IMMUNOLOGICAL EFFECT OF WATER SOLUBLE POLYSACCHARIDES OF *CINNAMOMUM ZEYLANICUM* ON HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS AND RAW264.7 CELLS.

A thesis submitted in partial fulfilment of the requirements for

the degree of

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IN

BIOTECHNOLOGY

By

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CERTIFICATE

This is to certify that the project entitled "**Immunological effect of water soluble polysaccharides of *Cinnamomum zeylanicum* on human peripheral blood mononuclear cells and RAW264.7 cells**" submitted by Harlovleen kaur in the partial fulfilment of the requirement for the award of degree of Master of Science in Biotechnology to Department of Biotechnology, Thapar University, Patiala, is a record of student's own work carried by her. The report has not been submitted for the award of any degree or certificate in this or any other University or Institute.

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled "**Immunological effect of water soluble polysaccharides of *Cinnamomum zeylanicum* on human peripheral blood mononuclear cells and RAW264.7 cells**" in the partial fulfilment of the requirement for award of the degree of Master of Science in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my work during the period of six months from January 2017 to June 2017, under the guidance of Dr. Manoj Baranwal, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other diploma or degree.

Harlovleen Kaur.

Place: Thapar university, Patiala

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Harlovleen Kaur

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ABSTRACT

Spices are known to possess several biological properties such as anti-inflammatory, anti-bacterial, anti-viral and anti-tumour. *Cinnamomum zeylanicum*is one of the oldest spice used for its medicinal properties. Present study focusses on the immunological assessment of water soluble polysaccharides in human peripheral blood mononuclear cells (PBMC) and RAW 264.7 cells. Water soluble polymeric substances obtained after performing Soxhlet extraction with four different solvents (hexane, dichloromethane, chloroform and methanol) was analysed for the presence of polysaccharides which confirmed the presence of sugar content. All four solvent extracts and the crude polymeric substance were subjected to the phytochemical screening for six different phytochemicals (alkaloids, phenols, terpenoids, flavonoids, quinones and tannins). Absence of phytochemicals in water soluble polymeric substances lead to conclusion that the extracts are rich in polysaccharides. The resultant crude polysaccharides were purified by adding saturated sodium bicarbonate solution and fractionating with equal volume of ethyl acetate. The crude and purified polysaccharides were found to have cell growth inhibition effect on mouse leukaemia macrophage cell line (RAW 264.7) with IC50 value 1120 ± 27 (crude) and 1780 ± 227 µg/mL (purified). In PBMC, the cell viability was found to be increase with increase in concentration for both ethyl acetate fraction and crude extract which is indicator of immune-stimulating activity. RAW cells were also assessed for the nitric oxide release and it was found that nitric oxide release was increased with concentration. The samples were assessed for IFN-Y production and an increasing trend was observed with concentration. Further, the crude sample and ethyl acetate fraction was assessed for antioxidant activity and it was found that in contrast with ethyl acetate fraction, crude polysaccharides showed free radical scavenging activity. Hence, it was suggested that water soluble polysaccharides possess cytotoxic, immunostimulant and antioxidant effect.

CHAPTER 1: INTRODUCTION

Plant based medicines are used since ancient times i.e. 6000 BC as described in the Ayurveda. These plants derived drugs can be formulated using either the entire plant or any part of the plant such as flowers, bark, root or the fruits (*Vangalapatiet al.*, 2012). Among 21,000 medicinal plants listed by the World Health Organization (WHO), approximately 2500 species are native to India, which makes India to stand at the first in the production of medicinal herbs (*Mahima* et al., 2012). This numerous treasure of medicinal herbs brings India the distinction of 'the botanical garden of the world'. The medicines derived from the plants are known as herbal or traditional medicines (*Narayanaswamyet at.*, 2011).

Spices such as garlic, ginger, turmeric, cinnamon and herbs such as oregano, rosemary, and poppy are used as an ingredient in food preparations where they are used to enhance the flavour and aroma. Along with their flavour enhancing properties, spices and herbs are also known to possess certain therapeutic advantages. The medicinal properties possessed by these spices have drawn interest of researchers towards the exploration of the compounds contributing towards these properties. Various compounds such as curcumin, eugenol and cinnamaldehyde have already gained importance in global market as they are used as active ingredient in cosmetics and medicines. In the third world countries, majority of the population relies on the herbal medicines (WHO 2002). *Cinnamomum zeylanicum* commonly known as Cinnamon or Dalchini is utilised across the world since several centuries for its flavour, aroma and therapeutic advantages. It belongs to the family Lauraceae of the genus *Cinnamomum*. It comes under a large genus of 250 species, most of which are aromatic. Apart from its use as a spice, it is also known to possess a wide range of pharmacological properties such as antibacterial and antifungal (*Seenivasanet al., 2001*), antioxidant (*Mazimbaet. al., 2015*), anti-inflammatory (*Honget al.,2012*), anti-tumour (*Kwonet. al.,2009*), anti-diabetic (*Desokyet. al.*, 2008), and anti-ulcer (*Jakhetiaet al.*, 2012). The bark of cinnamon is widely used component of the herbal medicine for treating common cold, cardiovascular diseases and chronic gastro intestinal and gynaecological disorders (NCCIH, 2001). Cinnamon is known to contain protein, fibre, potassium, vitamin A, vitamin C, iron as well as sodium (*Vanglapati et al., 2012*). It is used in the manufacturing of the toothpaste, mouthwash, lotion, pharmaceuticals, cosmetics, chocolates, candy, tea, alcoholic beverages and stimulating beverages.

Apart from various secondary metabolites which have been known for their medicinal

properties, other compounds such as polysaccharides and proteins isolated from plant based sources have recently attracted researchers to explore its properties. Polysaccharides from spices have been reported to have bioactive properties. Ginsan and panaxanes polysaccharides isolated from *Panax ginseng* spice *have shown enhancement in* nitric oxide cytokines production (Carbohydrate research, 2017). In addition to these activities, they have also reported to possess, phagocytotic and cytotoxic effect.

Present study is focussed on exploring the uses of polysaccharides isolated from *Cinnamon zeylanicum* which are useful in modulating the immune response. The effect of water soluble polysaccharides is evaluated on peripheral blood mononuclear cells (PBMC's) and mouse macrophage cell line (RAW 264.7).

CHAPTER 2: REVIEW OF LITERATURE

2.1 Traditional medicine

Traditional medicine can be explained as an outcome of the practice and knowledge which is native to a particular region or area and are exploited to maintain the health and also to cure, prevent or diagnose any kind of illness (WHO, 2003). Asian, African and Latin American countries have been using the traditional medicines for a long time and became an integral part of their household. These preparations are also termed as the "Non-Conventional medicines". These traditional medicines are also used in different countries very effectively:

Out of the total medicinal consumption, about 30-50% herbal preparation is used in China and they referred it as traditional Chinese medicine. Traditional medicines have shown a positive growth rate in Germany, 2014 because of its natural origin and very less side effects (Euromonitor International, 2015). In countries such as Nigeria and Ghana, traditional medicine has been used to cure about 60% of the people suffering from the fever as a result of malaria (WHO, 2015). In countries like London and South Africa about 75% people with HIV/AIDS depends on the traditional medicine (WHO, 2015). Global market for traditional medicine in US rose to $593 million in 2014 and it is expected to grow high (Euromonitor International, 2015). In developing countries like India, traditional medicine is being used for many decades and now more than 1.5 million practitioners are using these for treating the illness.

2.2 Spices and herbs

Spices and herbs are known to possess the medicinal, flavouring and preservation properties. In earlier 50,000 B.C, it was said that different parts of the plant like leaves, bark, root, stem, resins, essential oils and fruit contains different ingredients which play an active role in immunity (WHO, 2000).Our ancient medicinal system i.e. Ayurveda system is a good storehouse of knowledge for herbs and minerals having medicinal properties. Various medicinal plant**s** used in traditional medicine called Rasayanas that increases the body's immunity to protect from different infections. Herbal or plant therapy is generally called as "RasayanaChikitsa". The drugs obtained from this therapy confer with several properties like prevention of diseases, age delaying, and improving mental functions(*Sharma*, 1983).

Herbal plants are known to exhibit a number of biological activities such as anti-ageing, anti-stress and immunomodulatory activities. In the recent past, herbal plants have been reported

to help in conditions to those suffering from flu, cold, rheumatoid arthritis, infections of skin, hepatitis, heart diseases, and asthma. and also shown effective results in treating cancers (*Mathew et al.*, 2010; *Umashanker* and *Shruti*, 2011). Apart from these infectious and systemic diseases, herbal medicines are also effective for ear infections, wounds, and burns (*Mizaei-Aghsaghali*, 2012).

It has also been mentioned that more than 60% of the drugs for the anticancer and anti-inflammatory are now been made by the herbal preparations (*Kigenet. al., 2013).* Therefore, nowadays medicinal plants have become an essential source for the discovery of new drugs which are coming into the market.

2.3 Herbal preparations

Herbs are known for the flavour, scent or the medicinal properties that they possess. Different parts of the plant like stem, root, bark, leaves, fruit, and seed are used for the herbal or traditional preparations. Also, gums, resins, powders, juices and essential oils derived from them constitute the traditional medicines. These medicines can be processed by methods such as extraction, concentration, fractionation, heating, and soaking. Herbal preparations are easy to administer in the form of juices or powders. Some of the common examples of herbs possessing the medicinal properties are mentioned in Table 1:

Table 1: Examples of herbal plants and their properties (NCCIH, 2011)

HERB		PROPERTIES
Allium sativum(Garlic)		Anti-microbial, Anti-cancer
Aloe vera		Anti-inflammatory
Azadirachta indica(Neem)		Anti-bacterial, Anti-malarial
Papaver rhoeas(Poppy)		*Soothes bronchitis, asthma*
Ocimum sanctum (Tulsi)		Anti-bacterial, Anti-fungal

Lavundala angustifolia  Anti-septic

Zingiber officinale(Ginger)  Relieves cold and cough

Source: http://articles.mercola.com/sites/articles/archive/2014/09/01/medicinal-plants.aspx

2.4 Medicinal properties of herbal or traditional medicines:

Herbal medicines possess antibacterial, antifungal, antiviral and anti-inflammatory properties (*Rios and Racios*, 2015). They are also useful in reducing or preventing systemic or infectious diseases such as flu, rheumatoid arthritis, hepatitis, asthma and heart diseases. These medicines play a number of immunomodulatory functions like modulation of cytokine secretion, release of histamine, production of immunoglobulin, lymphocyte proliferation and promotion of phagocytosis (*Spelmanet al., 2006)*. They also help in the treatment and fighting against the cancer (*Kamiyamaet al., 2005).* These medicinal medicines are also given as adjuvants for enhancing the vaccine response (*Raghupathiet al.,2008).* Apart from the use in human health, it is used to treat number of diseases in animals as well (*Blechaet al.,2001)*. Number of active ingredients are present in the herbal plants which make them a potent herbal drug are given in Table 2.

