mimic

the action of major metabolic hormones and growth factors (Pusztai, 1993). The effects of some lectins on the gut and other parts of the body are especially important because lectins account for a relatively large fraction of plant proteins. The major consequences of lectin damage to the intestinal mucosa appear to be a serious impairment in the absorption of nutrients across the intestinal wall (Frokiaer *et al.,* 1997). This was first demonstrated *in-vitro* with isolated intestinal loops taken from animals that had been fed raw black beans, or a lectin purified there from displayed a significant decrease in the rate at which glucose was transported across the intestinal wall. It would appear that the effect of lectins on the absorptive ability of the intestine is most likely a consequence of changes in the intestinal permeability (Sjolander *et al.,* 1984; Greer and Pusztai, 1985). These findings may have important implications with reference to allergic reactions to foods containing lectins. Lectins can also affect brush border hydrolyses, which play a vital role in the digestion of proteins and carbohydrates (Triadou and Audran, 1983; Erickson and Kim, 1983). The interaction of lectins with brush border membranes resulted in inhibition of brush border peptidases (Kim *et al.,* 1976) and enterokinase (Rouanet *et al.,* 1983) *in-vitro*.

Lectin-induced damage to the intestinal mucosa alters their permeability so that normally innocuous intestinal bacteria, or the endotoxins that they produce, gain entrance into the bloodstream and produce toxic systemic effects (Pusztai *et al.,* 1989). It was also considered the possibility that lectins, either intact or partially digested may themselves enter the circulatory system to exert toxic reactions such as inhibition of protein synthesis, local or systemic immune hypersensitivity (Mitchell and Clarke, 1979), or tissue damage. The immunochemical demonstration of the presence of lectins in blood has provided support to this theory (Wang *et al.,* 1998). Lectins may influence systemic effects by two different but possibly simultaneous mechanisms. Lectins can indirectly influence the endocrine system of the body by binding to the neuroendocrine cells of the gut and stimulating the secretion of gut peptide hormones into systematic circulation (Liener, 1986; Pusztai, 1993). Alternatively, lectins can be transmitted through the gut wall into the blood circulation and thus may directly influence peripheral tissues and body metabolism by mimicking the effects of endocrine hormones.

The organs most often affected are the pancreas, skeletal muscle, liver, kidneys, spleen and thymus. Dietary lectins influence the structure and function of both enterocytes and lymphocytes (Liener, 1986; Pusztai, 1993), as they have potent anti-nutritional properties, and most of them are heat stable and resistant to digestive proteolytic breakdown in both rats and human subjects (Brady *et al.*, 1978) and have been recovered intact and biologically active in human feces (Brady *et al.*, 1978).

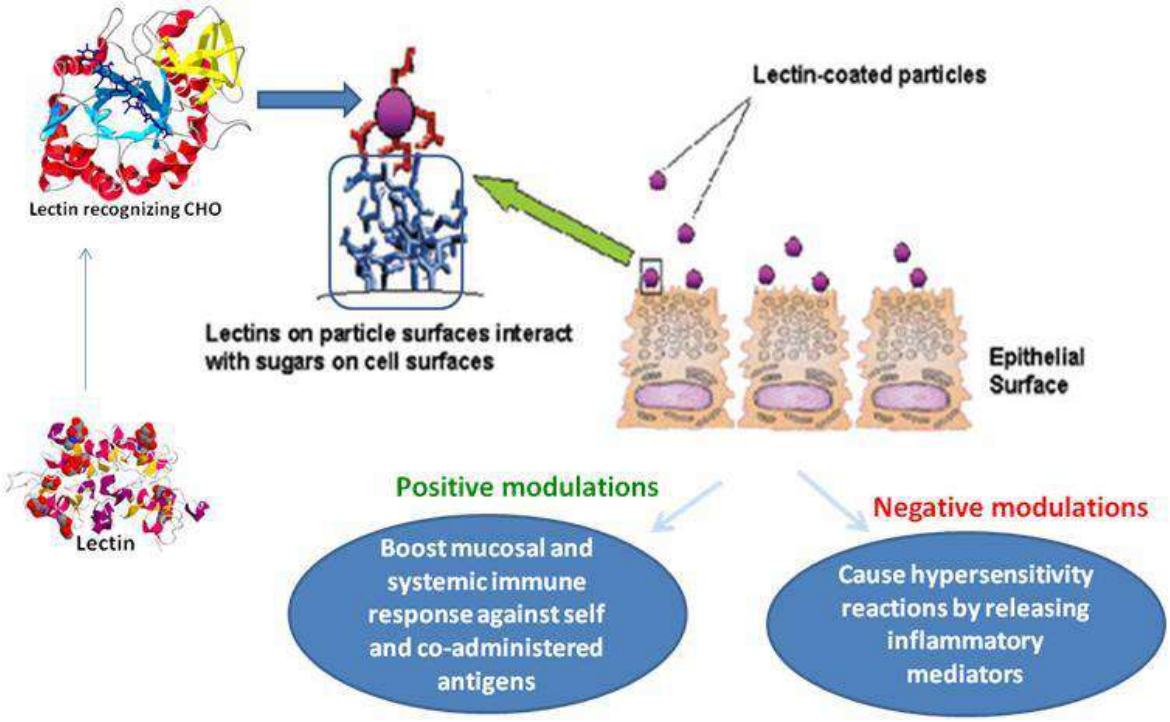


Fig.3: Lectins are immunostimulators: *A diagrammatic illustration of how dietary lectins may hypothetically interact with the gut and immune system*

Lectins, in general, bind surface glycans on gut and intestinal brush-border epithelial cells causing damage to the villi, which elicit functional changes, including increased permeability (Sjolander *et al.*, 1984; Greer *et al.*, 1985) which facilitate the passage of undegraded dietary lectins into systemic circulation (Pusztai, 1993). When the luminal concentration of intact dietary proteins is low, absorbed proteins generally elicit a minimal allergic response because of the limiting influence of T-suppressor cells. Due to their resistance to digestive proteolytic breakdown, the luminal concentrations of dietary lectins can be quite high; consequently, their transport through the gut wall can exceed that of other dietary antigens by several orders of magnitude (Pusztai 1989), and absorbed dietary lectins can be presented by macrophages to competent lymphocytes of the immune system (Hruby *et al.*, 1985; Ohba *et al.*, 2003).

Since dietary lectin escape proteolytic digestion and can cause increased gut and intestinal permeability, they are able to cross the gastrointestinal barrier rapidly and enter the systemic circulation intact and are able to induce local, systemic, and pathophysiological effects on immune cells.

2.4 Immunomodulatory dietary lectins in Onco-therapeutics

2.4.1 Dietary lectins interaction and immune function

Lectins may interact with the immune system in various ways. Dietary lectins may lead to generation of a specific anti-lectin response, but may also modulate immune response against co-administrated proteins, the so-called adjuvant effect. Moreover, lectins may also be capable of polarizing the immune response towards certain effector functions (Table 2). In addition, dietary lectins may give rise to mucosal (local) or systemic response (Kjaer and Frokiaer, 2005). It is evident that variety of plant lectins were found stable in the mouse gut and involve in interaction with the mucous membrane (Pusztai and Bardocz, 1996). Recently Clark *et al.,* Wang *et al.* have reported that plant lectins can move and interact throughout the gut lining in both mice and humans (Clark *et al.,* 1995; 1998). The finding that certain plant lectins interact with the mucosal epithelium and are translocated across the gut may have important applications in inducing mucosal and systemic immunity.

Dietary proteins usually give rise to specific downregulation of the immune response, due to induction of oral tolerance. Ingested lectins initially encounter the immune system and various plant lectins are capable of inducing a specific IgG response in mice (Di Aizpurua and Russel-Jones, 1988, Hjaer and Frokiaer, 2002, 2005). LEA and PHA showed high immunogenicity after nasal administration; LEA also gave rise to specific IgA and IgG responses (Lavelle *et al.,* 2000). High level of specific serum IgG was induced by oral administration of LEA to mice (Naisbett and Woodley, 1995), whereas nasally administered PHA only demonstrated an IgA response systemically and not mucosally (Lavelle *et al.,* 2001). Some of the earlier studies have shown that proteins with lectin/lectin-like properties are effective mucosal immunogens, and proposed a relationship between receptor binding in the gut and mucosal immunogenicity (De Aizpurua *et al.,* 1988). However, despite a number of studies on lectin binding, and evidence that plant lectins conjugated to antigens/haptens may enhance immune responses following oral (De

Aizpurua *et al.*, 1988) and nasal (Giannasca *et al.*, 1997) delivery, there is relatively little data on the comparative mucosal immunogenicity of plant lectins.

The immune responses against dietary lectins thus seem to be natural consequence of lectin ingestion, but whether this, in any way, has an adverse influence on the immune system is not fully elucidated. Also, in light of the intensive research in use of lectins as drug and vaccine delivery systems and as potential antileukemia agents (Gabor and Wirth, 2003; Lavelle, 2001), the immune response to orally ingested lectins needs further research.

The structural changes induced by dietary lectins on gut and intestinal epithelial cells elicit functional changes including increased permeability (Sjolander *et al.*, 1984), which may facilitate the passage of undegraded dietary lectins into systemic circulation (Pusztai, 1993). The interaction of numerous lectins, particularly PHA, with the rat small intestine has been extensively studied (Pusztai, 1991). These studies led to the following generalization: (a) to a significant extent, lectins reach the gut in a structurally intact and biologically active form, (b) lectins may bind to the gut wall and act as local growth promoters, but also as systemic toxins, and (c) the toxic effects are not observed in germ-free animals, suggesting bacterial overgrowth and toxin production is crucial (Brady *et al.*, 1978). In rats, dietary wheat germ agglutinin is rapidly transported across the intestinal wall into the systemic circulation where it is deposited in blood and lymphatic vessel walls (Pusztai, 1993).

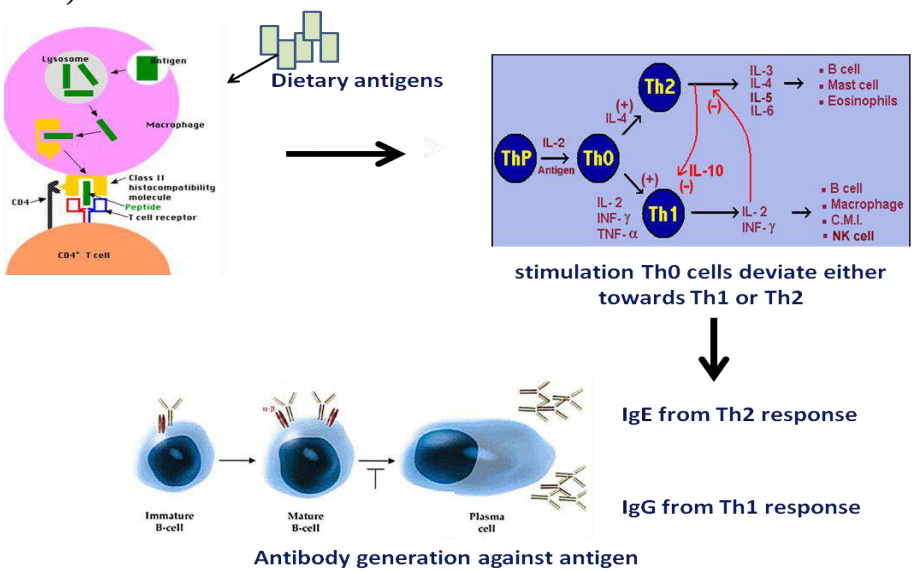


Fig.4: Dietary lectin induces humoral immune response: *Dietary lectin with leucoagglutinating property will interact with immune lymphocytes and induce humoral immune response against them self and co-administered dietary antigens.*

Table.2: Influence of dietary lectins on immune function: putative systemic and mucosal (local) immune responses and specific and general immunomodulation.

Immune response	Generation of lectin-specific response	Immunomodulating effects	Reference
Systemic response	Specific IgG, IgM and IgE antibodies	Adjuvant activity (immune response as opposed to tolerance) Polarizing effect (Th1 or Th2 polarization) Release of histamine and other bioactive mediators (allergy symptoms) Increase in NK cell activity	[Gollapudi *et al.*,1975; Lavelle *et al.*,2000; Xu-Amano *et al.*,1993]
Mucosal response	Specific IgA antibodies	Mucosal adjuvant activity (immune response as opposed to tolerance) Release of histamine and other bioactive mediators (allergy symptoms)	[Lavelle *et al.*,2001]
Parameter	**Direct effects (lectin interaction with the immune cells)**	**Indirect effects (lectin interaction with non-immune cells)**	**Reference**
Generation of lectin-specific immune Response	Cross-binding of lectins to B-cells (T cell-independent antibody response, mitogenesis)	Binding of lectins to red blood cells (particulate antigen) Binding of lectins to enterocytes (increased absorption)	[Di Aizpurua *et al.*, 1988]
Immunomodulation	Cross-binding of Fc receptors on mast cells (histamine release) Binding to APCs or T cells (mitogenesis, polarization and adjuvant effects) Increase in NK cell activity (increased cytotoxicity)	Intestinal Degeneration (Increased and/or altered absorption)	[Chandrashekhar et al.,2009; Wagner et al.,1999]

Only a few numbers of biologically active components have been discovered and reported to elicit an immune response, when administered orally or through other mucosal routes. Lectins are some of the few proteins that when administered by the mucosal route induces an antibody response. Although still sparsely documented, the type of immune response (local vs. systemic, tolerance vs. immunity) may be strongly dependent on the site of absorption in the gut. Uptake across Peyer's patches (PP) might induce immunity,

whereas, absorption through enterocytes might induce tolerance. Apart from indications that the binding activity of lectins can confer immunogenicity, there are very few investigations performed on what determines mucosal immunogenicity of plant lectins. Many different plant lectins have been shown to be mucosal immunogens in rodents (Di Aizpurua *et.al.,* 1988). In humans, banana lectin was found to induce a strong specific antibody response, especially of the IgG_4 isotype (Koshte *et al.,* 1992). PHA is highly immunogenic orally; in fact, orally applied PHA mounted an antibody response to the same level as seen in parenteral administration (Di Aizpurua and Rossell-Jones, 1988; Kjaer and Frokiaer, 2002, 2005). An increase of intraepithelial lymphocytes and jejunal lamina propria cells was observed in response to orally administered PHA (Banwell *et al.,* 1993), suggesting that absorption of PHA leads to T-cell proliferation in gut-associated lymphoid tissue (GALT).

2.4.2 Mucosal immunogenicity of ingested dietary lectins

In contrast to other dietary proteins, lectins are highly immunogenic and capable of eliciting a specific immune response after oral administration. Orally ingested lectins may, after absorption, affect the immune system systemically; however, it is also important to consider the local mucosa induced immune response, as the mucosa is where orally ingested lectins initially encounter the immune system. Natural serum antibodies (for ASA and alliinase present in garlic) against dietary proteins have been detected in humans (Tchernychev and Wilchek, 1996) and various lectins are capable of inducing a specific IgG response in mice (Kjaer and Frokiaer, 2002). Lavelle *et al.,* (2000) have investigated the mucosal and systemic immune response to a number of mucosa administered plant lectins. VAA-I showed the highest mucosal specific immune response. None of the lectins examined, viz., PHA, WGA, UEA-I, LEA and VAA-I gave rise to a strong mucosal response in the absence of systemic response (Lavelle *et al.,* 2000). WGA and UEA-I induced a stronger response when administered by the oral route as compared to intranasal administration (Lavelle *et al.,* 2000).

Dietary lectins may influence mucosal immunity against other co-administered proteins, *i.e.,* display adjuvant activity; co-administration of lectin with other proteins leads, in many cases, to an immune response against the co-administered protein. ConA complexed to ovalbumin (OVA) has been shown to enhance the formation of anti-OVA IgG and IgE in mice upon immunization (Gollapudi and Kind, 1975). Nasal and

subcutaneous administration of Con A has been found to induce IgE antibodies against Con A and against hapten-conjugated Con A (Mitchell and Clarke, 1979). PHA has been reported to affect the OVA-specific IgE response in mice injected with OVA and PHA (Astorquiza and Sayago, 1984). More recent studies have also demonstrated that lectins affect the immune response against mucosa administered OVA (Lavelle *et al.,* 2001; Watzl *et al.,* 2001). Plant lectins are, thus, not only mucosally immunogenic, but also have mucosal adjuvant activity, as tested by co-administration of OVA (a weak antigen) and different lectins (Lavelle *et al.,* 2001).

Oral ingestion of jackfruit seed lectin (jacalin) provoked an enhanced IgE response towards both OVA and the lectin after parenteral challenge (Restum-Miguel and Prouvost-Danon, 1985). Many plant lectins are known to induce IL-4 and IL-13, which promote the differentiation of T-helper cells towards Th2 cells (Abbas *et al.,* 1996; Finkelman *et al.,* 1990). Lavelle *et al.,* (2000) have found that mucosal administration of PHA, WGA and UAE-I gave rise to IgG1 antibodies indicative of a Th2 response in BALB/c mice. Intranasal immunization with LEA and VAA-I induced lectin-specific antibodies of the IgG1, IgG2a, IgG2b and IgA isotypes, whereas PHA induced only IgG1 and IgA in serum (Lavelle *et al.,* 2000; 2001). An important outcome of their study was, when using isotype antibodies as markers of Th1/Th2 immunity, that lectins are more Th2-skewing toward a Th2 response than is cholera toxin (Lavelle *et al.,* 2000), which is known to be a potent Th2-skewing immunogen (Xu-Amano *et al.,* 1993). The ability of lectins to promote polarization is relevant in connection with certain diseases, such as allergy, which is a Th2-driven disease. Literature survey shows that there is a spurt in the past few years, in research on immunomodulators of plant lectins. A number of plants of known therapeutic properties are evaluated on a battery of experiments to identify their mechanism of action at the molecular level.

2.4.3 Dietary lectins as mucosal adjuvant for oral vaccine delivery

Mucosal and particularly oral administration of antigen was frequently ineffective at stimulating strong and sustained immune responses. Many times, administration of several strong dosage was needed and the response that is elicited may only last a short span of time (Lamichhane *et al.,* 2014; Clement *et al.,* 2010). Adjuvants and delivery systems can improve the immune response induced by mucosally delivered antigen, by protecting the antigen targeting to the epithelium. One strategy for antigen targeting was to

use of molecules such as plant lectins, which bind specifically to mucosal epithelial cells. After feeding, it was discovered that many plant lectins were stable in the mouse stomach and interact with the mucosal membrane (Clement *et al.,* 2010). Studies that have shown the interaction of lectin with intestinal epithelial cells by selective labeling of antigen-sampling to M cells in the mouse Payer's patch by fucose-specific lectins (Guptha *et al.,* 2011; Chanadrashekar and Venkatesh 2009). Plant lectins have been shown to be intact in gut circulation and interact with gut mucosal epithelium to get transported acroos to induce immune response in both mice and humans (Kjaer and Frokiaer 2002; Zhao *et al.,* 1999). Mitogenic plant lectins including phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) are routinely used for activation of lymphocytes *in-vitro* (Cardoso *et al.,* 2007; Guptha and Vyasa 2011; Watzl *et al.,* 2001). High levels of specific serum IgG were induced by oral administration of tomato lectin in mice (Rhodes *et al.,* 1998). Since the binding of lectin in the gut trigger mucosal immunogenicity, it has been claimed that proteins having lectin or lectin-like properties are effective mucosal antigens (Lavelle *et al.,* 2002).

2.5 Anti-inflammatory effect of Dietary lectins

Many plant components are used traditionally by local communities in various nations, particularly in Africa and Asia, for pain treatment and inflammation reduction. According to estimates, the inhabitants of India and Sudan rely on traditional medicine for 65% and 90% of their healthcare, respectively. (Karar and Kuhnert 2017; Prashantkumar and Vidyasagar 2008). On the method of administration, plant lectins can exhibit either pro- or anti-inflammatory effects through lectin domain interaction. (Assreuy *et al.,* 1997; Assreuy *et al.,* 1999; Alencar *et al.,* 1999; Alenca r*et al.,* 2004). Butterfly pea (*Clitoria fairchildiana*) seeds contain lectins that have been isolated, identified, and their anti-inflammatory effect confirmed by Leite *et al.,* 2012. In the carrageenan-induced paw edema inflammation model, where there was a 64% reduction in edema, it was found that lectin showed anti-inflammatory effect. Araujo *et al.,* 2013 also assessed the monocot lectin's anti-inflammatory properties in *Channa limbata* seedlings. With the reduction of inflammation in the formalin test and neutrophil migration into the peritoneal cavity, the lectin demonstrated an anti-inflammatory activity.

Using an *in-vivo* model, Bezerra *et al.,* 2014 described the anti-inflammatory activities of the lectin from *Canavalia boliviana*. The presence of lectin at a dosage of 1 mg/kg also reduced the carrageenan-induced paw edema. In a different investigation, it was discovered that soybean agglutinin has an inhibitory effect on neutrophil migration, indicating an anti-inflammatory action (Benjamin *et al.,* 1997). The findings demonstrated that rats with various cavities were injected with soybean agglutinin (5–200 mg/cavity), which led to a normal inflammatory response defined by dose-dependent exudation and neutrophil migration 4 hours later. When administered intravenously, the lectin *Dioclea violacea* (Dvl), which was discovered in the Fabaceae family, was said to have anti-inflammatory characteristics because it prevents neutrophil migration in response to inflammation (Nascimento *et al.,* 2018; Freitas *et al.,* 2015). Due to its capacity to decrease renal vascular resistance, apoptosis, and ROS production, Freitas et al. 2015 revealed that Dvl had positive effects in treating acute kidney damage. The capacity of Dvl to prevent plasma protein extravasation and leukocyte infiltration in a rat model by reducing ICAM-1 expression has also been connected to its anti-inflammatory effects (Clemente-Napimoga *et al.,*2019). It has been demonstrated that a different lectin from *Canavalia grandiflora* (ConGF), a D-glucose/D mannose-specific lectin, inhibits the production of a variety of pro-inflammatory cytokines like IL-1 and TNF-α, affecting the leukocyte-endothelium interaction and neutrophil transmigration during inflammation in a dose-dependent manner (Nunes *et al.,* 2009). Plant lectins can therefore be employed to produce innovative anti-inflammatory medicines and new therapies to control inflammatory disease as a result of the aforementioned features.

2.6 Anti-tumor effects of Dietary Lectins

Studies using various lectins as antitumor or cytotoxic agents have shown diverse effect depending on source of lectins and cancer type or cell line. For example, Con A treatment triggered mitochondrial mediated apoptosis in human melanoma A375 cells by mitochondria dependent membrane potential collapse leading to release of cytochrome c and activation of caspase (Peumans *et al.,* 1996). On other hand, SNA (Sambucus Nigra Agglutinin) purified from *Sambucus nigra* found to activate the signaling pathways of Akt and ERK1/2 in ovarian carcinoma cells and mitochondrial outer membrane permeability induced in ROS generation and cytochrome-C release in the cytosol. The anxious mitochondrial respiration resulted in the G_2/M phase cell cycle arrest (Chowdhury *et al.,*

2017). Mistletoe Lectin (ML) from Chinese showed vital role on human T cells cytotoxicity, apoptosis and cytokine production. ML increased release of tumor necrosis factor TNF-α and also inhibiting the release of anti-inflammatory interleukin IL-10 (Gomg *et al.,* 2007). Likewise, some lectins react by altering the production of interleukins, although some other lectins exhibited to induce non apoptotic G_1 phase mechanism or G_2/M phase cell cycle arrest, through varying the cell cycle. Also, lectins show cytotoxic effects on various cancer cell lines like, PHA exhibits cytotoxic effects on MCF-7 cells, Con-A on A375 cells, Frutapin on HeLa cells etc. The ability of lectins to inhibit the cancer progression is well documented and some examples are listed in Table 3 (Lam *et al.,* 2010; Lam *et al.,* 2011). Hence, the promising therapy will be based on the selective elimination of the abnormal cells without disturbing the function of the normal cells.

2.7 Inflammation

The term inflammation, derived from the Latin word inflammation, is defined as a complex biological response of body tissues to injurious stimuli such as pathogens, damaged cells, or irritants (Turner *et al.,* 2014; Wiersinga *et al.,* 2014; Engelmann *et al.,* 2013; Mahindra *et al.,* 2010). It is regarded as a protective response that involves immunocompetent cells, blood vessels, and a broad range of molecular mediators derived from the various cells involved in the inflammatory process. The main role of inflammation is to eradicate the initial cause of cell injury, to mediate clearance of necrotic cells and damaged tissues from the original insult and inflammatory process, and to initiate tissue repair (Wiersinga *et al.,* 2014). The classical local signs of inflammation are redness, heat, swelling, pain, and loss of function. In addition, severe inflammation also elicits systemic effects, probably mediated, at minimal in part, by circulating soluble mediators arising from the local inflammatory process. Inflammation can be caused by a wide range of biologically very different conditions. Thus, understanding the inflammatory response and the immune mechanisms underlying the inflammatory process is essential in determining the detailed pathogenesis of human disease such as microbial infections, cancer, vascular disorders, and autoimmune reactions (Wiersinga *et al.,* 2014; Mahindra *et al.,* 2010; Roumen-Klappe *et al.,* 2002; McCoy *et al.,* 2015). As a protective strategy for the host, one of the main aims of inflammation is to restore cellular homeostasis in response to any damaging condition. The mechanism underlying the initiation of inflammation is therefore tightly coupled to the physiological state of homeostasis. And so, inflammation is appraised as an

'adaptive response' to any harmful effect terrifying the integrity of the cellular homeostasis. It is entirely plausible to understand that such an adaptive response operates at the expense of normal cellular functions (Medzhitov, 2010).

Inflammation is known to be a protective response of the host against infection and tissue damage, which can avert the outspread of pathogens or promote tissue repair (Dorward *et al.,* 2012; Sun 2017). In the early or acute stages of inflammation, pathogen-associated molecular patterns (PAMPs) are perceived by tissue macrophages or mast cells, activating the secretion of pro-inflammatory cytokines, chemokines, vasoactive amines, and eicosanoids, thus enhancing the immune response (Medzhitov 2008; Kono *et al.,* 2008; Nathan 2002). These pro-inflammatory mediators are known to expand vascular permeability, directing to a massive influx of plasma containing antibodies and other soluble components (Headland *et al.,* 2015). In addition, the injury site has been shown to discharge a variety of signaling molecules, including chemokines, cytokines, eicosanoids, and adhesion molecules, leading to the enlistment of neutrophils and monocytes (Medzhitov 2008; Wright *et al.,* 2010). As the inflammatory response progresses, monocytes and lymphocytes gather in the inflammation sites to neutralize harmful substances. Eventually, inflammatory cells experience apoptosis and cleared by macrophages. Therefore, the inflammatory process involves different types of cells and mediators, which can regulate cell chemotaxis, migration, and proliferation in a highly-programmed manner.

2.7.1 Inflammation and cancer

Course of the past decades, it has become evident that inflammation plays a censorious role in promoting cancer, in particular the tumorigenesis, a process of tumor development. In addition to cancer cells, various types of immune cells are frequently found within tumors. Fascinatingly, an inflammatory microenvironment is also more regularly found as a crucial part of all tumors (Mantovani *et al.,* 2008; de Visser *et al.,* 2006). It has been displayed that the inflammatory response altered by infection is also associated with rise of cancer risk (de Martel and Franceschi, 2009). New studies have revealed that lung tumorigenesis caused by tobacco smoke is indeed initiated by JNK1-mediated chronic inflammation, implying that the pathways of both tumorigenesis and inflammation are intently linked (Takahashi *et al.,* 2010). It is adhered that apart from the tumor-promoting inflammatory response, an alive anti-tumor immunity is also present in most tumor

microenvironments. Thus, the progression of tumorigenesis is based between tumour-promoting inflammation and anti-tumour immunity. Evidently, in entrenched tumour, anti-tumour is tremendously influenced by tumour-promoting inflammation (Smyth *et al.,* 2006; Lin and Karin, 2007). Many studies have shown that inflammation microenvironment not only involved in promoting of the cancer development but also expands mutation rates, maybe by producing reactive oxygen species and nitrogen intermediates which may cause DNA damage and genomic fluctuation (Grivennikov *et al.,* 2010). Genomic instability by inflammation is mainly observed by the enzyme activation-induced cytidine deaminase (AID). Overexpression of the enzyme leads to trigger the genomic instability in various cancer conditions, induced by the inflammatory cytokines (Okazaki *et al.,* 2007). Excepting genomic instability, other environmental factors like carcinogens, tobacco smoke, infectious microbes and inhaled pollutants can play a vital role in inflammation inducing cancers (Aggarwal *et al.,* 2009). Thus, the relation between inflammation and cancer does not function in one path as various studies proved that DNA damage-induced necrotic cell death can lead to the inflammation (Maeda *et al.,* 2005; Sakurai *et al.,* 2008).

Finally, tumour-associated inflammation can be initiated by the latest cancer therapy like radiotherapy and chemotherapy. These therapies are conjugated with a notable amount of necrotic death of both cancer cells and normal cells, which in turn activates the inflammatory response (Zong and Thompson, 2006). Therefore, based on the literature survey, it can be assumed that inflammatory response is merged with cancer biology from tumorigenesis to therapeutic intercessions. Inflammation and cancer are interlinked by two pathways: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is channelized by genetic events that cause formation of tumour. (a) Triggering various types of oncogene through mutation, (b) chromosomal dislocation or augmentation, (c) halting of tumour- suppressor genes. Cells which come under this manner produces inflammatory negotiators, which intern forms the inflammatory microenvironment in tumours for those which are not having inflammation condition (e.g., breast tumours). But, in the extrinsic pathway inflammation or infectious conditions increase the risk of developing cancer at assured anatomical sites (e.g., colon, prostate and pancreas). This pathway intersect, resulting in the activation of transcription factors, Nuclear Factor-B (NF-kB), Signal Transducer and Activation of Transcription 3 (STAT3) and Hypoxia-Inducible Factor-1 (HIF-1) in tumour cells. These transcription factors monitor the production of inflammatory negotiators, as well as cytokines, chemokines and Cyclooxygenase 2 (COX-2) which

induce production of prostaglandins). These factors engage and trigger various leukocytes, mainly myelomonocytic lineage. The cytokines activate the transcription factors in inflammatory cells, stromal cells and tumor cells; this increased in the production of inflammatory negotiators and creates the cancer related inflammatory microenvironment. Thus cancer-related inflammation has various tumour-promoting consequences.

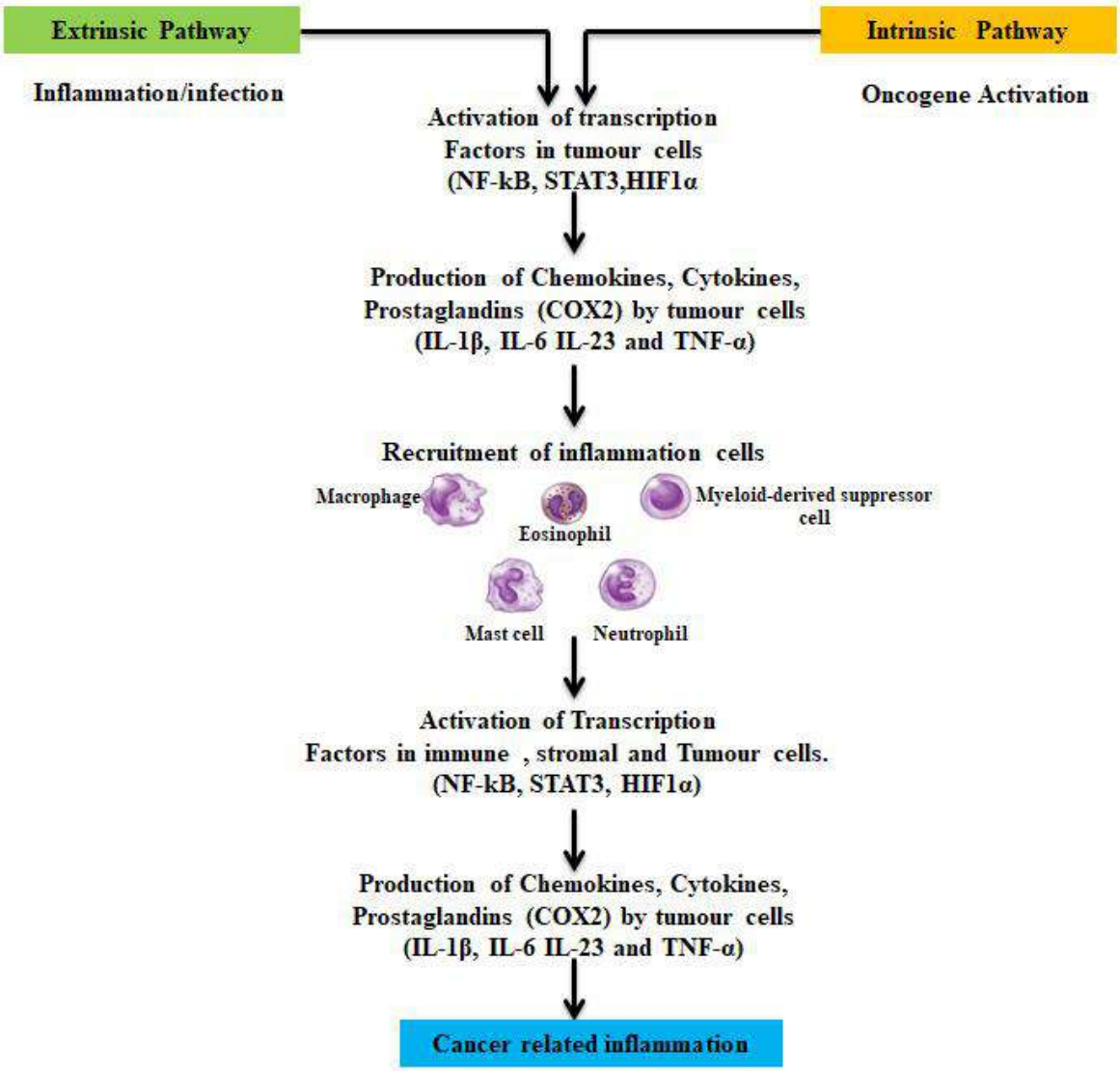


Fig.5: Inflammation and cancer related pathways*: Inflammation and cancer are connected by intrinsic and extrinsic pathway. In intrinsic pathway, genetic alterations prompt inflammation and cancer development. Whereas in extrinsic pathway, it is directed by the inflammatory or infectious conditions that expands tumour risk. Thus, activates transcription factors and inflammatory negotiators which in turn create cancer- related inflammation and tumour development. The graphical form of pathway was adopted from Digifico E et al., 2021.*

2.8. Tumour Immunology

Cancer is a second leading cause of death worldwide behind cardiovascular disease, accounting for nearly 10 million deaths in the year 2020, or nearly one in six deaths. The leading causes of cancer deaths are lung, liver, and gastric cancer in males and breast, lung, and colorectal cancer in females (López-Gómez *et al.,* 2013; Amri, 2014). The most important issue in cancer pathology is the distinction between benign and malignant tumors. If the cells are non-cancerous, the tumor is concluded as benign. It won't invade nearby tissues or spread to other areas of the body. Also, some types of benign tumors such as intestinal polyps are considered as precancerous and are removed immediately to prevent them becoming malignant. Benign tumors usually don't reoccur once removed, but if they do it is usually in the same place such as Moles, Fibroid cyst in breast or uterus, polyps of colon. Malignant means that the tumor is made of cancer cells and it can invade nearby tissues. Some cancer cells can move into the blood stream or lymph nodes, where they can spread to other tissues within the body this is called metastasis. Cancer can occur anywhere in the body including the breast, lungs, intestines, reproductive organs, blood, or skin (Kindt *et al.,* 2007). Despite the fact that the majority of cancers are caused by a combination of genetic and environmental risk factors and life styles (Brennan, Offiah, McSherry, & Hopkins, 2009), among environmental factors, Diet plays a vital role. It has been reported that some dietary components are the reason for reducing and inducing the risk of cancer (Garcia-Closas M *et al.,* 2014). There are certain, distinct means by which normal cells transform into cancerous ones. Oncological research in the latter half of the century has indicated that almost all cancerous cells display a relatively few numbers of acquired molecular, biochemical, and cellular features that result from alteration of key pathways (Kreeger and Lauffenburger, 2010). This may be a strong generalization considering that there are over 100 unique types of cancer, not including further subtypes of malignancies that have been identified (Hanahan and Weinberg, 2000). However, it is not such a stretch when realizing that the field of cellular biology emphasizes the similarity between all types of living cells. Mammalian cells, for example, are all alike in their mechanistic regulation of normal cellular processes, such as division, differentiation, and programmed cell death (apoptosis). Therefore, it is unsurprising that there are certain rules that govern the transformation of normal human cells to cancerous ones (Hanahan and Weinberg, 2000). The universal nature by which cancer occurs is further evidenced by the ongoing identification of specific mutation sites on the human genome that are found in

many forms of cancer. Researchers are also attempting to classify the genes crucial to carcinogenesis into specific classes by studying cancerous phenotypes in experimental models (Hanahan and Weinberg, 2000).

In 2000, Hanahan and Weinberg proposed six hallmarks of cancer that all together form the fundamental principle of this malignant transformation. Since tumor formation is a multistep process, normal cells evolve progressively to the neoplastic stage and along their way they acquire particular capacities that enable them to become tumorigenic. These basic hallmark capabilities, distinct and supplementary, are: [1] Sustaining proliferative signaling; [2] Evading growth suppressors; [3] Activating invasion and metastasis; [4] enabling replicative immortality; [5] Inducing angiogenesis and [6] Resisting cell death. Over the last decade, remarkable progress was made in the field of cancer research which led to a better understanding of these hallmark capabilities (Hanahan & Weinberg 2011). This also led to modifications and, ultimately, expansions of the original concept (Fig.6). Underlying theses hallmarks are genome instability and inflammation which contribute to multiple hallmark functions (Hanahan and Weinberg 2000; 2011). In 2011, more than a decade after the publication of the original cancer hallmarks paper, the next generation of cancer hallmarks were published, and two emerging hallmarks were proposed: reprogramming of energy metabolism and evading immune destruction (Colotta *et al.*, 2009).

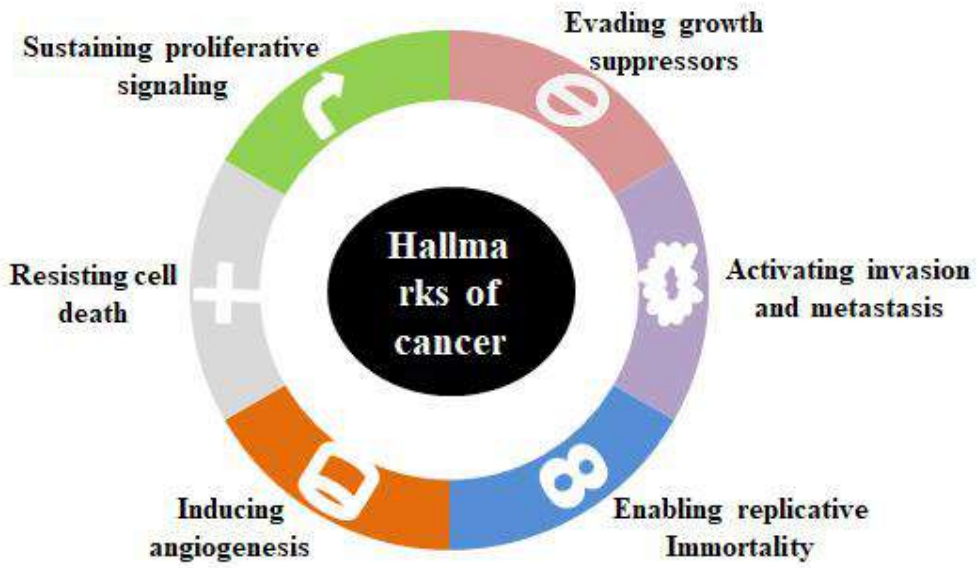


Fig.6: Hallmarks of cancer: *All cancer does show the same set of functional capabilities during their development, even through various mechanistic strategies.*

2.9. New dimensions: Expanding the frontiers of cancer biology

The next generation of cancer hallmark traits recognized the 'tumour microenvironment', or the cellular environment in which the tumour exists, as contributing to the acquisition of hallmark traits, adding another dimension of complexity to cancer progression (Colotta *et al.*,2009). The intent was to provide a conceptual scaffold that would make it possible to rationalize the complex phenotypes of diverse human tumor types and variants in terms of a common set of underlying cellular parameters. Initially they envisaged the complementary involvement of six distinct hallmark capabilities and later expanded this number to eight (Hanahan and Weinberg 2011). At this time, the eight hallmarks include the learned skills for maintaining proliferative signalling, dodging growth inhibitors, enabling replicative immortality, resisting cell death, inducing/accessing vasculature, activating invasion and metastasis, reprogramming cellular metabolism, and avoiding immune destruction. In the most recent elaboration of this concept, deregulating cellular metabolism and avoiding immune destruction were segregated as "emerging hallmarks". As such, the end result of cellular differentiation is in most cases antiproliferative and constitutes a clear barrier to the continuing proliferation that is necessary for neoplasia. There is increasing evidence that unlocking the normally restricted capability for phenotypic plasticity in order to evade or escape from the state of terminal differentiation is a critical component of cancer pathogenesis (Hanahan and Weinberg 2011; Feng *et al.,* 2021). There is, in addition, a case to be made for another apparently independent mode of genome reprogramming that involves purely epigenetically regulated changes in gene expression, one that might be termed "non-mutational epigenetic reprogramming". Indeed, the proposition of mutation-less cancer evolution and purely epigenetic programming of hallmark cancer phenotypes was raised almost a decade ago and is increasingly discussed (Baylin *et al.*,2016; Huang 2012, Darwiche 2020; Feng *et al.,* 2021; Nam *et al.,* 2021). For cancer, the evidence is increasingly compelling that polymorphic variability in the microbiomes between individuals in a population can have a profound impact on cancer phenotypes (Dzutsev *et al.,* 2017; Helmink *et al.,* 2019). Cellular senescence has long been viewed as a protective mechanism against neoplasia, whereby cancerous cells are induced to undergo senescence (Lee *et al.,* 2019). Senescence can be induced in cells by a variety of conditions, including microenvironmental stresses such as nutrient deprivation and DNA damage, as well as damage to organelles and cellular infrastructure, and imbalances in cellular signaling networks, all of which have been

associated with the observed increase in the abundance of senescent cells in various organs during aging (Birch *et al.*, 2020; Faget *et al.*, 2019; Gorgoulis *et al.*, 2019; He *et al.*, 2017; Kowald *et al.*, 2020). While the eight hallmarks of cancer and their two enabling characteristics have proved of enduring heuristic value in the conceptualization of cancer, the considerations presented above suggest that there may be new facets of some generality and hence of relevance to more fully understanding the complexities, mechanisms, and manifestations of the disease. By applying the metric of discernable if not complete independence from the 10 core attributes, it is arguable that these four parameters may well pursuant to further validation and generalization beyond the case studies presented become integrated into the hallmarks of cancer schematic (Fig.7)

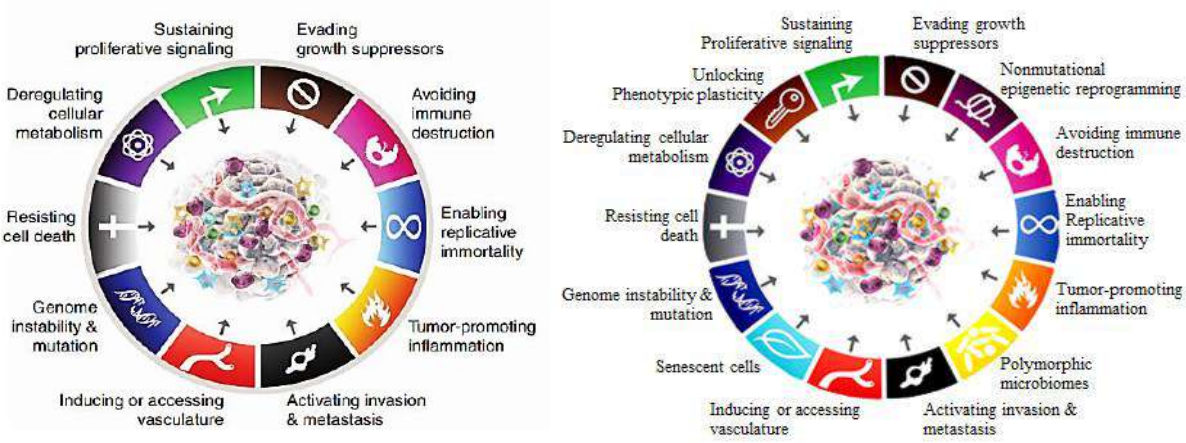


Fig.7: Hanahan and Weinberg disclosed the additional hallmarks of cancer: *New generation involve four more functional capabilities with standard six acquired capabilities. As refined by Douglas Hanahan and Robert Weinberg in 2011 and the graphical hallmarks of cancer was adapted from the Hanahan and Weinberg.*

2.9.1 Tumour Environment:

The hallmarks disputed above all capabilities surrounded by the tumour environment. This microenvironment involves pretendedly normal cells and cancer cells filled with abundant messenger molecules. Tumour promoting inflammation was caused by immune cells. Tumour environment includes growth factors that stimulate sustained proliferative signaling, evading growth suppressors and resisting cell death. ROS released contributes to genomic instability and mutation. Angiogenesis and activate invasion and metastasis was instigated by the release of proangiogenic factors and matrix-modifying enzymes.