Table 2: Active ingredients present in herbal drugs (Martins Ekor *et al*.,2000)

Herb	Active ingredient
Zingiber officinale(Ginger)	Gingerol, shagaol, paradol
Allium sativum(Garlic)	Alliin
Ephedra sinica	Alkaloid ephedrine, pseudoephedrine
Papaver rhoeas	Rhoeadine
Ginkgo biloba	Ginsenosides
Tussillago farfara	Pyrollizidine alkaloids
Aloe vera	Anthraquinone
Azadirachta indica(Neem)	Nimbidin, azadirachtin, gallic acid, mahmoodin
Ocimum sanctum (Tulsi)	Eugenol, ursolic acid

2.5 Benefits of herbal over conventional medicines:

Conventional medicines generally address the symptoms whereas the traditional medicines or herbal medicines can improve or reverse the condition thus, they treat the root cause. Being natural resources, they are safer and have lesser side effects. Traditional medicines have higher efficacy over time as compared to the conventional medicines. Conventional medicines are these days becoming more and more drug resistant due to the continuous use but herbal medicines do not show drug resistance and any of the environmental effects as well. Administration of these herbal drugs is also easy. Conventional medicines are very expensive and everyone cannot afford those, the herbal medicines are cost effective.

2.6 Polysaccharide from herbal plants:

Complex carbohydrates or polysaccharides present in all plants and fungi account for essential therapeutic properties. Polysaccharides are the polymeric carbohydrates which are made up of number of monosaccharide units which are attached to each other by glycoside linkages. They do not crystallize and are usually tasteless or are light sweet in taste. They appear brownish or white in colour.

Stimulation of immune system with immune modulators can elevate host resistance to the unknown pathogenic threats. Numerous bioactive polymeric substances have been isolated from a wide range of medicinal plants which have been shown to possess immunomodulatory activity though their ability to modulate the function of macrophages. Appropriate enhancement of innate immune system by bioactive compounds can increase host defence responsiveness thus, due to low level of toxicity and higher potency level, polysaccharides from plants becomes the ideal candidates for therapeutics with immunomodulatory effects (Igor *et al.,2008).*

Polysaccharides from different herbal plants like *Terminalia macroptera, Opuntia polyacantha* have been investigated earlier for biological activities. It has been identified by GC that in *Terminalia macroptera*, the composition of the polymer contained the neutral monosaccharides ie. Arabinose, rhamnose, galactose and glucose in different amounts (Yuan fengzou*et al.,2014).*

2.7 Spices and their immunomodulatory properties

Spices have been used since last many decades because of their taste and aroma. Several studies have proved their biological activities and efficacy (Jaw-MingChernge*t al*, 2008). Many spices have found to possess immunomodulatory properties which can be achieved either by Immunostimulant or immunosuppression. These activities depend on the part of the immune system on which they are acting for example they can stimulate the T-suppressor cells resulting in reducing the immune resistance or can increase the macrophages and NK cells. In some cases, they can also increase the production of antibodies as well. Spices like garlic, turmeric, clove and ginger are known as immune boosters as they stimulate the immune system. In a study, where black pepper significantly stimulates the T helper 1 cytokine release by splenocytes while cardamom suppress the T- helper 1 cytokine (Amin F. Majdalawieh*et al.*, 2010). Also, curcumin present in turmeric suppresses the immune system by down-regulating the expression of CD28 and CD80 (S. Sharma *et al.*,2006).

2.8 Introduction to Cinnamon

Cinnamon is one of the oldest known herbal medicines as mentioned in the Chinese literature. *Cinnamomum zeylanicum* was first introduced in West Africa (1970) by National Herbarium of Ghana (Boniface *et al., 2012). C. zeylanicum*belongs to the family Lauraceae of the genus *Cinnamomum* which is native to Sri Lanka, Myanmar, various parts of Asia and Australia. It is also used as a spice and flavouring agent.

Fig. 1: Cinnamon quills and powdered form

(Source: http://www.goldenpoppyherbs.com/media/Materia_ Pictures/cinnamon.png)

It is reported to possess various nutritional substances such as proteins, carbohydrates, minerals like magnesium, calcium, iron, zinc etc. and also vitamins (A, B_3, C, K) (Vangalapati *et al., 2012).*

2.9 Botanical elucidation

Cinnamon is evergreen tree which ranges from 20-40 ft in height. It propagates through its seed which are of different sizes ranging from less than 6.5 mm to more than 12.5 mm. The tree can survive the temperatures up to 32 ºF. It consists of yellow coloured flowers as shown in Fig. 2 and is characterised by the ovular shaped leaves which are 7-20 cm in length. Also, the fruits of are purple coloured and are pulpy (Fig. 3).

Fig. 2. Flower of cinnamon tree

(Source: https://strictlymedicinalseeds.com)

Fig. 3. Fruit of cinnamon tree

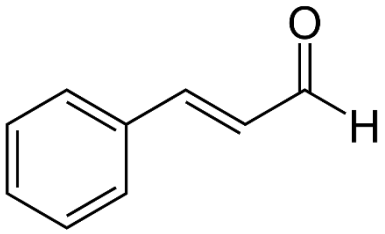
(Source:www.tradewindsfruit.com)

There are number of species of cinnamon, most of which are aromatic and contain volatile compounds (Jayaprakasha *et al.*,2002) and many of which are commercially used. Some common species are given in Table 3.

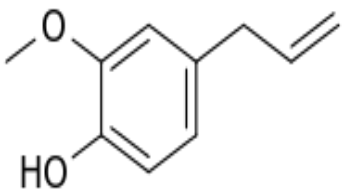
Table 3: Types of Cinnamon and their scientific names

Names	Scientific names
Ceylon cinnamon	*Cinnamomum zeylanicum*
True cinnamon	*Cinnamomum verum*
Indonesian cinnamon	*Cinnamomum burmanni*
Vietnamese cinnamon Saigon cinnamon	*Cinnamomum loureiroi*
Cassia or Chinese cinnamon	*Cinnamomum aromaticum*

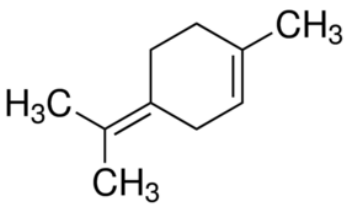
In the essential oil of *C. zeylanicum*, around 300 volatile compounds are reported. The major compounds present in the oil are trans-cinnamaldehyde, eugenol and linalool. Trans-cinnamaldehyde is a viscous compound which gives the cinnamon its characteristic colour, aroma and flavour. Also, it is reported to be effective against both gram negative as well as positive bacteria such as *E. coli* (gram negative) *and Salmonella enteric* (gram positive). Linalool and eugenol possess properties such as antibacterial, antifungal, and antiviral. These along with many other compounds possess pharmacological properties which makes cinnamon to be used in herbal medicines. Some of the structures of major compounds present in cinnamon are shown in Fig. 4.



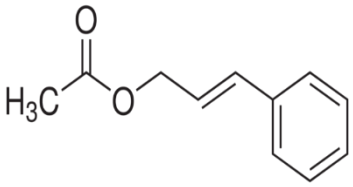
A: Cinnamaldehyde



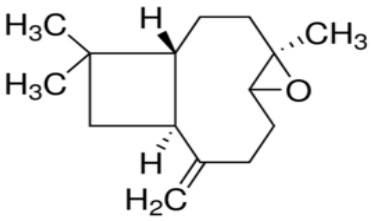
B: Eugenol



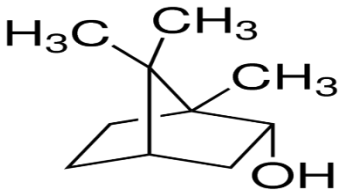
C: Terpinolene



D: Cinnamyl acetate



E: Caryophyllene oxide



F: L-borneol

Fig. 4: Structures of important compounds in cinnamon

Source: (Pasupuleti Visweswara Rao *et al.*, 2014)

Apart from the compounds which are present in the higher amounts, there are many compounds present in the lower amounts such as alpha-thujene, coumarin. Coumarin is a compound which is found naturally in *Cinnamomum zeylanicum*. The use of high level of coumarin is banned by US Food and Drug Agency in 1954 due to its carcinogenic and hepatotoxic effects seen on rats. It is determined that consumption of high level of coumarin can lead to kidney and liver problems. Food and Drug Administration (FDA) has set "Tolerable Daily Intake" of coumarin with respect to the weight of the body i.e. (0.1mg per kg weight) (Maheshwari *et al., 2013*).

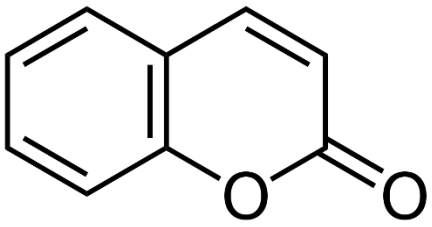


Fig. 5: Structure of Coumarin

(Source: https://upload.wikimedia.org/wikipedia/commons/thumb/ Coumarin_acsv.svg.png)

These different compounds are present in different parts of the plant such as root, bark, leaves, fruit, buds, flowers in different concentrations as shown in Table 4 (Vangalapati *et al., 2012).*

Table 4: Compounds present in different parts of cinnamon plant.

Part of plant	**Compounds present**
Root bark	Camphor (60%)
Bark	Cinnamaldehyde (65-80%) and Eugenol (5-10%)
Leaves	Cinnamaldehyde (1-5%) and Eugenol (70-95%)
Fruit	β- caryophyllene and Trans- cinnamyl acetate
Flowers	Cinnamyl acetate (41.98%), Trans-α bergamotene (7.97%), Caryophyllene oxide (7.2%)
Buds	Terpene hydrocarbons (78%), α-bergamotene (27.38%), α-Copaene (23.05%), Oxygenated terpenoids (9%)

2.10 Biological activities of *Cinnamomum zeylanicum*

Cinnamon, apart from being used as flavour ingredient in food has also been reported to possess certain biological properties.

Anti-oxidant activity: An antioxidant has a characteristic ability to trap free radicals. Highly reactive oxygen species are present in biological systems which may oxidise the biomolecules and initiate degenerative disease. In one study, cinnamon extracts were reported to show anti-diabetic activity by inhibiting hepatic glucose production (Chenga *et al.,* 2012).

Anti-microbial and antibacterial activity: To test the anti-microbial activity of cinnamon, three bacterial strains of *Paenibacillus* larvae (Chapadmalal, Mechongué and Cobo) were used by Gende *et al., 2008.* To determine the antibacterial activity, four gram-negative bacteria (*E.coli, Klebsiella pneumoniae, P. aeruginosa, Proteus vulgaris)* and two gram-

positive bacteria *(B. subtilis and S. aureus)* were screened against cinnamon extract which showed cinnamon to be a potential source of antibacterial agent.

Anti- inflammatory activity*:* Cinnamon helps to inhibit the unwanted blood platelet and also reduces the formation of arachidonic acid which is an inflammatory fatty acid. In a study, the oral administration of cinnamon water extract to mice decreases the levels of TNF-α and IL-6 in the serum significantly **(**Hong et al., 2012).

Anti-tumour activity: It is the activity which involves the growth inhibition of unwanted cells. Studies have shown that cinnamon and cinnamon oil treat tumours, leukemia, colon, gastric and lymphoma cancer by upregulating the pro- apoptotic molecules. (Kwon et al., 2009).

2.11 Immunomodulatory properties of *Cinnamomum zeylanicum*

According to a report by Balekar *et al., (2014), C. zeylanicum* was found to enhance cellular immunity. In the experiment different concentrations of *C. zeylanicum* (10, 25, 50mg/kg) were given to the mice who were treated with Sheep Red Blood Cells (SRBCs) as an antigen. An increase in the primary and secondary antibodies was observed in 25 and 50 mg/kg concentrations.

In vivo studies on mice induced with melanoma have reported that the aqueous extract of *C.zeylanicum potentiates* $CD8^+$ T cell activity (Kwon *et al., 2009*). Also, the mean number of peritoneal macrophages was found to increase in mice which were treated with poly-phenolic extract of *C. zeylanicum* (25 or 50 mg/kg) (Balekar *et al., 2014*).

Also, human peripheral blood mononuclear cells were reported to incubate with cinnamaldehyde (0-10 µg/mL) in the presence and absence of LPS. In this, the concentrations higher than 1µg/mL inhibited the secretion of immunosuppressive cytokines (IL-10) as well as inflammatory cytokines (TNF-α) (Kim *et al.,2010*).

2.12 Market of cinnamon

In 2012, industry of cinnamon has brought high revenue of approximately US$ 12,630 million. Out of the total spice industry only cinnamon contributes to over 55% having its biggest market in areas such as Mexico, Columbia, Peru and parts of USA. Products like cinnamon quills, chips, powders, bark and leaf oil, flavours, Tablets, perfumes, candles, etc. are quite popular throughout the world.

2.13 Immunomodulators in market

With the increase in the number of the diseases like asthma, cancer, allergic conditions, and multiple sclerosis, rheumatoid arthritis, the demand for immunomodulating drug has increased. In 2015, global immunomodulators market accounted for USD 131.7 billion(Immunomodulators Market Analysis by Product: Immunosuppressant, Immunostimulant, Vaccines, Antibodies), A large number of immunomodulators which are commercially available (Table 5).

Table 5: Immunomodulatory activity shown by commercially available drugs.

Drugs	**Activity**
Cyclosporine	Immunosuppressant
Azathioprine	Immunosuppressant
Prednisolone	Immunosuppressant
Antithymocyte globin	Immunosuppressant
Levamisole	Immunostimulant
Thalidomide	Immunostimulant

Thus, owing to the huge market of cinnamon, polysaccharides and immunomodulators, it would be very useful, if any polysaccharide is found which is isolated from the herb which is used in every household at a regular basis and is having the immunomodulatoy properties.

CHAPTER 3: OBJECTIVE

- Isolation and purification of water soluble polysaccharides from *Cinnamomum zeylanicum.*

- Assessment of cell growth effect of isolated polysaccharides in different immune cells.

CHAPTER 4: MATERIAL AND METHODS

4.1 Materials

List of chemicals and reagents required during the project:

Chemical/Reagent	**Company**
Hexane	EMPARTA ®Merck
Dichloromethane	EMPARTA ®Merck
Chloroform	EMPARTA ®Merck
Methanol	EMPARTA ®Merck
Foetal Bovine Serum (FBS)	Gibco® Life technologies
Histopaque-1077	Sigma-Aldrich
MTT	Sigma-Aldrich
Trypan blue	Himedia
Concanavalin A	Sigma-Aldrich
Glucose	Himedia
EDTA	LOBA chemic
Liquid nitrogen	
Phenol	Himedia
Sulphuric acid	LOBA chemic
Sodium hydroxide	Himedia
Sodium bicarbonate	Himedia
Bovine Serum Albumin (BSA)	Himedia
Dimethyl Sulfoxide(DMSO)	SRL
RPMI 1640	Himedia
Penicillin	Himedia
Streptomycin	Himedia
Glutamine	Himedia
DMEM	Himedia
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich
Trypsin	Himedia
Amphotericin	Himedia

4.2Preparation of *Cinnamomum zeylanicum* extract

Cinnamomum zeylanicum bark was procured from Indian Institute of Spices Research, Kerala, India. Bark was taken and weighed. Then these barks were washed in tap water to eliminate the impurities and then dried later at 37°C. The dried barks were transferred to the clean pestle and mortar and crushed them to fine powder using liquid nitrogen. This powdered form of bark was then subjected to Soxhlet extraction in four different solvents based on the increasing polarity index (Table 6) in order to isolate the water soluble polymeric substances (Y.C.Wong *et al., 2014*).

Table 6: Different solvents with increasing polarity index

Solvents	**Polarity**
Hexane	0.1
Dichloromethane	3.1
Chloroform	4.1
Methanol	5.1

Soxhlet extractor**:**Franz Ritter von Soxhlet invented this tool in 1879. It is a versatile tool which was originally designed for extraction of a lipid from a solid material. This is used when the compound has a limited solubility in the solvent.

Parts of Soxhlet apparatus:The apparatus is composed of mainly three parts namely Condenser, porous container and distillation pot. The function of the condenser is to keep the temperature low so that the solvent vapours cool down and return to liquid from. Porous container holds the thimble in which the sample is filled. The insoluble component is retained in the thimble itself and the soluble one is passed through it which helps in the extraction of the active compound. Distillation pot contains the required solvent which is placed on the heating mantle. It also acts as a reservoir for the concentrated sample (Fig. 6).

Soxhlet Extraction: Dried and powdered form of *C.zeylanicum* sample was transferred to the thimble after the weighing which was then placed in the extraction chamber of the Soxhlet

extractor. Glass beads were added on the bottom of the thimble. Then the extractor was connected with the cooling chamber on the top and the distillation pot on the bottom containing the 150mL of the desired solvent in it. The cooling chamber has 2 outlets, one for the water inlet and other for the outlet. This entire apparatus was placed on the heating mantle at the temperature 10°C less than the boiling point of the desired solvent. Twenty cycles were carried out at a particular temperature for a particular solvent (Table 7).

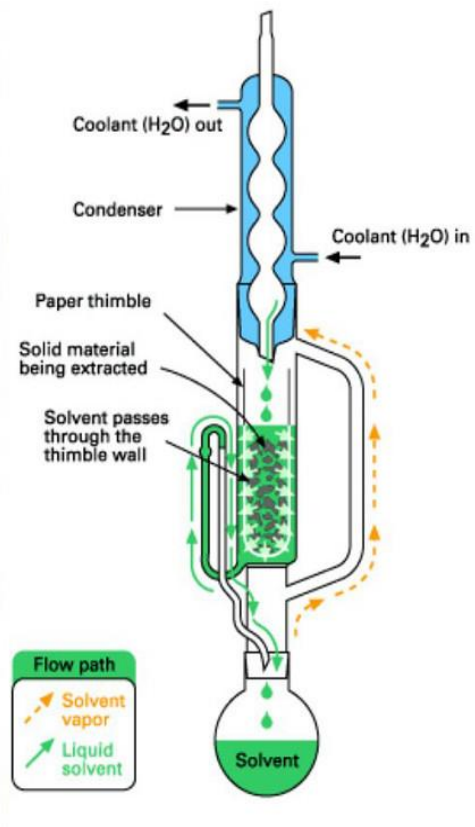


Fig. 6: Flow of solvent in Soxhlet extraction.

(Source: www.cremonatools.com/cremona_images/images/SOXLET%20scheme.jpg)

After the completion of twenty cycles, the extract was cooled down and the entire apparatus is dismantled. The extraction was done in the increasing order of polarity as shown in Table 8 in order to draw out the highly non-polar compounds at first. After the Soxhlet extraction was completed the remaining residue left in the thimble was dried down and further used for the isolation of the water soluble polymeric substances.