Eventually the hypoxic conditions contribute to deregulating cellular energetics. This highly complex microenvironment can be considered as an ecological system, where by a process of somatic evolution the most fit cancer cells are selected (Fig.8) (Merlo *et al.*, 2006).

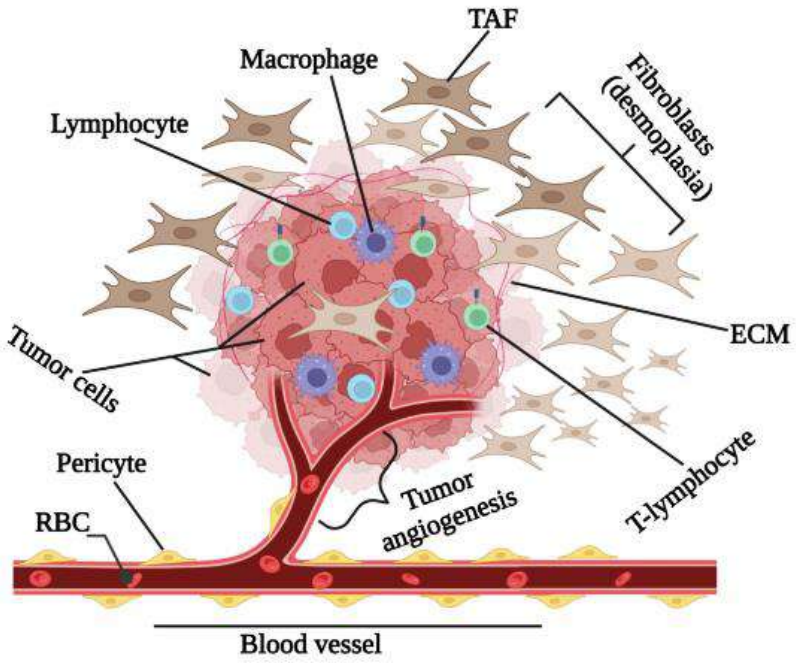


Fig.8: The tumor microenvironment contains cellular and non-cellular fractions*: It involves lymphocytes (T-cells, B-cells, NK cells), Tumour-associated fibroblast (TAF) and Extra cellular matric (ECM)The graphical form of tumour environment was adopted from Lauren B. Birkeness et al.,2022.*

2.10. Immuno-oncology- targeting immunosuppression

During cancer pathogenesis several components of the innate immunity are activated in efforts to minimize cancer mediated inflammation. This process also initiates adaptive immune responses for targeting the cancer via more specific immune mechanisms (Chen *et al.*, 2013; Dunn *et al.*, 2006). The alterations in the cancer cells, resulting in expression of tumor associated antigens which can be recognized by complement proteins and thereby predisposing the cancer cells to complement-mediated death (Pio *et al.*, 2014). While complement activation promotes mechanisms that aid in eradicating cancer cells, the presence of soluble and membrane-bound complement regulatory proteins (CRPs) that inhibit various steps in the multiple complement signaling pathways help protect cancer cells against complement-mediated injury. Inhibition of the complement cascade also hinders some of the effects of adaptive immune responses because complement proteins have also been reported to play a role in B and T cell activation/survival. Therefore, CRP-mediated complement inhibition may also result in insufficient activation and expansion of

B and T cells that can specifically target the cancer cells (Kwan *et al.,* 2012). Cell surface marker is MHC class I whose expression becomes altered or reduced in cancer cells that leads to activation of NK cells via activates their receptors such as NKG2D that binds to glycoproteins present on the tumour (Waldhauer *et al.,* 2008). NK-induced programmed cell death (apoptosis) can occur by several mechanisms such as tumor-necrosis factor-alpha- (TNF-α) dependent release of cytoplasmic granules (perforin and granzymes) that form pores in cell membranes; by antibody dependent complement cytotoxicity due to the presence antibody receptor (CD16) on NK cell surface; and by the release of cytokines such as IFN-γ which mediates activation and maturation of antigen-presenting cells such as dendritic cells. Cells are resistant to NK-mediated lysis due to normal expression of MHC class I that activates inhibitor receptors on NK cells which prevents NK cell induced apoptosis (Waldhauer *et al.,*2008). Additionally, some types of tumor cells have been known to prevent the antigen-presenting capabilities of dendritic cells, thereby inhibiting dendritic cell-dependent T cell activation (Palucka *et al.,* 2012). Interestingly, NKT cells' interaction with dendritic cells via CD40 ligand CD40 signaling, respectively, enables activation and secretion of IL-12. IL-12 can activate NK or CD8+ T cells for tumor lysis and suppression of cancer progression (Terabe *et al.,* 2008).

Similar to innate immunity, the adaptive immunity is comprised of several components that can either eradicate cancer cells or promote their proliferation (Chen *et al.,* 2013). This form of immune response is capable of targeting antigens specific to the cancer cells by exploiting the effector functions of antibodies, T cells, B cells, and antigen-presenting cells (Warrington *et al.,* 2011). The central dogma behind the cancer immunity concept involves the formation of neoantigens that are formed due to tumorigenesis/oncogenesis, which are phagocytosed by antigen-presenting cells (APCs) or pinocytosed by dendritic cells for antigen processing (Chen *et al.,* 2013). MHC class II molecules present exogenous peptides, whereas MHC class I molecules present endogenous peptides derived from cancer antigens (Warrington *et al.,* 2011). The processed tumor-associated antigens are then presented by MHC class II and MHC class I molecules on the APC to the antigen-specific T cell receptor on CD4+ T cells or CD8+ T cells, respectively (Chen *et al.,* 2013). Activation of CD4+ T cells by MHC class II on APC primes them for subsequent exposures to that particular antigenic peptide/MHC class II complex, thus forming memory T cells (Chen *et al.,* 2013; Harris *et al.,* 2013). IL-2 is also produced when T cells are activated and further promotes T cell proliferation (Minami *et*

al., 1993). While it is known that B cells can act as APCs to native T cells, activated CD4+ T cells (also known as helper T cells) can also interact with native B cells to promote their activation (Janeway *et al.*, 2001). Similarly, activation of CD8+ T cells occurs by interaction of antigen-specific T cell receptors with MHC class I/tumor antigen complexes leading to induction of cytolytic CD8+ T cell-mediated lysis of cancer cells (Chen *et al.*, 2013). Immune responses in oncogenic environment can also be suppressed by Tregs (Nishikawa *et al.*, 2010). Tumors/cancer cells can secrete chemokines like CCL22 that recruit Tregs to the oncogenic site and help suppress effector functions of other T cells that may be necessary to eradicate cancer cells (Nishikawa *et al.*, 2010; Gobert *et al.*, 2009). Collectively, the role of these innate and adaptive immune responses in oncogenesis serves to be the underlying basis for immune surveillance and cancer regression (Fig.9) (Schreiber *et al.*, 2011; Dunn *et al.*, 2006).

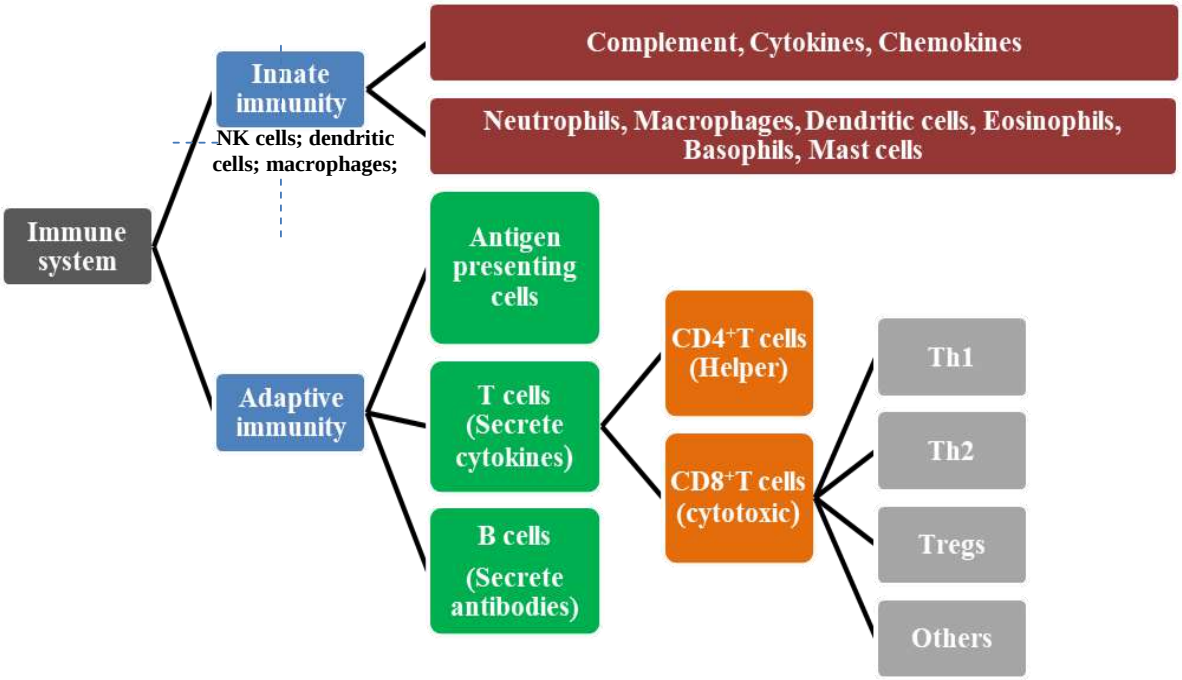


Fig.9: Overview of the immune system: *innate and adaptive immunity. An evolutionary bridge between both forms of immunity is observed due to the presence of T cells, NK cells, dendritic cells, macrophages, and complement proteins.*

2.10.1. Intending angiogenesis

The establishment and maintenance of a vascular supply is an absolute requirement for growth of normal as well as neoplastic tissue as a result of two main processes, vasculogenesis and angiogenesis, and that such neovascularization involves angiogenic growth factors. Vasculogenesis is the de novo *in-situ* differentiation of endothelial cells

(ECs) from mesodermal precursors in the embryo by association of endothelial progenitor cells (EPCs) or angioblasts, and their subsequent reorganization into a primary capillary plexus (Folkman 1971; Risau 1995).

Angiogenesis is the dynamic process through which new blood vessels are formed from pre-existing ones (Aydogan 2016). There are three types of angiogenesis, namely sprouting angiogenesis, which is the most common, intussusceptions or splitting angiogenesis, where a new wall grows inside an existing vessel, eventually dividing into two vessels, and looping angiogenesis, where vessel loops are mechanically dragged into the tissue (Aydogan 2016; Mirabelli 2019). The process of angiogenesis is strictly controlled during physiological processes, such as wound healing, tissue growth, and the female reproductive cycle. Therefore, in adult tissues, angiogenesis is mostly uncommon, and the endothelium is generally stable. In the resting vasculature, there are approximately 0.5% endothelial cells exhibiting mitotic activity and the angiogenic process requires the transition of endothelial cells from the quiescent state to an activated one (Duran *et al.,* 2017). The mechanism of angiogenesis involves several stages and a set of growth factors, substrate molecules, and multiple cell types. The factors involved in angiogenesis can be categorized into environmental, mechanical, and chemical factors. Specifically, environmental factors include hypoxia or increased amounts of nitric oxide produced by endothelial cells, which will further stimulate the release of angiogenic triggers. Additionally, mechanical factors, namely hemodynamic and shear stress have shown to stimulate the development of collateral vessel networks and maintain the patency of newly formed blood vessels. The mechanism of angiogenesis is mostly modulated by chemical stimuli, such as hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), angiopoietins (Ang), and vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF), insulin-like growth factor (IGF), transforming growth factor-beta (TGF-β), matrix metalloproteinase (MMP), and tumor necrosis factor (TNF) (Rust*et al.,* 2019; Kareva 2018; Moraga *et al.,* 2017; Taslimi *et al.,* 2018; Shoeibi *et al.,* 2018).

VEGFs are of disulfide-linked soluble secretory glycoproteins found in higher eukaryotes. By binding to specific soluble, membrane-bound vascular endothelial growth factor receptors (VEGFR) or co-receptors, they modulate a series of responses, such as cell proliferation and migration, metabolic homeostasis, and tubulogenesis (Lally *et al.,* 2016). The VEGF family comprises a series of structurally and functionally related proteins,

namely VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF). VEGFs exhibit their function by binding to three structurally related VEGFRs, namely VEGFR1, VEGFR2, and VEGFR3. VEGF binding to VEGFRs promotes the tyrosine kinase enzyme activation in the intracellular receptor domain and subsequently leads to the phosphorylation of the tyrosine residues, thus activating specific intracellular signaling pathways. VEGFR1 is expressed in monocytes, macrophages, hematopoietic stem cells, vascular smooth cells, and leukemic cells, VEGFR2, in vascular endothelial cells, endothelial progenitor cells, and megakaryocytes, and VEGF3, in lymphoid endothelial cells. VEGFs can also interact with other proteins, including neuropilins, integrins, cadherins, or heparan sulphate proteoglycans, enhancing VEGFR1 and VEGFR2 functions by guiding the migration of endothelial cells in angiogenesis (Yang *et al.,* 2018; Dehghani *et al.,* 2018; Melincovici *et al.,* 2018; Failla *et al.,* 2018). FGFs are secreted by stem cells and damaged cardiac myocytes and vascular endothelial cells. The angiogenic effects of the FGF family mostly rely on the activity of FGF-1 and FGF-2, which are key factors in wound healing, by stimulating the proliferation of endothelial cells and fibroblasts that will subsequently produce the granulation tissue necessary for the healing process (Shoeibi *et al.,* 2018; Inampudi et al., 2018; Henning 2016). Platelet-derived growth factors (PDGFs) and their receptors (PDGFRs) have served as prototypes for growth factor and receptor tyrosine kinase function (Saplacan *et al.,* 2017). PDGF signaling is implicated in a range of diseases. Autocrine activation of PDGF signaling pathways is involved in certain gliomas, sarcomas, and leukemias. Paracrine PDGF signaling is commonly observed in epithelial cancers, where it triggers stromal recruitment and may be involved in epithelial mesenchymal transition, thereby affecting tumor growth, angiogenesis, invasion, and metastasis (Medamana *et al.,* 2017; Lee *et al.,* 2018; Thiagarajan *et al.,* 2017). The Angiopoietin system acts as a vascular specific ligand/receptor system to control endothelial cell survival and vascular maturation. The Angiopoietin family includes four ligands (Angiopoietin-1, Angiopoietin-2 and Angiopoietin-3/4) and two corresponding tyrosine kinase receptors (Tie1 and Tie2). Ang-1 and Ang-2 are specific ligands of Tie2 binding the receptor with similar affinity and Tie2 activates the site of angiogenesis (Isidori *et al.,* 2016; Gnudi, 2016; Rak *et al.,* 2018; Parikh 2017). The adaptive response is regulated by hypoxia-inducible factors (HIFs) that are the key transcription factors that accumulate in response to low cellular oxygen levels. They bind to hypoxic-response elements (HREs) in the promoters or enhancers of numerous target genes that regulate cell metabolism and survival (Semenza, 2016). These

transcriptional induction of three transcription factors called HIF-1α, HIF-2α, and HIF-3α which dimerize with constitutively expressed beta chains that together form the HIF-1, -2 and -3 transcription factors. While these factors are normally expressed, the activity of HIF can simultaneously induce the expression of a spectrum of angiogenic factors as a response to hypoxia (Hashimoto *et al.,* 2015). Notably HIF-1α stimulates the expressions of all VEGF isoforms which in turn stimulates the expressions of other proangiogenic factors. Thus, the normal level of oxygen in cells and tissues, HIF-1α is degraded and inhibiting the expressions of VEGF gene and proteins. At last halts the cascade of proangiogenic factors (Fig.10) (Zimna *et al.,* 2015). Angiogenesis inhibitors are the chemical signals inhibit the formation of new blood vessels or supporting the removal of existing vessels (Angiogenesis). Some of the angiogenesis inhibitors are angiostatin, endostatin, platelet factor 4 (PF4), thrombospondin-1 and 2, interleukin-12, tumstatin, osteopontin, anti-angiogenic metargidin peptide, and endoglin silencing (Kareva *et al.,* 2018; Mousa *et al.,* 2017; Li *et al.,* 2018).

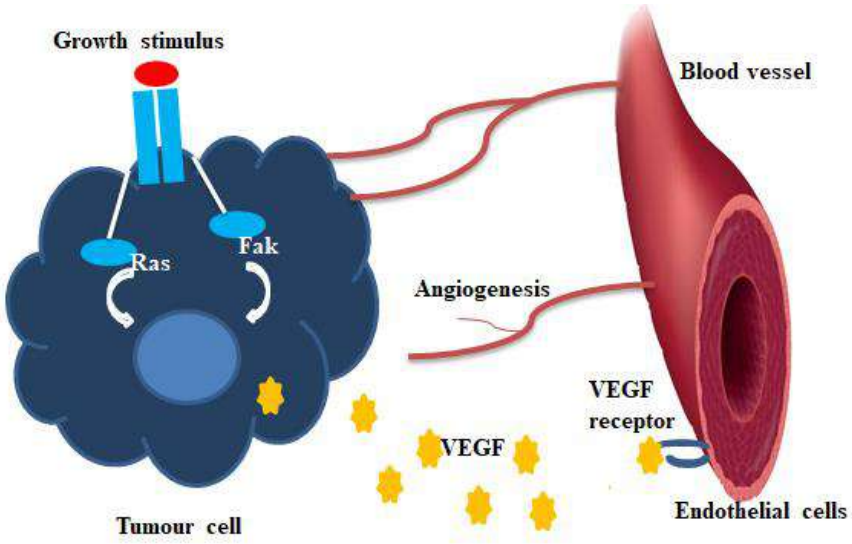


Fig.10: Schematic representation of Angiogenesis: *Tumour cells persuade a multiplex cascade of angiogenic signaling pathway and activates the downstream regulators which leads to angiogenesis.*

2.10.2. Tumor Angiogenesis

The nutrients, oxygen and other metabolic supplements are provided by a complex tumour microenvironment network. In the absence of angiogenesis, tumours are restricted to the smaller size and enters to latent stage, the anti-angiogenic which prevents the new vasculature required for the growth of tumours (Napione *et al.,* 2017; Bagley 2016). Tumor

angiogenesis is achieved through a series of sequential steps that further lead to cancer development (Sirover *et al.,* 2017). Once the tumour reached the specific size then cells undergo hypoxia. Hypoxia is the decreased oxygen level with leads to tumour angiogenesis (De Palma *et al.,* 2017; Marmé 2018; Najafi *et al.,* 2019). During hypoxic condition tumour cells secretes angiogenic molecules by utilizing those molecules tumour cells actuate the formation of new vessels. The most important proangiogenic factor is VEGF, which promotes angiogenesis within tumors through its highly mitogenic effects. After the VEGF-A level reaches a maximum concentration level at the leading edge of the vascular sprout; it binds to VEGFR2 and induces the migration of endothelial tip cells. Once activated, VEGFRs activates a series of downstream pathways involved in cell proliferation and survival, cytoskeleton rearrangement, and vascular permeability (Ricciuti *et al.,* 2019; Feng *et al.,* 2017). Furthermore, proliferate by the activating the extracellular kinases and MAP kinase signal transduction pathways. It can induce proteins that breakdown the basement membrane to allow endothelial cells to migrate and invade. These proteins include MMPs, urokinase type plasminogen activator (uPA) and its receptor uPAR as well as tissue type plasminogen activator. It makes vessels more permeable allowing molecules and fluid to leak out. MMPs secreted into the extracellular space it degrades the extracellular matrix to allow proangiogenic factors to reach the vasculature with the extracellular matrix degraded (Loizzi *et al.,* 2017; Fujita *et al.,* 2017). Proangiogenic factors including VEGF have receptors on the endothelial cells of blood vessels surrounding the tumour which stimulate angiogenic signal in the vessel. These allow the growth of new blood vessels into the tumour, this new blood vessels facilitate to new tumour growth. To overcome this many successful clinical trials are performed. Consequently, these features increase the hypoxia in the tumor microenvironment, thus promoting the formation of novel vessels and the induction of metastasis Bevacizumab is an antibody that binds VEGF and prevents to its receptors (Keith *et al.,* 2015; Stockmann *et al.,* 2014; Fukumura *et al.,* 2018). Many anti-angiogenic factors have been identified to reduce the growth of tumour cells.

2.11. Plant lectin with anti-cancer properties

Leukemia, sarcoma, hepatoma, and breast cancer have all been successfully treated with various plant lectins in the past as anti-tumor agents or anti-neoplastic medications (Li *et al*., 2008). Lectins have an anticancer effect because they have the ability to slow tumour growth by inducing apoptosis and autophagy, which reduces telomerase activity, inhibits

angiogenesis, and is deadly to cancer cells (Yau *et al.,* 2015); (Hamid *et al.,* 2013). Additionally, by identifying a modified glycan structure that is predominantly expressed in tumour cells, lectins are used to distinguish between benign and malignant cells (Haseenabeevi *et al.,* 1991).

Table 3. List of some lectins showing anticancer effects on Cancer cell lines.

Lectin and Sources	Abbreviations	Anticancer Cancer Activity	Cell lines	Reference
Concavalina A *(Canavaliaensiformis)*	Con A	Colon cancer	*Lovo,* HCT-15, SW837	[Kiss *et al.*,1996]
Jacalin *(Artocarpusheterophyllus)*	Jacalin	Colon cancer	A431, HT29 cells	[Vohra *et al.*,2015]
Mistletoe lectins *(Viscum album)*	ML	Breast cancer	HeLa-S3, MFM-223	[Knopfl-Sidler *et al.*,2005]
peanut agglutinin *(Arachishypogaea)*	PNA	Breast, colon cancer	HeLa cells	[Mukhopahyay *et al.*,2014]
Phytohemagglutinin-L *(Phaseolus vulgaris)*	PHA-L	Breast cancer, Hepatoma	MCF-7, HepG2	[Panda *et al.*,2014]
Pisum Sativum Agglutinin *(Pisum sativum)*	PSA	Lung Cancer	A594 cells	[Van Dammee*t al.*,1987]
Soybean agglutinin (*Glycine max*)	SBA	Breast cancer, Hepatoma	MCF7 and HepG2 cells.	[Lin *et al.*,2008]
wheat germ agglutinin (*Triticumvulgare*)	WGA	Pancreatic, Breast cancer	AR42J, MCF-7, T47D, HBL 100, BT 20	[Toribio*et al.*,1985; Mikkat*et al.*,2001]
Viciafaba agglutinin *(Viciafaba)*	VFA	Colon cancer	LS174T, SW1222 and HT29	[Jordison*et al.*,1999]

In addition to this, lectins work as a carrier to precisely target the tumor cells after being conjugated with chemotherapy drugs. For instance, Neutsch *et al* studies showed that functionalizing urothelial cells with WGA improved the nano carrier's binding affinity to

those cells. Additionally, this micro particle had improved anti-neoplastic efficacy (Neutsch *et al.,* 2013). Similar to this, Wistar rat intestinal mucosa showed increased drug delivery and absorption when exposed to WGA-tagged nanoparticles containing thymopentin (Yin *et al.,* 2006). When encapsulated in alginate microbeads, lectins obtained from Pisum sativum seeds demonstrated improved drug delivery to hepatocellular cancer (El-Aassar *et al.,* 2014). Con A related microsphere also revealed a rise in the drug's adhesion to and release from stimulated gastrointestinal fluids. (Bakowsky *et al.,* 2008; Jain *et al.,* 2014). Targeting human prostate cancer, Bauhinia purpurea agglutinin-bound liposomes were demonstrated to inhibit cell proliferation after specifically attaching to DU145 cells in mice (Ikemoto *et al.,* 2016). In spite of all the differences in the properties of these lectins, most of them exhibit a common biological function *i.e.,* they have antiviral, anti-insect, antifungal, anti-parasitic, immunomodulatory and antitumor activities.

2.12. Garlic as potential therapeutic dietary components with lectins:

Allium vegetables, which include garlic, onions, shallots, leeks, scallions and chives, contain high levels of flavanols and organosulfur compounds. From *in-vivo* and *in-vitro* studies, allium vegetables have been shown to have an anticarcinogenic potential of bioactive compounds, *e.g.*, allylsulphides. The antibacterial effect, which is attributed to garlic's thiosulfinate concentration, has recently been quantified (Wang *et al.*, 2012; Rafieian-Kopaei *et al.*, 2013). Studies have reported that garlic extract could inhibit tumour growth *in-vitro*. More general anticarcinogenic effects may derive from the organosulfur compounds in garlic that are responsible for its odour and flavor. Previous animal studies have also shown that a compound extracted from garlic that is diallyl sulfide, has strong cancer inhibitory properties. Because allium vegetables, especially garlic, have been shown to have anticarcinogenic potential in various literature reports, it has been hypothesized that lectin extracted from allium vegetable (garlic) is protective against cancer development.

2.12.1 Garlic and their pharmacological properties:

Garlic *(Allium sativum*) belongs to Plantae kingdom, Asparagales order, Amaryllidaceae family, Allioideae subfamily and Allium genus. Historically, garlic has possessed medicinal and dietary importance in different cultures for more than 4000 years. In addition, there is evidence that proves garlic has been consumed for various purposes

over the centuries. It is one of the most widely grown vegetable crops in Asia including China and is also known for its therapeutic applications and has been used as a flavoring agent since ancient times (Butt *et al.,* 2009). Garlic (*Allium sativum)* involves numerous therapeutic properties such as, Immunomodulators, hypolipidemic, antioxidant, antidiabetic, antithrombotic, antihypertensive, antimutagenic, antimicrobial and anticarcinogenic effects (Iciek *et al.,* 2009). Garlic (*Allium sativam)* is a traditional herbal food in cancer prevention and it is mostly recommended vegetable in the pyramid by the National Cancer Institute (Wang *et al.,* 2012; Rafi eian-Kopaei *et al.,* 2013).

Allium sativum contains many chemical ingredients, including 17 amino acids, more than 33 organosulfur components, 8 minerals (calcium, potassium, magnesium, germanium, selenium, copper, zinc, and iron), vitamins (A, B1, B2, B3 B6, B12, C, D, E), and some enzymes (allinase) (Rizwani *et al.,* 2011). Allicin as the major biologically active component of fresh garlic is a candidate in antitumor survey. Several studies have been conducted to determine the chemopreventive and anticarcinogenic role of garlic components. The *in-vitro* and *in-vivo* studies of Weisberger and Pensky, showed garlic thiosulfinate inhibit the tumor cell growth. Allicin (diallylthiosulfinate) which is quickly created from alliin using alliinase enzyme in freshly crushed garlic, is considered a very reactive thiosulfinate and the main ingredient responsible for biological functions.

2.12.2. Components of garlic show pharmacological properties:

Phytochemical ingredients and Organosulfur components originated from garlic such as allicin, ajoene, DADS, DATS, S-allyl cysteine (SAC) and S-allylmercaptocysteine (SAMC) are known for their pharmacological activities. Allicin (diallylthiosulfinate) present 2.3-7.7mg/g and shows antimalarial activity (Coppi *et al.,* 2006; Feng *et al.,* 2012), antiparasitic effect (Ankri et al., 1999; Lima *et al.,* 2011; Corral-Caridad *et al.,* 2012), anticancer activity and apoptosis induction in cancer cells (Lee *et al.,* 2013; Miron *et al.,* 2008; Cha *et al.,* 2012) antibacterial, antifungal and antiviral activity (Ankri *et al.,*1999). Alliin present in the amount of 5.3 to 30 mg/g is the highest organosulfur components present in whole garlic. Ajoene present 0.12-0.47mg/g and displays the most effective virucidal compound found in garlic (Weber *et al.,*1992) antifungal activity (Yoshida et al., 1987), reduce tumor size using apoptosis induction (Tilli *et al.,* 2003). DADS (diallyl disulfide) present at 0.06-0.89mg/g and have inhibit *in-vitro* proliferation of human A549 lung tumor cells (Sakamoto *et al.,*1997), inhibit the growth of human colon tumor cells

(Sundaram *et al.*,1996), apoptosis induction in human leukemia HL-60 cells (Kwon *et al.*,2002), antifungal activity (Yousuf *et al.*,2010; Avato *et al.*,2000). DATS (diallyltrisulfide) present 0.01-0.39mg/g and displays antiparasitic activity (Lun *et al.*,1994), inhibit *in-vitro* proliferation of human A549 lung tumour cells (Sakamoto *et al.*,1997), apoptosis induction in human prostate cancer cells (Xiao *et al.*,2004), antifungal activity (Avato *et al.*,2000). SAC (S-allyl cysteine) present at 0.36-0.60mg/g and exhibits anticancer activity (Yun *et al.*, 2014). SAMC (S-allylmercaptocysteine) shows antiproliferative effect on colon cancer cells, apoptosis induction by enhancement in caspase3 like activity (Shirin *et al.*, 2001), apoptosis induction in erythroleukemia cell lines (Sigounas *et al.*,1997). All these phytochemicals of garlic induce apoptosis via mitochondrial pathway in numerous cancer cells both *in-vitro* and *in-vivo.*

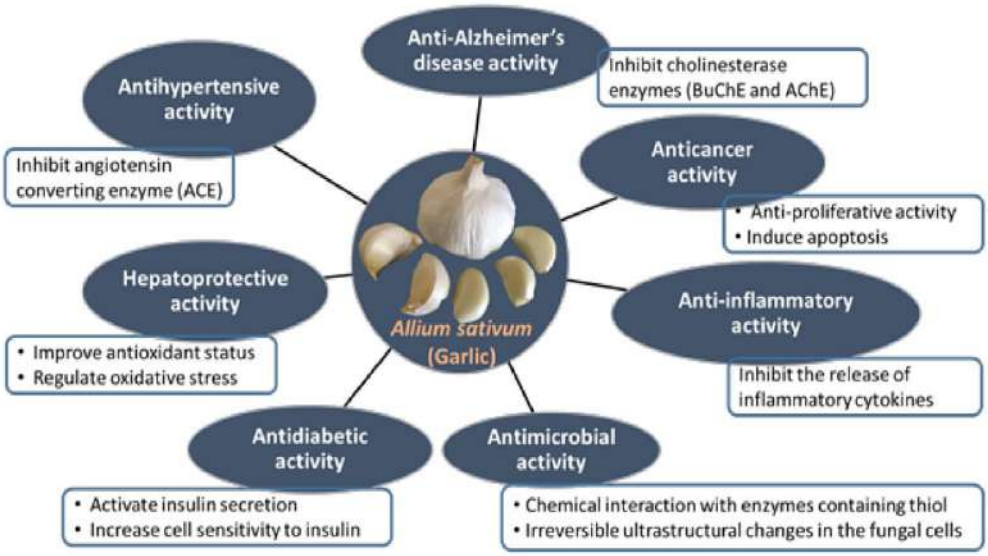


Fig.11: Schematic representation of garlic with potential pharmaceutical activities and mechanism of actions: *Garlic exhibiting varied pharmaceutical property by stimulating the immune system and protect the host.*

2.13. Biological functions of Garlic and its phytoconstituents

Natural goods such as fruits, vegetables, mushrooms, cereals, flowers, and wild fruits have all been studied for their antioxidant properties (Li *et al*., 2016). Liu 2018, reported that garlic has powerful antioxidant effects, according to a many research report. According to a study, raw garlic has higher antioxidant activity than cooked garlic (as measured by the 1,1-dipheny-l-2-picrilhydrazyl (DPPH) radical scavenging assay, 2,2'-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, and

ferric ion reducing antioxidant power (FRAP) assay). Locatelli *et al*., 2017 observed that antioxidant capacity of stir-fried garlic was also revealed to be higher (by-carotene bleaching), demonstrating that the processing might impact the antioxidant activity of garlic. DPPH and oxygen radical absorption capacity (ORAC) tests revealed that the ethanolic extract of garlic buds had higher antioxidant activity than the ethanolic extract of raw garlic in another research. In addition, DPPH, ABTS, FRAP, H_2O_2 scavenging, and $Fe2^+$ chelating tests revealed that aged garlic had stronger antioxidant capabilities than fresh garlic. Naji *et al*., 2017, in their study compared the efficacy of single and multi-clove garlic extract. They reported that single clove garlic extract exhibited a larger level of phenolic components and demonstrated stronger antioxidant activity than multi clove garlic extract. Organosulfur compounds, which have fascinating pharmacological and biological effects, are produced by plants of the genus Allium.

Kang *et al*., 2016, in an investigation on the nuclear factor erythrobia-2 related factor 2 (Nrf2)-antioxidant response element (ARE) pathway reported that aged garlic extract (AGE) induced the expression of several antioxidant enzymes, including heme oxygenase-1 (HO-1) and the glutamate-cysteine ligase modifier (GCLM) subunit, which protected human endothelial cells from oxidative stress. Garlic saponins have been shown to protect mouse-derived C2C12 myoblasts against H_2O_2-induced growth inhibition and DNA damage, as well as scavenge intracellular reactive oxygen species (ROS). Liu *et al*., 2018 reported that garlic and its active components (phenols and saponins) have antioxidant properties. The antioxidant activity of garlic was also altered by different processing methods. Raw garlic exhibited a higher antioxidant activity than cooked garlic, and fermented garlic; such as black garlic, had a higher antioxidant activity than crude garlic.

2.13.1. Garlic exhibiting Anti-Inflammatory Activity

Park *et al*., 2014 conducted study to see the anti-inflammatory effects of garlic and its bioactive components. In lipopolysaccharide-stimulated RAW 264.7 macrophages, ethyl linoleate in garlic inhibited the production of Nitric Oxide (NO) and prostaglandin E-2 by down-regulating the levels of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX2). Rabe *et al*., 2015 documented that in lipopolysaccharide-stimulated J774A.1 macrophages, the garlic 14-kDa protein reduced inflammatory mediators such as NO, *TNF* and interleukin *IL*-1 via blocking the transcription factor nuclear factor-kappa B (NF-κB)

signaling pathway. TNF-α and IL-1 receptor-associated kinase 4 levels were lowered and the activity of adenosine monophosphate-activated protein kinase (AMPK) was increased in the liver after AGE therapy.

2.13.2. Garlic with potential Antimicrobial Activity

Wallock-Richards *et al*., 2014 reported that garlic has antibacterial and antifungal effects across the board. The antibacterial properties of two particular garlic types from the Campania area of Italy, "Rosato" and "Caposele," were investigated. The Caposele variety was shown to have a considerable inhibitory impact on *Aspergillus versicolor* and *Penicillum citrinum* growth, whereas the Rosato variety had a larger inhibitory effect on *Penicillium expansum* growth. Furthermore, AGE inhibited *Burkholderi acepacian* growth. Li *et al*., 2014 found in their study that garlic oil demonstrated antibacterial properties, inhibiting the development of microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*. Garlic oil was discovered to suppress the fungus *Penicillium funiculosum*, most likely by entering into cells and organelles, disrupting cell structure and causing cytoplasm and macromolecule leaks. Fratianni *et al*., 2016 showed that garlic oil impaired *Candida albicans* normal metabolism, which is linked to the stimulation of critical genes involved in oxidative phosphorylation, the cell cycle and protein processing in the endoplasmic reticulum. Furthermore, they reported that raw garlic administration suppressed *Helicobacter pylori* in the stomach of individuals with *H. pylori* infection in a clinical investigation.

2.13.3. Garlic modulating immune system

Hassouna *et al*., 2015 described that garlic has a lot of bioactive chemicals that are good for the immune system. In RAW 264.7 macrophages, garlic polysaccharides have an immunomodulatory impact and influence the production of *IL*-6, *IL*-10, *TNF* and interferon. Fresh garlic polysaccharides are more powerful in immunomodulation than black garlic polysaccharides, which is most likely due to fructan components degrading during processing. Mohamed *et al*., 2016 reported that treating Wistar rats with garlic oil 30 minutes before giving them diazinon normalized various immunological parameters, including serum total immunoglobulin concentration and T-cell subtype CD4+. Furthermore, they reported that a mixture of garlic oil and levamisole dramatically balanced the T-helper-1 / T-helper-2 response. Lee *et al*., 2017 from their study on 14-day-

old chickens reported that selenylation modification of garlic polysaccharides improves its immune-enhancing function and selenizing garlic polysaccharides boosts lymphocyte proliferation, enhances interferon and *IL*-2 and raises serum antibody titer. Furthermore, consuming AGE has been shown to lower the frequency and severity of colds and flu, as well as increase immune system activities in people.

2.13.4. Cardiovascular Protection with garlic

Kwak *et al*., 2014, reported that the garlic powder uses successfully lowered blood pressure, total cholesterol, low-density lipoprotein, cholesterol and other cardiovascular disease risk factors. Natural remedies to protect the cardiovascular system are becoming more popular and garlic is one of the most promising alternatives.

2.14. Garlic lectin and its therapeutic potentials:

There are many prepared/processed plant dietary substances and food products contain garlic (*Allium sativum L*.) as one of the constituents (Block, 2010) as a flavor enhancer. Garlic was traditionally used in many therapeutic preparations to boost immunity and reports suggest that garlic contain immunomodulatory functional constituents (Moutia 2018). Consequential studies have shown that garlic exhibit wide range of pharmacological properties (Xia *et al.,* 2005; Wang *et al.,* 2001; Ghazanfari *et al.,* 2002; Banerjee *et al.,* 2002; Schafer *et al.,* 2014; Banerjee *et al.,* 2003). Earlier reports have been mentioned that, presence of mannose-binding lectins impart immunomodulatory functions of garlic. Detailed biochemical and molecular biological studies have demonstrated that two very homologous but not identical mannose-binding lectins (25 kD) are the most predominant proteins in garlic bulbs (Gorinstein *et al.,* 2005). The heterodimer *Allium sativum* agglutinin (ASA) contains two slightly different subunits of 11.5 and 12.5 kD, interestingly, both homologous subunits of ASA are derived from a single precursor containing two tandemly arrayed lectin domains, whereas the genes encoding ASA II contain only one lectin domain (Clement *et al.,*2010).

Notably, the primary immunomodulatory proteins in garlic have been identified as the lectins or agglutinins isolated from garlic bulbs with hemagglutinating and leucoagglutinating characteristics (Chandrashekar *et al.,* 2009; Clement *et al.,* 2010; Venkatesh, 2017). Glucose/ mannose-specific lectins derived from the related plant species exhibits the same structure but differs in the activation pattern of the human

lymphocytes. The garlic lectins have weak binding to glucose and strong affinity to mannose, oligomanosides and N-glycans (Dam *et al.,* 1998). The *in-vitro* immunomodulatory actions of raw garlic extract and garlic agglutinins (ASA I and II) on proliferation of lymphocytes and phagocytes have been previously reported (Clement *et al.,* 2010; Clement *et al.,* 2010a).

The mitogenic action of the lectins, appears to be involved in the presence of a variety of molecular forms in carbohydrate or glycan recognition. Small variation in the amino-acid sequence of the carbohydrate binding sites may have an impact on the specific binding pattern of the sugar in the cell membrane (Barral-Neto *et al.,* 1992). Plant lectins mitogenic actions represent specific carbohydrate-binding capacity; that to glycoconjugates on cell surface that are involved in the mitogenic process. In Contrast standard dietary antigenic proteins may activate 0.01-0.1% of the lymphocyte population, where mitogenic lectins can activate up to 20% of the lymphocyte population. Mitogenic lectins have been exhibited to be active in both gut and systematically after ingested through alimentary or parenteral routes (Carroll *et al.,* 2017). There is no available report on the mucosal immunogenicity or adjuvanticity and anticancer properties of garlic lectins. The garlic lectins are not known for the ability to induce an immune response against self- or co-administered antigens. Consequential studies have shown that garlic lectin exhibit antioxidant, antibacterial (Xia *et al.,* 2005), antifungal (Wang *et al.,* 2001), anti-diabetic, anti-obesity, digestive system protective, immunomodulatory (Ghazanfari *et al.,* 2002), cardiovascular protective, anticancer properties (Banerjee *et al.,* 2002; Schafer *et al.,* 2014; Banerjee *et al.,* 2003; Kodali *et al.,* 2015). Purified garlic lectin exhibits hemagglutinating and leucoagglutinating properties. Previous studies displays that the garlic lectin shows high affinity towards high mannose containing *N*-glycan than compared to free mannose (Dam *et al.,* 1998; Gupta *et al.,* 1996). Multiple recent studies have linked garlic intake with protective effects against a range of cancers, also showed that a garlic extract enhanced NK cell activity and cytotoxicity of macrophages towards tumour cells (Banerjee *et al.,* 2002; Schafer *et al.,* 2014; Banerjee *et al.,* 2003; Kodali *et al.,* 2015). There are no studies reported on the antitumor properties of garlic lectin. The aim of present study is the investigation is to study the immunomodulatory effect of garlic lectin on anti-tumour response in murine models. This current study, will provide insight about the dietary natural immunomodulator as a promising future potent biological molecule for promoting tumour regression.

3.0. Focus and Objectives of the Present proposed study

Lectins are the human dietary sources; provide humans with high quality protein, these are relatively anti-nutritional, biologically active hemo or leucoagglutinating lectins which play a vital role as therapeutic agents for cancer. Most of the studies that are available in the literature are on its structural characterizations and reports on their functional properties. However, among the biochemical and immunological importance of garlic lectins relevant to some tumour suppression and angiogenesis which is not yet studied so we like to study their significance and role in related to cancer.

1. Characterization of lectins from selected dietary source with varying carbohydrate specificity to investigate its glycoprotein interaction and digestive stability.
2. To study the biochemical manifestation of lectins induced immunomodulation on T and B cells, their immunogenicity in murine models.
3. To screen the effects of pure lectins for anti-cancer property targeting *in-vitro* cell models and *in-vivo* animal model system.
4. To elucidate the molecular mechanism of lectin induced tumour suppression and delineating role in cancer immunotherapy**.**

Materials & Methods

4.Materials and methods

4.1 Materials

The cell lines A375 (melanoma), B16F10 (melanoma), A549 (adenocarcinoma), HUH7 (hepatocytes), NIH3T3 (fibroblast cell lines), BEAS-2B (lung epithelial cells), EAC (Ehrlich Ascites carcinoma), were purchased from National Centre for Cell Science (NCCS), Pune (India) and ATCC, USA. Sephadex G-75, Ficoll histopaque, MTT, protease inhibitor cocktail and Freund's complete and incomplete adjuvants, alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG and BCIP/NBT liquid substrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI medium, antibiotic-antimitotic solution, Fetal Bovine Serum (FBS) from Gibco, New York. Ovalbumin, Schiff's Fuchsin-Sulfite Reagent, and other sugars were provided by Hi-Media Laboratories in India. Pre-stained protein marker was obtained from New England's Biolabs. Cell culture plastic wares were from corning, USA and Eppendorf, Germany. Fertilized hen's eggs were procured from the Veterinary College, Bangalore, India. Images were captured with a Sony steady shot DSC-W610 camera. Nitrocellulose membrane was obtained from Amershan Protran. Protein A agarose, p-nitrophenyl phosphate were procured from Bangalore Genei, Bangalore, India. All other experimental reagents and chemicals used in this study were of analytical grade and were procured from HiMedia, Sigma-Aldrich and SRL, India.

4.2 Collection and authentication of garlic lectin

The hybrid garlic (*Allium sativum*) was collected from the local market of Shivamogga, Karnataka state, India.

The website "The Plant List" lists the garlic plant, Allium sativum L.: http://www.theplantlist.org/tpl1.1/record/kew-296499 (retrieved October 19, 2018). The Department of Botany and Seed Technology, Sahyadri Science College, Kuvempu University, Shivamogga, Karnataka State, India, received a specimen of the dried garlic bulb and the garlic plant for authentication of the garlic bulb (voucher no. KU/SSC/BOT/209/1/2017-18) and the garlic plant (voucher no. KU/SSC/BOT/209/2/2017-18) dated November 10, 2017.

4.3 Preparation and purification of *AsL*

4.3.1 Preparation of Raw garlic extract (RGE):

Garlic bulbs (25 g) were peeled to remove the external skin (bulb coat), washed, cut into pieces, and homogenized by blending in 50 ml of (a) phosphate-buffered saline (PBS), pH 7.4 (for neutral pH buffer extract) or (b) distilled water (for aqueous extract) or (c) 50 mM sodium acetate buffer, pH 4.0 (for acidic pH buffer extract) to obtain a 50% w/v RGE. The extract was first filtered through a porous gauge and then through a muslin cloth after being kept at 4°C for two hours. The filtrate was then centrifuged at 5000*g at 4°C for 15 min. The resulting supernatant was clear and pale yellowish was stored at 4ºC; the precipitate was resuspended again in the same buffer and the procedure repeated twice for complete extraction.

4.3.2 Garlic powder extract (GPE):

To create a 50% w/v extract, commercial garlic powder (25 g) was suspended in 50 ml of PBS. With stirring, the extract was maintained at 4°C for two hours. After filtering the extract using a muslin cloth, the filtrate was centrifuged at 5000 x g for 15 minutes at 4 °C. The obtained clear light reddish supernatant was stored at 4ºC.

4.3.3 Heat processed garlic extract (HPGE):

Twenty-five grams of garlic bulb (after removing the bulb coat) were suspended in 50 ml of PBS, pH 7.4. The contents were boiled for 20 min; later, the boiled contents were allowed to cool at room temperature (5 ml of the clear boiled solution was stored as HPGE supernatant). Then the mixture was ground using mortar and pestle to obtain HPGE. The extract was initially filtered through a porous gauge and later filtered using muslin cloth followed by Whatman No. 1 filter paper. The filtrate was then subjected to centrifugation at 5000 x g at 4° C. The clear supernatant was collected and stored at 4°C. The HPGE supernatant obtained was concentrated by refrigerated Speed VAC (concentration system, RCT 60, Jouan) concentrator and stored at 4ºC. All the above extracts were used for determining the lectin content and biological activity, as assessed by glycoprotein binding assay and hemagglutination assay, respectively.

4.4. Isolation and Purification of garlic lectin (*AsL)*

The raw garlic extract prepared in phosphate buffered saline of pH 7.4 with high protein content and lectin activity was subjected further for isolation and purification of garlic lectin.

4.4.1 Ammonium sulphate precipitation

According to the previously described procedures with modifications, the clear garlic lectin extract was treated to consecutive 0% to 90% ammonium sulphate fractionation (Clement *et al.,* 2010). Briefly, 11.3g of ammonium sulphate was added pinch by pinch to 100ml of crude sample for 0-20% saturation at 4°C with gentle agitation using magnetic stirrer for 1h and allowed the mixture to stand at 4°C for overnight. Centrifuged at 3000 rpm for 20 min at 4°C, precipitate was collected and resuspended with 10mM PBS pH 7.4 where larger molecular weight proteins were precipitated. The supernatant was processed further with addition of 18.8g of ammonium sulphate to achieve 20–50% saturation at 4°C (Smeets *et al.,* 1997) with gentle agitation using magnetic stirrer for 1h and allowed the mixture to stand at 4°C for overnight. Centrifuged at 3000rpm for 20min at 4°C, obtained pellet was collected and resuspended with 10mM PBS pH 7.4 and supernatant was used for further isolation. About 29.2g of ammonium sulphate was added pinch by pinch to the supernatant obtained by previous process to achieve 50-90% saturation at 4°C for 1h on magnetic stirrer with gentle mixing, allow it to stand for overnight at 4°C and centrifuged at 3000rpm for 20min at 4°C were smaller molecular weight of proteins gets precipitated. This precipitated was collected and resuspended with 10mM PBS at pH-7.4.

4.4.2 Dialysis

The mixture was carried out in an Amicon stirred ultra-filtration unit (3 K cut-off membrane) for removal of salts and low molecular weight molecules. Amicon tubes were rinsed with milli-Q-water or buffer (to remove the glycerine from the membrane, if continuous interference of glycerine rinse with 0.1N NaOH and second spin of buffer or milli-Q-water). Added 3.5ml of Ammonium sulphate precipitated (50-90%) saturation fraction to the filter. Centrifuged at 7500g for 10-40 min at 4°C,

recover the concentrated solute by inserting the pipette into the bottom of filter device and withdraw the sample. Obtained sample was aliquoted and stored at -20°C.

4.4.3 Gel permeation chromatography

The sample concentrated from ultra-filtration was carried on the G-75 gel permeation chromatography for further purification.

Packing of column:

- Exactly, 9gms of sephadex G-75 was added to 100ml of 10 mM PBS buffer pH-7.5. Allowed the gel to swell overnight and washed with same buffer for at least three times.
- Cleaned the column, plugged the cotton to the base of the column and filled the column with 10mM PBS buffer pH-7.5. Then slowly lead the gel slurry from the top, allowed to settle down from the base.
- Before loading the gel adjusted the column flow rate to 3ml/8min (slow flow with drip controller) like a drop wise fall of buffer.
- Column was packed continuously without any break (never give a chance to dry, that allows air crack and air soluble insertion in the gel).

Equilibrating:

After column is packed equilibrate the column with loading and elution buffer (10mM PBS buffer pH-7.4). Allowed the buffer to flow through the column for at least 1 ½ column volume (if the bed volume is 100, it should be 150ml).

Sample loading:

2% of sample was slowly loaded to the top layer of the gel and allowed to enter into the column gel. Then immediately add 2ml of 10mM PBS buffer, connect the elution buffer to enter into the column.

Fraction collection:

- Started collecting the elution sample as 3ml fraction with the flow rate of 3 ml/8min.

- At least 2X of the bed volume was collected to make sure all the sample entered the column is eluted. The eluted fractions were monitored for protein by absorbance at 280nm. Plotted the graph and documented the peak obtained.

4.4.4 Reverse -phase High Performance Liquid Chromatography (RP-HPLC)

The HPLC equipment consisted of an auto-sampling injector model (Dionex Ultimate 3000, U.S.A) with solvent delivery. The active fraction (*AsL*) separated from sephadex G-75 gel permeation chromatography was finally purified by reverse-phase HPLC on a nucleosil C_{18} column (4.5x 250mm, particle size 5µm) equilibrated with solvent A (0.1% trifluoroacetic acid, pH 2.5). The elution was carried out for 60mins using binary gradient solvent A and solvent B (80% acetonitrile, 20% H_2O, 0.05% trifluoroacetic acid) both the column and solvents were maintained at 40°C. The flow rate was 1ml/min and the absorbance was recorded at 214-280nm. The reverse –phase HPLC were repeated for thrice. All hemagglutinating-containing peaks were pooled.

4.4.5 Protein quantification

Lowry's method was used for the protein quantification. Protein concentration was done by Lowry's method of protein assay using BSA (Bovine Serum Albumin) as standard (Lowry *et al.,* 1951).

4.4.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Equal concentration of protein was resolved on gel using Bio-rad protein mini vertical electrophoresis system (Bio-rad, USA) as illustrated below according to the method of Laemmli (Laemmli 1970).

- ***Plate setup:*** In order to cast 1 mm thickness gel, two glass plates (8 x 8 cm) containing 1 mm thickness inbuilt spacer were fixed together. The combined plates were clamped strongly in plate holders to avoid the leakage of gel solutions.

- ***Resolving gel:*** The percentage of resolving gel differs depending upon the molecular weight of desired protein, described in Table .Acrylamide-bisacrylamide solution (30% acrylamide and 0.8% N-N-bisacrylamide in distilled water), distilled water, Tris buffer A (1.5 M Tris-base, pH 8.8), 10% SDS, 10% ammonium per sulphate (APS), and N,N,N',N'-tetramethylethylenediamine were combined to create resolving gel solutions based on the molecular weight (TEMED).The contents were dispensed immediately into gel plate setup up to 3/4th level and left it for 15 min for gelling process at room temperature.

- ***Stacking gel:*** The concentration of Stacking gel solution is fixed (4.5%) was prepared by combining 0.75 ml of 30% acrylamide –bisacrylamide solution, 2.95 ml distilled water, 1.25 ml Tris buffer B (0.5 M Tris-base, pH- 6.8), 50 µl 10% SDS, 50 µl 10% APS and 10 µl TEMED. The stacking gel solution was dispensed immediately over resolving gel, plastic comb was interleaved into the stacking phase to make the well for loading proteins samples. It was allowed to form gel for 15 min at room temperature.

Table 4: Separating gel preparation for gel electrophoresis:

Components	**Volume (10ml of resolving gel preparation)**				
	6% gel	**8% gel**	**10% gel**	**12% gel**	**15% gel**
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
30% acrylamide/ bisacrylamide solution	2ml	2.6ml	3.4ml	4ml	5ml
1.5M Tris-HCl pH- 8.8	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% APS	100µl	100µl	100µl	100µl	100µl
10% SDS	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

- ***Loading well:*** Once the cast gel was polymerized, the comb was removed carefully from the gel avoiding any air bubbles and blockage. In case of any bubbles, the wells were cleared with a piece of filter paper.

- ***Sample preparation (for reducing gel):*** The 6X-sample buffer (250 mM Tris-base pH-7.0, 50% Glycerol, 9% SDS, 0.03% Bromophenol blue, and 5% -mercaptoethanol in distilled water) was combined with about 20µg of each protein sample.

- ***Sample preparation (for non- reducing gel):*** 20µg of each protein sample were combined with 250 mM 6X sample buffer. Tris-base pH-7.0, 9% SDS, 50% glycerol and 0.03% Bromophenol blue in distilled water) no boiling is done for non –reducing gel.

- ***Electrophoresis:*** These protein samples were briefly centrifuged using a microfuge for 30 seconds at 3000 rpm before being boiled for 5 minutes (Eppendorf, Germany). Followed by filling electrophoresis chambers with the running buffer (12.4 mM Tris-base, 192 mM glycine, 0.1% SDS, pH 8.0) and loading the protein samples into the gels, electrophoresis was carried out at 100 V for 2–3 hours. Pre-stained protein markers from New England Biolabs was used as standard proteins. The gels were then stained with respective staining protocols (Laemmli 1970).

- ***Staining protocol:***

a. Coomassie brilliant blue staining:

Before placing the gel for Coomassie brilliant blue staining, the gel was placed in the fixative solution (50% Distilled water, 40% Methanol, 10% glacial acetic acid) for 1hr, then discard the fixative solution and add Coomassie brilliant blue staining solution (0.1% Coomassie brilliant blue R-250, 50% Distilled water, 40% Methanol, 10% glacial acetic acid) for overnight with gentle agitation. Destain the gel in destaining solution (40% Distilled water, 50% Methanol, 10% glacial acetic acid). Replenish the solution several times until background of the gel is fully destained. Store the destained gel in storing solution (5% glacial acetic acid).

b. Silver staining:

- ***Gel fixation:*** After electrophoresis, placed the gel in a tray containing 100ml of fixer-1 (50% Methanol or ethanol in distilled water with 0.05% formaldehyde), Shaked for 30min. Decant the solution and added fixer-2 (25% methanol in distilled water with 0.05% formaldehyde) shaked for 20min. then washed the gel with distilled water for 3-5 min. Meanwhile prepare sensitizer solution and stain.

- ***Sensitizer:*** Decant the distilled water, poured 98ml of sensitizer solution *(0.02% sodium* thiosulphate in distilled water) to the gel (leaving behind 2ml solution in the flask for developing solution) shaked the gel for 1min. Decant the sensitizer solution and washed the gel for thrice with distilled water for 1min each.

- ***Staining:*** Added Staining solution (0.2% silver stain-0.076% *formaldehyde* to the gel. Covered the tray with plastic wrap and shaked for 25min (Meanwhile prepared the developing solution by 2ml sensitizer) drained the stain into a separate container washed the gel for thrice with excess distilled water for 1min each.

- ***Development:*** Added 150µl of formaldehyde for developing solution (6% *sodium carbonate* in 100ml of distilled water) mixed well by gentle swirling. Kept the gel for shaking and observed the stain development. Once the desired intensity obtained, decant the developer solution and added the stop solution (10% methanol, 5% glacial acetic acid and 85% of distilled water) promptly. Stored gel preferably in dark at 4°C or stored dry.

c. Periodic acid-Schiff (PAS) staining

By using PAS staining, the glycoprotein composition of the isolated *AsL* was examined. *AsL* was briefly resolved on a 15% SDS-PAGE under decreasing conditions and fixed for one hour in 12.5% TCA. Following incubation for 1 hr in 1% periodic acid at 4˚C /dark, the gel was washed thoroughly and allowed for color development using ice cold Schiff's Fuchsin-sulphite reagent. The reaction was halted with 7% glacial acetic acid after the development of bands, and the gel was then documented using Bio-Rad Gel Documentation. (Zacharius et al.,1969).

4.4.6.1 Native gel electrophoresis:

Equal concentration of protein was resolved on gel using Bio-rad protein mini vertical electrophoresis system (Bio-rad, USA) as illustrated below according to the method of Laemmli (Laemmli ,1970).

- ***Plate setup***: In order to cast 1 mm thickness gel, two glass plates (8 x 8 cm) containing 1 mm thickness inbuilt spacer were fixed together. The combined

plates were clamped strongly in plate holders to avoid the leakage of gel solutions.

- ***Resolving gel***: The percentage of resolving gel differs depending upon the molecular weight of desired protein, described in Table. Based on the molecular weight, resolving gel solutions were made by mixing distilled water, Tris bufferA (1.5 M Tris-base, pH 8.8), 10% ammonium per sulphate (APS), and N,N,N',N'- tetra methyl ethylene diamine with acrylamide-bisacrylamide solution (40 % acrylamide and 0.8% N-N-bisacrylamide in water) (TEMED). The contents were dispensed immediately into gel plate setup up to 3/4th level and left it for 15 min for gelling process at room temperature.

- ***Stacking gel:*** The concentration of Stacking gel solution is fixed (4.5%) was prepared by combining 0.5 ml of 40% acrylamide –bisacrylamide solution, 3.25ml distilled water, 1.25 ml Tris buffer B (0.5 M Tris-base, pH- 6.8), 60 µl 10% APS and 10µl TEMED. The stacking gel solution was dispensed immediately over resolving gel, plastic comb was interleaved into the stacking phase to make the well for loading proteins samples. It was allowed to form gel for 15 min at room temperature.

Table 5: Separating gel preparation for native gel electrophoresis:

Components	**Volume (10ml of resolving gel preparation)**				
	4% gel	**6% gel**	**8% gel**	**10% gel**	**12% gel**
H_2O	4ml	6ml	5.5ml	5ml	4.375ml
40% acrylamide/ bisacrylamide solution	1ml	1.5ml	2ml	2.5ml	3.125ml
1.5M Tris-HCl pH- 8.8	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
10% APS	50µl	50µl	50µl	50µl	120µl
TEMED	10µl	10µl	10µl	10µl	10µl

- ***Loading well:*** Once gel gets polymerized, comb was removed carefully from the gel avoiding any air bubbles and blockage. In case of any bubbles, the wells were cleared with a piece of filter paper.

- ***Sample preparation:*** The 4X-sample buffer (0.5 M Tris-base pH-6.8, 20% glycerol, and 0.02% bromophenol blue in distilled water) was combined with about 20 g of each protein sample.

- ***Electrophoresis:*** These protein samples underwent a 30-second centrifugation at 3000 rpm. The electrophoresis buffer (0.025M Tris-base, 0.192 M glycine, pH 8.3) must then be added to the electrophoresis chambers. After loading the protein samples onto the gels, electrophoresis was carried out at 100 V for 2–3 hours.

- ***Staining protocol:*** Stained the gel in the staining solution (0.1% Coomassie ***brilliant blue R-250,*** 50% methanol, 10% glacial acetic acid and 40% distilled water) for overnight with gentle agitation. After 24hr destained the gel using destaining solution (10% methanol, 10% glacial acetic acid and 80% distilled water) until the gel background becomes colourless and leaves the protein band coloured blue or purple and using the Bio-Rad Gel Documentation TM XR+ Imaging system, and documented.

4.5 Predicting Biological activity of purified garlic (*AsL)*

The garlic lectin was assessed for hemagglutination, leucoagglutination, pH and temperature stability and antioxidant properties. Both Raw garlic extracts and partially purified and purified lectins were subjected for the assays.

4.5.1 Haemagglutination assay for *AsL* samples

4.5.1.1Preparation of trypsinized 1%RBC suspension

Fresh 5ml Blood sample of Chicken were obtained from the nearby slaughter house Shivamogga, 5ml of Human (all blood groups) blood sample were obtained from the volunteer healthy person before getting signed to the consent form and rabbit blood sample was obtained from the farm house, Shivamogga. All the blood samples were collected in difference sterile tubes, containing an equal volume of Alsevier's Solution and centrifuged at 1000 X g for 10 minutes at 4 °C. The pelleted erythrocytes were washed three to four times with saline (0.9% NaCl), then eventually re-suspended in 10mM PBS, pH 7.4. The supernatant was then discarded. The RBC suspension was brought to 1%, added, and then incubated at 37°C for 10 minutes with 1% trypsin. In order to be used in the HA and HA inhibition assay, the trypsinized cells were then centrifuged at 1000 X g for 10 min at 4 °C, rinsed with saline, and readjusted to 1% concentration. (Burger 1974).

4.5.1.2 Haemagglutination activity

Using suspensions of 2% trypsinized chicken, human, and rabbit blood erythrocytes, the hemagglutinating activity (HA) of *AsL* was examined. A concavity agglutination plate with an equivalent volume of serially diluted protein solution and 50 µl of 2% erythrocyte suspension was gently mixed and incubated at 37° C for 1 hour to observe the agglutination. Lacking the protein, only erythrocytes solution incubated with PBS were used as control. The least amount of protein required to agglutinate is equal to the amount of protein at the maximum dilution.

4.5.1.3 Leucoagglutination assays

Human peripheral blood lymphocytes that had been isolated using Ficoll-Hypaque (density=1.077g/ml) were used for the leucoagglutination assay through density gradient centrifugation as described as product manufacture. About 2.5ml of histopaque solution was taken in the 15ml polypropylene centrifuge tube, slowly add 7.5ml of diluted blood sample (1:2) over the layer of histopaque. The samples were in the tubes were carefully placed in centrifuge for 400g at 4˚C for 15-30mins without mixing the contents. After centrifugation the tube consisting of four different layer such as plasma layer, lymphocytes layer (buffy coat), histopaque layer and pelleted erythrocytes and granulocytes layer. Carefully aspirated the buffy coat below the plasma layer and washed with 10mM PBS buffer for at least 3-4 times. Finally resuspended the buffy coat with 10mM PBS buffer, pH-7.5. After gently mixing the separated lymphocytes (100 l) in 10 mM PBS with an equivalent volume of *AsL* in a concavity agglutination plate, the mixture was incubated at 37 °C for one hour. The cells were stained using Leishman's reagent, and the results were recorded.

4.5.1.4 Stability of garlic lectins to various physicochemical conditions

a. ***Thermal stability:*** Hemagglutination activity was used to measure the thermal stability of AsL by incubating the lectin sample at various temperatures using the previously described procedures. (Siva Kumar and Rajagopal ,1986). About 50µg lectin sample were incubated for 10min at 4, 26, 37 and 100°C. At the end of the incubation period, the samples were gradually cooled to room temperature and measured for their hemagglutination activities.

b.***pH stability:*** The pH stability of *AsL* was also determined by hemagglutination assay using buffer ranges from 4-10. Various buffers used are 0.1 M sodium acetate for pH-4; 0.1 M sodium phosphate buffer for pH-6; 0.1M phosphate buffer saline for pH-7; 0.1 M carbonate buffer for pH-9. About 50µg lectin sample were incubated with various buffers for 30min at room temperature. At the end of the incubation time, the protein samples were tested for their hemagglutination activities and recorded (Suseelan *et al.,* 1997).

4.5.1.5 Glycocode estimation assay

By inhibiting HA, the glycocode or carbohydrate inhibition assay was identified. For carbohydrate-mediated HA inhibition, 0.1 ml of lectin solution was pre-incubated with various concentrations of carbohydrate/or glycoconjugate solutions in each of the wells for 1 hour at 37 °C before the addition of 0.1 ml of 1% trypsinized erythrocytes. Agglutination was observed, and the degree to which different sugars or glycoproteins inhibited the lectin's ability to hemagglutinate was determined (Dam *et al.,*1998).

4.6 Detection of pepsin-stable protein

Digestibility of AsL was determined by using pepsin at 2500 units of activity per mg, according to the method described by Ofori-Anti et al, 1974. Stimulated gastric fluid (SGF) was prepared as a solution of 0.084 N HCl and 35mM NaCl and the pH adjusted to 1,2 with dilute HCl and NaOH along with 2500 pepsin activity units per mg test proteins. The digests are performed by mixing 1.9ml of SGF containing 2500 pepsin activity units with 0.1ml of AsL (5mg/ml protein) and mixed well before placing on 37°C. Control sample was taken without the protein and protein mixed with SGF without pepsin was also performed to evaluate the auto-digestion. During incubation time at regular intervals of 0, 5, 10, 20, 40 and 60 min, 200 µl aliquots of the digest were taken out into a tube containing 70µl of NaHCO3 buffer (pH 11). 6X Sample dye was added to the samples obtained, mixed well and heated for 5min. Zero min digest is the solution of pepsin quenched before adding the test protein. All the aliquots were analysed by SDS-PAGE 15% gel under reducing condition. The results were observed and documented (Clement *et al.,*2010).

4.7 Pharmacological properties of *AsL*

The antioxidant, anti inflammatory properties of garlic extract and purified garlic lectin were studied for their pharmacological effects to modulate oxidative stress and inflammations.

4.7.1 Anti-oxidant activity of *AsL*

4.7.1.1 Estimation of free radical scavenging efficacy of AsL by DPPH anti-oxidant assays

AsL was screened for free radical scavenging activity by DPPH radical scavenging assay (Braca *et al.*,2001). *AsL* at varied concentration (0 -100 µg) were added to each test tube and volume was made up to 2 ml using the distilled water. The reaction was added with 3 ml of 0.004% DPPH in 95% ethanol was added and the mixtures were incubated at room temperature under dark condition for 30 min. The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm. Radical scavenging activity was calculated using the formula:

$$\% \text{ of radical scavenging activity} = ([(A_{control} - A_{test})/A_{control}]) \times 100.$$

Where $A_{control}$ is the absorbance of the control sample and A_{test} is the absorbance of the test sample. The DPPH radical scavenging activity of BHA was also assayed for comparison. Test was performed in triplicate and the results were averaged. (Randa *et al.*, 2013)

4.7.1.2 Lipid Peroxidation inhibition assay

In-vitro lipid peroxidation assay was carried out to estimate the lipid peroxidation inhibitory capacity of *AsL*. About 10% of liver homogenate was prepared using 0.15M KCl, 0.5ml of liver homogenate and 2ml of *AsL* at varied concentrations were taken in test tubes. Exactly 100µl of ferric chloride (0.2mM) was added to each test tube and incubated at room temperature for 30min to induce lipid peroxidation. The reaction was stopped by adding 2ml of ice-cold HCl (0.25N) containing 15% TCA, 0.38% TBA, and 0.5% BHA.

The content was mixed thoroughly and heated on boiling water bath for 60 min. Reaction mixture was cooled and centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 532 nm. Percentage of inhibition was calculated from the formula.

$$\% \text{ Of inhibition} = ([(A_{control} - A_{test})/A_{control}]) \times 100$$

Where $A_{control}$ is the absorbance of the control sample and A_{test} is the absorbance of the test sample. The test was conducted three times, and the outcomes were averaged.

4.8 Anti-inflammatory activity of *AsL*

4.8.1 *In-vivo* assays

4.8.1.1 Assay of *AsL* anti-inflammatory activity

a) Experimental animals

Wistar albino rats weighing 180–200g were kept in typical lab conditions and fed commercial rodent chow and water on a regular basis. The Institutional Animal Ethics Committee (IAEC) approved each experimental animals procured from Biogen Laboratory Animal Facility, Bangalore. All animals were handled in accordance with CPCSEA guidelines.

b) Treatment

Methotrexate

Methotrexate an oral anti-inflammatory tablet, was purchased and administered orally at dose rate of 50μg/kg/week by oral gavage (Meti, 2016).

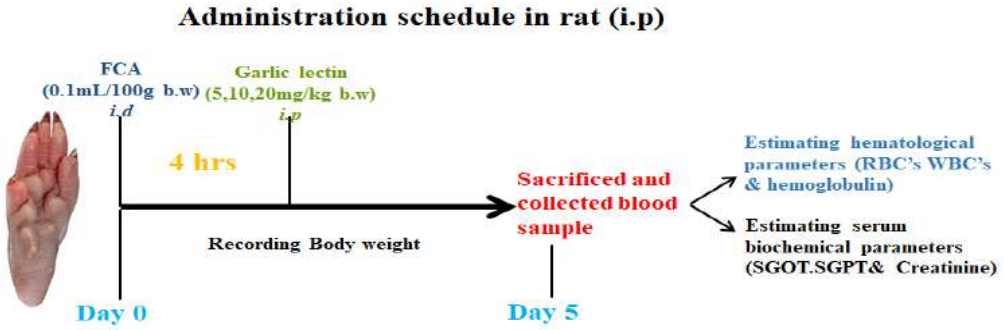


Fig.12: *Anti-inflammatory activity of garlic lectin by inducing edema to wistar albino rat by Freund's complete adjuvant through intradermal and treated with garlic lectin after 4hr on day 0. Blood sample were collected on day 5 to estimate the haematological and serum biochemical parameters.*

At the end of the experiment, all rats were sacrificed by giving overdose of Ketamine and Xylazine (as I/M injections). The hind paws were separated from the knee down and were fixed in 10% Neutral buffered formalin (NBF) for further studies.

c) Body weight

All the animals were monitored carefully by weighing the body weight on 0 and 5th day of the experiment using a digital weighing machine.

d) Paw volume measurement

Paw volume of all animals (treated and untreated) was measured using plethysmometer (INCO, Pvt Ltd) on 0 and 5th day of the experiment. The percentage inhibition between positive group and all treated groups was calculated as a change in paw volume

$$\% \text{ change} = \frac{\text{Mean paw volume of positive control group} - \text{Mean paw volume of treated group}}{\text{Mean paw volume of treated group}} \times 100$$

e) Paw thickness measurement

The inflamed hind paw (anterior-posterior) was measured using a digital vernier calliper (Ramprasath *et al.,* 2006). Measurements were done on 0 and 5th day post FCA injection. The percentage inhibition between positive group and all treated groups was calculated as a change in paw thickness.

$$\% \text{ change} = \frac{\text{Mean paw thickness of positive control group} - \text{Mean paw thickness of treated group}}{\text{Mean paw volume of treated group}} \times 100$$

f) Haematological parameters

The blood samples collected in EDTA vials from the animals on day 5th of the experiment were subjected for RBC's, WBC's and hemoglobulin estimation by using Erba hematology analyzer.

g) Serum Biochemical parameters

For serum biochemical parameters analysis, blood was collected on day 5th into serum vacutainers, centrifuged at 3000 rpm for 15 min and the serum separated was stored at -20°C till further analysis. Serum enzymes; SGOT, SGPT and creatinine using semi-automatic biochemical analyzer with Erba biochemical kits.

4.9 Characterization of *AsL*

4.9.1 Generation of polyclonal antibody against *AsL* in rabbits

New Zealand white rabbit experimentations were approved by the Institutional Animal Ethical Committee (IAEC), according to the CPCSEA rules for laboratory animal facilities, as determined by the committee (Ref No. KSHEMA/IAEC/01/2020 Date: 04.06.2020 in supervision of Research co-guide Dr. B.T. Prabhakar, Associate Professor, Department of Biotechnology, Sahyadri Science College, Kuvempu University, Shivamogga). Polyclonal antibodies against *AsL* were raised in rabbit by subcutaneous injection with l ml protein solution (0.1 mg/ml) in complete Freund's adjuvant. Booster doses with incomplete Freund's adjuvant were given 4 weeks after primary injection by subcutaneous at 15-day intervals (Towbin *et al.,* 1979).

In order to extract pre-immune serum one week before the first injection and immune serum one week after each booster dosage, the animal was bled via a marginal ear vein puncture.

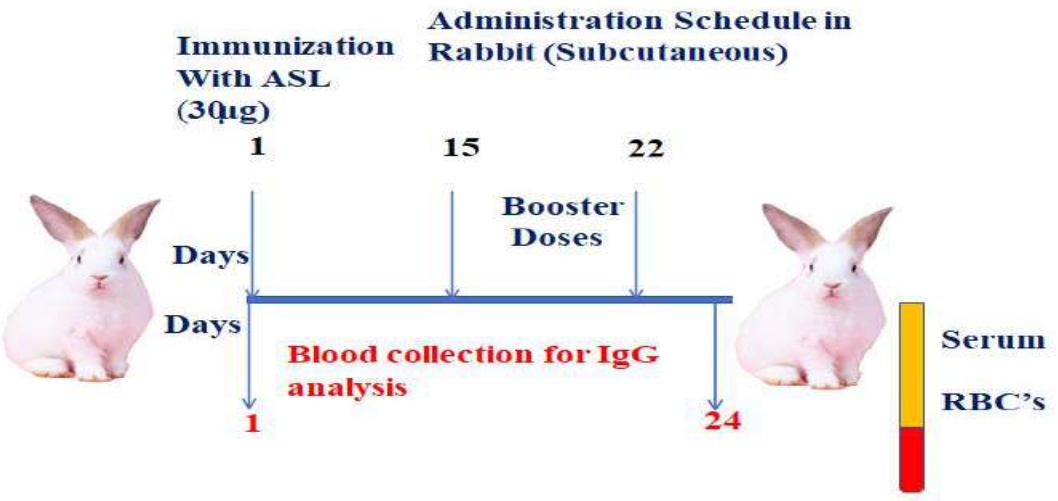


Fig.13: Immunogenicity of garlic lectin in New Zealand White rabbit; *initial immunization was at the day 1 and subsequent booster dose was administrated on day 15 and day 22. Blood sample were drawn on day 1 (pre-immunization) and day 24 (post-immunization) to examine the immune response.*

4.9.2 Purification of polyclonal antibodies from rabbit antisera.

4.9.2.1Ammonium sulphate precipitation

The immunoglobulin (IgG) from antiserum was purified by ammonium sulphate precipitate method. To 2ml of rabbit antiserum, ammonium sulphate was gradually added pinch by pinch with constant stirring at ice-cold temperature. At 4°C, the suspension was left overnight with just intermittent stirring. After centrifuging the mixture at 3000 rpm for 30 minutes at 4 °C, the supernatant was discarded. Pellet was resuspended with 10mM Phosphate buffer saline (Heide *et al.*, 1973). Sample was dialyzed against the 10mM Phosphate buffer Saline for 24hrs with frequent change of buffer. Dialyzed sample was collected and subjected to protein A agarose column chromatography for further purification.

4.9.2.2 Immunoaffinity chromatography

The dialyzed IgG was further purified by the protein A agarose column chromatography according to the manufacturers protocol (Bangalore Genei, Bangalore). About, 10 bed volume of 0.5M phosphate buffer saline was passed

through the protein A agarose column. Dialyzed IgG sample were thoroughly mixed with equal volume of 1X equilibration buffer and centrifuged at 10,000 rpm for 10mins.

Equilibrated clear fluid was loaded on to the protein A agarose column and connected the equilibration buffer at the rate of 3ml/min. twenty bed volume of 1X equilibration buffer was passed through the column and unbound proteins collected until the absorbance at 280nm reaches zero. Ultimately five bed volume of 1X elution buffer (0.05M citrate buffer, pH 3.0) was passed and fractions were collected at the flow rate of 3ml/min in tubes containing 20µl of neutralization buffer (1.5M Tris pH 10.0) and absorbance was read at 280nm. The bound fractions were pooled and stored at -20°C for further use.

4.9.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Purified and protein quantification was done by Lowry's method of protein assay using BSA (bovine serum albumin) as standard. Using a Bio-Rad small electrophoresis device, IgG was exposed to reducing 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) (Clement *et al.,* 2010).

4.9.3 Characterization of rabbit anti-*AsL* antibodies

4.9.3.1 Ouchterlony Double Immunodiffusion

The double immunodiffusion experiment was carried out in accordance with accepted standards. In a nutshell, 10mM PBS was used to make 0.8% agarose. (Ouchterlony and Nilsson, 1986). In a 30mm Petri plate, the solution was added and left to stand at room temperature. Wells were drilled into the gel after it had solidified. Wells were loaded with samples respectively; the gel was examined for an opaque precipitation line between the antigen and antisera wells during a 24-hour incubation period at 37°C in a humid setting.

4.9.3.2 Immunoblotting for anti-AsL antibodies

a) DOT Blot Analysis

Dot blot experiment was used to determine the immunoreactivity and specificity of rabbit polyclonal anti serum. The purified *AsL* (5-10 μg) sample was loaded on to the nitrocellulose membrane, allowed it to dry.

Membrane was blocked immediately by using blocking buffer (5% skimmed milk in TBST (10mM Tris base pH 7.5, 100mM NaCl, 0.1% Tween -20)) washed with TBST and the membrane was incubated with primary antibody (rabbit anti-*AsL* serum 1:500 dilution) for overnight at 4°C. The membrane was washed and incubated with secondary antibody (goat anti-rabbit IgG-ALP conjugated 1:30000) at 37 °C for 1 hr. After washing, the blots were developed using BCIP/NBT substrate solution. Pre-immune serum was used as negative control.

b) Western blotting

Depending on the molecular weight of the target protein, the protein samples (AsL) were resolved in 8–12% SDS–PAGE. The western blot procedure was used on the gels (Bair et al., 2009) with desired primary and secondary antibodies as explained below

- ***Wet transfer:*** Gels containing resolved proteins were sandwiched between nitrocellulose membrane (Amersham Protran) and rough and smooth pads from negative to positive charge during the wet transfer procedure. In the western blot unit (Bio-rad, USA) with pre-chilled running buffer (25 mM Tris base, 190 mM Glycine, 20% Methanol, pH adjusted to 8.0) in the direction of negative to positive charge, the plate containing the sandwich was inserted. Proteins were then added to membranes for 1 hour at 100 V and 4°C while being constantly stirred.

- ***Blocking:*** The membranes were removed from the transfer equipment and immediately blocked for 1 hour at room temperature in a gel rocker with blocking buffer (3% BSA in 10 mM Tris base pH 7.5, 100 mM NaCl, 0.1% Tween 20).

- ***Incubation with primary antibody:*** After thoroughly decanting the blocking buffer, the membrane was treated for an overnight period at 4°C with the required

primary antibody at diluted concentrations (rabbit anti-AsL serum 1:1000 dilutions).

- ***Incubation with secondary antibody:*** The membranes were rinsed with washing buffer (TBST of 10 mM Tris base pH - 7.5, 100 mM NaCl, 0.1% Tween 20) for 10 min three times after being probed with the chosen primary antibody. The membranes were re-probed with the desired dilution of anti-IgG secondary antibody (goat anti-rabbit IgG-ALP conjugated antibody 1;30000) at 37°C for 1 hour.

- ***Substrate incubation:*** The membranes' secondary antibody solutions were thoroughly decanted before being washed three times with washing buffer for ten minutes each. The membrane was exposed to the BCIP-NBT chromogen for 10 minutes at 25°C while it was dark. Washing the membrane with distilled water for 10 minutes put an end to the process.

- ***Result documentation:*** The Bio-rad Gel Documentation TM XR+ Imaging System was used to record the outcomes (Bio-rad, USA). Using ImageJ software, the protein expression patterns were assessed by contrasting treatment and control groups.

4.9.3.3 Determination of Antigen and Antibody titer

The immunoreactivity of polyclonal antisera were checked by direct ELISA. ELISA was carried out by coating 10µg protein in 100 µl of 0.1 M carbonate–bicarbonate buffer, pH 9.6 and left overnight at 4° C. After washing three times with TBST, the wells were blocked with 3% BSA/5% skimmed milk powder for 1 h at 37° C. Subsequent to washing with TBST, rabbit antiserum (primary antibody 1:1000 dilution) in TBST containing 1% BSA was added to the wells and the plate was incubated at 4°C for overnight. The wells were washed and goat anti-rabbit IgG–ALP conjugate (secondary antibody 1:30000 dilution) was added to the wells and incubated for 1 h. Following the addition of the substrate *p*-nitrophenyl phosphate (100 µl; 1 mg/ml), the absorbance at 405 nm was recorded in a microplate reader.

4.9.3.4 Determination of produced polyclonal antisera specific to AsL

The produced polyclonal antisera were checked for the specificity to AsL through direct ELISA. It is carried by coating 10µg (BSA, OVA, ConA and *AsL*) in 100µl of 0.1M carbonate-bicarbonate buffer, pH 9.6 and incubated overnight at 4°C. After washing three times with TBST, the wells were blocked with 3% BSA/5% skimmed milk powder for 1 h at 37 °C. Subsequent to washing with TBST, rabbit antiserum (primary antibody 1:1000 dilution) in TBST containing 1% BSA was added to the wells and the plate was incubated at 4°C for overnight. The wells were washed and goat anti-rabbit IgG–ALP conjugate (secondary antibody 1:30000 dilution) was added to the wells and incubated for 1 h. Following the addition of the substrate *p*-nitrophenyl phosphate (100 µl; 1 mg/ml), the absorbance at 405 nm was recorded in a microplate reader.

4.10 Humoral immune and adjuvant responses of *AsL*

4.10.1Experimental animals

The following study was undertaken after obtaining the clearance of the Institutional Animal Ethics Committee (IAEC approval #235/12). Six- to seven-week-old female BALB/c mice weighing around 22–25 g was obtained from the Central Animal Facility of the Indian Institute of Science (IISc), Bengaluru, India; they were maintained in the special room provided in the animal house facility present at the Department. All pertinent institutional policies and laws were adhered to throughout the experiments and research plan that involved the use and care of BALB/c mice. Mice were kept in cages in groups of six and had access to a special commercial diet (pellet) made mostly of grain goods. The ambient room conditions were maintained with a temperature at 23 ± 3 ◦C with a relative humidity of 50 ± 5% and a 10–12 h period of light/dark cycle.

4.10.2 Grouping of animals for immunization

The animals were grouped to study the adjuvant response in BALB/c mice, which were divided accordingly into four groups (n = 6) for intranasal and intradermal immunization. The chosen mice were divided into groups at random according to their body weight. To guarantee an equal response from each group of

mice receiving the antigen, the average weights of the mice in each group were kept constant. Saline was the sole substance injected into the first group of rats in the vehicle control condition to cause stress. Ova was given to the second group, while ASA I and ASA II were given to the third and fourth groups, respectively. All the antigens (OVA, ASA I, and ASA II) were administered in a dose-dependent manner by either the intranasal or intradermal route at the antigen concentration following the reported protocol (Lavelle *et al.,* 2001).