Table 7: Solvents with different extraction temperature and boiling points

Solvent	Boiling point	Extraction temperature
Hexane	69.1°C	58°C
Dichloromethane	39.8°C	29°C
Chloroform	61.5°C	51°C
Methanol	64.7°C	54°C

4.3Extraction of water soluble polymeric substances

The remaining dried residue after the Soxhlet was taken and stirred by adding the autoclave sterile water (pH: 6.0) using the magnetic bead. To avoid the contamination, the bottle was sealed with parafilm and also all the steps were done in laminar air flow. The stirring was done for 12hrs at a normal temperature. After 12 hrs, the stirring was stopped and the liquid extract was separated from the residue using the whattman filter paper in laminar air flow. The liquid extract was stored in 4°C and the remaining residue was again stirred using a magnetic bead for 12hrs at room temperature. Again, the separation of the liquid extract and residue material was done. The liquid extracts were combined in order to maximize the water soluble polymeric substances.

Then, the liquid extract was concentrated and chilled methanol was added (4 times the volume of concentrated liquid extract) for organic precipitation. As the dielectric constant of water is high (80.1), it does not form stable bonds between the ions. By adding ethanol having dielectric constant of 24.3, the attraction between the ions becomes strong and a stale precipitate is formed. After adding ethanol, the extract was kept on incubation for overnight at 4°C. The extract was then centrifuged at 8000rpm for 15 min at 21°C. The pellet was then resuspended in autoclave sterile water by retro pipetting and ultrasonication for well mixing.

4.4 Dialysis

The liquid extract was then proceeded for dialysis which involves the separation of the colloidal particles from the dissolved substances through the process of diffusion. The dialysis was done using the semi permeable dialysis membrane of pore size 12 kDa which was firstly activated by boiling it into a solution of 2% sodium bicarbonate for 10min and then in simple distilled water for another 10 min. After the dialysis membrane is activated the liquid extract is added into it and is tied with thread in the flask containing sterile water. It is stirred for 48 h and after the dialysis is completed, the liquid extract from the membrane is taken out.

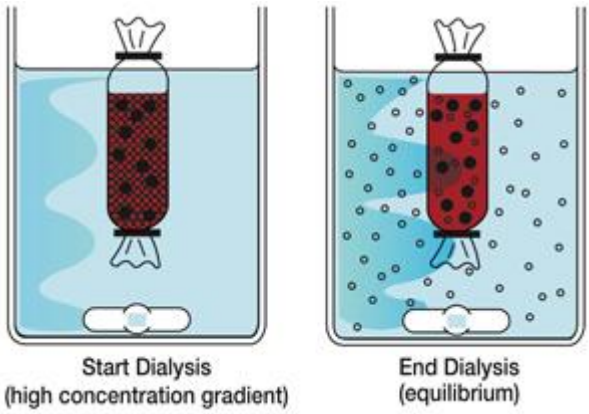


Fig. 7: Process of dialysis.

Source: http://spectrumLabs.com/dialysis/Fund2.jpg

To this dialysed extract, 20% of trichloro acetic acid (TCA) was added in order to remove the proteins from the sample. Then the extract was incubated for two h. After the incubation, the extract was centrifuged at 14000 rpm for 5 min and the supernatant was taken for lyophilisation and the pellet containing the proteins was discarded.

4.5 Lyophilisation

It is a process of freeze drying which includes the removal of water to get a dry (stable) product. It works at a temperature of -69°C or less. It involves the principle of sublimation (Nireesha *et al.,* 2013). This process has number of advantages like increased stability of product in dry state, solvent can be removed without high temperature and it makes the product storage easy.

4.6 Thin Layer Chromatography

Thin layer chromatography is a chromatographic technique which involves the separation of the different components of the mixture. This technique can be used to determine the number of compounds present in the mixture and their purity as well. This is very sensitive and very time-consuming technique. TLC mainly consists of three steps which include spotting of the sample, its development and finally visualisation. Firstly, spotting involves transferring of a small amount of sample on one of the ends of the TLC plate (aluminium sheet coated by silica gel). It can be done either by pipette or a capillary. Spotting is followed by the development which involves keeping the silica gel plate in a shallow pool of the development solvent also called as mobile phase which moves up via capillary action. Based on the polarity of different compounds present in the mixture, they move along with the mobile phase. The final step involves the visualisation of various compounds which may be present in the mixture of the solution. Firstly, the developed plate was observed in UV light to look for the presence of any compound. TLC was performed on a small aluminium sheet coated with silica. In order to run the sample on TLC, the lyophilised sample was dissolved in 400µl of sterile water and sonicated for the proper mixing. Then, equal amount of sodium bicarbonate and ethyl acetate were added to it. A nucleophilic reaction took place when few drops of hydrochloric acid were added to it by which all the organic compounds present in the sample came in the ethyl acetate layer. This ethyl acetate layer was withdrawn out and was run on TLC sheet using methanol as mobile phase and charring was done with $KMnO_4$ stain. This was prepared by dissolving 10g potassium dichromate, 1.5g of potassium permanganate and 1.25mL of 10% NaOH solution in 200mL of water.

4.7 Determination of carbohydrate content in water soluble polymeric extract of *Cinnamomum zeylanicum*

Phenol sulphuric acid method is the one of the best and easiest method for the analysis of carbohydrates present in the sample. In this method, the concentrated sulfuric acid breaks down any polysaccharides, oligosaccharides, and disaccharides to monosaccharides. Pentose and hexose are dehydrated to furfural and hydroxymethyl furfural respectively. These compounds then react with phenol to produce a yellow-gold colour.

For the analysis of sugar in *Cinnamomum zeylanicum,* different concentrations of glucose standard were prepared- 10, 20, 40, 60, 100µg/mL. In the 96 well plate, 50µl of the standards

and samples were prepared using distilled water to make up the volume. Then 150 µl of the sulphuric acid was added immediately followed by adding 30µl of phenol to it. After the proper mixing, it was incubated in water bath for 5min at 35°C. After the incubation, the reading was taken in ELISA plate reader at 490nm.

4.8 Estimation of protein content by Bradford's Assay

The Bradford's assay is one of the commonly used method for the determination of the total protein content present in the sample. In this, Bovine Serum Albumin (BSA) is used as a standard. It works on the principle of binding of Coomassie blue dye to the certain amino acids present in the sample and making a complex whose absorption can be measured at 595nm. The more the protein content is present in the sample the darker the blue colour appears.

Preparation of Bradford's reagent:100mg of Coomassie blue dye G250 was dissolved in 50 mL of 95 % ethanol. After this 100 mL of 85% phosphoric acid was added and the volume was made up to 600mL using distilled water. Then, the solution was filtered and 100mL of glycerol was added later and the final volume was made to 1000mL. The resultant solution is used after 24 hrs. The prepared reagent should be stored in 4°C till the future use.

For this, the test was done in 96 well microtiter plate. Different concentrations of standard were prepared- 10, 20, 40, 60, 100µg/mL. Similarly, BSA standard was prepared from 10µg/mL-100µg/mL. The volume was made up by distilled water to 50µl. 100µl of the Bradford reagent was added to the wells and reading was taken in ELISA plate reader at 592nm.

4.9 Assessment of different phytochemicals

Qualitative phytochemical tests were performed to check the presence of alkaloids (Wagner's test), flavanoids (Alkaline reagent test), phenol (Ferric chloride test), tannins (Braymer's test), terpenoids (Salkowski's test) and quinones (conc. HCl test) as per standard procedure (Aziz, 2015).

4.9.1 Ferric chloride test for phenols

20µl of solvent extracts and crude water soluble polymeric extract were treated with aq. 5% ferric chloride solution and was observed for the formation of deep blue/ black colour.

4.9.2 Braymer's test for tannins

Solvent extracts and crude water soluble polymeric extract were treated with 10% of alcoholic ferric chloride solution. They were observed for the formation of blue and greenish colour solution.

4.9.3 Wagner's test for alkaloids

Solvent extracts and crude water soluble polymeric extract were treated with 3-5 drops of Wagner's reagent (1.27 g of iodine +2 g of potassium iodide in 100 mL of water). The presence of alkaloids was observed by the presence of reddish brown precipitate.

4.9.4 Salkowaski's test for terpenoids

100µl of methanol was added to double the amount of each extract. Few drops of concentrated sulphuric acid (H_2SO_4) were added to the solution and reddish-brown precipitate was observed immediately for the presence of terpenoids.

4.9.5 Concentrated HCl test for quinones

Solvent extracts and crude water soluble polymeric extract were treated with drops of concentrated HCl and were observed for the presence of yellow coloured precipitate.

4.9.6 Alkaline reagent test for flavonoids

100µl of each extract was treated with few drops of 20% sodium hydroxide and it was observed for the presence of deep yellow colour.

4.10 Determination of free radical scavenging activity of water soluble polymeric extract of *Cinnamomum zeylanicum*

Antioxidant is a substance that inhibits the oxidation of other molecules. Its main characteristic is the ability to trap the free radicals which are present in biological systems and can cause genetic disorders like carcinogenesis and mutations (Singh and Singh, 2008) To determine the free radical scavenging activity, an antioxidant assay well known as DPPH assay was performed. DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) is an antioxidant assay which works on the principle of electron transfer that produces violet colour (Fig. 8). When DPPH (a stable freeradical) comes in the presence of an antioxidant molecule, it gets reduced which result in the change of violet colour to pale yellow colour (Damo.co., 2010). This assay is a very fast and an easy method to evaluate antioxidants with the help of

spectrophotometry.