4.10.3 Systemic (Intradermal) immunization

The intradermal (*i.d.)* immunization schedule is represented in Figure 14 (top portion). The grouped mice (n= 6) obtained an intradermal injection of a constant 30 µl of 1 mg/ml of the respective antigen on days 0 and 7 to the dorsum of each ear; the control animal was treated (without the antigen) and administered only 30 µl of saline to stress the animal throughout the experiment. After day 14, all mice were sacrificed following the standard protocol, and blood was drawn by cardiac puncture. Blood was also collected from the treated and untreated groups by retro-orbital venipuncture at specific intervals (days 7 and 14).

4.10.4 Mucosal (Intranasal) immunization schedule

Intranasal (*i.n.)* administration of antigens was performed in groups of mice (n = 6), as represented in Figure 1 (bottom portion). Mice were immunized on days 1, 7, 14, 21, 28, 35, and 42 with one of the following: PBS, OVA, ASA I, and ASA II. All the samples were prepared in sterile PBS and were made up to the concentration of 1 mg/ml. Mice were moderately anesthetized through the exposure to a mild dose of diethyl ether and were carefully monitored during the intranasal administration of the antigen by heartbeat observation. Using a micropipette, 30 µg of OVA or garlic lectins (ASA I and II) were delivered in 30 µL of phosphate-buffered saline (PBS) (for dosage administration, 15 µL was applied slowly to each nostril and allowed for inhalation). After the antigen exposure regime, on day fifty, all mice were sacrificed following the standard protocol and blood was collected by cardiac puncture. Blood was drawn from the mice during specific intervals from the treated and untreated animals by retro-orbital venipuncture at days 14, 35 and 50.

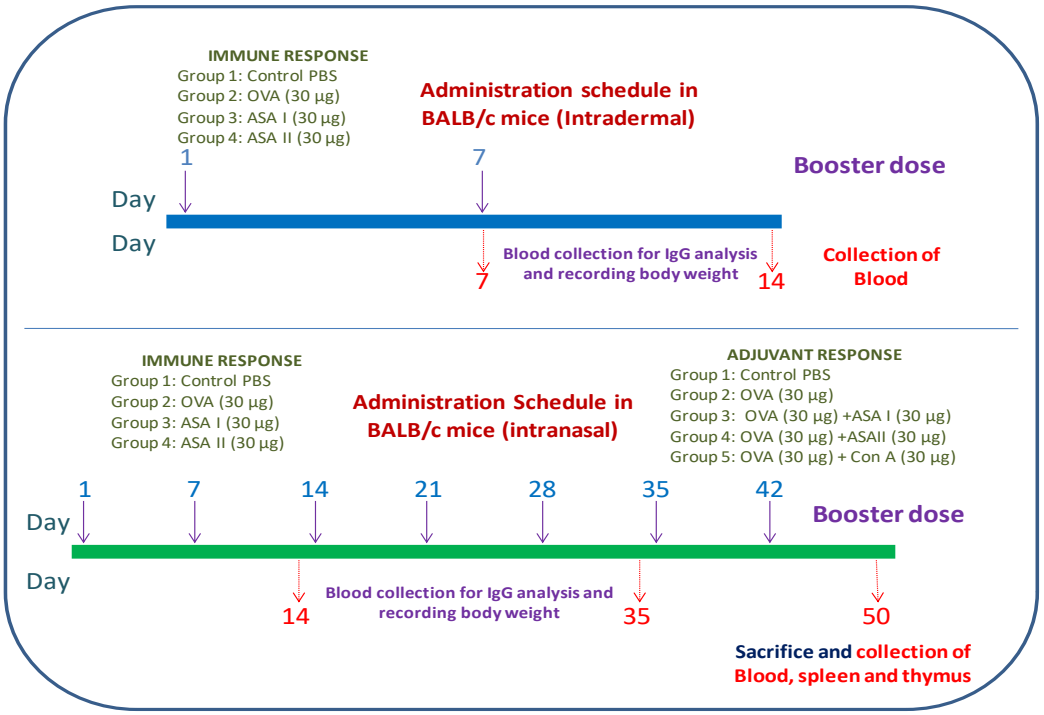


Fig.14: Systemic and mucosal administration schedule of garlic lectins in BALB/c mice model; *Intradermal systemic immunization schedule. The animals were given the initial dose on day 1, and the subsequent booster dose was administered on day 7. Blood was drawn on days 7 and 14 for examining the immune response; Intranasal mucosal immunization schedule. The animals were given the initial dose on day 1, and the subsequent booster doses were administered on 7th, 14th, 21st, 28th, 35th, and 42nd days. Blood was drawn on days 7, 35, and 50 for measuring the immune response.*

4.10.5 Body Weights, Splenic, and Thymic Indices

All animals were maintained in an ambient environment during the experiment and were fed with a commercial mouse diet. The weight of individual mice was monitored at selected time intervals during the progress of the experiments before the administration of test doses by the *i.d.* or *i.n.* routes. The weight of the animals was noted at a selected period of intervals, and the body weights were recorded on the 14th day for the intradermal (*i.d.)* group, and on the 50th day for the intranasal (*i.n.)* group. The change in the animal weights reflects the effect of administrated antigens on animal physiology, which further enermarates growth or retardation. The immunologically responding organs, like the spleen and thymus of the experimental animals, were isolated and collected separately after sacrificing the mice on the last day. The weights of the spleens and thymuses were recorded both in the treated and untreated groups.

The splenic index and thymic index were calculated and were based on the spleen and thymus weights and the body weight. The thymic and splenic indices were calculated as follows: splenic index is measured as spleen weight (mg)/body weight (g), and thymic index is measured as thymus weight (mg)/body weight (g).

4.10.6 Adjuvant Activity of Garlic Lectin against OVA through mucosal immunization

The animals selected for studying mucosal adjuvant activity were immunized as per the schedule on days 1, 14, 21, 28, 35, and 42. The groups were administered with PBS (control), ovalbumin (OVA) alone (30 µg), or OVA (30 µg) blended with either one of Con A (30 µg), ASA I (30 µg), ASA II (30 µg), or RGE (30 µg). The mice were strained and were administered with intranasal dosing of 30 µl of each sample (15 µl was dosed slowly through each nostril) with the help of fine tips attached to a micropipette. The animals were exposed to a low dose of diethyl ether for partial anesthesia and were clutched in place until the liquid was inhaled completely. The serum was isolated from the blood samples collected from the treated and untreated groups on the 14th and 35th days prior to immunization. On day 50, all mice were sacrificed with an overdosing of anesthesia and the blood was collected with cardiac puncture.

4.10.7 Collection of blood from experimental animals and serum separation

The animals were anesthetized, and blood samples were drawn from all the groups at specific intervals by retro-orbital venipuncture with the help of heparinized capillary tubes. On the last day, i.e., the 14th day for the intradermal group and the 50th day for intranasal group during the experiment schedule, the animals were sacrificed by an overdose of anesthesia followed by cardiac puncture. The heparinized bloods collected in the tubes were cotton plugged and kept at 24 ◦C for one hour for clotting. After, the tubes were centrifuged at 2500 rpm for 10 min in the refrigerator centrifuge with the temperature adjusted to 4 ◦C. The upper yellowish clear serum was pipetted to a new tube and stored at −20 ◦C; the same was used to measure the anti-lectin (ASA I and II)-specific IgG antibody response and the anti-ovalbumin (OVA)-specific IgG antibody response on the systemic, mucosal, and adjuvant experimental groups.

4.10.8 Detection of Anti-Lectin (ASA I and II) IgG and Anti-OVA-IgG Antibodies

The anti-lectin IgG and anti-OVA IgG responses on antigen administration through the systemic and mucosal routes was measured in the sera collected from the experimental group by ELISA. The detailed procedure for the experiment for the recognition of the IgG specific to garlic lectin (ASA I and II) and OVA in sera were described earlier (Chandrashekar *et al.*, 2012). Briefly, for the assay, 0.1 mg/ml of antigen samples were prepared in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6); the plates were then coated with 10 µg in 100 µl per well of samples and incubated at 4 ◦C overnight. After, the plates were washed with PBS containing 0.05% Tween (PBS-T) and the plates were further blocked with 2% gelatin prepared in 10 mM PBS and incubated at 37 ◦C for 2 h. Following incubation, the plates were washed and added with appropriately diluted serum samples as a source of primary antibody (mouse serum diluted 1:10 with a dilution buffer containing PBS-T with 1% BSA) and incubated at 4 ◦C overnight. The pooled serum derived from each individual animal of the groups was used as triplicate during the experiment. After the incubation, the plates were washed and incubated at 37 ◦C for 2 h by adding 100 µl/well of goat anti-mouse IgG conjugated to alkaline phosphatase (1:1000 dilution with PBS-T containing 1% BSA) as secondary antibody. Later, with extensive washing, the wells received 100 µl of AP substrate (p-nitrophenyl phosphate at 1 mg/ml prepared in 10% diethanol amine buffer, pH 9.4) and incubated for 20 min, and the reaction was arrested by the addition of 100 µl 3 N NaOH. The absorbance was read at 405 nm using microtiter plate reader.

4.11 Anti-cancer activity of *AsL*

4.11.1*In-vitro* assays

4.11.1.1Lymphocyte proliferation of Garlic lectin (ASL):

4.11.1.1.1 Preparation of complete RPMI-1640 media for proliferation assay

For all experiments, RPMI-1640 cell culture media was employed. For incomplete medium, powdered medium was added to filtered triple-distilled water and gently stirred to dissolve. To completely dissolve the medium, the pH was

brought down to 4.0 using 1 N HCl, and then the pH was brought back up to 7.2 with 1 N NaOH. After making the final volume with water and bringing the pH down to 7.2, tissue culture grade sodium bicarbonate was added. Incomplete medium was supplemented with 10% v/v fetal calf serum, 1% sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml of streptomycin to obtain a complete medium, which was used for proliferation assay (Mosmann 1983).

4.11.1.1.2 Isolation of human peripheral blood lymphocytes (PBLs)

Ten milliliters of venous blood were drawn from healthy normal subjects using a disposable syringe. Five milliliters of each sample were transferred to HiAnticlot vials (heparin-coated flat-bottom polystyrene vials with polypropylene caps from HiMedia Laboratories Ltd., Mumbai, India). To prevent coagulation, the vials' contents were carefully swirled. After a short while, the heparinized blood was carefully deposited onto Ficoll-hypaque, which was housed in a 15 ml graduated polystyrene tube and had a density of 1.077 g/ml. For the purpose of separating erythrocytes, lymphocytes, and plasma according to density, the tubes were maintained at 25°C for 90 min. The tubes were then centrifuged at 250 g for 20 min at 25 °C. Centrifugation at a lower temperature (4°C) was avoided since this result in cell clumping and poor recovery (Colic and Savic, 2000). After centrifugation, below the plasma layer, a circle of white translucent coat containing lymphocytes was aspirated carefully using a Pasteur pipette. The cells were then gently aspirated and resuspended in isotonic phosphate-buffered saline (PBS). The buffy coat contains lymphocytes cleaned using PBS four or five times at 4°C before being finally deposited in full RPMI-1640 media.

4.11.1.1.3 Isolation of murine splenocytes and thymocytes

Spleen and thymus were collected under aseptic conditions from normal BALB/c mice (23-25 g, 12-weeks-old) after sacrification, placed in isotonic phosphate buffered saline (PBS) (Colic *et al.*, 2002). These tissues were separately minced using a pair of scissors and passed through a fine steel mesh to obtain a homogenous cell suspension. The cells were pelleted to remove the tissue debris. After centrifugation (380 x g at 4°C for 10 min), the pelleted cells were washed three

times with PBS (400 x g at 4°C for 10 min) and finally resuspended in complete RPMI-1640 medium.

4.11.1.1.4 Removal of contaminating erythrocytes from the cell suspensions

In order to eliminate the erythrocytes, the pellet produced after washing with PBS was resuspended in a modified ammonium chloride buffer (150 mM NH_4Cl, 10 mM KHCO3, pH 7.4 containing 10 mM sodium edetate) (Boyam, 1968). The cells were centrifuged for 20 minutes at 400 g at 4° C. the pellet was mixed in physiological salt solution (PSS) [137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.6 mM $CaCl_2$, 10 mM HEPES, 0.05% gelatin and 6.45 mM NaH_2PO_4 at pH 7.4], washed three times in the same buffer, and finally resuspended in RPMI-1640 medium.

4.11.1.1.5 Counting of lymphocytes and determination of viability

The isolated lymphocytes from normal human subjects and mice spleen and thymus were counted using crystal violet stain. An aliquot (5 µl) of cell suspension was taken and diluted with 250 µL of diluent buffer (PBS with 1% BSA) to which 10 µL of crystal violet stain (stock) was added. A clean, fine pipette tip was used to charge the mixture to the hemocytometer after it had been let to stand at room temperature for a minute. Leukocytes were counted in the hemocytometer's outer four chambers while being examined through a 10X low power eyepiece. For the proliferation assay, the cell concentration was increased to 2.5 x 106 cells/ml. Trypan blue exclusion was used to determine the percentage of lymphocytes in the isolated cell suspension that were viable. An aliquot of the cell solution was removed, mixed with 0.2% Trypan blue at a 1:1 dilution, and left at 25°C for around two minutes to determine the vitality of the cells. Trypan blue-infused cell slurry was added to a hemocytometer before being examined under a microscope. Dead or partially damaged cells stand out against a light blue backdrop as dark blue because they absorb the dye. The healthy cells seem clean and unmarked by any stains.

4.11.1.1.6 Lymphocytes mitogenicity assay

a. Lymphocytes treatment: Lymphocyte proliferation for *AsL* was done following previously published protocol (Ranganatha *et al.*,2013). Briefly, peripheral lymphocytes were isolated from healthy blood based on lymphotrap density gradient separation method. Isolated cells were cultured in complete RPMI medium as describe (Clement *et al.,* 2010). The cells were adjusted to 2.5 $x10^5$ cell/ml and were seeded as 100µl of cell suspension in 96 well microtiter tissue culture plates and challenged with crude and *AsL* in aCO_2incubator at 37°C with 5% CO_2. Following incubation, $1/10^{th}$ volume of MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were added and further incubated for 4 hr at 37° C, 5% CO_2. After the incubation, 200µl of DMSO was added to each well to dissolve the insoluble blue formazan crystals. The absorbance at 570nm was measured in a microtiter plate- ELISA reader.

b. Effect of AsL in malignant cell proliferation: The cells A375, B16F10, A549, HUH7, NIH3T3 and BEAS-2B were grown in complete RPMI -1640 medium incubated at 37° C ,5% CO_2 and 98% humidity. The anti-proliferative effect of *AsL* (0-100µg/ml) was evaluated by MTT for 48hr as describe above . For every set of experiment 5-flurouracil was used as a positive control and analyzed with compared to the positive control. Each experiment was repeated thrice (Al-Ghorbani *et al.*,2015).

c. IL-2 determination through ELISA: AsL was used to grow and treat the lymphocytes. Supernatant was taken from each group following treatment in order to measure the levels of secreted IL-2. On a 96-well microtiter ELISA plate, 100 ml of serum and 100 ml of supernatant were coated individually with coating buffer 0.05 M carbonate-bicarbonate buffer, pH 9.6, and incubated for an overnight period at 4° C. After washing three times with TBST, the wells were blocked with 3% BSA/5% skimmed milk powder for 1 h at 37° C. Subsequent to washing with TBST, primary antibody 1:1000 dilution of anti-IL-2 in TBST containing 1% BSA was added to the wells and the plate was incubated at 4° C for overnight.

The wells were washed and anti IgG–ALP conjugate (secondary antibody 1:10000 dilution) was added to the wells and incubated for 1 hr. Following the addition of the substrate *p*-nitrophenyl phosphate (100 µl; 1 mg/ml), reaction was terminated by 50µl of 0.1 N NaOH. The absorbance at 405 nm was recorded in a microplate reader.

4.11.2 *In-vivo* assays

4.11.2.1 In-Ova CAM assay

The preliminary Angio modulatory effectiveness of garlic was investigated by *in-Ova* CAM assay. Fertilized hen's egg was procured from Veterinary College Bangalore, Karnataka, India. Before incubation, all of the eggs were cleaned with 70% alcohol. The fertilized eggs were then nurtured for a further 37 hours in a humid incubator. 5 days into the incubation period. On eggs, a window was cut out using sterilized scalpels. These were re-incubated for 24 hours after being sealed with sterile coverslips and cello tapes. After incubation, CAMs were divided into five systems and treated with extracts as doses 1, 2, 3, control alone with rVEGF165, and control without AsL and rVEGF165 were added to the developing fertilized eggs. All in-Ova CAM systems were re-incubated for 48 hours at a humidified incubator following treatment (Vijay Avin *et al.,* 2014). Angiogenesis modulation on *in-Ova* CAMs of treated and untreated were photographed and analyzed.

4.11.2.2 Tumour models and treatment

4.11.2.2.1 Experimental animals

All the animals (Swiss albino mice) experiments were approved by the Institutional Animal Ethical Committee (IAEC), in accordance with the committee for the purpose of CPCSEA guidelines for laboratory animal facility. (Ref No. KSHEMA/IAEC/01/2020 Date: 04.06.2020).

4.11.2.2.2 Ehrlich ascites carcinoma (EAC) tumour model

a) Determination of the Lethal Dose 50 (LD_{50}) of AsL

A "staircase" approach was utilised to determine the LD_{50} of AsL. (Lorke 1983). Swiss albino male mice weighing 27–30g were used, and they were divided into six groups (n = 6 per group). *AsL* was dissolved in 10mM PBS and injected intraperitoneally (*i.p.)* into animals at doses of 100, 200, and 500 mg/kg body weight (*b.w.).* The mice were then monitored continuously for 4 hours for general behavioural, neurological, and autonomic profiles, then every 30 minutes for the following 4 hours, and finally for mortality at the end of 24 hours. Thus, the highest non-fatal and lowest deadly dosages were established.

b) Cell transplantation and tumour development

Swiss albino mice aged 6–7 weeks, weighing 25–30 g, were housed under standard laboratory conditions for acclimatize. the regular maintenance of EAC cell lines in the mice peritoneum through serial transplantation. EAC cells bearing- mice were sacrificed and the EAC cells along with ascites fluid were harvested and ascites volume was recorded. Then, subjected to centrifugation at 3000 rpm for 5 min. Then the cells were washed with ammonium chloride solution to remove the RBC's followed by thrice washing with sterile 10mM PBS to completely remove the ammonium chloride solution. And the pelleted EAC cells were re-suspended in 10mM PBS and stored in cold condition. Proximately 5x 10^{6} cells/ mice (0.5ml) were transplanted to the peritoneum of mice via intraperitoneal (*i.p*) route of administration, the cells were allowed to grow in the mice peritoneum and monitored the weight on daily basis.

c) AsL treatment

After the 4^{th} mice were divided into 3 groups as control, dose-1 (*AsL* 25mg/ kg *b.w*) and dose-2 (*AsL* 50mg/kg *b.w*) on every alternative day.

d)Tumour growth

After treatment, Tumour growth was monitored carefully by weighing the body weight of tumour bearing animals. Finally, tumour growth rate of each group

was determined by subtracting initial body weight from final day body weight. The tumour growth inhibition was calculated by the following formula,

$$\text{Growth (\%)} = \frac{\text{Treated tumour growth (g)}}{\text{Control tumour growth (g)}} \times 100$$

e) Ascites volume

On the 10th day of tumour transplantation, EAC bearing mice of all the group were scarified and collected the ascetic fluid and the volume of ascetic fluid was recorded.

f) Cell count by trypan blue dye exclusion assay

The EAC cells pelleted from the above step were washed with 10mM PBS thrice by centrifugations. The pelleted EAC cells were re-suspended in 10mM PBS and placed on ice. About 10µl of 0.4% trypan blue dye was added each cell suspension and cells were counted using haemocytometer. Total numbers of viable cells were counted by entering number of viable cells present in four squares of haemocytometer using following formula.

$$\text{Total viable cells} = \frac{A+B+C+D}{\text{Total no. of chambers counted}} \times \text{Dilution factor} \times 10^{4} \text{ cells/ ml}$$

g) Survivability

EAC tumour was developed by above described procedure. After onset of tumour establishment, animals were treated with and without *AsL* (0,25,50mg/kg *b.w*) for three doses on every alternative days. After 3rd dose, mice were left and monitored for the maximal survival of animals and was documented in the form of kaplan-Meier survivability curve.

4.11.2.3 Solid tumour model development and treatment

a) Ehrlich ascites carcinoma (EAC) development

Swiss albino mice aged 6–7 weeks, weighing 25–30 g, were housed under standard laboratory conditions for acclimatize. EAC cells bearing- mice were sacrificed and the EAC cells were harvested, then, subjected to centrifugation at 3000 rpm for 5 min. Then the cells were washed with ammonium chloride solution to remove the RBC's followed by thrice washing with sterile 10mM PBS to completely remove the ammonium chloride solution. And the pelleted EAC cells were re-suspended in 10mM PBS and stored in cold condition. Proximately 5x 10^{6} cells/ mice (0.5ml) were re-injected to the right hind limb thorough subcutaneously (*s.c.*) to develop EAC solid tumour. Then, the size of tumour was measured using vernier calipers in daily basis and recorded (Vijay Avin et al., 2014).

b) AsL treatment

After the visible development of the tumour, mice were administered with six doses of *AsL* (0, 25 and 50 mg/kg b.w. *i.p.*, n = 6) for every alternate day and tumour progression was monitored (Vijay Avin *et al.*, 2014).

c) Tumour size

After the 35th day, mice from all groups were killed by cervical dislocation, and the EAC tumour were then detached using sterile forceps, scissors, and scalpels. By using a weighing balance, tumour weight was calculated. All tumour size groups were compared, and photos were taken.

d) Serum collection

For the collection of serum, the blood was collected from all the treated and untreated groups and allowed to clot for 30-40min at 37˚ C. Serum was separated from the clot and any residual insoluble materials removed by centrifugation at 4˚ C for 10 min at 10,000 rpm and used for quantification of RBC, WBC, urea, creatinine, SGOT and SGPT (Prasanna *et al.*, 2008)

A. Acute toxicological studies

i) RBC and WBC count

To collect blood samples, the jugular vein of mice from the normal, *AsL* untreated and treated groups were incised with sterile scalpel and blood was collected separately in fresh tubes (Heparin tubes) containing 0.3% EDTA. the blood samples were used for the counting of erythrocytes and leucocytes levels with haemocytometer using Leishman's staining.

ii) Estimation of urea

Utilizing the serum of experimental animals from AsL-treated and untreated groups, the rate of urea was calculated. 2 grammes of Diacetylmonoxime (DAM) were dissolved in 100 milliliters of 2% acetic acid to create the reaction mixture (2% DAM). Every sample was held in a boiling water bath for exactly 20 min before being cooled and vortexed. At 480 nm, the purple complex was detected against a white background. Using a standard graph, the amount of urea in the serum sample was determined.

iii) Estimation of creatinine

For the creatinine quantification the serum from normal, *AsL* treated and untreated animals following to modified Jaffe's method (Husdan and Rapoport, 1986). The standard creatinine solution was prepared in the concentration of 1mg/ml in distilled water and used as the standard. Working standard creatinine solution was added in microliter plate in the 0-1mg/dl range. The standard solution of different concentrations and serum samples were mixed with 1N NaOH and 1% picric acid. The reaction mixtures were mixed by vertexing for 1min and incubated at room temperature for 15min. the reaction mixture was measured at 500nm using Bio-spectrophotometer (Eppendorf, Germany). The creatinine was measured through the standard curve by taking concentration of creatinine along X-axis and absorbance along Y-axis.

iv) Estimation of SGOT and SGPT

For SGOT and SGPT quantification the serum from the normal, *AsL* treated and untreated animals were collected. Sodium pyruvate standard solution was prepared in distilled water used as the standard. Working standard was added in microtiter plate in the 0-1mg/dL range. Substrate buffers for SGOT (L-Aspartate and α- Ketoglutarate) and SGPT (L-Alanine and 2-Oxoglutarate) were prepared in 0.1M sodium phosphate buffer pH 7.4. The standard solution of different concentrations, control (only substrate buffer) and test sample (substrate buffer + serum sample) were incubated for 15mins at 37 °C. Followed by the addition of 2,4Dinitrophenyl hydrazine (2,4 DNPH) and incubated for 10mins at room temperature.

The reaction was halted by the addition of 0.4N NaOH, reaction mixture was observed at 540nm in Bio-spectrophotometer (Eppendorf, Germany). SGOT and SGPT was measured through the standard curve by taking concentration of sodium pyruvate along X-axis and absorbance along y-axis.

B. ELISA

The in-vivo cytokines secretion of Interleukin-1 (IL-1), Interleukin-6 (IL-6), Tumour necrosis factor (TNF-α) and VEGF-A levels were quantified by ELISA methods as previously described (Prabhakar *et al.,* 2006) the protocol explained below and experiment were carried out thrice independently.

- ***Sample preparation:*** The serum from with or without *AsL* treated ***animals were collec***ted by cardiac puncture after sa*cri*fice. The blood sample was centrifuged at 3000rpm for 20min and serum was carefully aspirated to evaluated the serum IL-1, IL-6, TNF-α and VEGF-A.

- ***Coating:*** In 96 well microtiter ELISA plates were coated with 100µl of ***serum*** using multichannel pipette (Eppendorf, Germany) with c***o***ating buffer (0.05M sodium bicarbonate, pH-9.6) and incubated at 4°C for 16hrs.

- ***Blocking:*** The incubated plates were washed thrice with TBST (0.05M TBS pH-7.4, 0.1% Tween 20) for every 10min. Followed by blocking with

blocking buffer (5% skimmed milk powder in TBST) to the plates for about 1hr at room temperature.

- ***Primary antibody binding:*** After blocking, blocking buffer was discarded and washed thrice with TBST buffer for 10 mins each and plates were incubated with 100µl of respective primary antibodies mix (1:1000 of anti-IL-1, anti-IL-6, anti- TNF-α and anti-VEGF-A with blocking buffer, pH-7.4) to each well for 2hr at 37° C.

- ***Secondary antibody binding:*** Plates were washed with TBST for 10 min each and incubated with 100µl secondary antibodies mix (1:10000 of anti-IgG conjugated with ALP: blocking buffer, pH -7.4) for 2 hr at 37° C.

- ***Quantification:*** Finally, plates were washed thrice with TBST buffer for 10 min each and incubated 100µl of pNPP substrate solution. The reaction was ended using 50µl of 0.1N NaOH and absorbance was read at 405nm in ELISA reader (Rayto, India).

C. Survivability

The number of animals that survived and the duration after the treatment regimen were documented in a separate analysis in the form of kaplan-Meier survivability curve.

D. Hematoxylin and Eosin (H&E) stain for EAC treated tumour tissue

The H & E staining was performed to quantify the EAC treated and untreated tumour groups and analyzed.

a. Preparation of microsections

The formalin (10%) fixed tissues were treated with the alcohol (70%, 80% and 95%) for about 2hr. Then tissues were placed in xylene (80% and 95%) for 1hr to remove the alcohol and tissues were immersed in the paraffin wax for 1hr. After 1hr, Using Mayer's glycerol-albumin combination, the segment was mounted to the microscopic slides after being cut at a thickness of 5 m. To stain the tissues, slides were submerged in xylene (95%, 80%, and 70%) for 5–10 minutes to dissolve the

paraffin wax, followed by hydration with alcohol (95%, 80%, and 70%) and finishing with distilled water.

b. Staining

The staining was carried out first with haematoxylin for 30 min and rinsed with tap water and dipped with 0.5% HCl for few seconds. The slides were place in 0.2% ammonia water and washed with running tap water. Slides dipped in 95% alcohol followed by eosin for 1min and slides were placed in the alcohol for 2-5min and lastly with xylene. The slides were fixed with DPX with coverslip and observed under the microscope and documented.

4.12 Statistical analysis

The data were analyzed and graphs were made using MS Excel version 10. Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Student's t-test. Values are expressed as mean of triplicate values± standard deviation (S.D.). Values of $*p< 0.05$ and $** p< 0.01$ were considered statistically significant.

Results

5. Results

5.1 Raw Garlic Extract (RGE) with potent lectin activity mitigates neoplastic malignancy targeting angiogenesis and cell death

5.1.1 RGE extract exerts lectin activity:

Fresh Garlic bulb extracts were prepared and were checked for protein isolation and the presence of garlic lectin the extractions. The extraction of lectin was confirmed by hemagglutination activity.

5.1.1.1 Preparation of crude garlic extract:

About 100g of garlic bulbs were extracted with double distilled water, 10mM phosphate buffer saline pH-7.4, 50mM sodium acetate buffer pH 4.0 and also crude garlic extract obtained through heat process. The results were displayed in the Table.6. Concentration of protein were estimated using lowry's method as described above and obtained protein concentration are 8.6mg/g, 12.5mg/g, 7.5mg/g and 3.5mg/g. the results clearly exhibit that RGE extracted with 10mM PBS contains slightly increased in the concentration than compared to the RGE extracted with double distilled water, 50mM sodium acetate buffer and heat processed. In 50mM sodium acetate buffer, protein concentration was slight less than compared in 10mM PBS buffer due to its acidic nature protein may reduce its activity. Heat processed crude raw garlic shows declined protein concentration. When garlic processed with heat, it gets clamped due to its clamping nature some proteins are insoluble and shows less protein concentration. Eventually, using 10mM PBS extracted RGE yields more protein concentration.

Table 6. Extractions of crude garlic and their haemagglutination activity:

Extraction Method	**Protein yields (mg/g)**	**HA activity (Units/mg/protein)**
RGE (Double distilled water)	8.6	22.6
RGE (10mM PBS pH 7.2)	12.5	25.9
RGE (50mM sodium acetate pH 4.0)	7.5	19.7
Heat processed RGE (10mM PBS pH 7.2)	3.5	9.1

5.1.1.2 Haemagglutination activity of crude garlic:

To confirm the presence of the lectin in RGE extract hemagglutination assay was done using erythrocytes of chicken, human (all blood groups) and rabbit. About 5ml of chicken and human blood samples were collected in the 5ml of Alsevier's solution separately; about 2ml of rabbit were also collected in equal volume of Alsevier's solution. In all blood samples, erythrocytes were dissociated by 1% trypsinization. Using these erythrocytes haemagglutination activity was examined. Where the observed results infer that RGE significantly agglutinated the erythrocytes from rabbit (Fig 14). An experiment on the chicken and human erythrocytes does not show any visible agglutination (Fig 15A & B). There may be possible difference in the glycosylation on the surface of erythrocytes and the representation of terminal sugar moieties on the RBC's. These clearly explain the specificity of RGE towards rabbit erythrocytes. The summarised result was shown in Table 7.

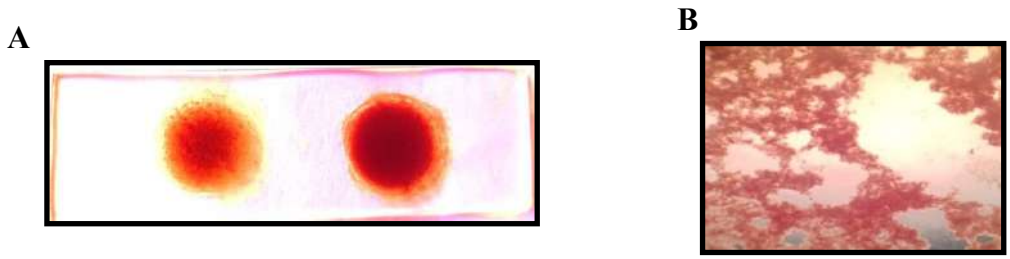


Fig.15: Rabbit erythro-agglutinating activity of RGE: [A] *Agglutination of 1% trypsinized rabbit erythrocytes by extracted RGE sample at two different concentrations (a) 10mg (b)20mg.* ***[B]*** *Microscopic view of agglutinated rabbit blood.*

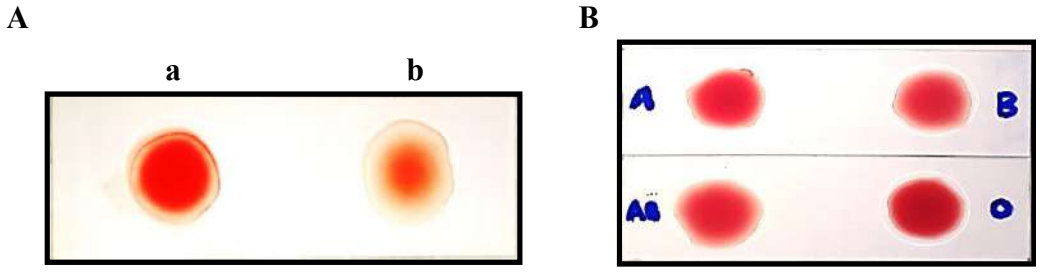


Fig.16: [A] Investigation of hemagglutination in chicken RBC's: *(a) No agglutination of 1% trypsinized chick erythrocytes by RGE. (b) No agglutination is visualized in chick RBC's (which is suspended only in phosphate saline buffer) by RGE extract.* ***[B] Human erythrocytes:*** *No agglutination of 1% trypsinized human RBC's by RGE extract. RGE extract not agglutinating erythrocytes of different human blood types.*

Table 7. Comparative specificity of garlic lectin towards RBCs and peripheral lymphocytes agglutination in raw garlic extract (RGE)

Cells types	Agglutination	Agglutination visibility
Rabbit RBCs	+++	High Visible agglutination on slides
Chicken RBCs	-	No agglutination
Human A group RBCs	-	No agglutination
Human B Group	-	No agglutination
Human O Group	-	No agglutination
Human peripheral Lymphocytes	++	Microscopic agglutination visibility

5.1.2 RGE enhance immune cell proliferation in in-vitro

Human PBL's were separated from the blood sample through density gradient centrifugation using Ficoll-hypaque (density=1.077g/ml). Obtained white translucent buffy coat containing lymphocytes below the plasma layer were collected and the cell concentration was adjusted to $2.5x10^6$ cells/ml. The seeded 100µl of cell suspension in 96 well microtiter tissue culture plates and treated with RGE, ConA and RGE+ConA. The lymphocyte interaction and proliferation were assessed by MTT assay at an increasing concentration of 0.01, 0.1, 1 and 10 µg/mL (Fig17).

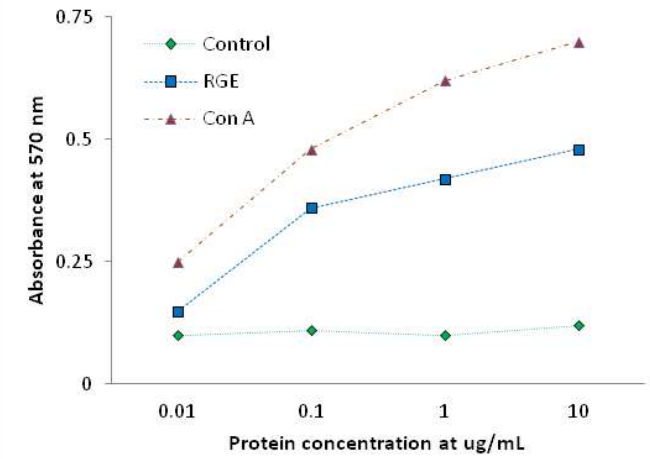


Fig.17: The protein concentration used in the range of 0.01 to 10 µg/ml. *ConA was used as a reference positive for lymphocyte proliferation and control is not added with any protein. the results confirm the immunogenicity of RGE similar to that of ConA.*

Obtained results were clearly explained the immunoproliferative properties of RGE. RGE (5µg/ml and 10µg/ml) triggered the potent mitogenic response to the human lymphocytes upon dose dependent manner, which shows more equivalent to the Con A. Con A was used as the positive control, treated along with RGE and the results was very significant and displays the lymphoproliferative properties (Fig 18).

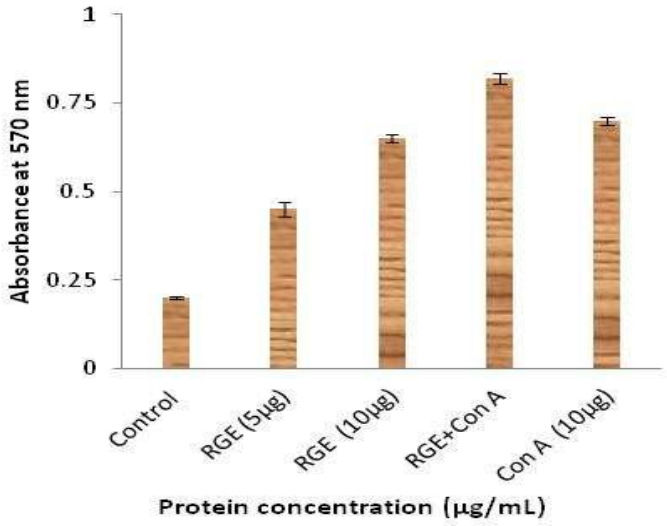


Fig.18: The immunomodulatory effects of RGE (10µg/mL) on human peripheral blood lymphocytes (PBLs): *Both RGE and ConA treated independently shows the significant responses. ConA treated along with RGE represents a fold increase response as compared to that of control. Con A was used as a positive control.*

5.1.3. RGE inhibits cancer cell proliferation

RGE was examined for cytotoxicity and anti-proliferative properties against a variety of cancer cells, including human A375, A549, HUH7, and BEAS-2B cells, as well as murine B16F10 and NIH3T3 cells. According to three independent MTT assays, the IC_{50} values against each of the cell lines were A375 - 15.5 g, B16F10 - 18 g, A549 - 14 g, HUH7 - 19 g, NIH3T3 - 96 g, and BEAS-2B - 89 g. (Table 8). The findings make it clear that all four malignant cell lines were extremely susceptible to RGE, which hindered the ability of the aforementioned cells to proliferate. In contrast, even at a concentration of 50 g/ml, RGE had no effect on the non-cancerous NIH3T3 and BEAS-2B cells.

Table.8: IC_{50} value of RGE against various human and mouse cancer cell lines

Cell lines	IC $_{50}$ values (µg)	5-Fluorouracil (µg)
A375	15.5±1.5	4.5±0.6
B16F10	18.0±0.8	10.0±2.0
A549	14.0±0.5	7.5±0.4
HUH7	19.0±1.7	5.13±0.16
NIH3T3	96.5±3.9	85.0±4.3
BEAS-2B	89±2.5	60.0±1.5

5- Fluorouracil is used as positive control. Cytotoxicity was measured by MTT assay against cell line in three independent (n=3) DMSO was used as a vehicle control which showed very negligible cytotoxicity. Values are indicated in mean ± SD.

5.1.4 Anti-angiogenic activity of RGE

VEGF is one of the most censorious and specific angiogenic factor of vascular endothelial cells for regulating angiogenesis. Specific, reliable angiogenesis assays simultaneously assessed antiangiogenic target activity (CAM-Chorioallontoic membrane assay) which are induced by $rVEGF_{165}$. Fertilized hen's eggs were incubated at 37°C in a humidified incubator. On the 5^{th} of incubation, a small window was made and treated with $rVEGF_{165}$ and RGE at two different concentrations (5µg/mal and 10 µg/mL). The windows were closed using sterilised coverslips and incubated for 48hr at humidified condition. All treated and untreated were opened and observed the for the visible vascularization results. The zone of a vascularization shows that RGE reduced the neovasculature by 86% and 75%, respectively, in the developing embryos in ova CAM with increasing concentration. The results were shown in Fig 19A and B.

Ova CAM images clearly indicate the decrease in the neovascularization as appeared with decreased angiogenesis intensity. The blood vessels intensity was compared for garlic lectin with the positive treated rVEGF. The Graphical images illustrate percentage anti-angiogenic potential of RGE. Treatment with RGE displayed 86% and 75% inhibition of CAM vessel density. RGE treated displays the inhibition of vessel density in CAM where the concentration of RGE was increased the density of the blood vessel drops.

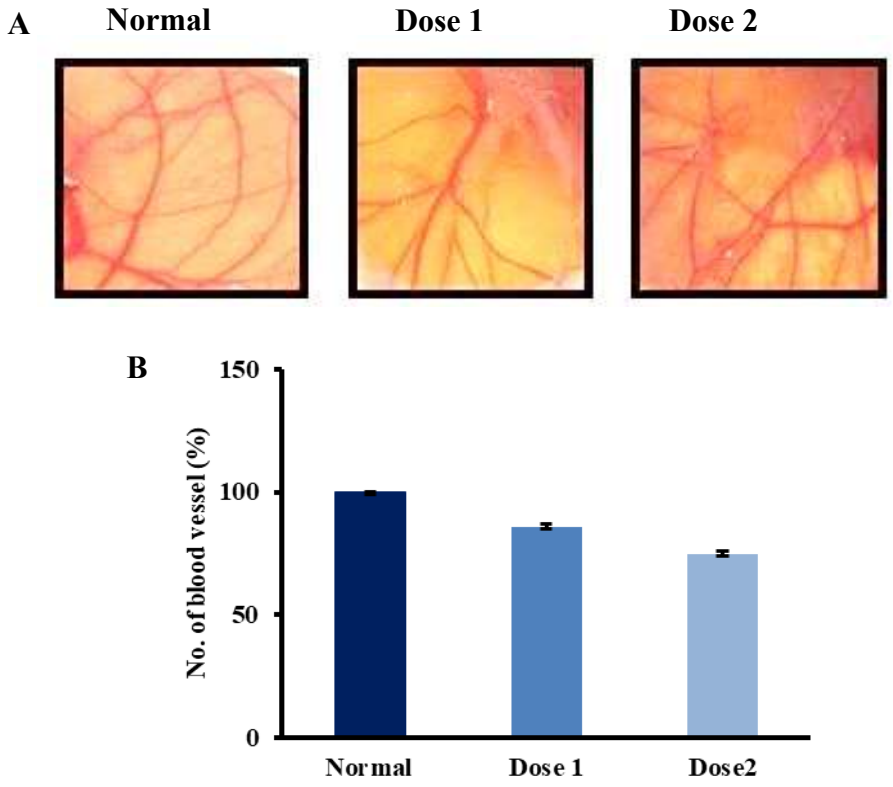


Fig.19: Antiangiogenic activity of RGE in Ova angiogenic assay models. *Ova CAM and Graphical images with anti-angiogenic potential. Treatment with RGE displayed 86% and 75% inhibition of CAM vessel density. RGE treated displays the inhibition of vessel density in CAM where the concentration of RGE was increased the density of the blood vessel drops. Results are the means of three determinations, each conducted in triplicates. Statistically significant values are *p< 0.05; **p< 0.01.*

5.1.5 Anti-tumour activity of RGE in EAC *in-vivo* model

RGE extracts' cytotoxic effects were subsequently confirmed in *in-vivo* tumour models. By administering Ehrlich ascites carcinoma, ascetic tumour models were created (EAC). Ehrlich ascites carcinoma, which forms the ascetic tumour, is usually maintained in the peritoneum in mice by serial transplantation. Swiss albino mice were administrated with EAC cell lines and the cell were allowed to grow in the mice peritoneum in a form of ascetic tumour. The animals were observed for the increase in the body weight on daily basis up to day 4. After day 4, mice were treated with RGE (0, 25,50mg/kg *b.w*) for three doses *(i.p)* on alternative days.

Tumour growth was monitored by weighing the body weight of tumour bearing animals. On the day 10 all tumour bearing mice were sacrificed, EAC cells along with ascetic fluid were harvested. In ascites tumour model, administration of RGE extracts regressed dose dependent decrease in the tumour weight ~35% (Fig 20A) Decreased tumour volume corresponds to dose dependent reduction in ascites secretion to final 4.5ml compared to 8.5ml in untreated, show 2-fold decreasing tumour growth compared to the control (Fig 20B). The tumour volume and ascites secretion synchronized with the cell count number. As RGE dosage is increased, the number of cells decreases, demonstrating RGE's anti-tumor properties (Fig 20C).

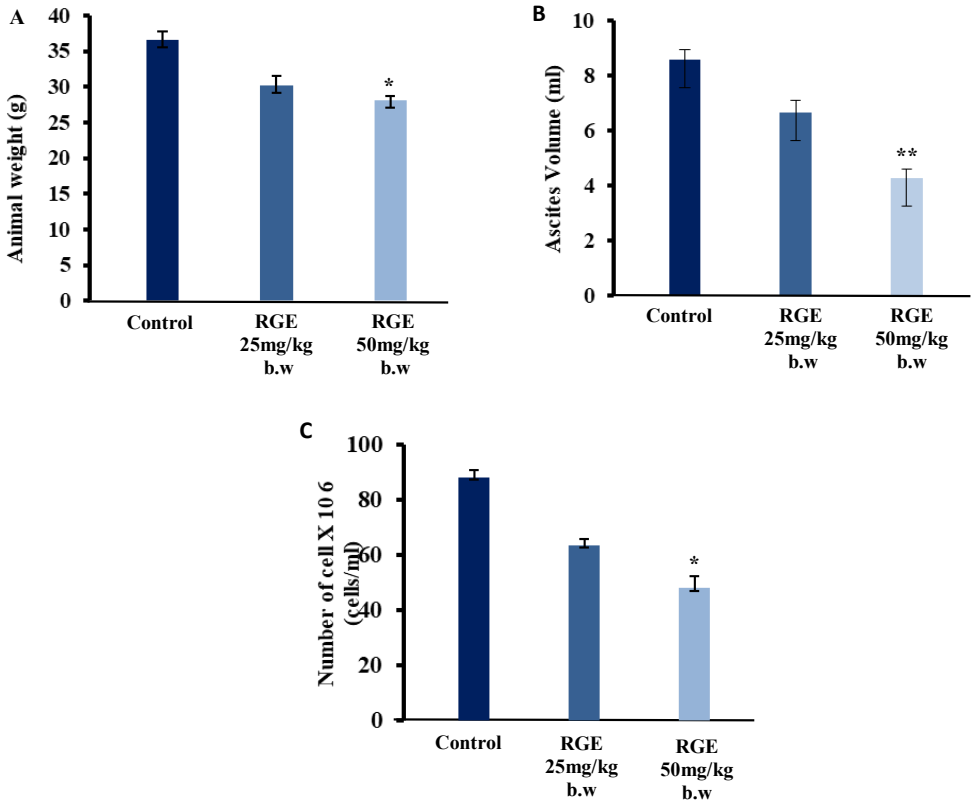


Fig.20: Ascites tumour was induced in Swiss albino mice by injected EAC cells (5 x 10^6 cells/mice) intraperitoneally. *[A] Regressed tumorigenic index in the dose dependent manner indicative of the reduction in the tumour growth. [B] Decrease in ascites secretion. [C] Declined tumour cell population. All the parameters were evaluated in comparison to the relevant control tumour bearing mice. Results are the means of three determinations, each conducted in triplicates. Statistically significant values are *p<0.05; **p< 0.01.*

The mice were allowed to the maximum survival, survivability was noticed and documented in the form of Kaplan-Meier survivability curve. The anti-tumour activity has concomitantly influenced the survivability of the animal which has extended the survival rate from 10days to 36days (Fig 21). The RGE had considerably increased the survivality days for the mice.

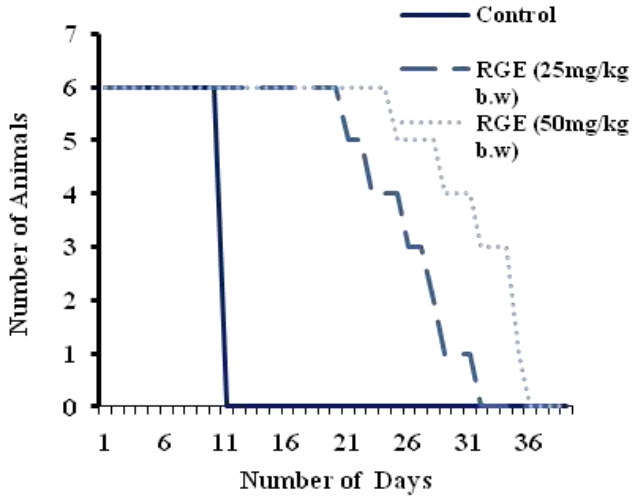


Fig.21: Survivability of treated and untreated mice: *Increased the survival rate as compared to that of control from day11 to day36. Kapler-Meier graph depicting the RGE-treated rats' increased lifespan in a dose-dependent way. The findings represent the average of three determinations that were made in triplicate for each.*

5.2. Purification of garlic lectin (*Allium sativum* lectin-*AsL*) from RGE:

The garlic lectin (*AsL*) was isolated and purified from raw garlic extract. Initially, RGE PBS extract was subjected for Ammonium sulphate precipitation with percent fractionation. The isolation and presence of garlic lectin in the fractions were confirmed by hemagglutination. The purity was checked by SDS-PAGE. Further, the garlic lectin (*AsL*) was purified through Sephadex G-75 and the fractions were confirmed and analysed for homogeneity by C18 protein HPLC.

5.2.1.RGE fractionation by Ammonium Sulphate Precipitation (ASP) and dialysis

Garlic lectin (*AsL*) was partially purified from the crude garlic bulb extract RGE, initially through ammonium sulphate precipitation at different saturation fraction such as, 0-20%, 20-50% and 50-90%. Thus, 0-20% saturation was achieved by the addition of 11.3g of ammonium sulphate with gentle mixing on magnetic stirrer and 20-50% saturation was achieved by 18.8g of ammonium sulphate addition. Larger proteins get precipitated at 0-20% and 20-50%. Finally, 50-90% saturation was made by the addition of 29.2g of ammonium sulphate. At 50-90% saturation fraction smaller proteins get precipitated. The resolubilized precipitates were further followed up through dialysis for all the three different saturation fractions and the dialysed fraction were concentrated using Amicon ultracentrifuge tubes for concentration of proteins and the removal of salts and low molecules. Samples obtained were stored at -20°C for further use. All the saturation fractions of ammonium sulphate precipitated and dialyzed samples were confirmed for the consistent activity of lectin by hemagglutination assay (Fig 22). The dialysed fraction samples with 50-90% saturation had shown higher agglutination activity that indicate the isolation of sample in tat fraction.

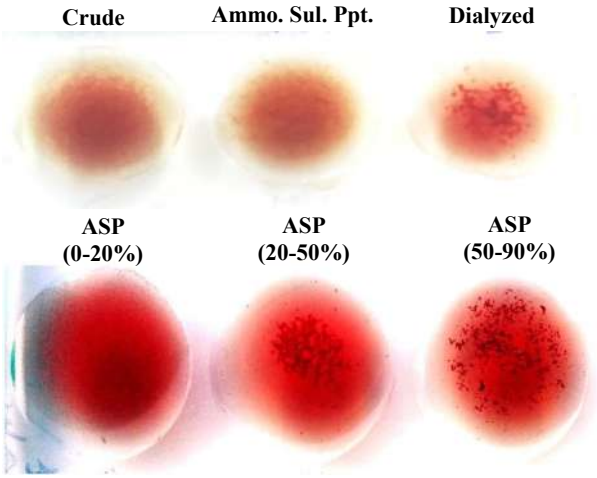


Fig.22: Erythro-Agglutinating activity of AsL: *AsL potently agglutinates 2% trypsinized rabbit RBC's at different purification steps during ammonium sulphate precipitation.*

Protein concentrations were measured by lowry's method and comparative concentration and HA activity was shown in the Table. 9. As protein turns into the purified form, the concentration of a protein gets increased with increased hemagglutination activity that indicated the presence of partially purified garlic lectin (PP *AsL*) in the fractions. The highest amount of protein isolation was found in the fraction with 50-90% ammonium sulphate precipitation and the same fraction was also found to exhibit highest hemagglutination activity. The dialysed fraction after concentration had shown high protein amount after removal of salts and also had considerable increase in the hemagglutination activity.

Table.9. Protein yield and HA activity at various steps of protein fractionation

Extraction Method	**Protein (mg/g)**	**HA activity (Units/mg/protein)**
ASP (0-20%)	7.8	20.4
ASP (20-50%)	10.5	27.4
ASP (50-90%)	18.8	49.16
Dialyzed and concentrated 50-90% ASP	25.6	66.94

5.2.2. SDS-PAGE of ASP fractions for protein profile analysis

The protein profile of the fractions and molecular mass of the fractionated protein was estimated by the performing 15% SDS-PAGE (reducing) after subjecting to the Coomassie brilliant blue for visualization. Dominant two bands were visualized at 12kDa and 80kDa (Fig 23). 12kDa protein is confirmed based on the literature evidence as protein representing garlic lectin (*AsL*) as it was appeared as thick dominant band. Other 80kDa was predicted as the Allinase, components in the RGE. The partially purified AsL represented at lane 5 after dialyses 0f 50-90% ASP fraction has a very minor contaminant band from 100 kDa to 30 kDa and a doublet large band at 12 kDa that represent the garlic lectin.

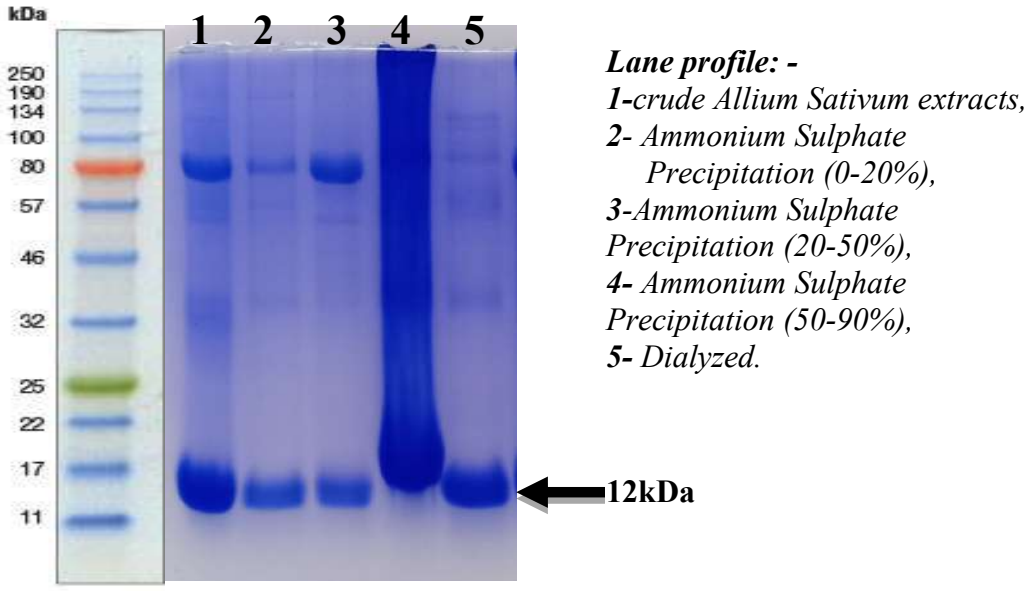


Fig.23: Partially purified garlic lectins protein SDS-profile: *Molecular mass determination of partially purified AsL by SDS-PAGE under reducing condition. SDS-PAGE subjected to the Coomassie brilliant blue staining to visualize the bands. Dominant protein band determines the presence of lectin at molecular weight of 12 kDa.*

5.2.3. Sephadex G-75 gel permeation chromatography:

The dialysed PP*AsL* was concentrated by Amicon ultra-filtration. The concentrated PP*AsL* was further subjected to the gel permeation chromatography for purification of lectin. About 2% of ultra-filtrate lectin sample was loaded to the preequilibrated column by 10mM PBS buffer pH-7.4. Fractions were collected as 3ml with the flow rate of 3ml/8min. 2X of the bed volume was collected to make sure sample entered the column was collected. The eluted fractions were monitored for protein by absorbance at 280nm. The results demonstrate with the, among four peaks, Peak-1 is the Allinase peak, peak-2a and peak-2b (Shoulder peak) are garlic lectin peak of two subunits of slight similar molecular weight (Fig 24). Peak-3 represents the low molecular weight protein. Peak-2a and Peak-2b contains doublet band representing slightly variation in the molecular weight with a similar molecular weight of 12 kDa and 12.5 kDa with two homo or heteromeric subunits of garlic lectin as *AsL*-2a (ASA I) and *AsL* (ASA II).

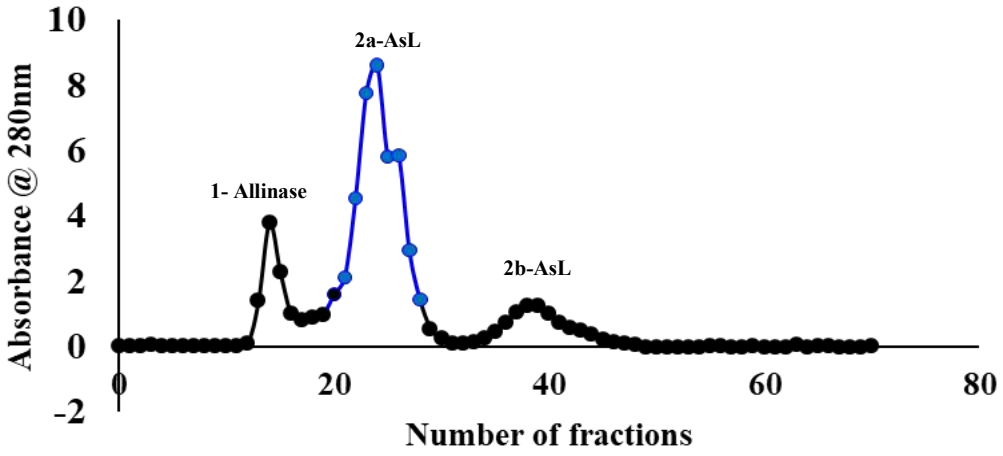


Fig.24: Partially purified garlic lectins gel permeation Chromatography: *Gel permeation Chromatography of AsL purification on Sephadex G-75 Column using 10mM PBS pH 7.4 as eluent at 25°C. Protein elution was monitored at 280nm.*

5.2.4 Final purification by High performance liquid chromatography:

A peak obtained in Gel permeation chromatography was further subjected with C18 HPLC to check the homogeneity along with SDS PAGE. The purified fraction from Peak-2a appears as a single peak with a retention time of 20.4 mins and the result of HPLC was shown in Fig 25. The purified garlic lectin protein was identified as 95% homogeneity.

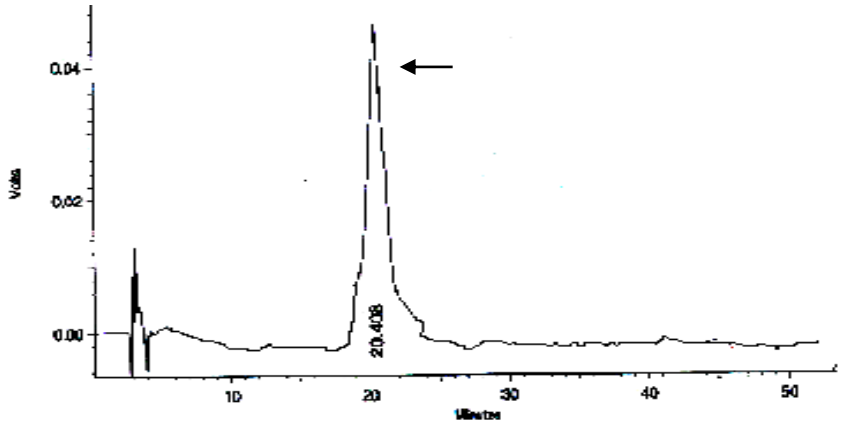


Fig.25: Reverse-phase HPLC (C_{18}) profile of purified garlic lectin AsL: *Protein load- 1.6 µg in a volume of 20 µL. Protein detection: 230 nm. The arrow represents the major peak with a retention time of 20.4 min. Column: C_{18} (4.5 x 250 mm; particle size 5µm). Elution: binary gradient of solvent A (0.1% TFA) and solvent B (80% acetonitrile in 0.05% TFA) at a flow rate of 1 mL/min.*

5.2.5. Comparative HA activity and protein yield during *AsL* purification steps

The specific hemagglutination activity and the protein yield during the purification steps was monitored and represented in Table 10. The final *AsL* protein yield from 100 g of garlic bulb is 42.66 mg. The specific hemagglutination activity of *AsL* was increased with each purification steps confirming it increased purity. The *AsL* HA activity was increased from 20 units/mg (crude extract) to 160 units/mg (*AsL*) at the ASP purification level. The yield of *AsL* was approximately 114.02mg from 100g of garlic bulbs. The HA specific activity of purified pooled fraction of Sephadex G-75 is 628 HA Units/mg with a recovery percentage of 10.53% of protein.

Table 10: Purification of garlic lectin from garlic bulb (100g)

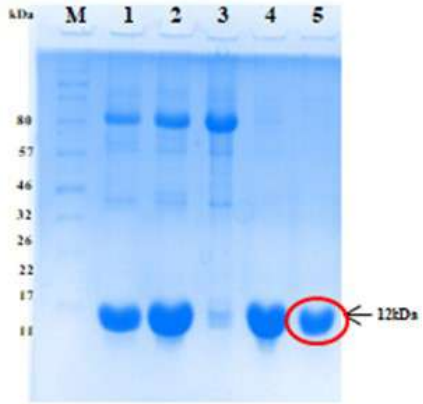
Extraction Method	Total HA activity (Units)	Concentration (mg)	Specific Activity (Units/mg)	Recovery (%)
Crude Extract (50% w/v)	64,780.0	3239.0	20	100.0
Precipitation by Ammonium Sulphate at 50%- 90% saturation rate	28,034.4	350.43	80	43.27
Post-Dialyzed by Amicon Ultracentrifuge	18,243.2	114.02	160	28.16
Sephadex G-75 Pooled peak 2a & peak -2b	6,825.2	42.66	628.00	10.53

5.3. Electrophoretic analysis of purified garlic lectin (*AsL*)

The purified garlic lectin *AsL* was analysed by reducing and non-reducing SDS-PAGE to confirm the subunit nature of the lectin. Further the lectin was assessed by native Page and SDS-PAGE with silver staining and PAS staining to confirm its purity.

5.3.1. Purified *AsL* SDS-PAGE protein profile under reducing condition:

Pooled components of peak-2 and peak-3 obtained from the sephadex G-75 gel permeation chromatography including the active *AsL* were subjected to the SDS-PAGE under reducing condition to know the protein profile of the *AsL*. An intense band obtained at the molecular weight 12 kDa represents the *AsL* Fig 26.

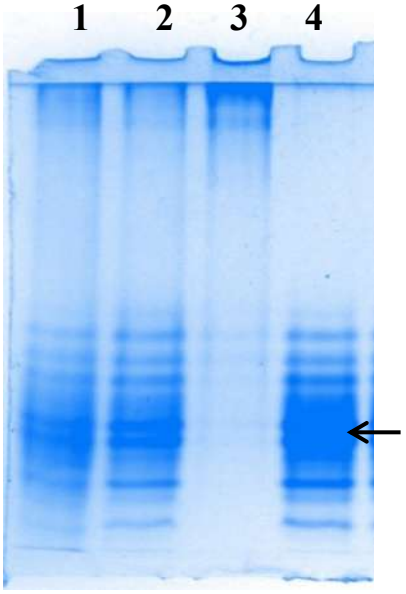


Lane Profile: -
M*- Pre-stained protein marker,*
1 *- Crude Allium Sativum extracts,*
2 *- Dialyzed sample,*
3 *- Peak-1,*
4 *- Peak-2a (AsL),*
5 *- Peak-2b (AsL).*
protein fraction with apparent masses ranging from 12kDa.

Fig.26: SDS-PAGE reducing (15% gel) of AsL: *Components obtained in sephadex G-75 gel permeation chromatography. The molecular weight was monitored by the pre-stained markers. The intense band at molecular weight 12 kDa is AsL.*

5.3.2. Purified *AsL* SDS-PAGE protein profile under native condition:

Native gel electrophoresis was performed to observe the protein profile of the *AsL* in its native state. The protocol was followed as described for native PAGE (12%) with slight modification. The bands obtained in their native form having various isoforms. A thick band obtained between the two thin bands was considered as *AsL* and was identified with negative charge. The protein resolved due to slight change in charge and isomeric nature and moved extensively toward positive charge pole Anode indicating the *AsL* protein subunits are negatively charged and acidic in nature Fig 27.

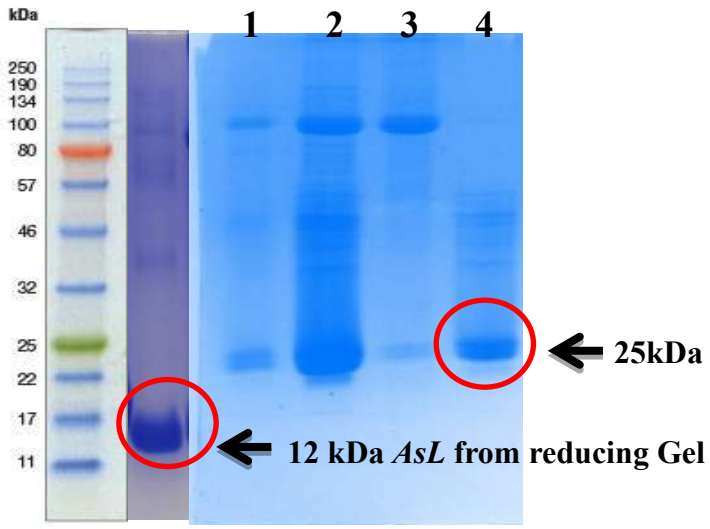


Lane Profile: -
1 *- Crude Allium Sativum extracts,*
2 *- Dialyzed sample,*
3 *- Peak-1,*
4 *- Peak-2.*
protein fraction with apparent masses ranging from 12 kDa.

Fig.27: Native gel electrophoresis*: The protein profile was observed under the native state of the AsL. Obtained thick band at the molecular weight ~12kDa between the two thin bands ranging 10-17 kDa was considered as AsL.*

5.3. 4. Purified *AsL* SDS-PAGE protein profile under non-reducing condition:

Under non- reducing condition the purified *AsL* protein profile was observed with its native form without breaking their disulphate bond with β-mercaptoethanol. *AsL* can also clearly observed at non-reducing condition. The *AsL* displayed the molecular mass of 25 kDa in the protein profile represented in the Fig 28. The comparative reducing gel with a band representing molecular mass of 12 kDa was shown in the same figure.

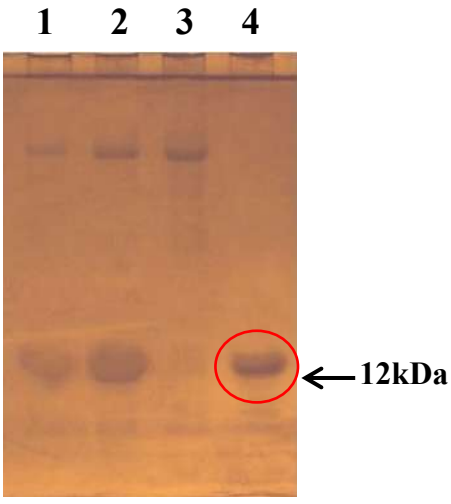


Lane Profile:-
1 *- Crude Allium Sativum extracts,*
2 *- Dialyzed sample,*
3 *- Peak-1,*
4 *- Peak-2.*
protein fraction with apparent masses ranging from 25 kDa.

Fig.28: Non-reducing gel electrophoresis: *AsL was purified by passing through gel permeation chromatography. And peak obtained are loaded on SDS-PAGE (15%) under non –reducing condition to visualize the band pattern of the sample. It clearly visualized at the molecular mass of 25 kDa AsL was purified.*

5.3.5 Purified *AsL* with reduced SDS-PAGE and silver staining:

Purified *AsL* was loaded in SDS-PAGE (15%) under reducing, and subjected to silver staining (sensitive staining). On staining with silver stain the purified fraction of *AsL* showed a single homogenous band at 12 kDa represented in Lane 4 of Fig 29.

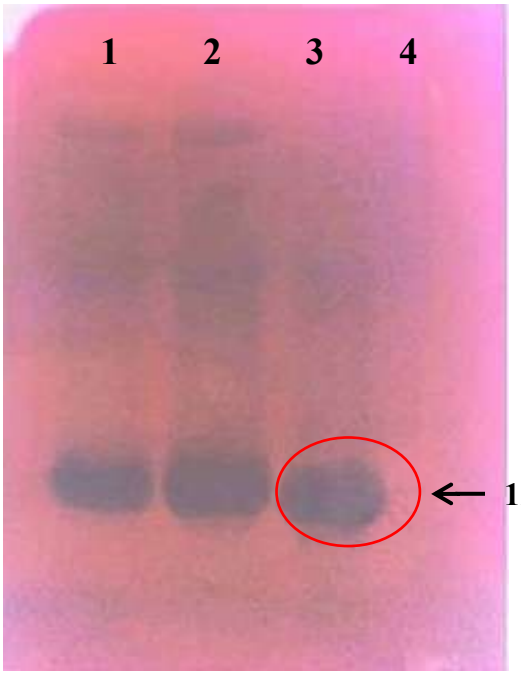


Lane Profile:-
1 *- Crude Allium Sativum extracts,*
2 *- Dialyzed sample,*
3 *- Peak-1,*
4 *- Peak-2.*
protein fraction with apparent masses ranging from 12kDa.

Fig.29: Silver staining gel electrophoresis: *SDS-PAGE (15%) under reducing condition with AsL and various purified fraction from gel permeation chromatography were observed under silver staining. The band was intensive and common at 12 kDa.*

5.3.6. Purified *AsL* SDS-PAGE protein (Periodic acid Schiff) PAS staining:

Peaks obtained in the gel permeation chromatography were examined for the presence of glycoprotein by subjecting to the PAS staining. On PAS staining *AsL* band appears pink in colour upon the clear background and exhibits the presence of glycoprotein. BSA was used as negative control (Fig 30).



Lane Profile: -
1 *- Crude Allium Sativum extracts,*
2 *- Dialyzed sample,*
3 *- Peak-2,*
4 *– BSA.*
protein fraction with apparent masses ranging from 12kDa.

Fig.30: PAS staining gel electrophoresis: *Peaks of AsL obtained by gel permeation chromatography, representing the molecular mass of 12 kDa at various SDS-PAGE conditions. PAS staining exhibits the presence of glycoprotein nature of AsL.*

5.4. Purified *AsL* exhibits potent biological activity

The Purified *AsL* was further studied to assess the biological activity with hemagglutination and leucoagglutination assay to identify its glycocode to predict sugar specificity. The study further assessed for thermal stability, pH stability, pepsin digestive stability, antioxidant and immunogenic property.

5.4.1. Confirming the lectin activity of purified *AsL* protein HA assay:

The eluted protein fractions from Sephadex G-75 gel permeation chromatography was immediately analyzed for the lectin activity, surprisingly peak-2a and peak-2b displayed cell agglutination activity in contrast to the flow through fraction (Fig 31)

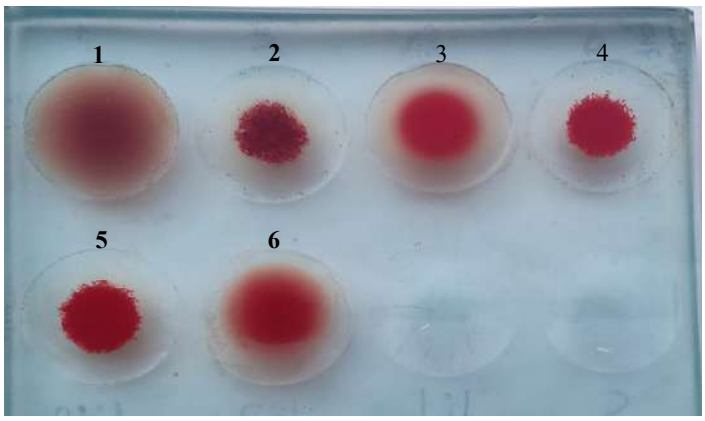


Fig.31: Hemagglutination activity: *[A] Agglutination of 1% trypsinized rabbit erythrocytes by extracted garlic sample. 1-Crude sample (20µg), 2- Dialyzed Sample(10µg), Sephadex G-75 gel permeation chromatographic purified peaks (3- Peak-1, 4- Peak-2a, 5- Peak-2b, 6-Peak-3).*

The microscopic view of agglutinated 2% rabbit erythrocytes with and without *AsL* was shown in Fig 32. The Peak-2 representing *AsL* fraction showed highest hemagglutination activity and the same was shown in the microscopic wherein clear appearance of RBC cells agglutination with lectin sample.

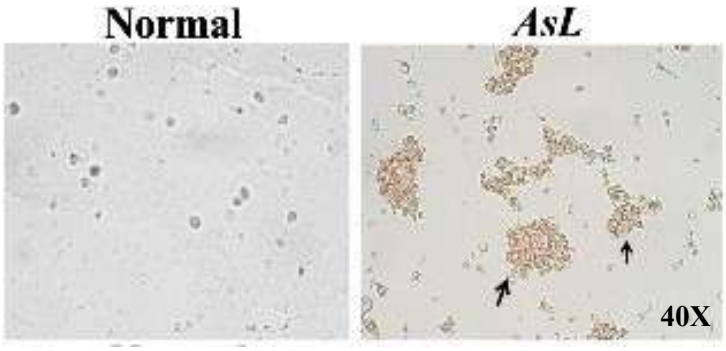


Fig.32: Microscopic view of erythro-agglutinating AsL: *AsL induced agglutinated 1% rabbit erythrocytes visualized at 40X optical magnification.*

5.4.2 Leucoagglutination of *AsL* with Human Peripheral Lymphocytes (PBLs)

Human blood samples were collected in a EDTA coated heparin tubes. Diluted with 10mM PBS buffer as described in the protocol and subjected with ficoll-hypaque (density=1.022g/ml) to separate the lymphocytes from blood components, clearly aspirated the white translusent buffy coat above the plasma layer using a pipette and washed thrice with 10mM PBS buffer . lymphocytes and AsL induced lymphocytes were stained with leishman's staining and observed under microscope at 40X optical magnification (Fig 33).

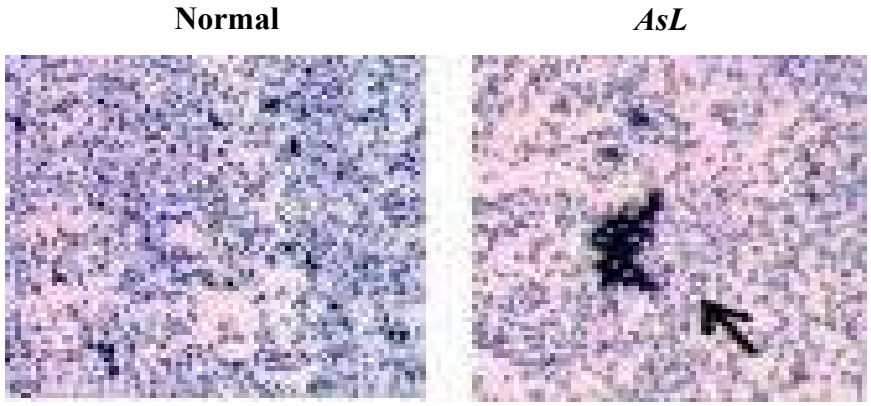


Fig.33: AsL displays leucoagglutination in concentration dependent manner: *Leucocyte agglutination is shown by AsL in a concentration-dependent way. Leucocytes were separated using density gradient centrifugation, and a 40X optical magnification was used to view a microscopic view of AsL-induced agglutinated leucocytes.*

5.4.3 Concentration kinetics of *AsL* for Specific HA activity of purified lectin

The purified garlic lectin (*AsL*) was serially diluted with 10mM PBS in concavity slide and was added with 2% rabbit erythrocytes and observed 1hr for visible agglutination. Serial dilution was done to know the maximum and minimal concentration of *AsL* required to agglutinate 2% rabbit erythrocytes and was started with an initial concentration of 400 µg/well and it was 2X diluted with 1:1 using PBS. The visible agglutination was observed for the purified *AsL* at 1.56 µg indicating the minimum concentration of *AsL* required to produce visible agglutination with for 2% rabbit RBC.

The agglutination was shown in Fig 34. The concentration was considered as the amount of lectin required to produce Hemagglutination Unit (HA Unit). If 1.59 ug is one unit for 1 mg the HA units were 628 HA Units. The specific hemagglutination activity for the purified *AsL* is computed as 628 HAU/mg protein.

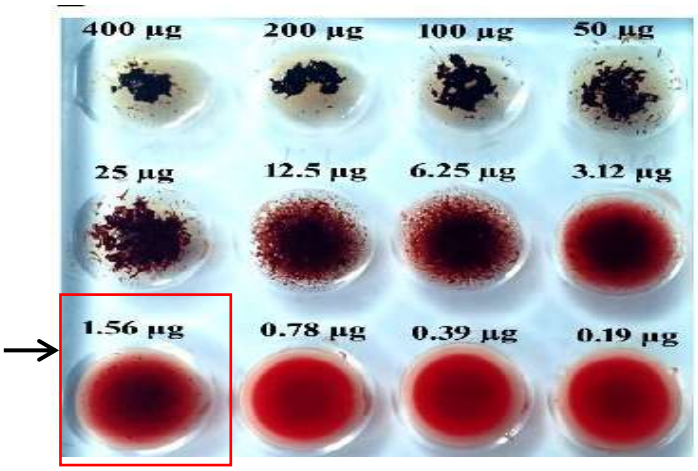


Fig.34: Concentration Kinetics of AsL: *Haemagglutination assay using a 400 g concentration of AsL serially diluted in a concavity assay plate. The minimum extract concentration needed to cause visible agglutination at the greatest dilution is represented by the AsL titer value of ~2 µg.*

5.4.4. Thermal and pH stability of Purified *AsL*:

AsL agglutinating activity indicated that it was highly thermostable even at temperature of 20° to 40° C. Whereas at 100° C, the agglutinating activity of *AsL* was completely inhibited indicating inactivation of lectin. The result of lectin thermal stability was shown in Fig 35.

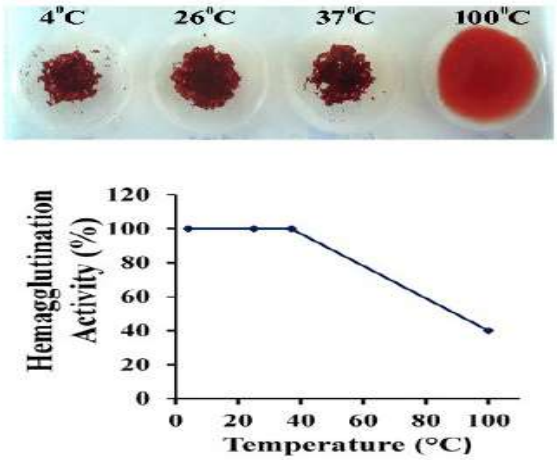


Fig.35: Thermal stability of AsL: *AsL displayed 100% haemagglutination activity up to 40˚ C and upon increasing the temperature it gradually lost the lectin activity with increasing temperature.*

The lectin pH stability was considered with four various pH buffers (sodium acetate pH-4, sodium phosphate pH-6, phosphate buffer saline pH-7 and carbonate-bicarbonate buffer pH-10). These were used to determine the pH stability of *AsL* and the lectin retained its visible agglutinating activity even in the extreme pH at 4 and 10 pH (Fig 36). AsL was discovered to be stable even under high pH alterations and modest temperature changes.

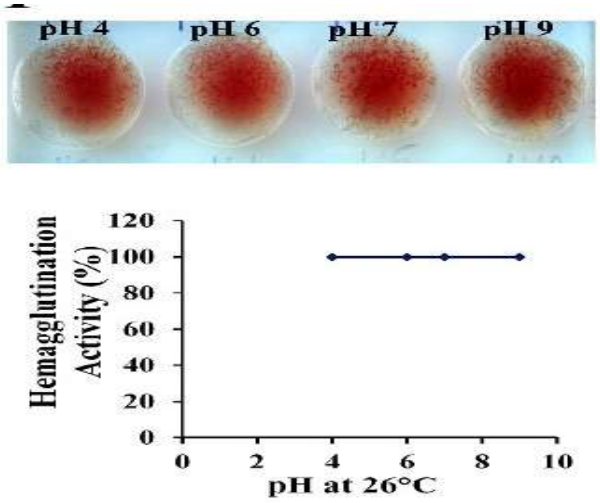


Fig.36: pH stability of AsL: *AsL heamagglutinating activity was stable for various pH buffers from pH-4 to pH-9 at 26° C.*

5.4.5. Determination of glycocodes and sugar specificity for *AsL*:

To confirming the lectin sugar and glycan specificity, Purified *AsL* were screened for glycocode identification through HA inhibition assay using major different free carbohydrates and glycoconjugates. *AsL* agglutination was strongly inhibited by Glucose, Mannose and Maltose indicating the lectins specificity (Fig 37).

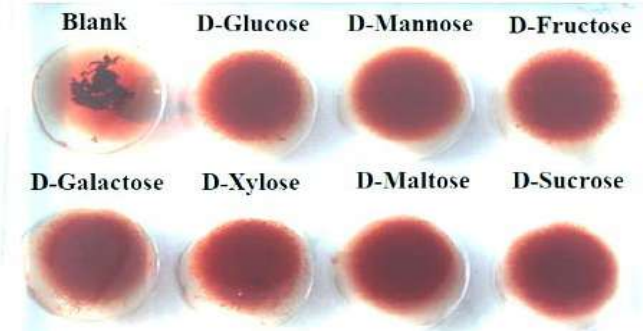


Fig.37: Determination the glycan specificity: *AsL was pretreated with various carbohydrates and glycoconjugates to ascertain the glycan specificity, and then an agglutination assay employing 1% trypsinized erythrocytes in concavity haemagglutination plates was conducted.*

Surprisingly, only carbs like glucose, mannose, and maltose showed a strong HI activity, demonstrating *AsL* selectivity. Table.11 contains the inhibitory concentrations.

Table.11: Evaluation of carbohydrate specificity for the purified *AsL*

Sl.No	Inhibitors (Selective glycoproteins and Carbohydrates)	Inhibitory Concentration(µg)
1.	D- Glucose	125
2.	D- mannose	25
3.	D-maltose	75
4.	D-Fructose	not inhibitory
5.	D-Galactose	not inhibitory
6.	D-xylose	not inhibitory
7.	D-Sucrose	not inhibitory
8.	D-Lactose	not inhibitory
9.	Ovalbumin	160
10.	Fetuin	130

AsL Specifically inhibiting the hemagglutination for carbohydrates such as glucose, maltose and mannose. Thus, AsL exhibits stronger sugar specificity towards glucose and mannose. It had also shown inhibition to for the glycoproteins indicating the interaction.

5.4.6. Pepsin digestive stability of purified garlic lectin (*AsL*)

AsL mixed with SGF (including 2500 pepsin activity units per mg test proteins) and incubated at 37° C. During incubation time intervals (0, 5, 10, 20, 40 and 60 min), about 200µl of sample were taken out and placed at 75° C to stop the reaction. The aliquots were analyzed by SDS-PAGE 15% gel under reducing condition. The result presented in Fig 38 shows the stability of *AsL* up to 2hr when mixed and incubated with pepsin in SGF at 37° C. Digestion products were separated in SDS-PAGE 15% gels under reducing condition. The purified *AsL* resist the digestion up to 1.5 hr and the digestion was appeared with the generation of peptides only after 10 min.

The Complete disappearance of *AsL* band was visualized at 2 hr. This clearly indicate the *AsL* is digestive resistant and has capability to escape pepsin induced gastric digestion of proteins. This stability can provide the lectin an opportunity to interact with cells.

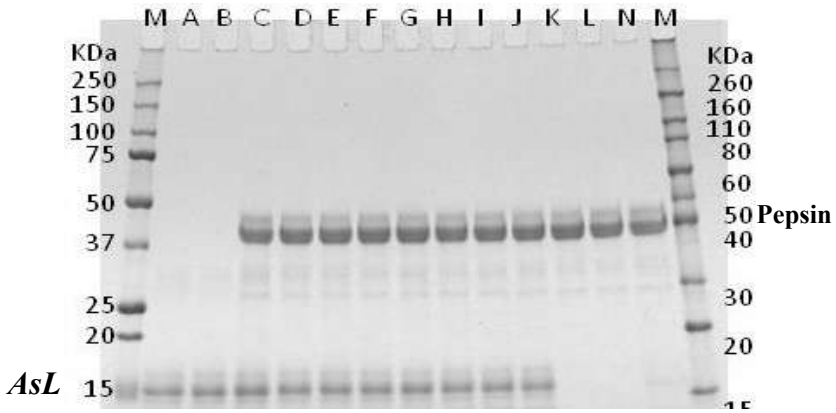


Fig.38: AsL exhibits pepsin digestive stability: *Sample ASL were digested in pepsin at pH 1.2 with 2,500 pepsin activity units per mg test protein. Lanes C-N represent SGF-digests at different incubation times: 0, 0.5, 2, 5, 10, 20, 30,40, 50, 60 and 120 min, respectively. Lane A and B in panel a indicates Test protein in the absence of pepsin.*

5.4.5 DPPH antioxidant assay with purified *AsL*:

AsL was screened for free radical scavenging activity using the DPPH method and it is based on the measurement of the reducing ability of antioxidants on DPPH free radical (Fig 39). The *AsL* was 55% effective as an antioxidant compare to standard Ascorbic acid. The lectin has antioxidant property and can provide partial protection against oxidative induced damage to the cells.