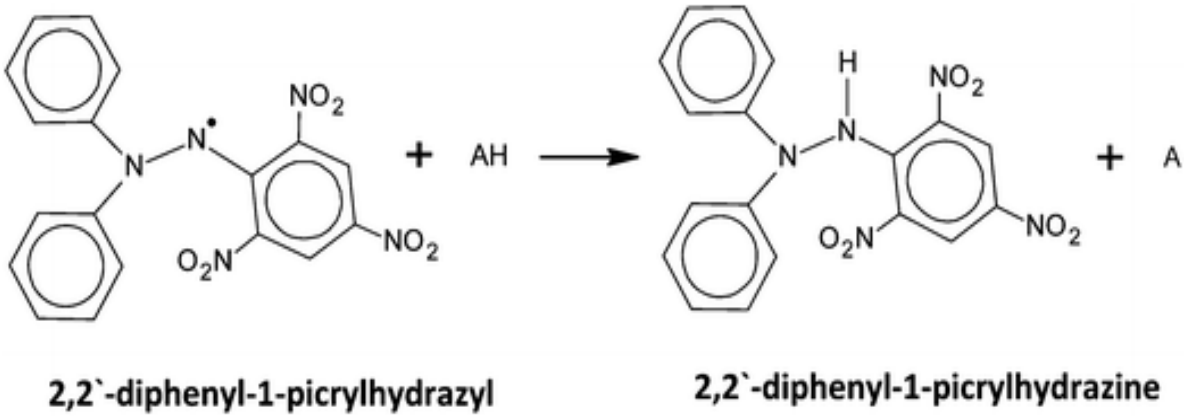


Fig. 8: Principle of Antioxidant assay.
(Source: Krystyna Pyrzynska and Anna Pękal, 2013)

For this, different concentrations (250, 500, 750, 1000µg/mL) of water soluble polymeric extract and ethyl acetate fraction were mixed with 150µl of DPPH (100µM) in methanol. Ascorbic acid (100µg/mL) was taken as a positive control. The 96 well microtitre plate was then kept at 30ºC for 45 minin dark and then the change of violet colour to pale yellow was measured at 517 nm using ELISA plate reader. Free radical scavenging activity was expressed in terms of inhibition percentage which was calculated using formula:

Free radical scavenging activity= $\{(A_{control} - A_{sample})/A_{control}\} \times 100$

4.11 Handling and maintenance of cell lines

4.11.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC were isolated from the blood of healthy donors from Rajendra hospital, Patiala. They were isolated using the ficoll density gradient method (Kumar *et al., 2009*). In a centrifuge tube, 5 mL of histopaque-1077 was added on to which 5 mL of blood was carefully layered. Then it was centrifuged at 400x g for half an hour at room temperature. After the centrifugation, the yellow coloured plasma layer was carefully removed and the buffy coat was taken out carefully containing the PBMC. Then the cells were washed using 10 mL of 1X PBS centrifuged at 250X for 10 min twice. After the washing the pellet was suspended in the 1mL of RPMI -1640 media.

4.11.2 Enumeration of cells

After these steps, the cells were counted in haemocytometer using trypan blue dye (S. Debey *et al., 2004*). The basic principle is that the dead cells accumulates the dye and appears blue in colour whereas the living cells appears to be shiny. The slide was prepared by adding 10µl of trypan blue dye, 10 µl cells and 80 µl of media. The cells were finally counted in all the four boxes under 40X magnification of light microscope using the following formula:

Cell count = {(A+B+C+D)/4} x dilution factor $x10^{-4}$

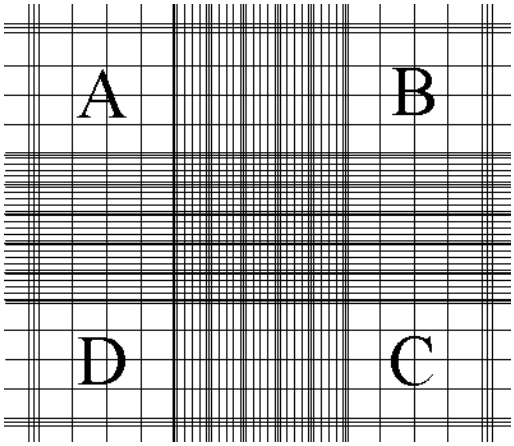


Fig. 9: Haemocytometer

Source: https://home.cc.umanitoba.ca/~adam/lab/Haemocytometer.htm

Where A, B, C, D are cell counts in A, B, C, D boxes respectively (Fig. 9).

4.11.3 Preparation of Phosphate Buffer Saline (PBS)

8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.2g of KH_2PO_4 was added in 800mL of double distilled water for the preparation of 1X PBS. pH was adjusted to 7.4 using NaOH or HCl and then the volume was made up to 1litre using double distilled water. Then it was autoclaved at 121°C for 20min. After autoclaving, it was filtered and stored at 4°C.

4.11.4 Preparation of RPMI Media

RPMI media was prepared by dissolving the RPMI powder in sterile water. To it, sodium bicarbonate, glutamine and the antibiotics such as Penicillin (100 IU/mL) and streptomycin (100µg/mL) were added. Then it was filtered using 0.22µm filter and stored at -20°C. Just before the use, media was supplemented with 10% FBS and 10mM HEPES buffer.

4.11.5 Preparation of Dulbecco's modified Eagle's media (DMEM):

DMEM is used in a wide range of cell culture applications. It is basically a modification of Eagle's minimal essential media which contains four times higher concentration of vitamins and amino acids and two to four times much as glucose concentration. AT065A DMEM media was used.

Media was prepared by dissolving 9.6 gm of powdered media in 900 mL of Milli Q water with continuous stirring. pH of the media was adjusted to 4. Then, 26.5mL of 4% $NaHCO_3$ solution and 20 mL of 200mM glutamine solution was added to the media. The final pH was then adjusted to 7.4 with the help of 1N hydrochloric acid or sodium hydroxide solution. 10mL of antibiotics solution was added to the media along with the other nutrients. Then the volume of the media was made up to 1000mL with the help of Milli Q water. In the end, the media was filtered sterilized through 0.22 µm filter membrane under vacuum condition. The sterile media was then stored in -20ºC till use. 10% FBS was added at the time of use.

4.11.6 Revival of cell lines

The frozen cells stored at -80°C were thawed continuously till they are completely turned into liquid. Then these cells were suspended in 8mL of DMEM growth media supplemented 10 % FBS which was followed by centrifuge at 2000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 5mL of 1xPBS buffer. 1mL of DMEM media was added to the pellet. Then, the cells were counted by haemocytometer using trypan blue exclusion assay. The cells were seeded in T25 cell culture flask containing 5mL of DMEM media and left for incubation at 37ºC with 5 % CO_2 in CO_2 incubator. The flask was checked after regular intervals for any change in pH and the growth of cells.

Maintenance of cell lines: Cell lines which are anchorage dependent need to be maintained by changing the media after every two or three days as required so that they remain in their exponential phase. For this, the media was removed and washing was given by carefully rinsing the flask with 3mL of 1x PBS. In order to remove cell debris tapping was done. After tapping the PBS was removed and the fresh media (DMEM) was added to the flask.

Sub-culturing of cells: Sub-culturing was done when the flask was 70-80% confluent. Warm trypsinisation method was used for sub-culturing. In involves trypsin which is a protease that cleaves the integrins to detach the adherent cells. For this, media was discarded and the flask

was washed with 3mL of 1x PBS. Equal amount of 1x trypsin solution was added to the flask and was incubated at 37ºC in CO 2 incubator for 5-6 min. After the incubation, equal volume of DMEM media was added in the flask to stop the effect of trypsin. Then the solution was taken out of the flask and was centrifuged at 2000 rpm for 10 min. The pellet was resuspended in 1mL of complete media and counting was done by trypan blue dye exclusion method using haemocytometer. The cells were then seeded in either T25 or T75 flask. The cells were regularly monitored for their morphology, pH change, contamination and growth.

4.12 Assessment of PBMCs and RAW 264.7 using MTT:

The effect of Extracellular polymeric substances on peripheral blood mononuclear cell proliferation was assessed by MTT (3- 4, 5 dimethylthiazolyl-2- 2-5, diphenyltetrazolium) based calorimetric assay. This works on the principle in which the MTT reduces to formazan (purple coloured product) due to an enzyme succinate dehydrogenase which is secreted from mitochondria of the metabolic active eukaryotic cells (Fig. 10). Crystals are formed as a product of this which are then dissolved by dimethyl sulfoxide (DMSO). MTT assay is originally used to determine the cell proliferation but if cells undergo apoptosis or necrosis, the cell viability can also be measured indirectly. The linear relationship between number of cells and signal produced is established for each reaction, thus allowing an accurate quantification of changes in the rate of cell proliferation.

For PBMC, 2×10^5 freshly isolated cells were seeded in 96 well microtiter plate per well. EPSs were added in the wells with concentrations varying from 250µg/mL to 1500µg/mL. 2µl of Concanavalin A was added as a positive control and then finally the volume was made up to 200µl by RPMI 1640 media.

Mouse leukaemia macrophage cell line (RAW 264.7) was procured from NCCS (National Centre for Cell Sciences), Pune. For RAW 264.7, 1×10^4 cells were seeded in 96 well microtiter plate per well. EPSs were added in the wells with concentrations varying from 250µg/mL to 1500µg/mL as well. 2µg/mL LPS was added in the wells which acts as a positive control. Finally, the volume was made up to 200µl by DMEM. Overnight incubation was done before adding the sample. The plate was then incubated at 37ºC in CO_2 incubator with 5% CO_2. After the incubation, 20µl MTT was added in each well. Following the addition, the plate was then again incubated for another four h.

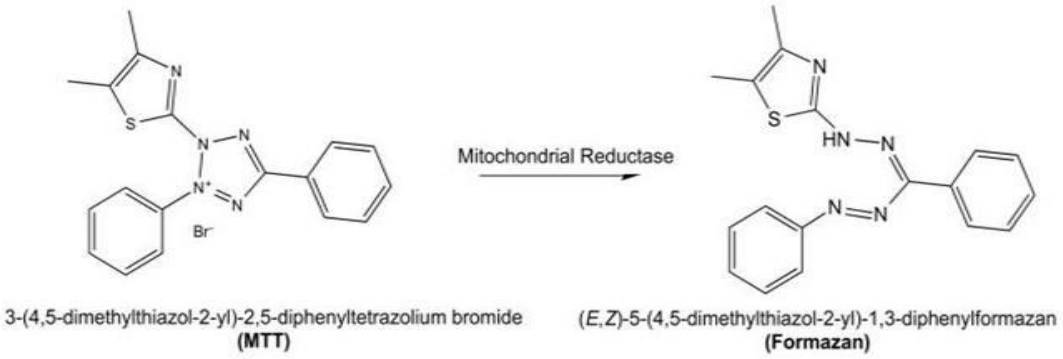


Fig.10: Conversion of MTT to formazan in MTT assay

After incubation, the plate was centrifuged for 10 min at 2000 rpm to settle down the crystals. 170µl of media was discarded and 100 µl of DMSO was added in each well. Similar steps were done for PBMC as well. Finally, the absorbance was recorded at 570nm taking reference wavelength at 620 nm on ELISA plate reader. Three independent experiments for both PBMC and RAW 264.7 were designed to confirm the results and also, all the experiments were performed in triplicates as well.