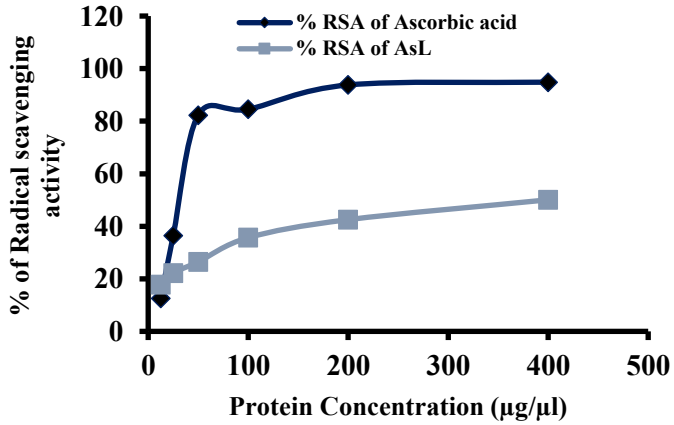


Fig.39*: **Antioxidant property of AsL:** Represents the percentage of DPPH scavenging activity of AsL, where AsL exhibited significant radical scavenging activity which increases with increasing concentration of AsL.*

5.4.6. *AsL* inhibits the lipid peroxidation

The results of *AsL* to inhibiting lipid peroxidation were shown in Fig 40. The lipid peroxidation inhibitory percentage increased with the increased concentration of *AsL* indicating the lectin ability to prevent lipid oxidation and provide protection.

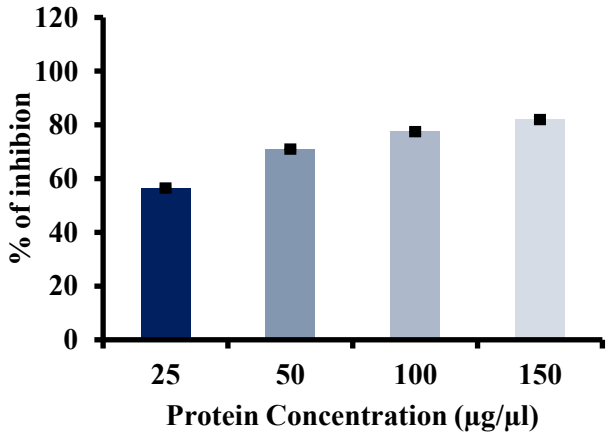


Fig.40: AsL inhibits the lipid peroxidation: *The result of the AsL to inhibitory lipid peroxidation were shown in figure the lipid peroxidation inhibitory percentage increased with the increased concentration of AsL.*

5.4.7. Garlic lectin (*AsL*) exhibits anti-inflammatory activity in reducing paw edema

Paw edema was induced by Freund's complete adjuvant and treated with garlic lectin (*AsL*) with three different concentrations at regular intervals of time. Eventually *AsL* exhibits anti- inflammation property by gradually reducing the edema as similar to that of methotrexate. Methotrexate was used as positive anti-inflammatory control. The mean paw thickness of *AsL* (20 mg) showed a significant ($p< 0.05$) decrease in the paw thickness with a value of 9.23±0.02 mm in comparison to vehicle control at the end of 5^{th} day. However, the mean paw thickness values of *AsL* (20 mg) remained significantly ($p< 0.05$) increased compared to normal vehicle control rats. Among the treatment groups *AsL* significant ($p<0.05$) improvement in the paw thickness was observed in *AsL* (5 &10 mg) rats compared to *AsL* (20 mg) rats.

Table.12: The mean (±SE) paw volume (cu.cm) and paw thickness (mm) values of different groups

Name of the groups (n =6)	Paw volume (cu. Cm)		paw thickness (mm)	
	DAY 0	DAY 5	DAY 0	DAY 5
Vehicle control PBS	0.35±0.01	0.35±0.01	2.26± 0.04	2.26±0.05
Positive control	0.33±0.01	1.35±0.01	2.23± 0.05	10.48±0.14
AsL (5mg)	0.33±0.01	1.31±0.00	2.21± 0.05	10.20±0.06
AsL (10mg)	0.33±0.01	1.25±0.01	2.23± 0.03	9.40±0.039
AsL (20mg)	0.33±0.01	1.23±0.00	2.23± 0.05	9.21±0.05
Methotrexate	0.33±0.013	1.26±0.00	2.21± 0.04	9.23±0.02

The mean paw volume values of untreated remained significantly (p<0.05) increased compared to the normal vehicle control rats. Among the treatment groups treated with *AsL* (20 mg) significant (p<0.05) decrease in the paw volume was observed in the rats compared to *AsL* (5 &10 mg) treated rats.

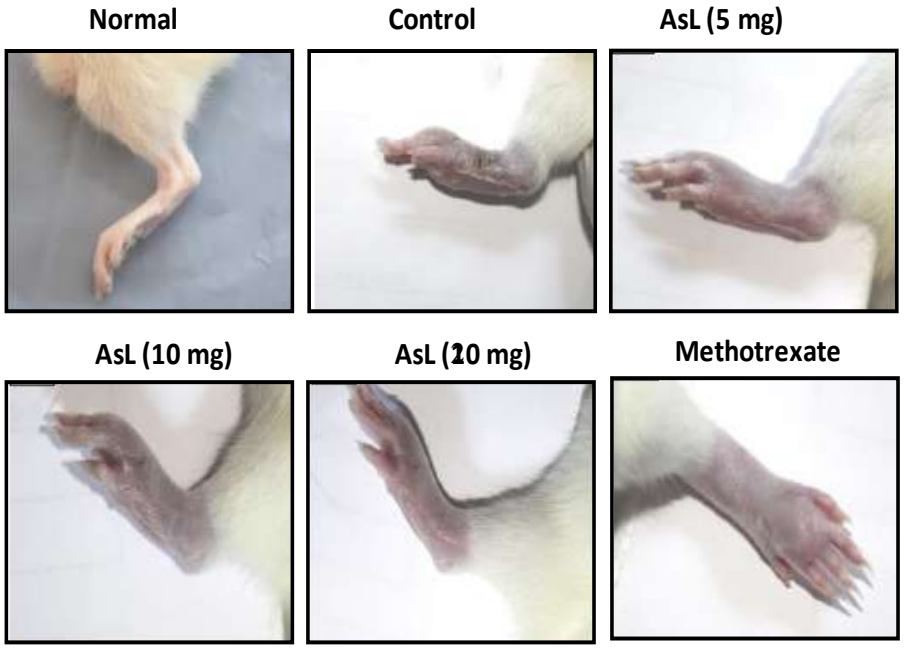


Fig.41: Anti-inflammatory property of AsL: *The vehicle control rats examined for inflammatory study of the experiment did not reveal any gross abnormalities in left hind paw. Positive control showed significant progressive swelling and erythema, as well as a deep ulcerated lesion at the paw that was filled with an ulcerated mass and the floor was covered with profuse and offensive slough with no evidence of healthy granulation tissue and the edge was slightly inflamed with no drainage fluid. The animals of group treated with AsL (5,10 & 20 mg) showed progressive decrease in the severity of gross lesion as compared to positive control.*

Table.13: The mean (±SE) hematological and biochemical parameters values of different groups

Name of the group (n =6)	Haematological parameters			Biochemical parameters		
	WBC (10^3 /µl)	RBC (10^6 /µl)	Hb (g/dl)	AST (IU/L)	ALT (IU/L)	CRT (mg/dl)
Vehicle control	9.05± 0.21	8.80± 0.10	14.13± 0.60	84.19±0.94	53.64±3.11	0.38±0.003
Positive control	18.76± 0.29	5.65± 0.21	8.25± 0.27	129.00±2.22	65.00±1.46	0.69±0.007
AsL (5mg)	18.06± 0.04	7.85± 0.12	11.85± 0.16	112.00±2.02	52.33±0.6	0.56±0.02
AsL (10mg)	16.70± 0.13	8.27± 0.04	14.06± 0.09	86.50±5.74	50.47±2.9	0.55±0.01
AsL (20mg)	15.01± 0.22	8.78± 0.09	15.26± 0.18	80.22±2.056	53.00±0.93	0.54±0.04
Methotrexate	16.31± 0.14	7.98± 0.10	13.73± 0.22	82.96±4.10	55.94±2.15	0.56±0.01

The mean WBC's, RBC's and hemoglobulin of *AsL* (5,10& 20mg) treated remained significantly ($P < 0.05$) increased compared to the normal group in the present study. The treatment with *AsL* (5, 10& 20mg) groups a significant ($p<0.05$) improvement in the mean AST, ALT and creatinine values was observed compared to normal vehicle control group rats (Table 14). The haematological and biochemical parameters infers that the *AsL* behaved similar to methotrexate in regulating the inflammation induced during paw edema.

5.5. Immunogenic property of *AsL* in rabbit to produce humoral immune response

Polyclonal antibodies were raised against gel permeation chromatography purified *AsL* in New Zealand White rabbit. Initially rabbit was immunized with *AsL* co-administrated with freund's complete adjuvant and followed by the booster doses with incomplete freund's incomplete adjuvant. Pre immune serum was collected before immunizing the rabbit with *AsL*. Post immune serum was collected 1 week after booster doses. The serum was analyzed and characterized for the anti-*AsL* antibody to assess specificity, sensitivity to understand its immunogenic property.

5.5.1. Anti-*AsL* IgG antibody sensitivity and immuno specificity

The immunized serum collected was screened for Antibody titer with antigen concentration of 0.5, 0.25 and 0.125 mg/mL to assess the optimal titer dilution for immune serum to a dilution up to 1:10000 dilution. The sensitivity of antibody was observed up to 1:1000 dilution (Fig 42) and the immune sera had high titer which observed and compared with non-immune sera (Fig 43). The results indicate the *AsL* induce anti-AsL antibody the sensitivity of antigen reorganization is high with observed titer values.

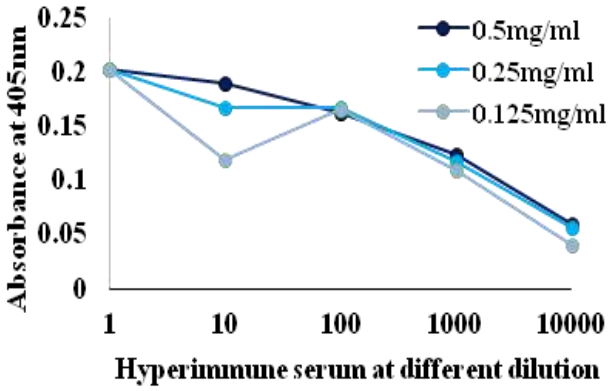


Fig.42: Antibody titer for hyperimmune serum: *Rabbit were immunized with purified AsL, after 3rd immunization animals were bled and serum was labelled as Ab1. Titer value of Ab1 was determined by ELISA. The dilution of the serum 1:1000 shows the minimal antigen and antibody interaction.*

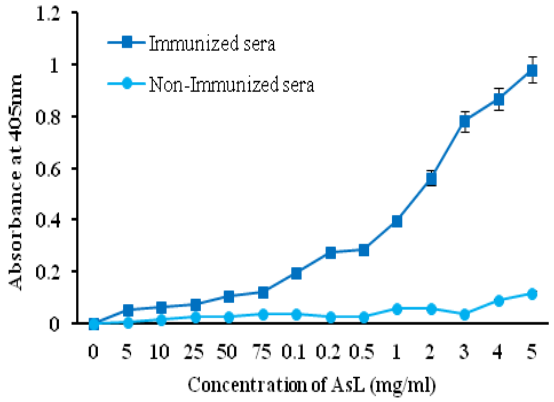


Fig.43: Antigen titer for hyperimmune serum: *Titer value of Ag was determined by ELISA. The dilution of the serum1:500 shows the minimal antigen and antibody interaction.*

5.5.2. Immunogenic potential and isolation of anti-AsL antibodies:

The immunized serum samples obtained were processed for purification with initial ammonium sulphate precipitation and dialysis. Finally Dialyzed immune serum sample were further purified by passing through affinity (Protein A-agarose column) chromatography technique. The antibody elution profile was presented in Fig 44.

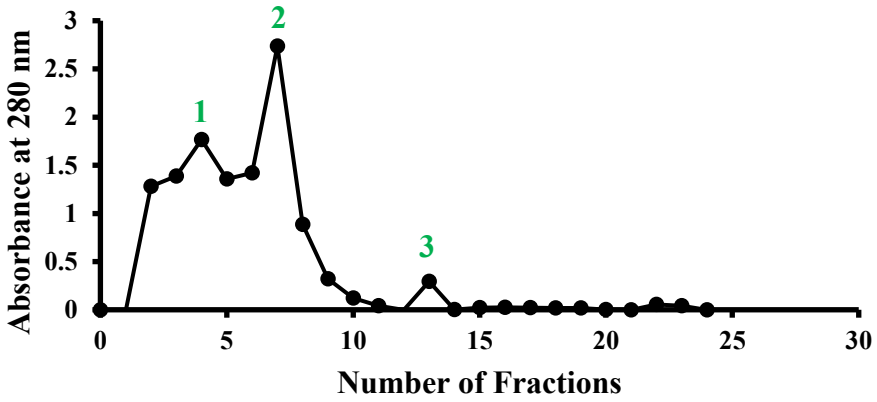
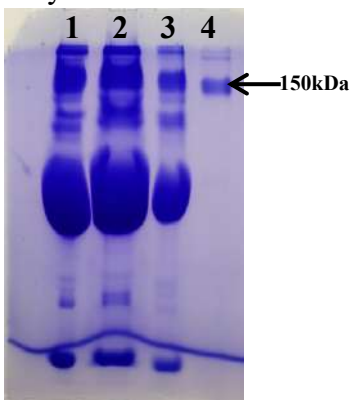


Fig.44: Purification of IgG by Protein A agarose chromatography: *Dialyzed antibody was chromatographed on protein A agarose column. Fractions of 1ml at flow rate of 1ml/3min were collected. The protein elution profile was monitored spectrophotometrically at 280 nm. The first peak was unbound peak and second peak was bound peak (IgG), which was eluted by changing the pH of the buffer.*

5.5.3 Analysis of purified anti *AsL*-IgG antibodies by SDS-PAGE:

All the purification fractions were subjected to SDS-PAGE to visualize the band pattern of IgG antibody (Fig 45). Peak-2 fraction showed the Ani-*AsL* antibodies which confirmed by 12% SDS-PAGE.



Lane Profile: -
1 *- Crude serum,*
2- *$(NH_4)_2SO_4$ precipitated serum,*
3 *– Dialyzed serum,*
4 *– Affinity purified serum.*

Fig.45: Antibody profile by SDS-PAGE (8% gel): *Under non-reducing condition various purification fraction were loaded and subjected to Coomassie brilliant blue (R-250) shows molecular mass at 150 kDa. Purified IgG from immunoaffinity chromatography was appeared in a single band under reducing condition.*

5.5.4. Characterization of rabbit anti-*AsL* antibodies specificity

Specificity of *AsL* and rabbit anti-*AsL* antibodies were determined by the immunoblots (western blot and dot blot). In western blot antigen were transferred to the nitrocellulose membrane and in dot blot antigen was coated. Both the blots were placed for blocking for an hour and followed by the addition of primary antibody (rabbit anti-*AsL* antibodies) and incubated at 4° C for overnight. After incubation blots were washed and incubated with secondary antibody (goat anti-rabbit IgG-ALP conjugated). Bands were visualized by the addition substrate. Immunoblot analysis showed that rabbit anti-*AsL* antibodies reacted very strongly with *AsL* The bands clear displays the specificity of *AsL* and rabbit anti-*AsL* antibodies (Fig 46 A & B).

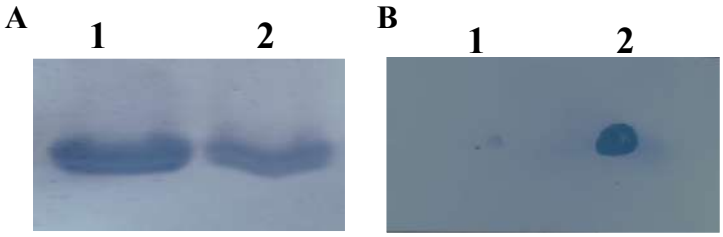


Fig.46: Immunoblot analysis: *[A] Immunoblot analysis of rabbit anti-AsL polyclonal antibodies. Lane 1-crude garlic lectin, 2-purified garlic lectin. [B] Dot blot analysis for the rabbit anti-AsL polyclonal antibodies 1.BSA and 2. Purified Garlic lectin. BSA was used as the negative control.*

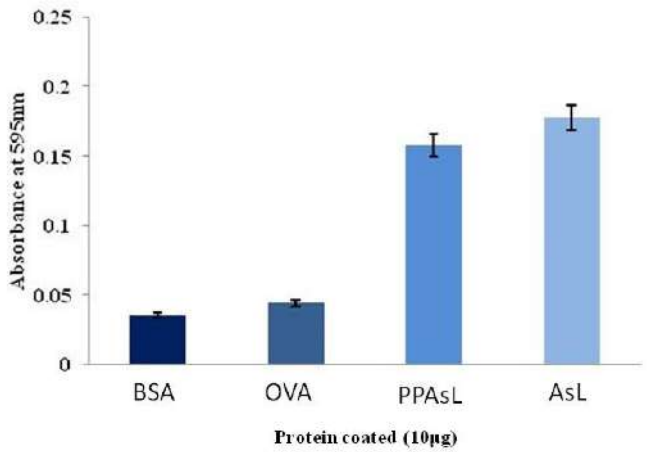


Fig 47: Specificity of antigen and antibody: *AsL shows the strong interaction with immunized serum sample than compared with globular and glycoprotein.*

The anti-*AsL* antibodies were checked for the antigenic specificity by ELISA at protein coating concentration of 10 µg/well. The results are shown in Fig 47. The Anti-*AsL* IgG specific to PP*AsL* and *AsL* where as BSA and OVA exhibit very weak affinity (Fig 47). The results clearly indicate the antibodies were very specific to immunized *AsL* by dot blot, immunoblot and ELISA. This infers the *AsL* is immunogenic and induce humoral response on systemic administration.

5.5.5. Confirmatory assay for antigen and antibody specificity:

The Ouchterlony double immunodiffusion technique was used to further examine the immunoreactivity of anti-*AsL* antibodies to the crude *AsL* and purified lectins. This method produced a precipitation line, demonstrating that the purified protein is immunologically identical to that present in both the crude and purified lectin (Fig 48).

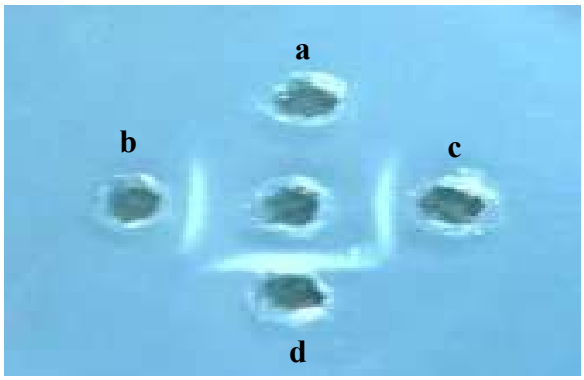


Fig.48: Ouchterlony Double Diffusion: Antigen Antibody interaction at different stages of purification was analyzed through immuno precipitation. *a-pre immune serum, b-crude serum, c-ammonium sulphate precipitation serum, d-Dialyzed serum. The precipitation line indicates the identical proteins from the crude, ammonium sulphate precipitation and dialyzed sample against antigen (center well).*

5.6. Systematic and mucosal humoral immune response to *AsL* in BALB/c mice

The AsL which is also called as Allium sativum agglutinin (ASA) was administered intradermally in BALB/c mice following the protocol mentioned in the methods section. The systemic immune response was measured in terms of the anti-*AsL*/ASA antibody response in the mice after 7^{th} and 14^{th} day to assess after two intradermal (*i.d*) dose administration. The mucosal administration was done through intra nasal administration of lectin following the protocol and the induction of systemic immune response infer the ability of lectin to recognize and interact with immune cells to induce anti-lectin antibodies.

5.6.1 Systematic humoral immune response of garlic lectin

The garlic lectin was administered through intra dermal route to study the systemic influence on immune cells to induce humoral immune response. The animals were monitored for 14 days with dosage and serum collection at 7th and 14th day for checking antibody titer. The animals were evaluated for change in body weights, spleenic and thymic weights, indices along with the titer induction of anti lectin antibodies. Ovalbumin was used as a control weak antigen for comparison of the immune response.

5.6.1.1. Body weights of the animals after intradermal administration of garlic lectin

The weights of the individual experimental animals in both the treated and control groups were recorded. Mice body weights were measured prior to the administration of each antigen dose and the average animal weights in each were managed in the range of 25 ± 1 g. The body weights of the mice belonging to the experimental groups that were administered antigen by the intradermal route are presented in Table 15. There is no observed significant difference in the body weights of the mice in comparison with those of the control group. Though there is a marginal increase in the weight (~0.3 to 0.8 g) in both treated and control groups, the results are not found to be significant.

Table 15: Body weights of mice at different intervals of experiment during intradermal (i.d.) administration for systemic response

Group [a]	**Body Weight [b] (g), mean ±SD**		
	Day 1	**Day 7**	**Day 14 [c]**
Control	23.47 ±1.62	23.70±1.75	24.10 ± 2.32
OVA	24.26± 2.47	24.48±1.91	24.74± 2.98*
ASA I	24.62 ±2.12	24.57±1.72	24.68± 2.24
ASA II	24.53 ± 1.63	25.03± 1.29	25.36±2.13*

*[a] n = 6 animals in each group; OVA, ovalbumin; ASA, Allium sativum agglutinin. b the body weight is presented mean ± SD of mice in a group.[c] Versus day 1; * Significant at p < 0.005*

5.6.1.2. Anti-Garlic Lectin IgG immune response upon systemic administration

The systemic effect on the administration of OVA or garlic lectins (ASA I and II) in BALB/c mice through the intradermal route was studied for a targeted immune response without an adjuvant. The ovalbumin (OVA) is generally accepted as a weak antigen and served as control. The serum IgG response to the garlic lectins (ASA I and II) was compared to the anti-OVA IgG response and was considered as an indicator of the systemic immune response, as shown in Fig 49. The anti-OVA IgG immune response on day 7 and day 14 were almost cognate; however, both garlic lectins (ASA I and II) showed a considerable increase in anti-lectin IgG antibodies on day 14 in comparison to day 7. On day 14, the anti-lectin IgG antibodies titer was found to be ~4-fold increased, and found significant at $p < 0.005$ for ASA I. It was 2.5-fold higher for ASA II as compared to the IgG response to the OVA control.

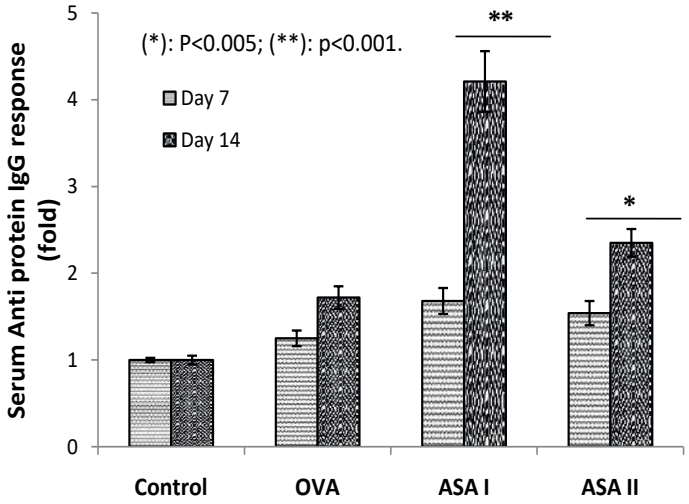


Fig.49: *Serum IgG systemic immune response to ovalbumin (OVA) and garlic lectins (ASA I and II) after intradermal administration in BALB/c mice. Antigens (100 µl/well) were coated overnight, washed, blocked with BSA, and incubated with test serum. Detection with goat anti-mouse IgG-AP and PNPP substrate. The IgG response is represented as a fold increase in comparison with control, taking the absorbance of the control as 1. Panel indicates IgG immune response against OVA, ASA I, or ASA II as assessed by ELISA using the serum of experiment animals obtained at day 7 and day 14 by the intradermal route. Coated antigen amount: 10 µg/well; IgG serum dilution, 1:10 (volume: 100 µl); * $p < 0.005$; ** $p < 0.001$.*

5.6.2 Mucosal humoral immune response of garlic lectin

The garlic lectin was administered through intra nasal route to study the mucosal immune response and to check the ability of lectin to cross mucosal barrier to interact with immune cells to induce humoral immune response. The animals were monitored for 14 days with dosage and serum collection at 7th and 14th day for checking antibody titer. The animals were evaluated for change in body weights, spleenic and thymic weights, indices along with the titer induction of anti lectin antibodies. Ovalbumin was used as a control weak antigen for comparison of the immune response.

5.6.2.1 Body weights of the animals after intranasal administration of garlic lectin

The body weights of the animals in each experimental group which were administered the antigen through the intranasal route are shown in Table 16. From day 0 to day 50 in the experiment, both the untreated control and the treated groups showed slight changes in the body weight, and these observed differences may due to the varied physiological and metabolic responses of the animals to the administered antigens in the respective experimental groups.

Table.16: Body weights of mice at different intervals of experiment during intranasal administration for mucosal response.

Group [a]		**Body Weight** [b] **(g), mean ±SD**		
	Day 1	**Day 14**	**Day 35**	**Day 50**[c]
Control	25.33 ±3.29*	25.73 ±4.50	26.17 ±3.57	27.03 ± 2.74*
OVA	25.58 ± 1.55	26.24 ±2.41	26.94 ±2.13	26.80 ± 2.56
ASA I	25.68 ± 1.39*	25.24 ±1.86	25.28 ±1.36	25.22 ± 1.59*
ASA II	25.44 ± 1.26	25.66 ± 0.85	25.66 ± 0.89	25.38 ±1.33

[a] n = 6 animals in each group; OVA, ovalbumin; ASA, Allium sativum agglutinin.
[b] The body weight is expressed as mean weight of animals in a group. [c] Versus day 1;
** Significant at p < 0.005*

5.6.2.2. Anti-Protein IgG immune response upon mucosal administration

The weak antigen OVA and immunogenic garlic lectins were administered to BALB/c mice through the intranasal route to study the anti-protein IgG mucosal immune response. Serum containing protein-specific IgG antibodies were collected at different intervals of time from day 1 to day 50. The sera were used for measuring the anti-OVA and anti-lectin IgG immune responses, with the results represented in Fig 50. Ovalbumin (OVA) showed a very small increase in IgG antibodies specific to OVA from day 14 as compared to the control, which was not significant. This suggests that the anti-OVA response showed an increase after the second booster dose at day 50 ($p<0.01$). The IgG response specific to garlic lectin (ASA I and II) is considerably higher and most significant as compared to the anti-OVA IgG response ($p<0.01$). The anti-ASA I IgG response is evidently strong (2.5–3-fold increase), whereas in the case of anti-ASA II IgG, the response is ~2-fold higher as compared to the OVA group.

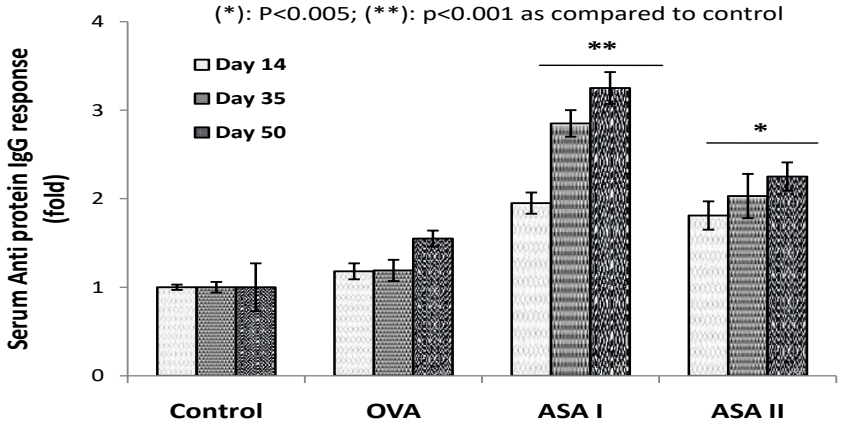


Fig.50: *Mucosal immune (anti-OVA and anti-lectin) IgG response analyzed in the sera obtained from BALB/c mice after intranasal administration of antigens. Antigens (100 µl/well) were coated overnight, washed, blocked with BSA, and incubated with test serum. Detection of IgG antibody response by using goat anti-mouse IgG-alkaline phosphatase-conjugated antibody and p-nitrophenyl phosphate (PNPP) substrate. The IgG response is represented as a fold increase in absorbance over control, wherein the absorbance of the control was taken as 1. Coated antigen amount is 10 µg/well; primary antibody source, mice sera dilution was at1:10 (volume, 100 µl); * p < 0.005 vs. control; ** p < 0.001 vs. control. Each value is represented as mean ± S.D. (n=3).*

5.6.3 Splenic and Thymic indices in control and treated groups

After sacrificing the animals on the 14th day for the intradermal (*i.d.*) group, or the 50th day for the intranasal (*i.n.*) group, the spleens and thymuses were isolated. The weights of both spleens and thymuses were measured to obtain information on the possible stimulation of the thymus or spleen induced by the administered antigens (OVA and garlic lectins), the observed result are shown in Table 16. It is seen that the thymus and spleen weights were observed to be slightly increased in the garlic lectin-treated groups; however, a considerable or significant observable difference was not seen in the splenic or thymic weights between the OVA and the garlic lectin groups. The splenic and thymic indices are shown in Fig 51 and Fig 52 respectively; the thymic and splenic indices are found to measure the differences accurately among the control and treated groups. The ASA I group significantly stimulates both the thymus and the spleen as compared to the control, OVA, and ASA II group.

Table.16: Weights of spleen and thymus of BALB/c mice by intradermal (*i.d.*) and intranasal (*i.n.*) administration of antigen (OVA, ASA I and II) samples.

Group [a]	**Intradermal Route** [b]		**Intranasal Route** [c]	
	Thymus Weight (mg) mean ± SD	**Spleen Weight (mg) mean ± SD**	**Thymus Weight (mg) mean ± SD**	**Spleen Weight (mg) mean ± SD**
Control	43.45± 2.13	106.83±07.99	40.42± 4.63	107.47±9.91
OVA [d]	46.83± 6.21	131.95±08.97	42.61±3.52	116.43±7.64
ASA I [d]	55.32±2.84	138.56±06.13	49.98±5.84	128.89±15.39
ASA II [d]	51.27±3.80	128.45±10.12	44.12±2.71	117.18±12.05

[a] n = 6 in each treated group; OVA, ovalbumin; ASA, Allium sativum agglutinin. [b] Spleen and thymus were collected for weight on day 14. [c] Spleen and thymus were collected for weight on day 50. [d] Versus the control ($p < 0.01$).

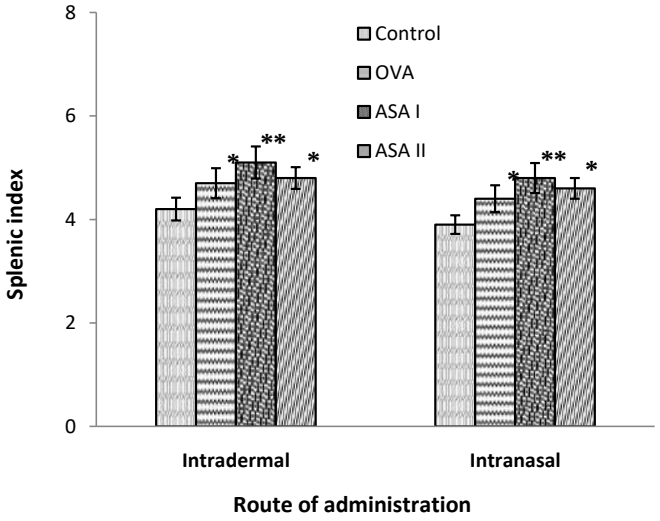


Fig.51: *Splenic indices were measured in the control and test experimental groups after intranasal and intradermal immunization of OVA and garlic lectins in BALB/c mice. Splenic index values for OVA, ASA I, or ASA II experimental groups on intradermal or intranasal administration of antigens. Spleen was collected on day 14 for intradermal groups and on day 50 for intranasal groups. Each value is represented as mean ± S.D. (n = 6); * p < 0.05; ** p < 0.01.*

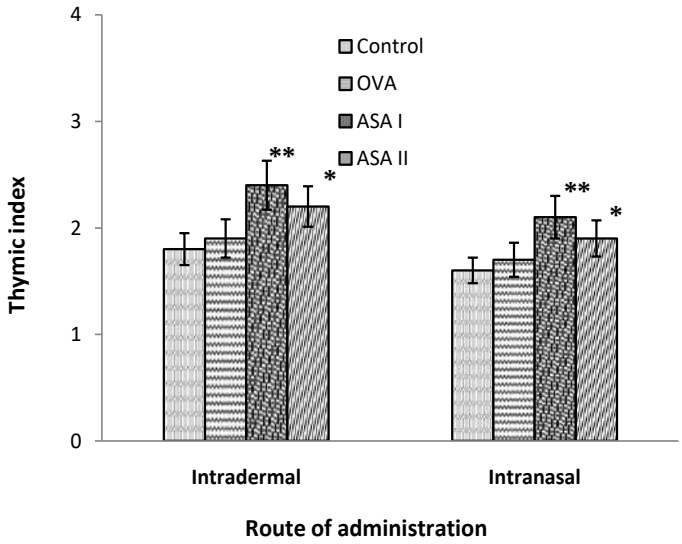


Fig.52: *Splenic indices were measured in the control and test experimental groups after intranasal and intradermal immunization of OVA and garlic lectins in BALB/c mice. (B): Thymic index values for OVA, ASA I, or ASA II experimental groups by intradermal or intranasal routes of antigen administration. Thymus was collected on day 14 for intradermal groups and on day 50 for intranasal groups. Each value is represented as mean ± S.D. (n = 6); * p <0.05; ** p <0.01.*

5.7. Humoral Adjuvant Property of Garlic Lectins (ASA I and II)

The mucosal adjuvant potential of garlic lectins (ASA I and ASA II) was assessed with a weak antigen OVA. The OVA was administered in combination with ASA I and II by the intranasal route and were examined for their ability to induce the humoral immune response to self and OVA. ASA I and ASA II were further analyzed for their effectiveness in interaction with immune cells to enhance the IgG antibody response against OVA in order to assess their adjuvanticity. The results of the body weights of the mice in the experimental groups of the control and those treated with OVA alone, OVA with ASA I, and OVA with ASA II were studied for their physiological effects on the growth of animals during the experimental regime. The anti-OVA IgG response for OVA alone and OVA with garlic lectins (ASA I and ASA II), Con A, and RGE were also assessed so to understand. the humoral response and adjuvant potential of ASA I and ASA II, which were presented as follows:

5.7.1 Body weights of experimental animals during mucosal adjuvanticity study

The experimental mice in the control and treated groups were monitored for body weights after the intranasal administration of the antigens. The change in body weights of the control group were compared with the OVA alone or the OVA with garlic lectins in the adjuvanticity study to mark the significant impact of the antigen administration in the growth and development of the animals. The results are presented in Table.17. There is no observed significant difference in the body weights of the mice in the test groups at day 0, 14, 35, and 50 as compared to the body weights of the mice in the untreated control group. Similarly, there is no observed significant difference in the body weights of the animals on day 50 vs. day 0 in each group. This justifies that the administered antigens did not demonstrate any growth retardancy in any of the experimental group.

Table.17: Body weights of BALB/c mice at different intervals of time after antigen administration by intranasal route in adjuvanticity study.

Group [a]	Body Weight (g), mean ±SD			
	Day 0	**Day 14**	**Day 35**	**Day 50[b]**
Control	23.26± 2.06	25.35 ± 2.15	24.29 ±1.39	24.76 ± 1.86
OVA	23.93 ± 1.47	23.84 ± 2.17	24.47 ± 2.04	24.38 ± 2.89
OVA + ASA I	24.68± 2.08	24.12 ± 1.73	24.45 ± 2.23	23.89 ± 2.68
OVA +ASA II	23.47 ± 1.91	24.57 ± 2.09	25.17 ± 1.82	25.89 ± 2.13
OVA + ConA	24.68± 1.29	23.79 ± 1.87	24.46 ± 2.81	24.29 ± 1.78
OVA +RGE	24.67 ± 1.42	24.72 ±1.35	25.97 ± 1.85	25.96 ± 2.46

[a] n = 6 in each group; intranasal administration of each antigen at 30 µg in 30 µL volume; OVA, ovalbumin; Con A, concanavalin A; RGE, raw garlic extract. [b] Versus day 0; not significant at p<0.05

5.7.2. OVA-Specific IgG Response in the Garlic Lectin Adjuvanticity

The adjuvanticity property of purified garlic lectins (ASA I and ASA II) was studied for the OVA antigen. OVA alone or OVA with ASA I/ASA II were administered by the intranasal route to the BALB/c mice in the experimental groups. Ovalbumin (OVA) was considered as an experimental weak antigen and the IgG response against OVA administered with garlic lectins was measured to determine the adjuvant property of ASA I and ASA II lectins. Comparing to the adjuvant effect of garlic lectins, ConA with Glc-/Man specificity was used as a prototype lectin, and raw garlic extract (with all the components of raw garlic) was also considered so to understand and compare the adjuvanticity effect. The OVA-specific IgG response measured from the sera collected at different interval periods of the treatment (14th, 35th, and 50th day) is shown in Fig 53.

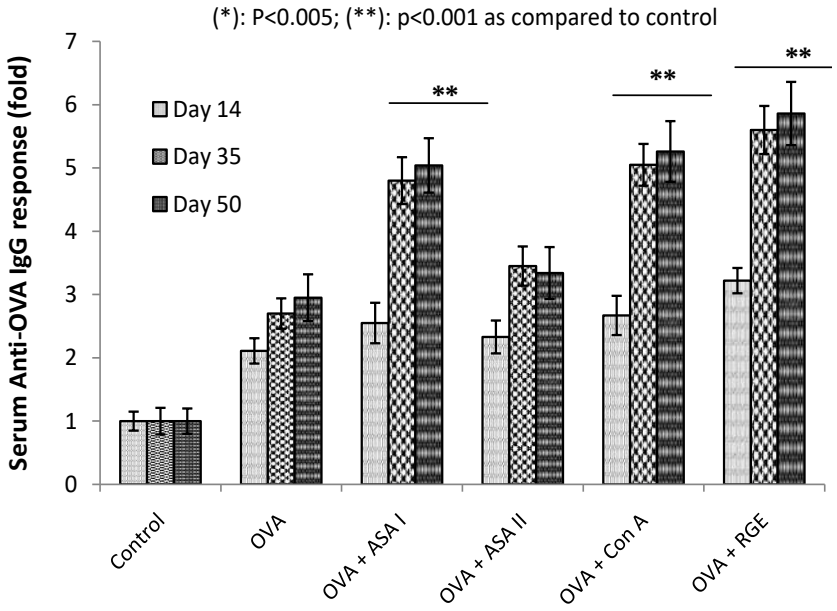


Fig.53: Ovalbumin (OVA)-specific IgG mucosal immune response to intranasal administration of OVA alone, or after OVA, co-administered with garlic components*: (ASA I or ASA II or RGE) or ConA by intranasal route at different times of administration. Coating antigen (10 µg OVA in 100 µl/well) overnight, washed, blocked with BSA, and incubated with test serum. Detection was with goat anti-mouse IgG-alkaline phosphatase conjugated antibody and PNPP substrate. The IgG response is represented as a fold increase taking the absorbance of the control as 1; serum dilution: 1:10; ** p<0.001 vs. control. Each value is presented as mean ± S.D (n = 3).*

During the adjuvant experiment, in the experimental group where the intranasal administration of OVA was done along with ASA I and ASA II, the lectin ASA I group showed an increase in the anti-OVA IgG response on days 35 and 50 in comparison with the anti-OVA IgG response of the OVA group. Compared to the OVA-alone group, the OVA + ASA I group showed around a two-fold high (p<0.005) OVA-specific IgG immune response (Fig 54). However, the OVA + ASA II group showed only a marginal increase (not significant) in comparison to the OVA-alone group. The OVA + ConA group showed almost identical observations in the IgG response as in OVA + ASA I. However, the OVA + RGE group showed the highest OVA-specific IgG response (marginally higher to ASA I and ConA groups) possibly, due to the synergetic stimulation of immune cells with garlic compounds.

5.7.3. Anti-OVA IgG titer in adjuvanticity with and without lectins

The sera obtained from the adjuvanticity study groups was assessed for the anti-OVA antibody (IgG) response in the OVA-alone and adjuvant-treated groups (OVA + ASA I/ASA II/Con A/RGE) and was measured for its antibody titer, and the observations are presented in Fig.54. The IgG antibody titer in the sera obtained on the 50th day after the final sacrifice of the animals of both the control and treated groups was measured and compared for the assessment of adjuvanticity. It has been identified that the OVA-specific IgG antibody response was stronger in the OVA + ASA I as well as the OVA + Con A groups in comparison with the OVA and OVA + ASA II groups. The limit of antibody detection was observed at the dilution titer of 1:10,000 of serum in the case of the OVA + ASA I, OVA + Con A, and OVA + RGE groups. In the case of the OVA and OVA + ASA II groups, the antibody detection was found significant at a serum dilution of 1:100. These observations made during the antibody titer detection clearly emphasize that ASA I, Con A, and RGE significantly enhance the induction of IgG antibody production against the weak immunoantigen (OVA) and signifies that they stimulate the immune cells involved in humoral immunity as an adjuvant to increase the IgG immune response to the co-administered antigen OVA.

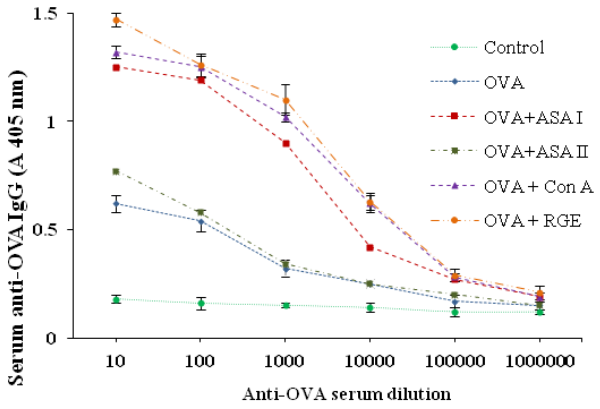


Fig.54: Anti-OVA IgG antibody titer (ELISA absorbance value at 405 nm) detected for sera obtained from untreated control and adjuvant treated experimental groups (OVA, OVA + ASA I, OVA + ASA II, OVA + ConA, OVA + RGE): *Control and test proteins (100 µL/well; 0.1 mg/mL concentration) were coated overnight, washed, blocked with BSA, and were incubated with test serum. Detection with secondary antibody conjugate (goat anti-mouse IgG-AP) and PNPP substrate. The initial dilution of serum with 1:10 and was further serially diluted by 10-fold (10^{-1} to 10^{-5}) using the dilution buffer.*

5.8. Proliferation and mitogenic properties of purified garlic lectin AsL

The ability of purified garlic lectin (*AsL*) to interact with immune cells and induce proliferation was studied. The isolated lymphocytes from human peripheral blood, spleen and thymus were induced to study the immunomodulatory functions with purified *AsL* to confirm possible interaction with the immune cells. The immunomodulatory functions of *AsL* were inferred with the following results.