4.13 Estimation of nitric oxide production in the mouse leukaemia macrophage cell line (RAW 246.7)

Nitric oxide production in the mouse macrophage cell line was estimated using Griess reagent. For this, cells were seeded at a density of 2.5×10^5 cells/well and incubated overnight for the adhesion. Media was changed on the next day and samplewas added in the concentration range of 250 to 1500µg/mL. LPS (10 µg/mL) was used as a positive control.The experiment was carried out in triplicates. Then the plate was incubated for 48 h at 37ºC in 5% CO_2 in CO_2 incubator. Production of nitric oxide in the supernatant was measured in terms of nitrite production as it is the stable product. 100 µl of the supernatant was taken out and was mixed with equal amount of Griess reagent (1% sulphanilamide in 5% H_3PO_4 and 0.1% N-naphthyl ethylene-diamine-di-hydrochloride in 5% H_3PO_4). Incubation was done at room temperature for 10 min and then the absorbance was recorded at 570 nm.

4.14 Determination of the immune response by estimating the cytokines produced in the PBMC

Firstly, PBMC were cultured in the density of 2×10^5 cells/well in a 96 well microtiter plate and samples were added in varying concentration as described earlier. After the incubation of 48h at 37ºC in CO_2 incubator with 5% CO_2, 100µl supernatant was collected in the micro-centrifuge tubes for analysis of cytokine production. The supernatant can be stored at -20ºC.

IFN-γ secretion in the culture supernatant was measured by sandwich ELISA (L. Darwich*et al*., 2008) in 96-well ELISA plate as per manufacturer's instruction. 100 µl of capture antibody (1µg/mL in PBS) was added to each ELISA plate well and incubated overnight at room temperature. The wells were aspirated to remove liquid and the plate was washed four times with 300 µl wash buffer (0.05% Tween-20 in PBS) per well. After the last wash, the plate was tapped in inverted position to remove residual buffer on paper towel. 300 µl of blocking buffer (1% BSA in PBS) was added to each well and the plate was incubated for 1 hour at room temperature. After washing the plate four times with wash buffer, 100µl of the test sample and the IFN-γ standard were added to each well in triplicate. The plate was incubated at room temperature for overnight. The plate was washed 4 times and 100 µl of detection antibody (1µg/mL in sample diluent buffer) was added to each ELISA plate well and incubated at room temperature for 2 h. Plate was washed four times and 100 µl of diluted avidin-HRP conjugate (1:2000) in sample diluent was added and incubated for 1h. Plate was again washed four times and 100 µl of ABTS substrate solution was added to each well. The plate was wrapped in a foil and incubated at room temperature for 15 min for colour development. Absorbance was recorded at 405 nm with wavelength correction set at 650 nm in ELISA plate reader (Tecan, Austria).

4.15 Statistical analysis:

The data were expressed as the mean ± standard error of mean of three independent experiments. Data were analysed using analysis of variance (ANOVA) and the means were compared using Tukey's test at $p < 0.05$.

CHAPTER 5: RESULTS

5.1 Yield of water soluble polymeric substance from *Cinnamomum zeylanicum* bark

15gm powder of *Cinnamomum zeylanicum* bark was taken for sequential Soxhlet extraction with different solvents (hexane, DCM, chloroform and methanol). Twenty cycles for each of the solvent was run keeping the extraction temperature 10°C lower than their boiling temperature. The residue left after the Soxhlet extraction was 12.6 g which was used for isolation ofpolymeric substances. The water soluble polymeric substances were subjected to lyophilisation and the final yield was found to be 110 mg which was then dissolved in the autoclave sterile water. The crude sample was assessed for its solubility in various non-polar and polar organic solvents (dichloromethane, chloroform, ethyl acetate, methanol) but no solubility was found which led to the extraction by partial purification in ethyl acetate. The partially purified ethyl acetate fraction was analysed by TLC where mobile phase was kept 100% methanol. The developed spots were detected by charring the TLC plateusing $KMnO_4$ as the detection dye which confirmed the presence of polar polysaccharides (Fig. 11).

Fig. 11: TLC analysis of the Extracellular polysaccharides

(Ethyl acetate (stationary phase). The mobile phase was kept 100% Methanol charred with KMnO4).

5.2 Sugar and protein contentin different solvent extract and water soluble polymeric substances

5.2.1 Sugar content:

Sugar estimation was done by phenol sulphuric method using glucose as standard in different solvent extracts and water soluble polymeric substances of *Cinnamomum zeylanicum* (Table 8). Glucose content was calculated by plotting standard curve against different concentration of glucose. Highest glucose content was foundin crude water soluble polymeric substances following ethyl acetate fraction of polymeric substances which has less sugar content than crude. When compared with water soluble polymeric substances, all

these fractions had relatively less glucose content. Amongst the fractions namely hexane, dichloromethane, chloroform and methanol, hexane and methanol found to have the lowest and the highest glucose content respectively (Fig. 12).

Table 8: Sugar content of different solvent extracts and water soluble polymeric extract

Solvents	**Mean ± SEM**
Hexane	8.36 ± 1.92
Dichloromethane	10.41 ± 0.176
Chloroform	37.4 ± 5.036
Methanol	41.51 ± 4.432
Crude	239.4 ± 19.87
Ethyl acetate fraction	85.26 ± 4.309

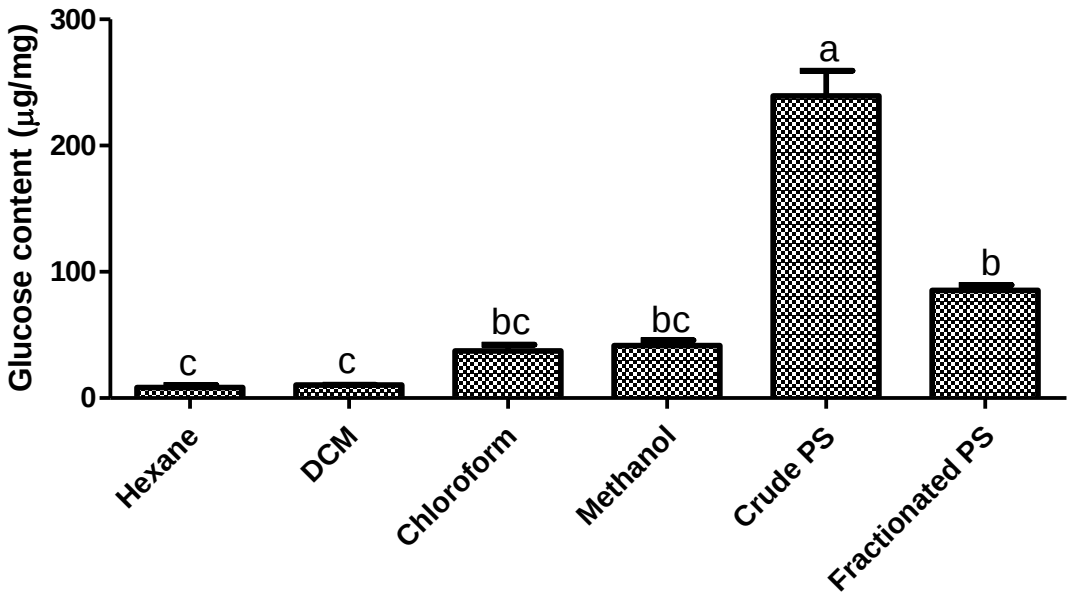


Fig. 12: Sugar content in different solvent extracts and water soluble polymeric substances. PS: Polymeric Substances. Bars with the same letters are not significantly different at $p>0.05$.

5.2.2 Protein content:

Further, solvent extract and polymeric substances was assessed for proteins. For this, Bradford assay was performed using BSA as a standard in water soluble polymeric substances and different solvents namely hexane, dichloromethane, chloroform and methanol. Protein content was checked by plotting standard curve of BSA with different

against concentration (Fig.13). Bradford's result has shown that there was no protein content present in any of the extract confirming the TCA precipitation (Table 9).

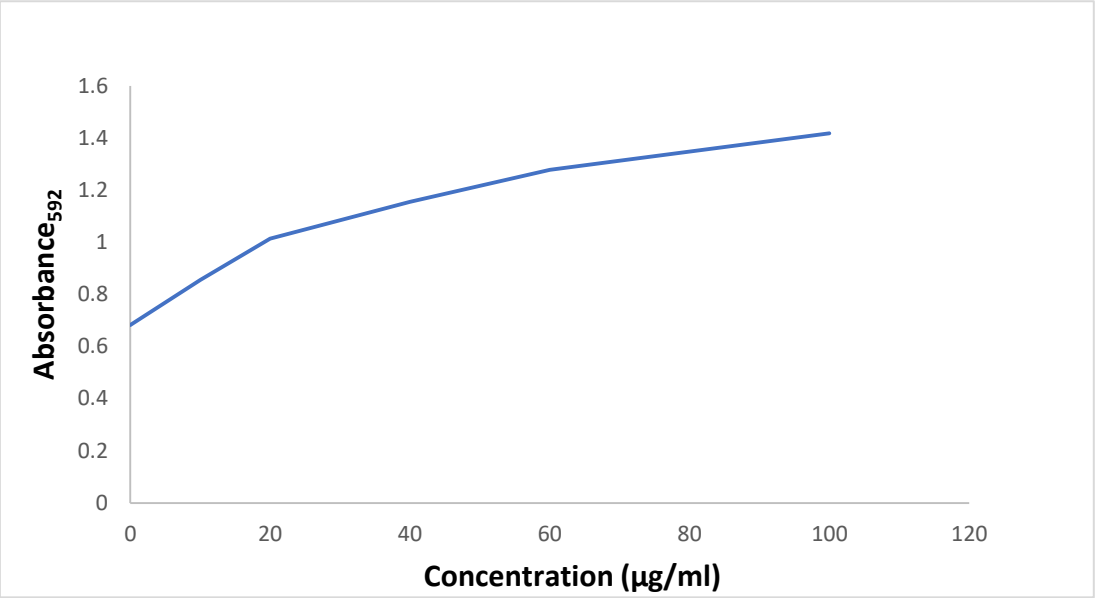


Fig. 13: Standard curve of BSA

Table 9: Protein content in different solvents and Crude water soluble polymeric extract of concentration (250µg/mL)

Solvents	Protein content
Hexane	-113.5
Dichloromethane	-113.49
Chloroform	-113.48
Methanol	-113.519
Crude water soluble polymeric extract	-113.715

5.3 Phytochemical assessment in different solvent extract and water soluble polymeric substances

Phytochemical screening of alkaloids, flavanoids, phenol, tannins, terpenoids and quinones were done in different extracts. Screening results confirm the presence of different phytochemicals in the *Cinnamonum*solvent extracts and crude water soluble polymeric extract. Alkaloids and flavanoids were found to be present in all four solvent extracts while quinones and tannins were found to be present only in methanol extracts (Table 10). Methanol extract have shown the presence of all the phytochemicals. However, no

phytochemicals were found to be present in water soluble polymeric extract.