5.8.1 Mitogenic stimulation of human PBLs by purified garlic lectins (*AsL)*

The dietary lectin from garlic bulb (*AsL*) was purified to homogeneity and was tested for their ability to proliferate human PBLs. ConA, which is a known lymphocyte mitogen was used for comparison. Initially, the AsL lectin along with positive reference mitogen, ConA, were checked in the concentration range of 0.01 to 10 μg/mL concentration. The results are shown in Fig 55. Garlic lectin AsL showed stimulatory effects with human PBLs at concentration from 1 to 10 μg/ml. *AsL* behaves similar to ConA in its potential to cause proliferation of human PBLs.

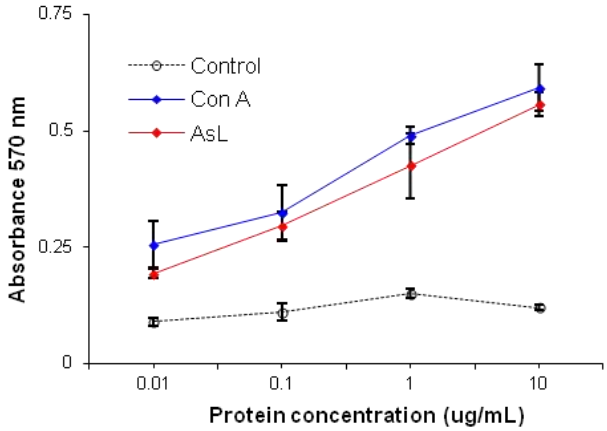


Fig.55: AsL proliferates the lymphocytes isolated from human and compared with known mitogen (ConA): *The immunomodulatory effects of garlic lectin (AsL)on human PBLs in the concentration range of 0.01 to 10 μg/ml. ConA was used as a reference positive for lymphocyte proliferation.*

5.8.2 Mitogenic and co-mitogenic effect of purified lectin *AsL*

Garlic lectin, *AsL* was found to be mitogenic and co-mitogenic for human PBLs when added with a known mitogen. The results are shown in Fig.56. *AsL* showed a similar effect as that of the known mitogen ConA, and an increased response is seen in the presence of ConA (significant at $p \leq 0.001$).

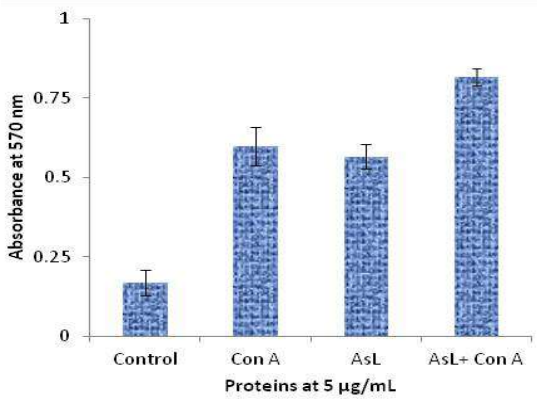


Fig.56: Synergistic activity of AsL with ConA: *Mitogenic and co-mitogenic effects of garlic lectin (AsL) on human PBLs. All the lectins were used at 5 µg/mL concentration. Cells used: 1x10^5 cells/ml.*

5.8.3 Comparative Proliferative index on PBLs

The proliferative effect of garlic lectins on human PBLs are shown in terms of proliferation index in Fig 57. The index for control (untreated cells) taken as 1.0, and for others are represented as fold increase or decrease over the control. Proliferation index is calculated by dividing the absorbance of test by absorbance of the control. *AsL* and ConA shows 3.5 fold increase *AsL* is a mitogenic.

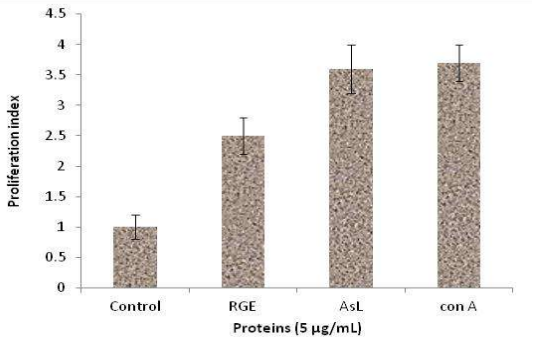


Fig.57: AsL proliferates the human lymphocytes and compared with RGE and ConA: *Proliferation index of purified garlic lectin and raw garlic extract on human PBLs. The extract was used at 10 µg/ml, purified lectins and ConA and AsL at 5 µg/ml concentration. Proliferation index was calculated by dividing absorbance of the test by absorbance of control.*

5.8.4 Modulatory effects of garlic lectin on murine splenocytes

Murine splenocytes were isolated from the spleen obtained from adult BALB/c mice. The splenocytes were tested for proliferation by garlic lectin *AsL* at 0.1 - 10 μg/mL concentration. ConA and PHA which are known T-cell mitogens were used as reference positive mitogens, and the cells in the absence of any lectin served as control. The result of splenocytes stimulation is shown in Fig 58. There is a significant difference (at $p \leq 0.001$) in the proliferation of splenocytes by *AsL* at 1 and 10 μg/ml, and this effect is comparable to those of the reference mitogens.

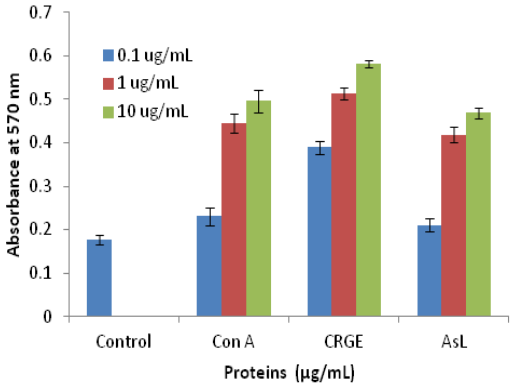


Fig.58: AsL proliferates the splenocytes isolated from murine and compared with RGE and ConA: *Immunostimulatory effects of garlic lectin AsL on murine splenocytes. The concentration range of lectins tested is 0.1 to 10 μg/ml. ConA a known T-cell mitogens, and represent reference positives.*

5.8.5 Modulatory effects of garlic lectin on murine thymocytes

Murine thymocytes were isolated form the thymus obtained from adult BALB/c mice. Thymocytes were stimulated by garlic lectins ASA I and ASA II at 0.1 - 10 μg/ml concentration. The results are shown in Fig 59. The stimulatory response of garlic lectins for murine thymocytes seems to be more as compared to human PBLs and murine splenocytes. Similar trend of activation by garlic lectins is seen as in the case of murine splenocytes. The effect of *AsL* is comparable to that of ConA, however, *AsL* showed a slightly higher degree of activation for thymocytes compared to splenocytes.

The result indicate that the garlic lectin (*AsL*) has more specificity to T-lymphocytes and can modulate both humoral and cell mediate immunity.

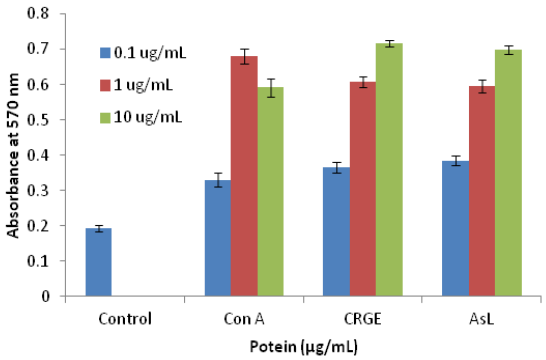


Fig.59: AsL proliferates the thymocytes isolated from murine and compared with RGE and ConA: *Immunostimulatory effects of garlic lectin AsL on murine thymocytes. The concentration range of lectins tested is 0.1 to 10 µg/ml. ConA is a known T-cell mitogens, and represent reference positives.*

5.8.6 Comparison of the effect of garlic lectins on murine lymphocytes

Both the garlic extract and garlic lectin stimulate murine lymphocytes to varying degrees. The comparative results of the effect on these lectins on PBLs, splenocytes and thymocytes are shown in Fig 60. *AsL* showed stronger stimulatory effect with 3fold higher compared to normal in PBLs, splenocytes and thymocytes. Raw garlic extract (RGE) has significant modulatory effect on both human and murine lymphocytes.

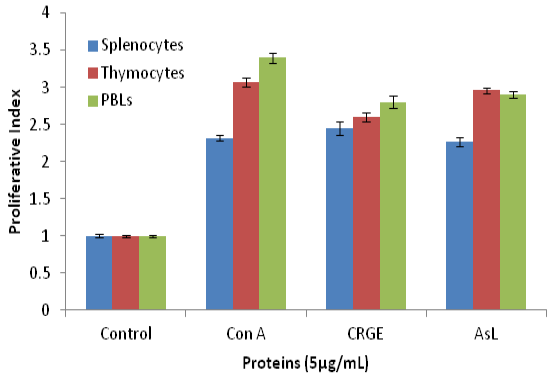


Fig.60: AsL proliferates the lymphocytes, splenocytes and thymocytes isolated from human and murine, compared with RGE and ConA: *Comparison of the proliferation index of garlic lectin on human PBLs, murine splenocytes and thymocytes. All lectins are used at 5 µg/ml concentration. Cell concentration used is 1 x 10^5 cells/ml.*

5.8.7. *AsL* induces IL-2 from activated lymphocytes-mitogenic signalling cytokine

The cell supernatants of human peripheral blood lymphocytes (hPBL) that were incubated with purified garlic lectin (*AsL*) were analyzed for the presence of self-mitogenic signalling cytokine IL-2. The *AsL* treated hPBLs showed increased concentration of IL-2 in the cell supernatant indicating their mitogenic effect on hPBLs. *AsL* stimulated lymphocytes and increased IL-2 synthesis at 5µg/ml and 10µg/ml concentration compare to the non-treated normal and control samples (Fig 61).

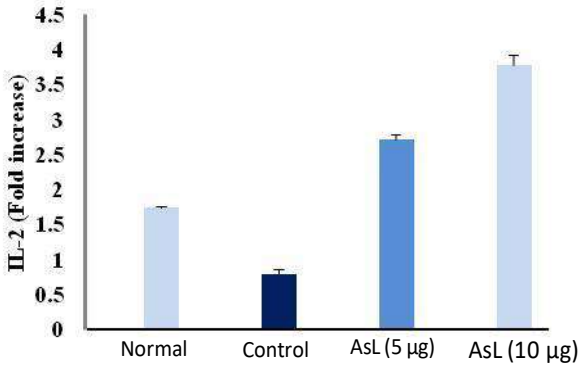


Fig.61: *Fold variation between the control and AsL-treated animal's blood IL-2 levels. The level of IL-2 in the control animal is declining, whereas AsL at 25 and 50 mg/kg b.w. administered intravenously causes a substantial increase in IL-2 levels.*

5.9. Evaluation of *in vitro* anti-cancer property of purified garlic lectin (*AsL)*

The purified garlic lectin which exhibited Leucoagglutination and lymphocyte proliferation was studied for the potential anticancer properties with cell culture with targeted cancer cells lines and in vivo in a tumour induced mice model system. The *AsL* had induced synthesis of self-mitogenic cytokines IL-2 in activated lymphocytes that indicate stimulation of immune cells. The IL-2 is a known antiangiogenic molecule and probably involve in sustaining tumour growth. Presently, selected tumour cells were screened for cytotoxicity assay with purified *AsL* and the cell viability and cytotoxicity effect were analysed by MTT cell viability assay. The *AsL* was studied against both human and murine cell lines. The *In-vitro* anticancer activity of *AsL* was verified and was further extended for antiangiogenic assay to study the ability of *AsL* to restrict neovascularation and suppress tumour growth.

5.9.1. Cytotoxic effect of AsL on human melanoma cell line A375

In the present study, the screening of cytotoxicity effect of *AsL* was analysed by conducting MTT cell viability assay against human melanoma cell lines A375. The *in-vitro* anticancer activity of *AsL* was verified by cytotoxic assay. The cultured A375 cell lines were treated with different concentrations of *AsL* and the effect on the survivability of cells was observed. Results showed the decreased cell viability against *AsL* treatment with approximate IC_{50} value of ~12.5µg/ml. Detailed results is shown in Table.18.

Table. 18: Effect of purified garlic lectin (*AsL*) in A375 cell proliferation:

Concentration (µg/ml)	Average Absorbance+ SD	Normalized Viability (%)
0	2.517±0.102	100±4.059
3.12	2.352±0.283	93.470±11.237
6.25	2.347±0.043	93.272±1.691
12.5	1.256±0.048	49.907±1.888
25	1.213±0.141	48.212±5.584
50	1.094±0.063	43.470±2.493
100	1.083±0.150	43.020±5.965

Cytotoxicity was measured by MTT assay against cell line in three independent (n=3) DMSO was used as a vehicle control which showed very negligible cytotoxicity. Values are indicated in mean ± SD.

5.9.2. Screening of *AsL* induced cytotoxity against cancer cell lines and IC_{50} value

Cytotoxicity and anti- proliferation activity of *AsL* were tested against various cancer cells of different origin which include mouse B16F10, NIH3T3 and human A375, A549, HUH7 and BEAS-2B cells. The obtained IC_{50} value against each of the cell lines were A375 - ~12.5µg, B16F10- ~13µg, A549- ~10µg, HUH7- ~15µg, NIH3T3-~93µg and BEAS-2B- ~89µg as assessed by three independent MTT assay (Table 19). It is apparent from the results that all four malignant cell lines were sensitive to *AsL* which have inhibited the proliferation potency of above mentioned cell lines. In contrast *AsL* did not show any changes to the non-cancer NIH3T3 and BEAS-2B cells even at the concentration of 50µg/ml.

The comparative cytotoxic effect of *AsL* with normal and cancer cells were shown in Table 15. The lectin behaved similar with the 5-flurouracil which was used as a positive anti-cancer control.

Table.19: IC_{50} value of *AsL* against various human and mouse cancer cell lines

Cancer Cell lines	IC $_{50}$ values (µg)	5-Fluorouracil (µg)
A375	12.5±1.9	4.8±0.8
B16F10	13.0±0.6	10.5±2.4
A549	10.0±0.9	7.2±0.9
HUH7	15.0±1.5	5.20±0.19
NIH3T3	93.5±3.9	86.0±4.3
BEAS-2B	86±2.5	65.0±2.0

5-Flurouracil is used as positive control. Cytotoxicity was measured by MTT assay against cell line in three independent (n=3) DMSO was used as a vehicle control which showed very negligible cytotoxicity. Values are indicated in mean ± SD.

5.9.3 Quantified the VEGF secretion upon treated with AsL

The A375 cancer cell lines were cultured and treated with *AsL* (5mg/ml and 10 mg/ml). The conditioned media from the *AsL* treated cell culture were subjected to the quantification of VEGF level by ELISA method as previously described (Prabhakar et al., 2006). *AsL* treated conditioned media down regulates the VEGF synthesis at 5mg/ml and 10mg/ml concentration to that of control (Fig 64). Measurement of secreted VEGF levels were abrogated which is confirmed through ELISA after *AsL* treatment as measured in cell supernatants.

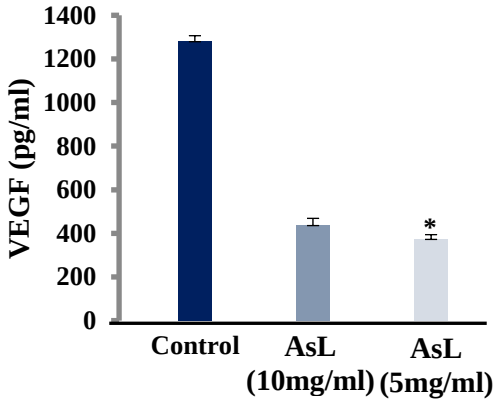


Fig.62: *Reducing the secretion of VEGF levels in conditioned media upon treating with AsL (5mg/ml and 10mg/ml) than compared to the secretion of VEGF level in control. Results are the means of three determinations, each conducted in triplicates. Statistically significant values are *p< 0.05; **p< 0.01.*

5.10. *In-Vivo* anti-cancer effect of Garlic lectin (*AsL*)

By using specific, reliable angiogenesis assays that are induced by $rVEGF_{165}$, antiangiogenic target activity was simultaneously assessed. Fertilized hen's eggs were incubated at 37° C in a humidified incubator. On the 5th of incubation, a small window was made and treated with $rVEGF_{165}$ and *AsL* at different concentrations. The windows were closed using sterilised coverslips and incubated for 48 hr at humidified condition. All treated and untreated were opened and observed the results.

5.10.1. *AsL* displays anti-angiogenic property in Ova- CAM assay

Garlic lectin (*AsL*) was treated with 10 µg and 20 µg concentration to observe its effect on neovascularization in growing chick embryo. *AsL* regressed the development of new blood vessels and observed to inhibit neovascularization in the developing embryo in ova CAM (Fig 63A). The *AsL* inhibited angiogenesis at increasing concentration of 10 and 20 µg by 62% and 55% respectively, as evident from the zone of a vascularization (Fig 63 B).

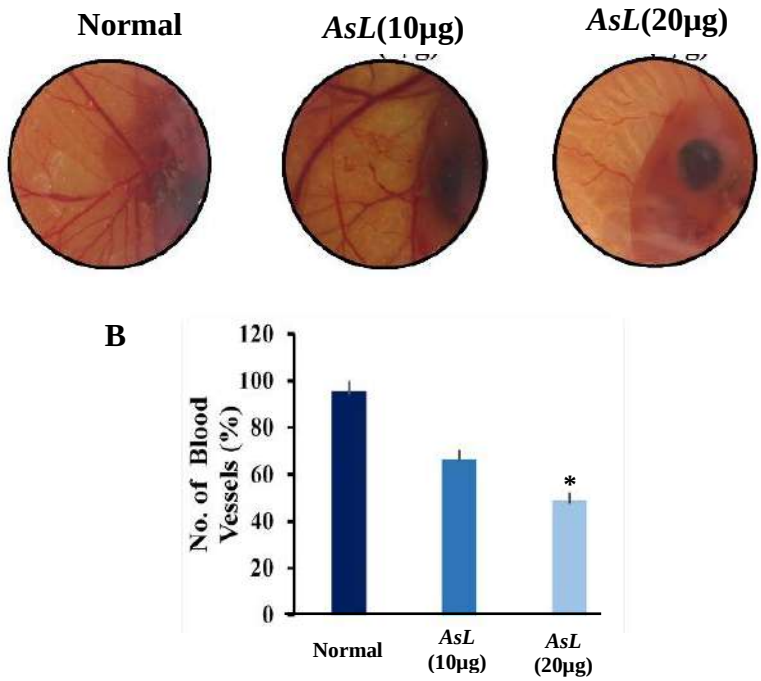


***Fig.63**: CAM: Antiangiogenic activity of AsL in Ova angiogenic assay models: [A and B] Ova CAM and graphical image with anti-angiogenic potential. Treatment with AsL displayed 62% and 55% inhibition of CAM vessel density. AsL treated displays the inhibition of vessel density in CAM where the concentration of AsL was increased the density of the blood vessel drops. Significant p value<0.0001.*

5.10.2. Garlic lectin (*AsL*) exerts anti-cancer effect on ascites tumour model.

EAC cells bearing- mice were sacrificed and the EAC cells along with ascites fluid were harvested. Then, cells subjected to centrifugation and washed with ammonium chloride solution to remove RBC's further washed with 10 mM PBS buffer pH 7.4. Proximately 5 x 10^{6} cells/ mice (0.5 ml) were transplanted to the peritoneum of mice via intraperitoneal (*i.p*) route of administration; the cells were allowed to grow in the mice peritoneum and monitored the weight on daily basis. Using *in-vivo* tumour models, the lethal effect of *AsL* extracts was further confirmed. The mice were given EAC cell lines and *AsL* treatment. to research *AsL's* impact on ascitic tumour models. Administration of *AsL* extracts reversed a dose-dependent reduction in the tumour weight of approximately 35% in an ascites tumour model (Fig 64 A) Reduced tumour volume is correlated with a dose-dependent decrease in ascites secretion to a final level of 4.5 ml as opposed to 8.5 ml in untreated patients (Fig 64 B). The number of cells correlated with the size of the tumour and the release of ascites (Fig 64 C).

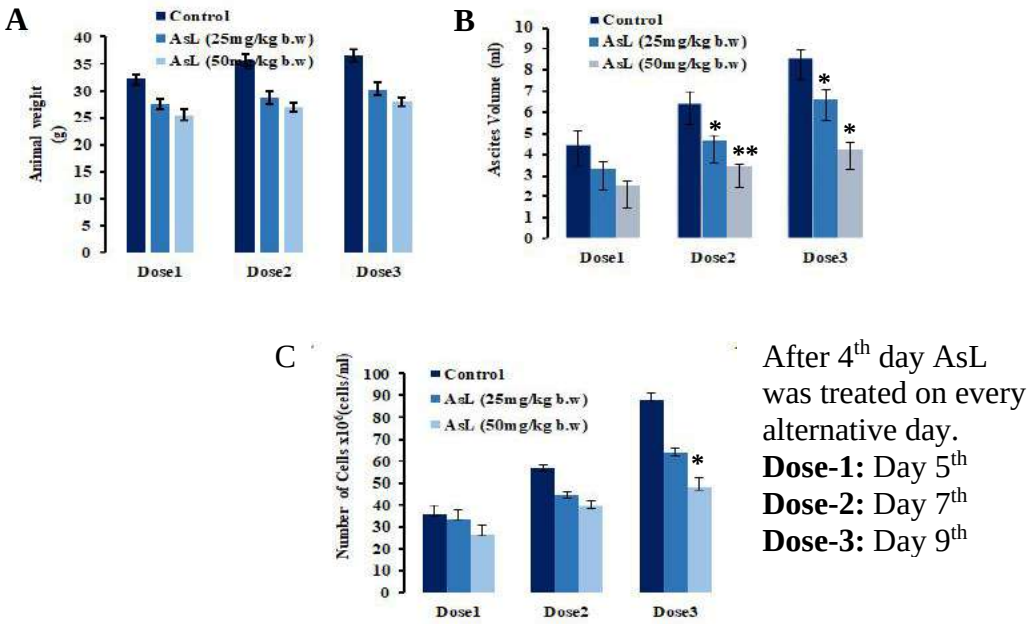


Fig. 64: Ascites tumour was induced in Swiss albino mice by injected EAC cells (5 x 10^{6} cells/mice) intraperitoneally. *[A] Regressed tumorigenic index in the dose dependent manner indicative of the reduction in the tumour growth. [B] Decrease in ascites secretion. [C] Declined tumour cell population. All the parameters were evaluated in comparison to the relevant control tumour bearing mice.*

5.10.3. Garlic lectin (*AsL*) increases the survival rate of the ascetic tumour mice

The survival rate of the mice on treatment with *AsL* at 25 mg/kg body weight and 50 mg/kg body weight had significantly increased the survival period of the mice compared to untreated mice used as control. The observed results indicate that *AsL* exhibit anti-tumour activity and contained the tumor growth and has concomitantly influenced the survivability of the animal. The survival period was extended to 20 days in a dose administered at 25 mg/kg body weight and about 25 days at dose administered at 50 mg/kg body weight Fig 65). This infers that the lectin *AsL* extended the survival rate from 10 days to 36 days which is very significant and indicate its *in-vivo* anti-tumour property against EAC cells.

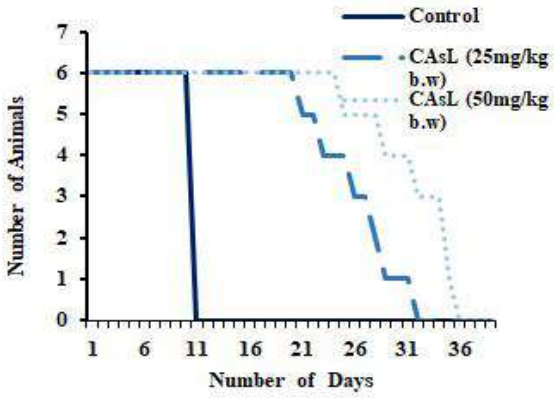


Fig.65: Survivability of treated and untreated mice: *Increased the survival rate as compared to that of control from day 11 to day 38. Kapler-Meier graph showing the prolonged life span of AsL treated animals upon dose dependent manner. Results are the means of three determinations, each conducted in triplicates.*

5.10.4. *AsL* downregulates VEGF level in EAC tumor model

The VEGF level was examined by the serum of all treated and untreated animals through ELISA. *AsL* significantly reduces the VEGF level where, the control animal shows 1270 pg/ml. *AsL* (25 & 50 mg/kg b.w) treated reveals 400 and 380 pg/ml. There is an observed 2-fold decreased in the synthesis of VEGF in *AsL* lectin treated group with respect to the untreated control.

The down regulation of VEGF synthesis induced by lectin signalling had reduced the tumour growth progression that increases the survivality of the mice. This indicates garlic lectin *AsL* had potential anti-tumor properties.

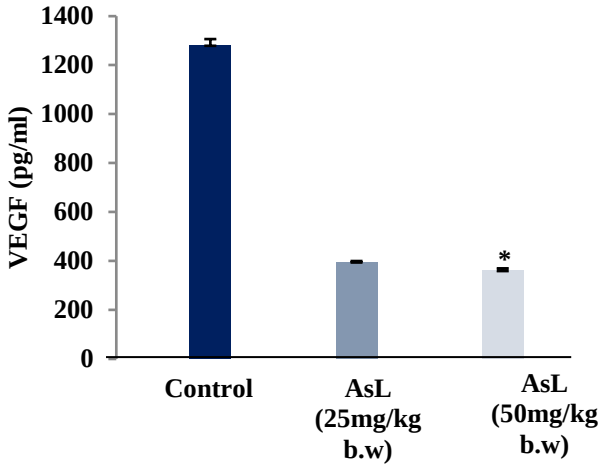


Fig.66: AsL decreases the secretion of VEGF: *Reducing of secreted serum VEGF levels in AsL treated AsL mice in-vivo. Results are the means of three determinations, each conducted in triplicates. Statistically significant values are *p<0.05; **p<0.01.*

5.11. Development of EAC solid tumour model and anti-tumor property of *AsL*.

EAC cell lines were administrated to the right thigh of mice through subcutaneously (*s.c*) to develop EAC solid tumour. Then, the size of tumour was measured using vernier calipers in daily basis and recorded (Fig 67). The tumour size increased day by day and had decreased the survival of the mice.

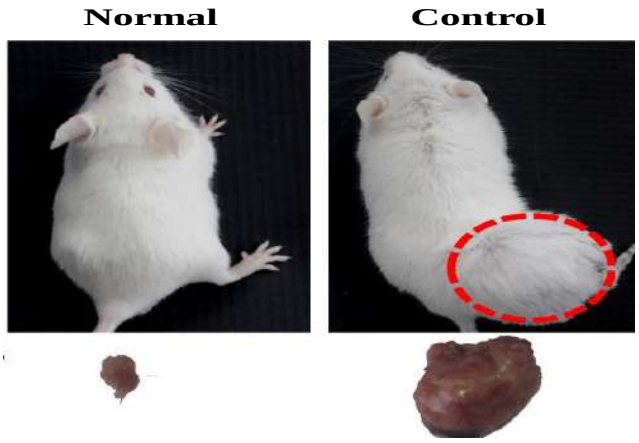


Fig.67. Development of EAC cells in mice: *The EAC solid tumour was successfully established in the mice thigh. It had increased in size drastically compared to the normal mice.*

5.11.1 Anti-tumor effect of the *AsL* on solid tumour growth

The solid tumour growth inhibitory activity of purified *AsL* was studies at two concentrations of *AsL* (25 mg/kg body weight and 50 mg/kg body weight). The mice without induced solid tumor and administered saline had served as normal. The mice with EAC solid tumor with saline administration had served as tumor control. The mice bearing solid tumor and injected with *AsL* was test groups. As seen in (Fig 68 A), The *AsL* treated group had markedly reduced in the EAC solid tumour growth in a dose dependent manner and as observed by visible morphology. The extracted tumour size was also reduced considerably and the same was observed with the size of Liver and spleen indicating the increased immune function to contain or restrict the tumor through increased spleen action (Fig 68 B).

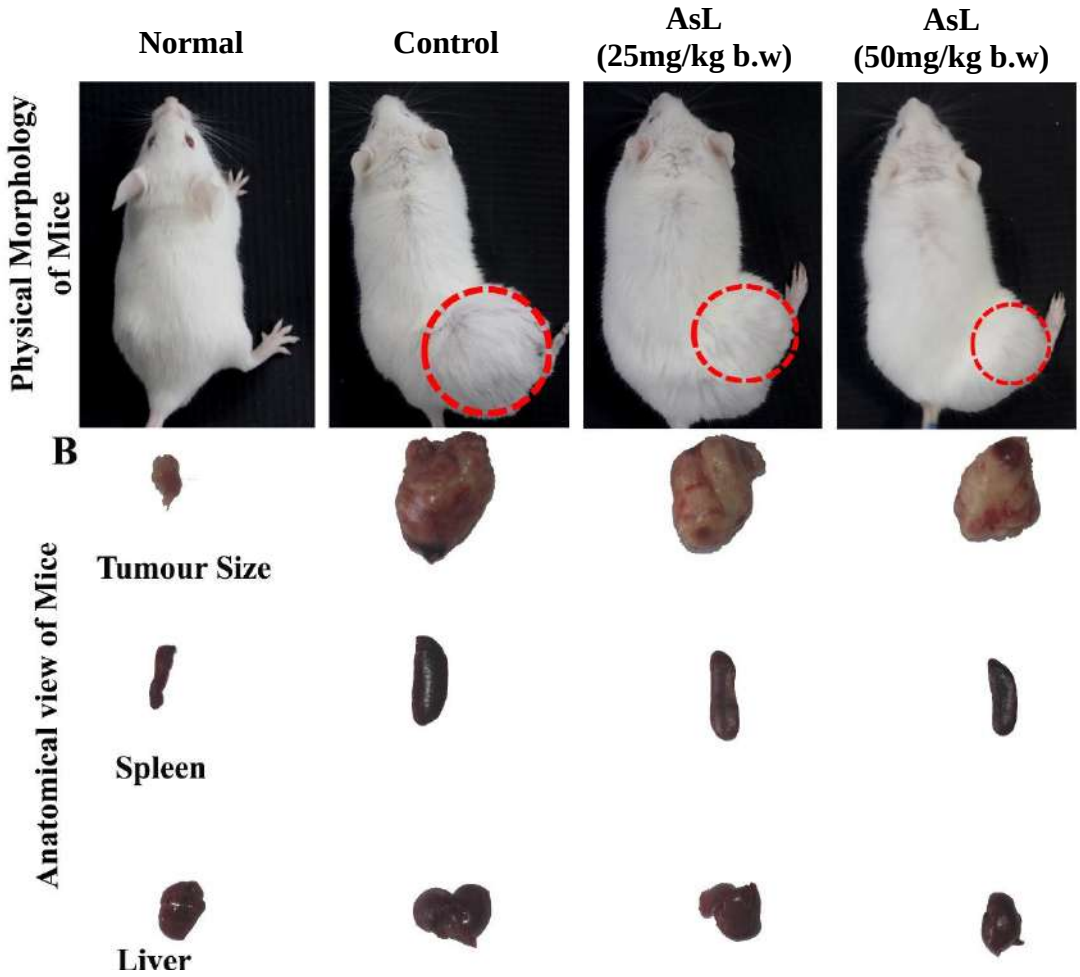


Fig.68: Effect of ASL on EAC Solid Tumour: *Tumour-bearing mice received six doses of AsL at 25 mg/kg and 50 mg/kg b.w. on alternate days. [A] Morphology of the EAC induced mice. [B] Gross appearance of the excised tumour.*

5.11.2 Dose dependent inhibition of solid tumor growth by *AsL*

The tumour inhibitory activity of *AsL* has also reduces the EAC solid tumour growth. The final growth inhibition of up to 65% was observed as measured by vernier caliper. The volume was reduced from 5.5 cm^3 to 2.7 cm^3 and the reduction was seen in a dose dependent manner. There was substantial decrease in the tumor volume at 50 mg/kg *AsL* dose. It had restricted the growth of a tumor and contained its development. The 25 mg/kg *AsL* dose also effective and had performed better to prevent the tumor growth (Fig 69). It has noticed that the tumor had grown substantially to higher volume in control group without any treatment.

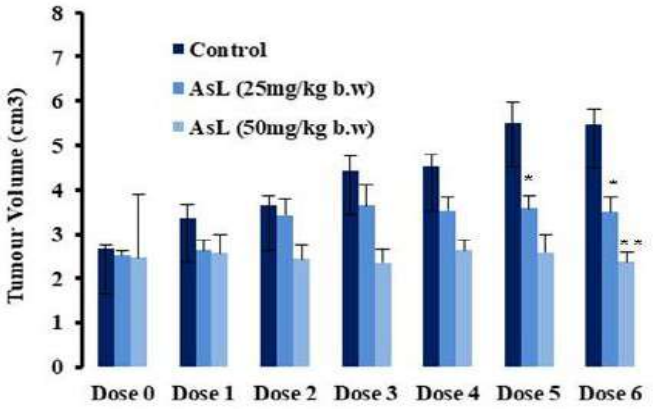


Fig.69: Effect of AsL on EAC Solid Tumour: *Tumour-bearing mice received six doses of AsL at 25 mg/kg and 50 mg/kg b.w. on alternate days. [A] The inhibitory effect of AsL on tumour progression at different time points.*

5.11.3. Comparative weight of EAC tumor development in *AsL* treated groups

The weight of the tumour was comparably smaller with 10.5 g in *AsL* treated group and the control tumor had weighed up to 15.5 g (Fig 70). The size of the tumor was contained and reduced by the action of *AsL* by resisting tumor growth.

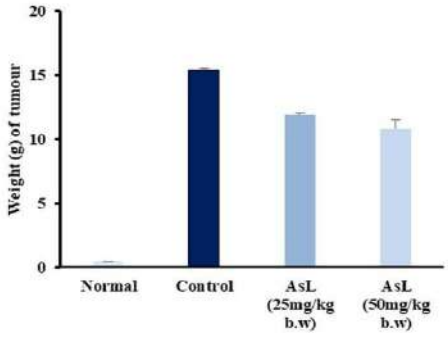


Fig.70: Effect of ASL on EAC Solid Tumour: *Weight of the excised normal thigh, control and AsL treated tumours in gram.*

5.11.4 The administered *AsL* increased the survival rate of the solid tumour mice

After onset of tumour establishment, solid tumour treated with *AsL* (25 and 50mg/kg *b.w*) for six doses on every alternative day. After 6thdose, mice were left and monitored for the maximal survival of animals and was documented in the form of kaplan-Meier survivability curve. The untreated control mice survived for 40 days without any treatment. The mice treated with 25 mg/kg *b.w* had increased the survival period to 80 days and the mice dosed with 50 mg/kg *b.w* increased up to 85 days. This indicates a considerable increase in the survival period of mice administered with *AsL*.

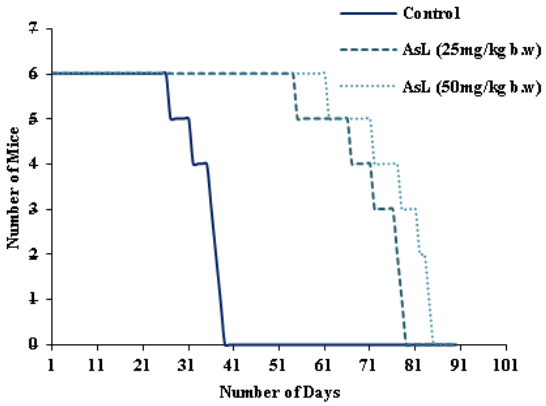


Fig.71: Survivability of AsL treated and untreated mice: *Increased the survival rate as compared to that of control from day 40 to day 84. Kapler-Meier graph showing the prolonged life span of AsL treated animals upon dose dependent manner.*

5.11.5. Haematoxylin and eosin (H&E) stain for EAC treated tumour tissue

The H & E staining was performed to quantify the EAC treated and untreated tumour groups and analyzed. Decreased number of blood vessels indicated the effect of *AsL* treated to solid tumour. Histological section of tumour was observed under the microscope at 40X optical magnification (Fig 72 A) and graphical representation of blood vessels (Fig 72 B). The *AsL* treated groups showed decreased in blood vessel density compared to control groups.

This indicate that, the garlic lectin *AsL* down regulates the VEGF and inhibited neovascularization that suppress the tumour growth. The *AsL* induces antiangiogenic. It also cytotoxic and reduced the tumour size by inhibiting the growth of the solid tumour.

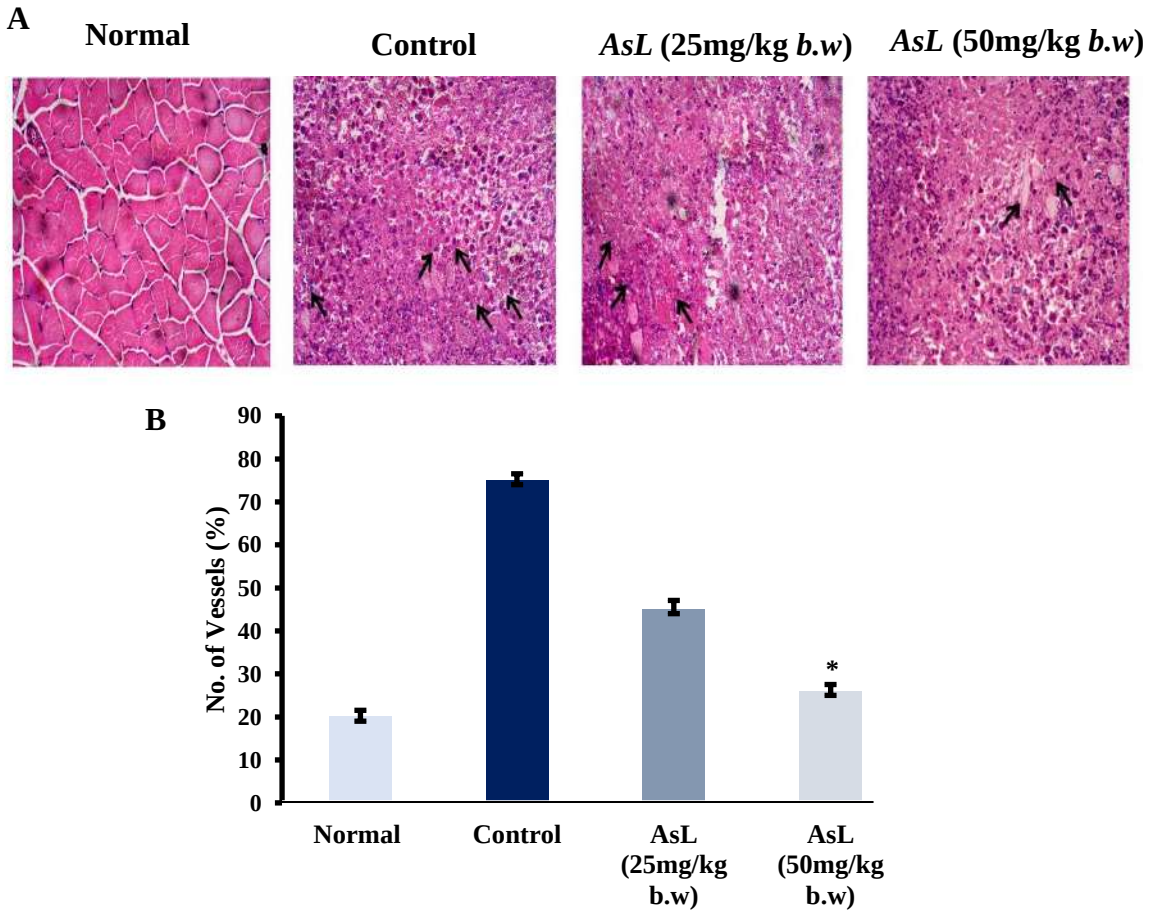


Fig.72: *Haematoxylin and Eosin (H & E) Staining: - [A]H & E stained representative of histological sections of tumour. Images depicting the representative histological section of tumour at 40X optical magnification. [B] Graphically representing number of blood vessels. Significant p-value <0.0001.*

5.11.6. *AsL* downregulates VEGF level

The vascular endothelial growth factor (VEGF) levels were examined and measured from the serum obtained from all the *AsL* treated and untreated control animals through ELISA. *AsL* group showed significantly reduced circulatory VEGF level where, the control animal shows higher value.

The VEGF in control group were 1270±25 pg/ml whereas the *AsL* 25 and 50mg/kg *b.w* treated reveals 400±12 and 380±14 pg/ml respectively that is 2-fold decreased level with respect to the control. The observed decrease in the circulatory VEGF level indicates that *AsL* down regulate VEGF and inhibit new blood vessels formation in the sight of tumor development. The anti-angiogenic property of *AsL* induce ant-cancer properties.

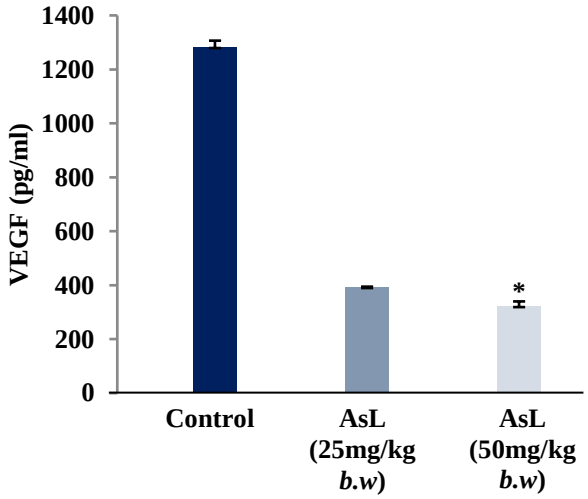


Fig.73: AsL reduces the secretion of VEGF: *Reducing of secreted serum VEGF levels in AsL treated AsL mice in-vivo. Results are the means of three determinations, each conducted in triplicates. Statistically significant values are *p<0.05; **p<0.01.*

5.12. Evaluation of biochemical and signalling molecules from the treated and control solid tumour bearing animals.

The haematological and certain biochemical parameters representing organ function were examined to compare the levels in the treated and untreated group to assess *AsL* induced toxicity. The serum from animals induced with solid tumour and treated with *AsL* were checked for haematological parameters like RBC, WBC, SGOP, SGPT, creatinine and urea. Further, the treated and control sera from animals were assessed for cytokine levels to understand the mechanism of cell signalling during *AsL* induced tumour suppression. The IL-1, IL-6, TNF-α and VEGF-A were evaluated by immunogenic assay and to compare and elucidate possible mechanism.