Table 10:Qualitative analysis of phytochemicals in *Cinnamomum zeylanicum* extract

		Hexane	Dichloromethane	Chloroform	Methanol	Crude PS
Phytochemicals	Alkaloids	+	+	+	+	-
	Flavanoids	+	+	+	+	-
	Phenols	-	+	-	+	-
	Terpenoids	-	+	+	+	-
	Quinones	-	-	-	+	-
	Tannins	-	-	-	+	-

5.4 Effect of water soluble polysaccharides on mouse macrophage cell line (RAW 264.7)

Both crude and Ethyl acetate fraction of water soluble polymeric extract were evaluated for their effect on the growth of mouse macrophage cell line and proliferation index was calculated. For this, different concentrations of polymeric extract (250, 500, 750, 1000, 1500µg/mL) were taken and MTT assay was performed using lipopolysaccharide as a positive control to determine the effect of these extracts on RAW 264.7 cell line for crude (Table 11) and Ethyl acetate fraction of water soluble polymeric extract individually (Table 12). Proliferation index were found to be decreased with increase in concentration in both crude and ethyl acetate fraction of polysaccharides. IC50 value of crude and Ethyl acetate PS was found to be 1120 ± 27 and 1780 ± 227 µg/mL respectively. Although, the ethyl acetate fraction showed the decreasing trend, proliferation index remained above 0.6 (Fig. 15) which is not the case with crude as the proliferation index dropped down to 0.2 at higher concentration (1500 µg/mL) (Fig 14). Proliferation index was found to be close to LPS at 250 µg/mL in both extracts indicating stimulatory effect at lower concentrations.

Table 11: Effect of different concentrations of crude extract of polymeric substances on the growth of mouse macrophage cell lines (RAW 264.7)

Sample (µg/mL)	Mean ± SEM
LPS (2µg/mL)	1.0200 ± 0.0582
250	1.1356 ± 0.2789
500	0.8717 ± 0.1773
750	0.8140 ± 0.0859
1000	0.7422 ± 0.1477
1500	0.3573 ± 0.0520

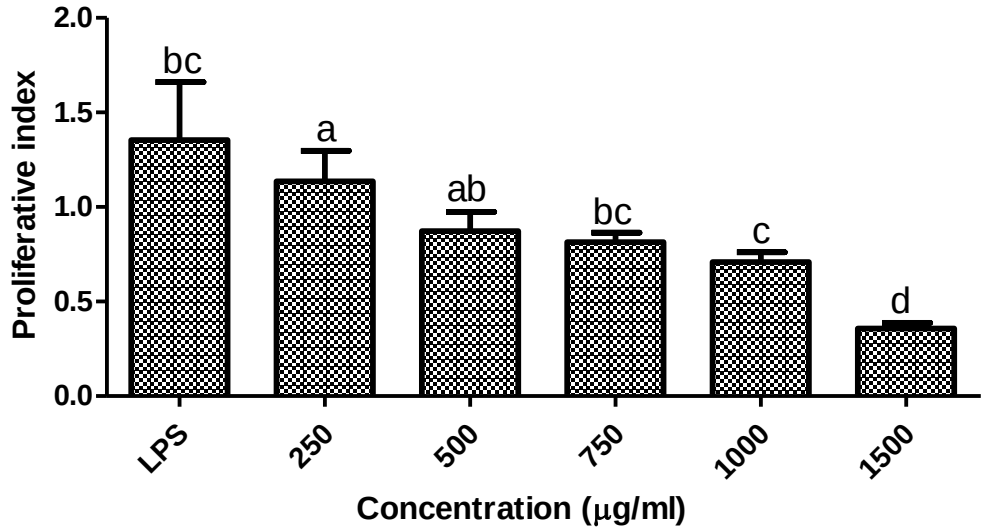


Fig.14: Effect of crude extract of polymeric substances on the proliferation index of mouse macrophage cell lines (RAW 264.7). Bars with the same letters are not significantly different at $p>0.05$. LPS: Lipopolysaccharide (2 µg/mL) was used as a positive control. Proliferation index is the ratio of absorbance of the LPS/EPS treated cell and the untreated cells (Cells only).

Table 12: Effect of different concentrations of ethyl acetate fraction of polymeric substances on the growth of mouse macrophage cell lines (RAW 264.7)

Sample (µg/mL)	Mean ± SEM
LPS (2µg/mL)	1.3571 ± 0.5067
250	1.1822 ± 0.2696
500	0.9924 ± 0.0560
750	0.9073 ± 0.2288
1000	0.7833 ± 0.1465
1500	0.6705 ± 0.0368

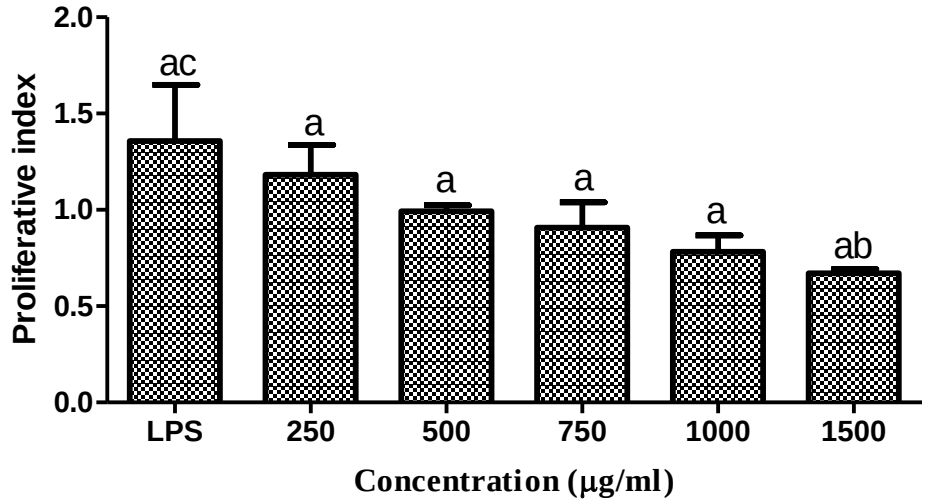


Fig.15. Effect of ethyl acetate fraction of polymeric substances on the proliferation index of mouse macrophage cell lines (RAW 264.7). Bars with the same letters are not significantly different at p>0.05. LPS: Lipopolysaccharide (2 µg/mL) was used as a positive control. Proliferation index is the ratio of absorbance of the LPS/EPS treated cell and the untreated cells (Cells only).

5.5 Effect of Polymeric substances of *Cinnamomum zeylanicum* on the peripheral blood mononuclear cells

The crude and Ethyl acetate fraction of water soluble polysaccharides in different concentration (250, 500, 750 and 1000 µg/mL) were assessed for their effect on the peripheral blood mononuclear cells through MTT assay using concanavalin A as a positive

control (Table 13 and 14). Proliferation index were found to increase with increase in concentration in both extracts showing stimulatory effect. At higher concentration (> 750 µg/mL), the proliferation index was found to be close to concanavalin A representing stimulant effect. However, in case of ethyl acetate fraction, it was observed that the proliferation index is high (Fig. 17) as compared to the crude (Fig. 16) suggesting that the fraction is better.

Table 13: Effect of different concentrations of crude polymeric substances on the peripheral blood mononuclear cells

Sample (µg/mL)	Mean ± SEM
Con A(5µg/mL)	1.7870 ± 0.1843
250	0.8547± 0.3225
500	1.0626 ± 0.1150
750	1.3469 ± 0.4152
1000	1.4915 ± 0.5640

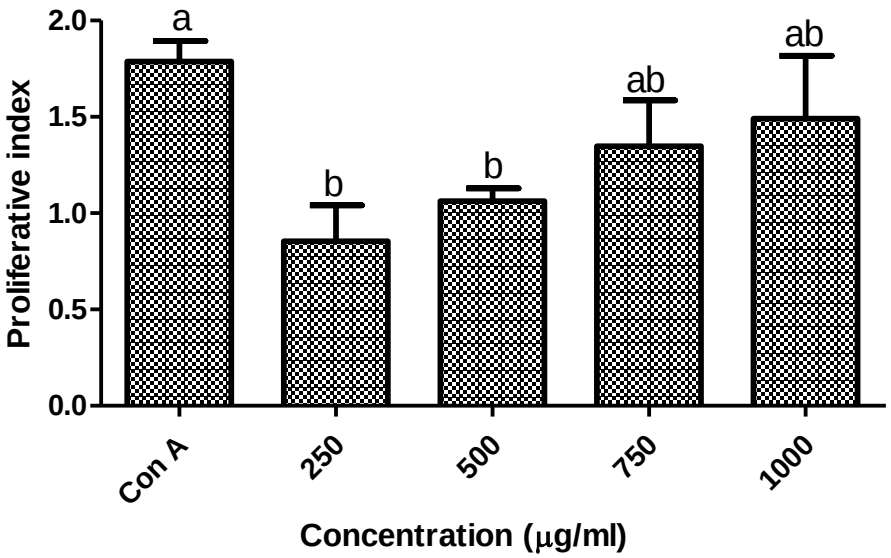


Fig.16: Effect of crude polymeric substances on the proliferation index of peripheral blood mononuclear cells. Bars with the same letters are not significantly different at p>0.05. ConA: Concanavalin A (5 µg/mL) was used as a positive control. Proliferation index is the ratio of absorbance of the LPS/EPS treated cell and the untreated cells (Cells only).