5.12.1. *AsL* exhibits no toxicological effect in solid tumour bearing animals

The toxicological parameters were analysed after treatment with *AsL* on EAC. *AsL* in spite of being a most effective anti-tumour molecule, had more restricted or no undesirable side effect as confirmed by serum and haematological parameters from mice bearing EAC tumour. The level of creatinine and urea in *AsL* treated were not significantly altered compared to normal and control. Haematological profile postulated that *AsL* not modulated the rate of RBC and WBC in blood corresponded to control and normal mice. SGOT and SGPT used as indicator for Liver functions remains unaltered as compared to that of normal and control. The results are summarized in Fig 74. The results observed indicate that there is no observed difference in the haematological and organ function markers in the treated groups compared to control. This suggest that *AsL* is not toxic and does not show any side effects in the mice even after six doses of administration. The garlic lectin *AsL* does not harm any normal cells and organs and target the tumour cells to act as potential anti-angiogenic and anti-tumour functions.

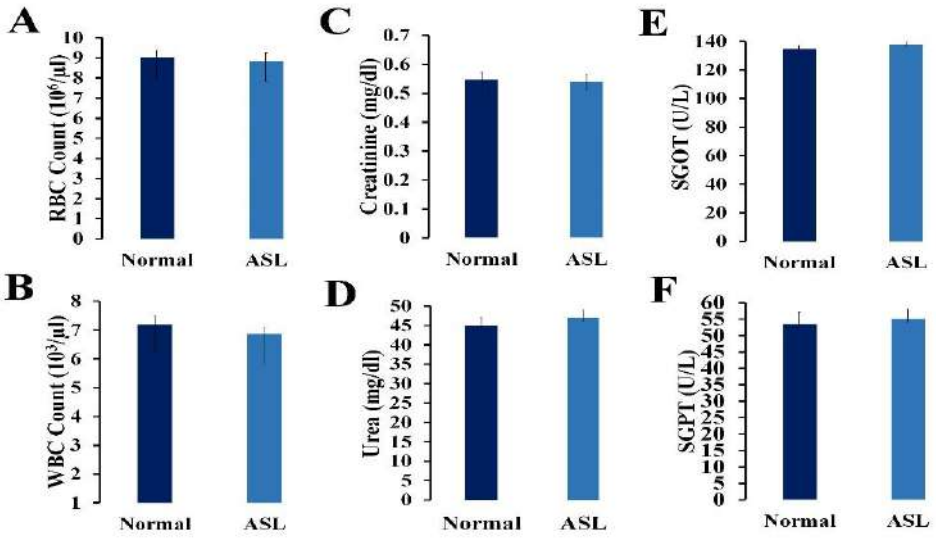


Fig.74: AsL exerts no toxicological effect on experimental mice: *[A&B] The hematology tests, WBC and RBC were analyzed. [C&D] Urea and Creatinine as indicators of renal function. [E&F] Biochemical tests, SGOT, SGPT are used as indicators of liver function.*

5.12. 2. *AsL* gated the secretion of cytokines

The inhibition of cytokines was examined by the serum of all treated and untreated animals through ELISA. *AsL* significantly reduced the level of TNF-α, with the control group showed 900 pg/ml and the *AsL* treated group showed 390 pg/ml. There was marginal difference in the dose of 25 mg/kg & 50 mg/kg *b.w* with 400 pg/ml and 350 pg/ml respectively. The similar results were observed with other cytokines and the observed results confirmed the potent anti-cancer nature of *AsL* through inhibition of IL-1, IL-6 and VEGF. The decreases in these cytokines were observed near to 2- and 2.5-fold inhibition at 50mg/kg *b.w* treated group respectively comparing to control group (Fig 75), where we observed increased TNF-α, IL-1 IL-6 and VEGF in control.

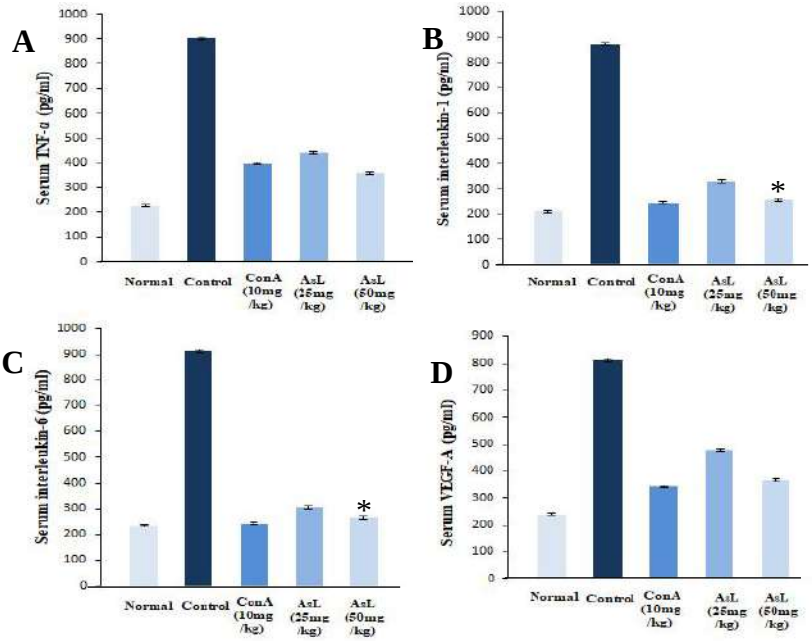


Fig.75: AsL suppresses cytokine secretions: *Reduced secretion of TNF-a, IL-1, IL-6 & VEGF-A of the AsL treated tumour bearing mice. Results are the means of three determinations, each conducted in triplicates. Statistically significant values are *p<0.05; **p<0.01.*

The garlic lectin *AsL* had demonstrate anti-angiogenic and anti-cancer function without inducing any toxicological effects in the murine EAC solid tumour model. The lectin was earlier observed with potential IL-2 inducer from activated lymphocytes that confirms the ability of *AsL* to induce lymphocyte stimulation. The *AsL* induced down regulation of VEGF, stimulate IL-2 and intern activated cell mediated immunity and reduced inflammatory cytokines. This establishes the lectin mediated signalling mechanism in inducing anti-angiogensis and tumour suppression.

Discussion

6. Discussion

Lectins are proteins with a high degree of stereo specificity to recognize various sugar structures and form reversible linkages upon interaction with glycoconjugate complexes. Lectins not only bind to the oligosaccharides on cells but also to the free floating glycans including monosaccharides. They are known to agglutinate various blood groups of erythrocytes, leucocytes and malignant transformed cells. These are abundantly found in nature (plants, animals and many other species) and are found in a whole variety of commonly consumed foods. It appears likely that eliminating lectins from the diet will soon become the next "food trends" for alternative practitioners. During the past two decades dietary lectins have become powerful and indispensable research tools in biology. Lectins have broad application in cell research mainly because of their high specificity and sensitivity during cellular interaction, which allow selective detection and signaling modulation that induce various cellular and biochemical manifestation. The majority of the well-characterized plant lectins have been isolated from the seeds of dicotyledonous species. But lectins of non-seed origin from other species are also emerging as promising tools chiefly because of two reasons: (i) a good number of them might contain novel sugar-binding sites; and (ii) they can provide valuable information regarding the biological roles of plant lectins, which still remain elusive (Van Damme 2014). Further, due to the unique carbohydrate recognition property, lectins have been extensively used in many biological functions that make use of protein-carbohydrate recognition like detection, isolation and characterization of glycoconjugates, histochemistry of cells and tissues, tumour cell recognition and many more (Berg *et al.,* 2002).

Lectins can endure gastric and intestinal digestion and can persist for several days in the digestive tract unaltered. They may attach to intestinal epithelial surface glycoproteins or glycolipids, impairing the ability of the digestive organs to absorb nutrients (Campion *et al.,* 2009). Additionally, lectins are conceived of as antigenic molecules and can stimulate the immune system in a manner similar to immunomodulation. Especially plant lectins show anti-cancer properties. Predominantly, due to alteration in the glycosylation in the cancer cell membrane.

Plant lectins preferentially attached to the membranes of cancer cells or their receptors to cause cytotoxicity in cancer cells by triggering apoptosis, autophagy, or necrosis as well as by reducing cell growth (Lei and Chang, 2009). As everyone understands, there have been many distinct, complicated, assorted biological processes during tumour progression *in-vivo.* Moreover, it is no doubt that those processes should be much variable among several tumour groups or even in individual. Presumably, aberrant glycosylation on specific glycoproteins or glycolipids is deeply related to this diversity of tumour behaviour; therefore, understanding the biological functions and significances of glycosylation including carbohydrate determinants and lectin interactions in tumour will reach the goal of cancer research. So far, it has been very difficult to find the detailed carbohydrate structure on certain glycoproteins or glycolipids as well as the binding partner for several lectins (Quach *et al.,* 2010). In line with previous reports (Smeets *et al.,* 1997), we have purified a well-known mannose specific *Allium Sativum* lectin from garlic bulb and the lectin from garlic owing hemagglutination property. Garlic lectin carbohydrate specificity was found, that mannose/maltose shows inhibition to hemagglutination for garlic lectin. This confirms garlic lectin belongs to mannose specific class, which is strictly specific to terminal mannose residue (Van Damme *et al.,* 1991; Bachhawat *et al.,*2001; Van Damme *et al.,* 1992). Since malignant cells must survive in a favourable microenvironment and avoid the host's anti-tumour immune response, it is clear that immunomodulatory medication types are important in oncotherapeutics (Quach *et al.,* 2010). Garlic lectin can prevent and manage different cancers. These anticancer mechanisms include the regulation of carcinogen metabolism, inhibition of cell growth and proliferation, induction of apoptosis, suppression of angiogenesis, and inhibition of invasion and migration. Garlic can also diminish the negative effects of anticancer therapies. Therefore, our present investigations on tumour regression behaviour of immunomodulatory garlic lectin.

The immunomodulatory potential of *AsL* in the current study led us to focus more of our research on solid tumour, a trustworthy *in-vivo* tumour model that has the potential to have effects on mice. *AsL* successfully regressed the tumour characteristics of the EAC *in-vivo*, according to the results, which also showed an increased survival rate. Unless the altered cells develop angiogenic capacity, they are not tumorigenic. One of the key processes in malignant growth is tumour neo-vascularization, which is

characterised by precocious capillary sprouting and excessively twisted, distorted, and vascular branching (Hanahan and Weinberg, 2011; Bhat and Singh, 2008). We observed, seemingly high disordered blood vessels in both the EAC ascetic tumour and the EAC solid tumour. Our study revealed impressive findings that *AsL* potently regressed the tumour-induced neo vasculature, which positively correlates with non-tumour anti-angiogenic models.

Four alternative processing techniques, including aqueous extraction, neutral buffer extraction, acidic buffer extraction, and heat processed extraction, were used to prepare garlic bulbs extract. The protein contents of raw garlic extraction were calculated, and a lectin confirmation test using a haemagglutination assay was carried out (Table 6) to determine whether lectins are present and haemagglutinate the samples. RGE demonstrated a significant ability to agglutinate rabbit erythrocytes, strongly indicating the presence of lectins (Fig 15). Thus, RGE does not show any visible agglutination for both chicken and human erythrocytes (Fig 16). Comparative studies showed the heamo and leuco agglutinating property of RGE via rabbit blood (Table 7). RGE enhance the immune cell proliferation in *in-vitro* when compared with ConA and control. RGE significantly shows the lymphocyte proliferation as similar to positive control (ConA) (Fig 17). The immunomodulatory effects of RGE on human peripheral blood lymphocyte shows a significant response of RGE and ConA when treated independently. RGE treated along with ConA exhibits a fold increased response as compared to that of control (Fig 18). The angiopreventive effect of RGE encouraged our investigation to extend cytotoxic assessment of RGE on various human and murine cancer cell lines. The exposure of RGE showed cell specific action against the cancer cell proliferation (Table 8) with prolonged activity, Hence, RGE may become a therapeutically active and specific candidate in the cancer treatment. Angiogenesis, the formation of new vessels from pre-existing vasculature is an important mechanism used by tumours to promote oxygen supply for growth and metastasis (Fong, 2009; Wang *et al.,* 1995; Kaelin, 2005; Madanecki *et al.,* 2013). This process is promoted by various angiogenic stimulators including VEGF which is one of the most critical and specific angiogenesis factors regulating normal physiological and tumour angiogenesis (Fong 2009; Folkman 1972; Brown and Giaccia 1998). It is very clear from the results of *in-Ova* non-tumour angiogenic models that RGE has effectual potentiality to regress the

$rVEGF_{165}$ induced neo vessel formation (Fig 19). Moreover, RGE was capable of reproducing the angiopreventive effect in *in-vivo* non-tumour angiogenic models. Tumour angiogenesis is the proliferation of existing blood vessels penetrating into the tumours to supply nutrients, oxygen and is essentially required for the growth and metastasis of solid tumours (Folkman 1972) therefore, inhibition of micro vascular density in tumour is an important aspect in cancer prevention (Bhat and Singh 2008; Al-Ghorbani *et al.,* 2015). Antiangiogenic effect of RGE was reproduced in Ova-CAM model resulting regressed neovascularization. As consequences, the tumour growth parameters such as tumour growth, ascites secretion and survivability were also altered which relied on neovascularization (Fig 20 and 21). Further lectin was purified by passing through ammonium sulphate precipitation at three different saturation fractions (0-20%, 20-50% and 50-90%). The entire obtained protein fractions were again confirmed with the confirmatory assay that clearly exhibits the presence of lectin in all the three saturation fractions. 50-90% saturation fractions show a fold increased in the rate of agglutinating rabbit erythrocytes when compared with that of 0-20% and 20-50% saturation fractions. Thus, confirms the lectins presence (Fig 22). Salting out process was performed in the Amicon ultracentrifuge tubes (3K cut off) via dialysis partially purified garlic lectin was obtained. Protein concentration of various ammonium sulphate saturation fractions and dialyzed samples were estimated and HA activity was determined. As protein concentration increases, HA activity also increases upon purification (Table 9). Achieved samples were subjected to SDS-PAGE to determine the protein profile of the garlic lectin under reducing condition and stained with Coomassie brilliant blue (R-250). The intensive band at 12 kDa determines the presence of garlic lectin (Fig 23).

Furthermore, garlic lectin was purified by sephadex-75 gel permeation chromatography. Three peaks were obtained peak-1 larger molecular protein Allinase get separated first. Followed, by the peak -2a and shoulder peak-2b eluted with slight variation in molecular weight of 12 kDa and 12.5 kDa protein. Representing *AsL*-2a (ASA-I) and *AsL*-2b (ASA-II) (Fig 24). Peak-2a was subjected to Reverse-phase HPLC projected a single peak of garlic lectin (Fig 25) and comparative HA activity was done for entire sample of purification step and obtained yield was mentioned.

The specific HA activity of *AsL* was increased at each purification steps confirms it increased purity (Table 10). Molecular mass determination of purified lectin by SDS-PAGE under assorted conditions was executed. Under reducing condition *AsL* visualized with intense band at molecular weight of 12 kDa (Fig 26). Chased by native gel electrophoresis and observed its native isoforms. The intense band attained between two thin bands with negative charge is considered as AsL. The protein resolved due to slight change in charge and isomeric nature and moved extensively toward positive charge pole Anode indicating the *AsL* protein subunits are negatively charged and acidic in nature (Fig 27). After reducing status, Molecular mass was also determined under non-reducing condition the purified *AsL* protein profile was observed with its native form without breaking their disulphate bond with β-mercaptoethanol. The *AsL* can also clearly observed at non-reducing condition. The *AsL* displayed the molecular mass of 25 kDa in the protein and the comparative reducing gel with a band representing molecular mass of 12 kDa (Fig 28). Protein profile was again confirmed with the silver staining which is highly sensitive than compared to the Coomassie staining. On staining with the silver nitrite, the intensive band of *AsL* can be visualized at the 12kDa (Fig 29). Lectins are the glycoproteins which consist of carbohydrate attached to the protein. As we isolated the garlic lectin, it should display the presence of glycoproteins. The peak-2a obtained from the gel permeation chromatography, show the presence of glycoproteins by subjecting to PAS staining. Glycoprotein appears pink in colour with clear background. BSA was used as control (Fig 30).

Apart from this, the garlic also have abundant lectins and diverse metabolites with multifunctional therapeutic capabilities (Read and Northcote, 1983; Won et al., 2014), despite which there were negligible studies relating to it till date. Lectins are a broad set of proteins with the ability to detect cell-surface carbohydrates with particularity, and as a result they play crucial roles in biological processes (Lis& Sharon,1998; Liener,1991). Haemagglutination is a straightforward test for lectin content in different purification processes (Fig 31 and 32). Both erythrocytes and leucocytes exhibited high AsL agglutination, strongly indicating the presence of lectin (Fig 33). *AsL* shows a very minimal concentration to produce visible agglutination through concentration kinetics (Fig 34). The thermal stability of *AsL* was stable up to 37° C, but on increasing temperature it gradually lost its stability (Fig 35). *AsL* exerts pH stability from acidic to

the basic buffers (Fig 36). The carbohydrate and glycoconjugate binding property of *AsL* indicated that it has a strong preference and higher interaction capacity towards mannose and maltose (Fig 37), and concentration kinetics represented the agglutination was strongly inhibited by glucose, maltose and mannose carbohydrate (Table 11). *AsL* exhibiting the pepsin digestive stability up to 2hr. Digestive products were separated in SDS-PAGE 15% gels under reducing condition (Fig 38).

Antioxidants are defended to play a vital role in body defense mechanism against free radicals or Reactive oxygen species (ROS) which are catastrophic by products raised during aerobic activity in normal cells. Antioxidants can prevent oxidative stress that may cause various generative diseases. Uptake of various types of antioxidants through diet can maintain the normal physiological functions of living system. Several edible fruits and vegetables are abeyant to decrease the hazardous effect of several diseases, like inflammation and cancer (Gaber *et al.,* 2020). The lectins present in the dietary constituents were known to have antioxidant property providing with possible protective role in disease tolerance (Siti *et al.,* 2011). Elevated expression of free radicals induced such as peroxides contribute to neovascularization and thereby increase progression of the malignancy. Comprehensive studies have emphasized the vital interrelationship among immunomodulatory and angiogenesis. The substantial evidence from many experiments demonstrates that T-cells are the key for anti-angiogenic immune response. This recommends the conjugative approach embracing immunostimulatory and anti-angiogenic aspects has convincing immunotherapeutic strategies for treating cancer (Heine *et al.,* 2011; Mengfeng *et al.,* 2008) with immunostimulatory, anti-angiogenic dietary constituents. AsL was screened for free radical scavenging activity using the DPPH method and it is based on the measurement of the reducing ability of antioxidants on DPPH free radical Fig 39. represents the percentage of DPPH scavenging activity of *AsL*, where *AsL* exhibited significant radical scavenging activity which increases with increasing concentration of *AsL.* Lipid peroxidation is the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in oxidative stress induced cell damage.

The results of the *AsL* to inhibiting lipid peroxidation were shown in Fig 40. The lipid peroxidation inhibitory percentage increased with the increased concentration of *AsL*. Anti-inflammatory effects of *AsL* was demonstrated by paw edema to wistar albino rats. Induced paw edema with freund's complete adjuvant and treated with *AsL*. Paw volume and paw thickness was slightly reduced upon treatment with *AsL* compared to the control (Table 12). Serum sample were further used to determine the hematological parameters exhibiting significant count of RBC's, WBC's and hemoglobulin. Also determined the biochemical parameters showing slight significant values of SGOT, SGPT and Creatinine compare to control (Table 13). Gross morphological appearance of paw edema shows the inflammation decreased upon treating with *AsL* on increased concentration. Thus, *AsL* exhibits potent anti-inflammatory properties (Fig 41).

AsL also exhibiting the immunogenicity when produced against rabbit and purified using protein A agarose chromatography (Fig 44). Varied purification fraction was loaded on 8% SDS-PAGE under non-reducing condition. Polyclonal antibodies produced having molecular weight of 150 kDa protein (Fig 45). Anti-*AsL* IgG antibodies and *AsL* antigen showcase a strong specificity to each other and were confirmed through immunoblot analyses (Fig 46). Specificity was also confirmed by comparing with globular proteins and glycoproteins, Thus anti-AsL antibodies shows strong specificity towards *AsL* (Fig 47). Ouchterlony double diffusion analysis expresses the immunoprecipitation line of *AsL* with varied purification steps of anti-*AsL* antibodies (Fig 48). Antigen and antibody titer instructed the minimal dilution fractions required for the antigen and antibody interaction (Fig 42 and 43). The ingested dietary components should pass through the digestive tract, which is the largest immunological organ of the body, where dietary molecules interact with the immune cells. It possesses the largest interactive surface area through intestinal villi that are exposed to the external milieu, and thus confronted with the biggest immuno antigenic load, comprising pathogens, dietary molecules and proteins, and pathogens and commensal organisms (Hasegawa *et al.,* 2015). The 'gut' immune system has the capability to distinguish foreign (toxic) proteins and nutrient proteins (Lamichhane *et al.,* 2014).

Gut-associated lymphoid tissue (GALT) is very much selective in recognizing dietary antigens; the unique gut immune microenvironment provides ambience to stimulate and regulate an immune response to food antigens by the suppression of immunocompetent cells.

Current information indicates that the administration of immunogenic antigen proteins by intradermal or intranasal routes induces vigorous anti-protein IgG immune responses (Lavelle *et al.,* 2000; Roth-Walter *et al.,* 2004). The observations drawn in the present study demonstrated that garlic lectins (ASA I and ASA II) are effective immunogenic proteins which stimulate humoral antibody (IgG) responses following the systemic (intradermal) and mucosal (intranasal) administration of antigens. While the antigenic delivery of ASA I or ASA II induces a higher antibody titer on both the systemic and mucosal IgG immune response in comparison with OVA, the magnitude of the IgG response was found to be different for OVA, ASA I, and ASA II, and was very high for lectin ASA I, indicating its ability to interact efficiently to stimulate immune cells to induce response. The administered antigens had no toxic effect on the growth and development of the experimental animals, and no adverse effects were observed on the mice upon either intradermal or intranasal delivery. It may be recalled here with the report that the oral administration of garlic also resulted in the induction of significant immunogenicity in BALB/c mice (Clement *et al.,* 2010). It was observed that both the garlic lectin, ASA I and ASA II, groups showed a 3–4 fold increase in IgG antibody response on day 50 as compared to the control. Further, garlic lectins were found to be potent immunogenic proteins, and were found equivalent to phytohemagglutinin (lectin from red kidney bean) in immune recognition and stimulation (Clement *et al.,* 2010).

In this study, it is observed that ASA I or ASA II showed reasonable immunogenic properties by inducing IgG immune response against self- and co-administered antigens, thus indicating that garlic lectins have intrinsic immunogenicity despite the absence of an adjuvant. Such antibodies produced were likely to be similar to ‘natural antibodies’ which have been reported for many dietary proteins and several plant lectins, including garlic lectin (Venkatesh *et al.,* 2013).

Human systems have a tendency to have natural antibodies circulating in the serum that have a characteristic broad reactivity and which appear to protect humans against a variety of pathogens not previously encountered. The presence of these food proteins or lectin-triggered natural antibodies in human circulation prior to viral or bacterial infections were of great importance in providing infection tolerance and resistance to pathogenic diseases (Zhou *et al.,* 2007).

OVA is well reported in studies as an antigen with poor immunogenic properties (Kjaer *et al.,* 2002; Lavelle *et al.,* 2000). The results from the present study indicate the same with the observed low anti-OVA IgG antibody titer and a significantly high anti-ASA I IgG (~2.5- to 3-fold) immunogenic response in comparison with the reference antigen (OVA), suggesting that ASA I is a strong immunogen. Anti-ASA II IgG response (~1.3- to 2-fold) showed moderately higher IgG response than OVA, which suggests that ASA II is also a weak immunogenic protein. Since ASA II showed around a 3-fold lower binding efficiency to glycan structures than ASA I (Clement *et al.,* 2010; Dam *et al.,* 1998), this confirms the interrelationship of glycan-binding ability to possible immunogenic properties. Many reports suggest that the food lectins ingested through the diet will efficiently interact with the intestinal villous enterocytes based on their glycan recognition. Most of these lectins will cross the intestinal barrier and enter into the systemic circulation, where they encounter immune cells to activate the humoral immune response by inducing lectin-specific IgG responses. The dietary lectins entering the circulation via uptake across the Peyer's patch for internalization through follicle-associated epithelium instigates the intestinal IgG and IgA immune responses in the intestinal-associated lymphoid follicles (Guptha *et al.,* 2011; Shan *et al.,* 2013; Srivastava *et al.,*2015). The interactive receptors in gut cells, to which lectins bind and interact for internalization, are not well known, but may appear to be the key factors in determining the lectin-induced mucosal immunogenicity (Pasetti *et al.,* 2010). The presented results in the study explore the adjuvanticity potential of lectins from garlic bulbs (ASA I and ASA II) which are co-administered with weak immunogenic antigen OVA through the intranasal route of administration. Garlic lectins exhibit an effective adjuvant property by showing a considerable increase in the OVA-specific IgG antibody titer response in the sera obtained from the OVA + ASA I group as compared to OVA-alone and OVA + ASA II groups.

The observed results clearly indicate that among the garlic lectins, ASA I possesses significant adjuvant properties. RGE administered with OVA showed a slightly higher IgG antibody response than purified ASA I; this might be due to the possible synergistic adjuvant effects of the active garlic components (organosulfur compounds and fructans) in the RGE or the aged garlic extract (Chandrashekar *et al.,* 2012; Chandrashekar *et al.,* 2011; Chandrashekara *et al.,* 2016). Many proteins with an affinity for eukaryotic cell surface molecules are weak mucosal immunogens in mice. Different routes of administration may result in varied immune responses (Srivastava*et al.,* 2015; Smart 2004), as it is likely there may be possible differences in the immune cells present in the nasal-associated lymphoid and gut-associated lymphoid tissues (Pasetti*et al.,* 2010)

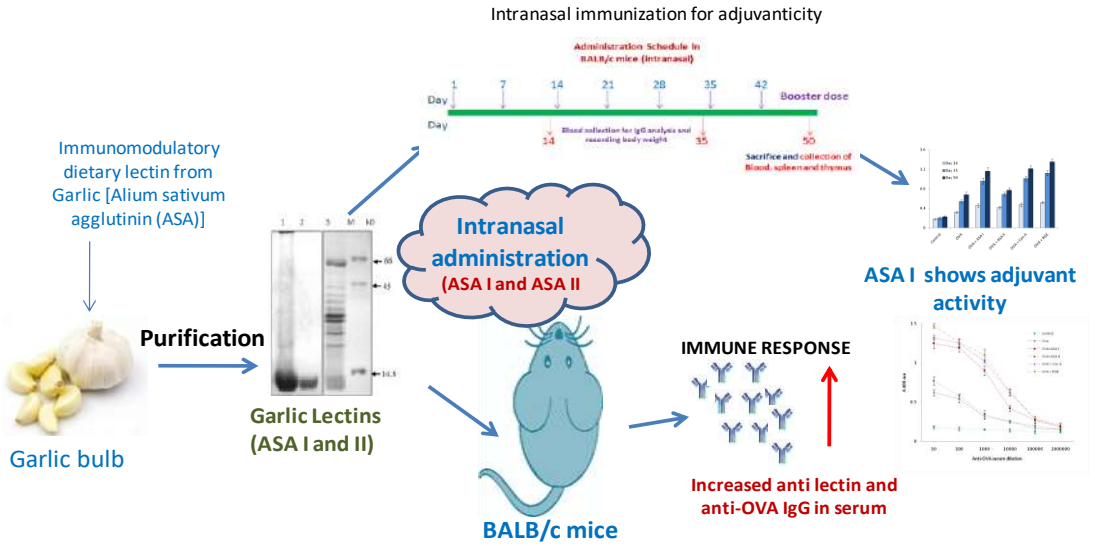


Fig.76: *Illustration of the immune and adjuvant response of garlic lectins (ASA I and ASA II) on intradermal and intranasal administration in BALB/c mice. This explains the ability of garlic lectin, ASA I, as potential mucosal adjuvant for delivering and eliciting immune response against a weak antigen such as ovalbumin, through intranasal administration.*

AsL with lectin activity was examined for its immuno-potentiating potential because various plant lectins cause polyclonal activation of lymphocytes, which leads to proliferation and differentiation into cytokine-producing cells (Qing *et al.,* 2011). The mitogenic action of certain plant lectins is intimately related to leucocyte agglutination (Beeneedicte *et al.,* 1998).

Intriguingly, *AsL* with cell agglutinating ability dramatically induced lymphocyte in vitro proliferation and IL-2 secretion. IL-2 is essential for promoting T and NK cell proliferation and activation as well as controlling these cells effector capabilities against tumour cells (Balkhi*et al.* ,2015). *AsL* having an immunomodulatory effect on the human peripheral blood lymphocytes at increased concentration range, as similar to that of ConA (Fig 55). *AsL* was found to be mitogenic and co-mitogenic for human peripheral lymphocytes with known mitogen (ConA). *AsL* shows the analogous effects that of ConA and increased mitogenicity of *AsL* when co-administrated with ConA (Fig 56). Mitogenicity of Purified *AsL* and RGE was further compared with the known mitogen (ConA). Upon same concentration of both *AsL* and RGE, AsL shows a fold increased as similar to the known mitogen, than compared to the RGE (Fig 57). Immunostimulatory effects of both *AsL* and RGE on murine splenocytes. RGE shows a similar stimulatory effect as ConA, *AsL* displays slight decreased mitogenic property on murine splenocytes than compared to that of ConA and RGE at increased concentration (Fig 58). Immunostimulatory effects of both *AsL* and RGE on murine thymocytes, *AsL* and RGE shows a slight increased mitogenic effects in murine thymocytes independently than compared to that of known mitogen (ConA) (Fig 59). Comparative mitogenic effects of *AsL* and RGE with known mitogen (ConA) on murine lymphocytes, splenocytes and thymocytes. *AsL* exhibits more effective about 3 fold increased as compared to normal lymphocytes, splenocytes and thymocytes. RGE shows a significant effect on both murine and human lymphocytes (Fig 60). The conditioned media obtained from lymphocytes treated with *AsL* in cell culture, parade 2-fold increased secretion of cytokine IL-2 of *AsL* treated animals compared to the control (Fig 61).

As evidenced from our study, stimulated immune system could have eventually lead to the *AsL* anti-proliferative and anti-tumour activity against malignant cells *in-vivo* (Table). Our study examines the active ingredients in *AsL* responsible for the agglutination and antiproliferative activity revealed cytotoxicity against A375 cells *in-vitro* with the IC_{50} value at ~12µg in a similar fashion like that of the *AsL* (Table 18). This process is promoted by various angiogenic stimulators including VEGF which is one of the most critical and specific angiogenesis factors regulating normal physiological and tumour angiogenesis (Fong 2009; Folkman 1972; Brown and Giaccia

1998). It is very clear from the results of in-vitro non- tumour angiogenic models that *AsL* has effectual potentiality to regress the $rVEGF_{165}$ induced neovessel formation (Fig 63).

AsL was then subjected to in-vivo anti-tumour studies using Ehrlich ascites carcinoma tumour model. Our results indicate that *AsL* significantly reduced the tumour progression which is evident from the tumour weight and cell count (Fig 64). Ascites supports the tumour cell proliferation and its secretion can lead to the further aggravation of disease (Thirusangu *et al.,* 2016a and 2016b). In our study, *AsL* treated mice showed significant reduction in the ascites secretion which was evidently linked to the significant tumour inhibition in mice with prolonged survival (Fig 65). The level of VEGF will be high in the secreted ascites of tumour bearing animals and it is potentially liked to the tumour cell proliferation and neo angiogenesis (Vijay Avin *et al.,* 2014c; Thirusangu *et al.,* 2017a). Results henceforth indicate that *AsL* has down regulated the level of VEGF which inhibited the angiogenesis thereby halting the tumour growth (Fig 66). Therefore, it is clear from this observation that *AsL* potently inhibited the tumour cell proliferation most evidently through the neo vasculature blockade. In addition, altered cells do not develop tumorigenic potential until they develop angiogenic potential (Tandle *et al.,* 2004). One of the key processes in malignant growth is tumour neo-vascularization, which is characterised by precocious capillary sprouting and excessively twisted, distorted, and vascular branching (Hanahan & Weinberg, 2011; Folkman, 1990; Bhat& Singh, 2008). It is commonly known that EAC causes severe vascular diseases that make a person more invasive. *AsL* Significantly reduced the tumour progression which is evident from the tumour weight (Fig 70). Regression of the tumour size and increased the survival rate of the *AsL* treated mice (Fig 71). Our current study revealed that *AsL* treated showed very low MVD count as evident from the microscopic evaluation of H&E stained solid tumour tissue section (Fig 72). The level of VEGF will be high in the secreted ascites of tumour bearing animals and it is potentially liked to the tumour cell proliferation and neo angiogenesis (Vijay Avin *et al.,* 2014c; Thirusangu *et al.,* 2017a). Results henceforth indicate that *AsL* has down regulated the level of VEGF which inhibited the angiogenesis thereby halting the tumour growth (Fig 73). *AsL* exerts no toxicological effect on experimental

mice (Fig 74). Also reduces the secretion of cytokines such as Tumour necrosis factor-α, interleukins-2, interleukins-6 and VEGF-A (Fig 75).

Overall, the above observations, study reports that the lectin from the garlic presents an immunostimulatory characteristic with significant anti-proliferative activity targeting angiogenesis which is the core hallmarks of cancer. The creation and identification of such molecules may have a potential role in preventing cancer, as some plant components are reportedly of utmost relevance to immune function. With the help of these studies, we recommend that *AsL* be investigated as a potential cancer treatment candidate targeting angiogenesis via immune system modulation. Future research involving pure protein fraction and in-depth mechanistic studies would undoubtedly open up a new area for the creation of novel cancer agents and drugs, particularly lectins derived from plant sources.

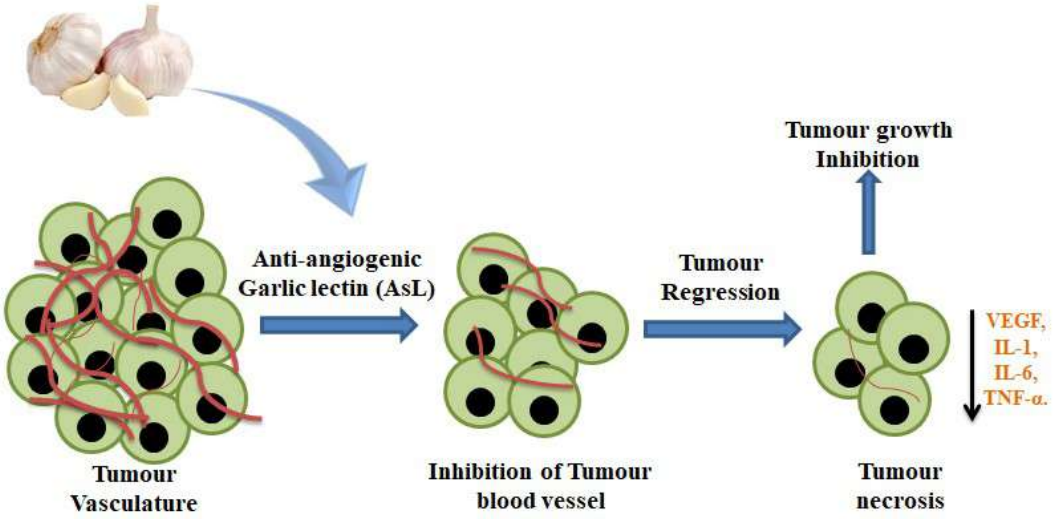


Fig.77: *Schematic representation of garlic lectin upregulating the secretion of some cytokines (VEGF, IL-1, IL-6 and TNF-α), in turn downregulate the tumour growth.*

General Summary & Conclusion

7. General summary and conclusion

Allium sativum. L (*AsL*) "garlic", one of the most interesting medicinal plants, has been suggested to contain compounds that could be beneficial in numerous pathological situations including cancer. However, *AsL* was identified as a promising anti-cancer with multiple modes of action such as antiproliferative and anti-angiogenic, its consumption through healthy diet may have beneficial effects on cancer. We attempted to isolate lectins from the garlic and to evaluate the agglutinating proteins for immunostimulatory and anti-angiogenic efficacy. *AsL* significantly deduced strong lectin activity by cell agglutinating behaviour. *AsL* agglutinated rabbit erythrocytes. *AsL* haemagglutination activity was inhibited only by carbohydrates such as glucose, mannose and maltose indicating *AsL* specificity for saccharides. *AsL* have a stability at extreme high pH buffer, but at increased temperature *AsL* loses its activity. *AsL* acts as an anti-oxidant agent by determining the Malonaldehyde (MDA) through DPPH assay and lipid peroxidation assay. *AsL* exhibits potent anti-inflammatory property when used to treat paw edema for rats. *AsL* acts as a potent immunogen by producing antibody against New Zealand white rabbit. Specificity of anti-*AsL* antibodies and *AsL* antigen exhibits the immunogenicity of *AsL.*

Garlic bulb lectins, specifically ASA I, exhibit a strong mucosal and systemic immune response by inducing anti-lectin IgG antibodies by both intradermal and intranasal routes of administration. ASA I also has the ability to produce a marked stimulation and proliferation of splenocytes and thymocytes in order to enhance the efficacy of weakly immunogenic 'subunit' vaccines (recombinant), there is an unmet need for specific adjuvants and targeted delivery systems that are efficacious when given mucosal (either by oral or nasal routes). ASA I, has all the properties to function as a mucosal adjuvant and can boost the humoral immune response to co-administered weak immunogenic antigens. This can be a potential dietary adjuvant for future consideration in targeted delivery by weak antigens for immunization through mucosal administration.

The observed results in our study demonstrate that garlic lectins (at 30 µg dose by *i.n.* administration) elicited humoral immunogenic response by inducing the increase in serum IgG titer in BALB/c mice, thereby revealing their intrinsic immunogenicity. Further, garlic lectins displayed humoral adjuvant immune response for the poor immunogenic antigen OVA (a reference model weak antigen), as is evident by the

observed increase in the serum anti-OVA IgG antibody on days 35 and 50. The thymic and splenic indices of the garlic lectins administered through the intranasal route in the mice group were significantly higher than that of control or OVA groups. All in all, it appears that the intranasal administration of garlic lectins leads to the production of natural antibodies (serum IgG), which acts as mucosal adjuvants for the delivery of weak antigens. Therefore, garlic lectins (especially ASA I) are dietary immune system boosters and have the potential to stimulate immune cells involved in humoral immunity and are of great use as mucosal adjuvants for various experimental antigens (oral or nasal vaccine candidates with weak mucosal immunogenicity) in future studies on humans. The studies have some limitations in deciphering the mechanisms of mucosal immunogenicity through the activation immune cells and in understanding the impact of specific cytokine signalling. Further, it will require additional studies with a dose-dependent adjuvant and lectin combination so to optimize the garlic lectins for mucosal antigen delivery. However, the present study is the first proof identifying garlic lectins as a mucosal adjuvant to deliver antigens efficiently through oral or nasal mucosal routes. In addition, it has ability to activate immune cells to boost immune response and has no side effects with the present chemicals or with other immunoadjuvants. Hence, garlic lectins are promising future potential biological adjuvants of choice for antigen delivery through mucosal routes for efficient immunogenicity.

AsL showed immunomodulatory effect by stimulating mitogenicity in murine and human peripheral blood lymphocytes. Verifying the role of secreted cytokines by *AsL* triggered PBL's in cultured media indicated that il-2 levels were significantly high. *AsL* exerted cytotoxicity and anti-proliferative effect on EAC, A375, A549, B16F10, HUH7 cells as assessed by MTT assay. *AsL* potently affected the vital tumour parameter in mice thereby leading to the regressed the tumour cell expansion in Ehrlich ascetic carcinoma and solid tumour. Moreover, it is observed that *AsL* has directly affected the growth and proliferative potentials of malignant cells *in-vivo* by the modulating the immune system and regressing the tumour vascular supply. Further studies on purified protein provided tangible evidences that lectin from the garlic is decisively responsible for immuno-potentiating ability and tumour regression by targeting angiogenesis.

Allium Sativum L. was evaluated for anti-proliferative activity against EAC cells. Although the pathological effect of plant lectins is well-documented. For the first time, to our knowledge. this study illustrates the critical role of Garlic lectin as anti-angiogenic molecule in cancer therapeutics. Future experiments with purified protein fraction and an in-depth mechanistic study would definitely open a new perspective field for novel agent and drug development for cancer specifically lectins from plant sources.

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Annexure I & II

9. List of Publications:

1. **Shruthishree D. Padiyappa**, Hemavathi Avalappa, Madhusudana Somegowda, Shankarappa Sridhara, Yeldur P. Venkatesh, Bettadatunga T. Prabhakar, Siddanakoppalu N. Pramod, Mona S. Almujaydil, Shadi Shokralla, Ashraf M. M. Abdelbacki, Hosam O. Elansary, Ahmed M. El-Sabrout and Eman A. Mahmoud (2022). *Immunoadjuvant and Humoral Immune Responses of Garlic (Allium sativum .L) Lectins upon Systemic and Mucosal Administration in BALB/c Mice.* **Molecules**, 27, 1375. **(Impact factor - 4.4). doi.org/10.3390/molecules27041375**

2. **Shruthishree D. Padiyappa**, Hemavathi Avalappa, Yeldur P. Venkatesh, Nagaraj parisara, Bettadatunga T. Prabhakar, Siddanakoppalu N. Pramod (2022). *Characterization of antioxidant, Anti-cancer and Immunomodulatory functions of partially purified garlic (Allium sativum .L) lectin.* **Biomedicine**, 42nd volume**. (Impact factor - 0.06).**

10. List of paper presentation in international/ national conference:

1. **Shruthi Shree D P,** B. T Prabhakar, S.N. Pramod. *Purified lectin from Allium sativum L. induced anticancer potentiality in EAC solid tumour,* International webinar on Current trends and alternative approaches to target covid-19, 2021, Department of chemistry in co-ordination with IQAC, Sahyadri science college, Shivamogga, India.

2. **Shruthi Shree D P,** Pramod S.N, Prabhakar B.T. *Purified lectin from Allium sativum L. as potential cancer therapeutic in EAC solid tumour,* 42nd Annual conference of Indian Association of Biomedical Scientists (IABMS-2021), CRL-KSHEMA & NUCSER, Nitte, Mangaluru, India.

3. **Shruthi Shree D P**, Prabhakar B.T, Pramod S.N. *Purification and production of anti-rabbit polyclonal antibody against purified lectin from garlic (Allium sativum),* National conference on Impact of research development in life sciences, 2022, Kuvempu University, Shankarghatta, Shivamogga.
   [BEST ORAL PRESENTATION AWARD]