Table 14: Effect of ethyl acetate fraction of polymeric substances on peripheral blood mononuclear cells

Sample (µg/mL)	Mean ± SEM
Con A(5µg/mL)	2.296 ± 0.50646
250	1.263± 0.4519
500	2.0781 ± 0.2351
750	2.4388 ± 0.2232
1000	2.8090 ± 0.4660

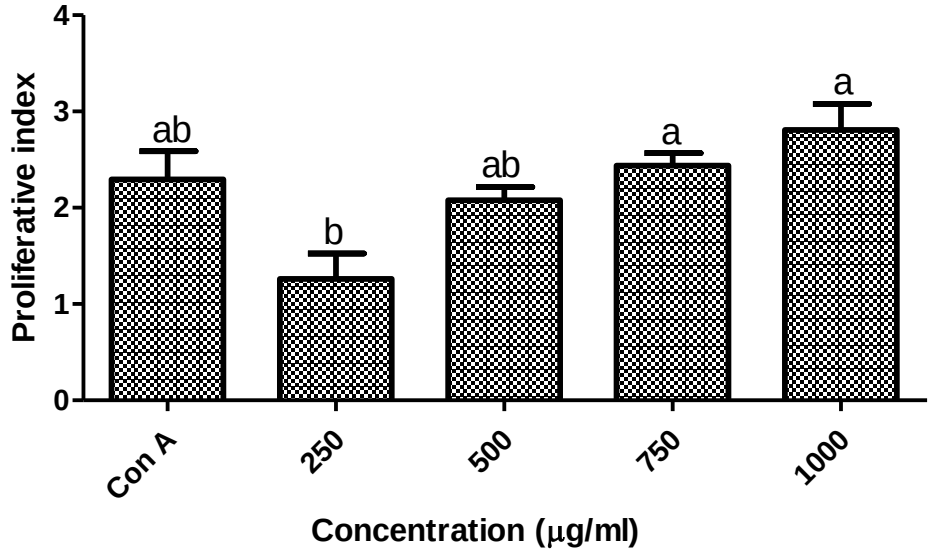


Fig. 17: Effect of ethyl acetate fraction of polymeric substances on the proliferation index of peripheral blood mononuclear cells. Bars with the same letters are not significantly different at p>0.05. ConA: Concanavalin A (5 µg/mL) was used as a positive control. Proliferation index is the ratio of absorbance of the LPS/EPS treated cell and the untreated cells (Cells only).

5.6 Effect of crude water soluble polymeric substances on the production of nitric oxide

Nitric oxide production by crude water soluble polymeric substances in RAW 264.7 was estimated using Griess reagent. Different concentrations of the crude polysaccharide extract were taken (250, 500, 750, 1000, 1500µg/mL) and lipopolysaccharide (LPS) was taken as a control (Table 15). It was found that release of nitric oxide is increasing with increase in concentrations (Fig. 18). Nitric oxide release was found to be more in all concentrations when

compared with control (cells only) but significantly pronounced at concentration (> 1000 µg/mL).

Table 15:Effect of different concentrations of crude water soluble polymeric substances on nitric oxide production

Sample (µg/mL)	Mean ± SEM
Cells only	0.0047 ± 0.00244
LPS (10 µg/mL)	.0055 ± 0.00031
250	0.006733 ± 0.00158
500	0.0071 ± 0.0011
750	0.009133 ± 0.00067
1000	0.001203 ± 0.00128
1500	.02193 ± 0.00133

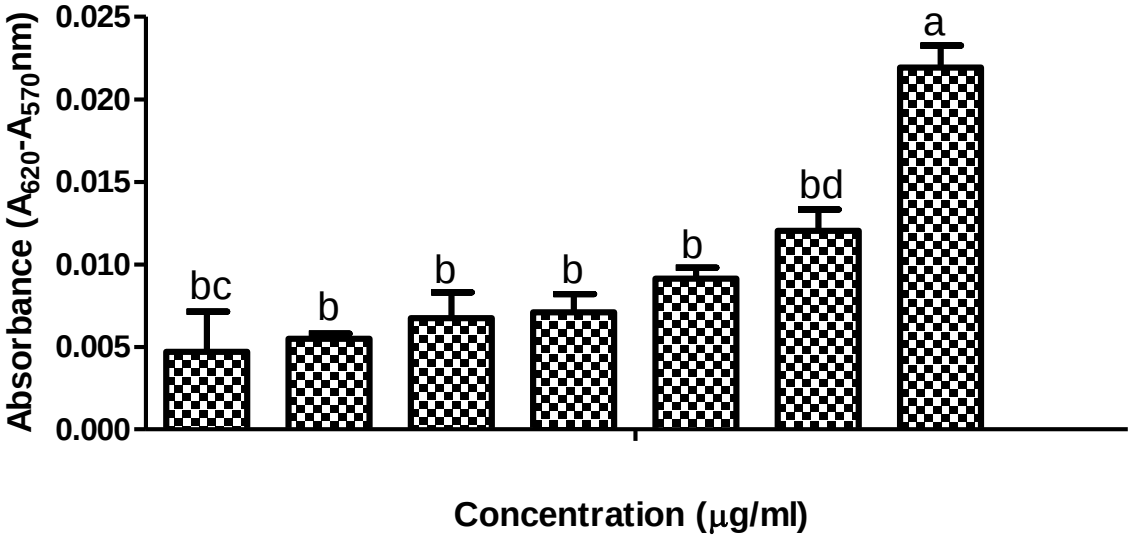


Fig. 18: Effect of crude water soluble polymeric substances on the nitric oxide production. Bars with the same letters are not significantly different at p>0.05. LPS: Lipopolysaccharide (10 µg/mL) was used as a positive control.

5.7 Effect of ethyl acetate fraction of polymeric substances on the production of cytokine: IFN-γ

The effect of ethyl acetate fraction of water soluble polysaccharide on the production of IFN-γ was estimated using IFN gamma kit using Con A as a positive control. Different concentrations (250, 500, 750 and 1000 µg/mL) were assessed for their effect (Table 16). It was seen that production of IFN-γ increased with the increase in concentration (Fig. 19)

Table16: Effect of ethyl acetate fraction of polymeric substances on the production of IFN-γ

Sample (µg/mL)	Mean ± SEM
Con A (5µg/mL)	.06653 ± 0.00933
250	0.0444 ± 0.001343
500	0.05327 ± 0.0000152
750	0.06467 ± 0.002171
1000	0.08243 ± 0.004218

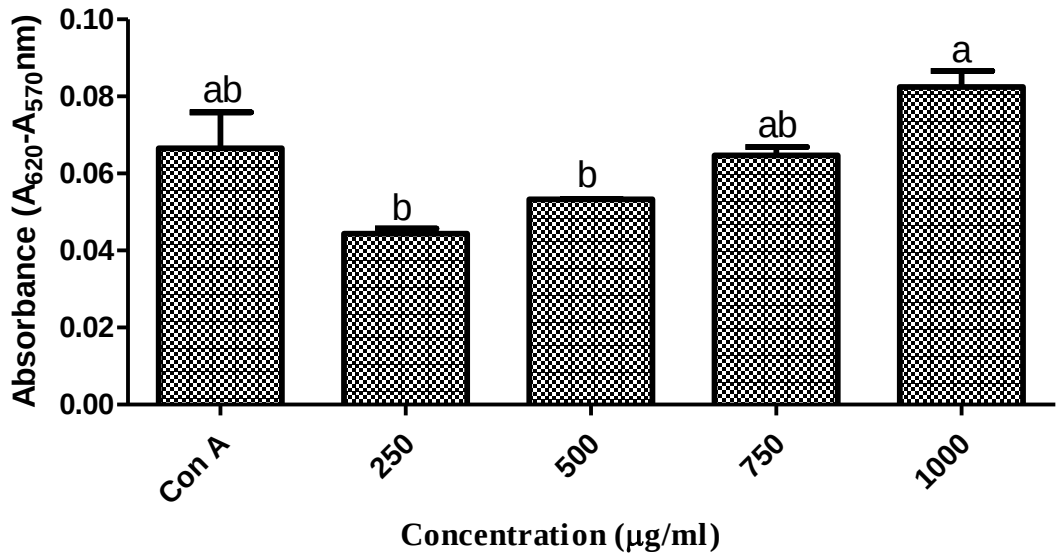


Fig. 19: Effect of ethyl acetate fraction of polymeric substances on the production of IFN-γ. Bars with the same letters are not significantly different at p>0.05. ConA: Concanavalin A (5 µg/mL) was used as a positive control.

5.8 Assessment of free radical scavenging activity of crude and ethyl acetate fraction of polymeric substances.

The crude and Ethyl acetate fraction of water soluble polymeric extract in different concentrations (250, 500, 750 and 1000 µg/mL) were assessed for their free radical scavenging activity using DPPH assay (Table 17 and 18). Ascorbic acid was used as a positive control.It was observed that antioxidant activity increased with concentration in case of crude sample (Fig. 20) but no profound effect was seen in ethyl acetate fraction of polymeric substances (Fig. 21).

Table 17: Free radical scavenging activity ofcrude water soluble polymeric substances

Sample (µg/mL)	**Mean ± SEM**
Ascorbic acid	86.25 ± 0.3192
250	24.92 ±0.6329
500	25.53 ± 1.074
750	30.21 ± 0.7414
1000	37.34 ± 0.3568
1500	44.11 ± 0.54

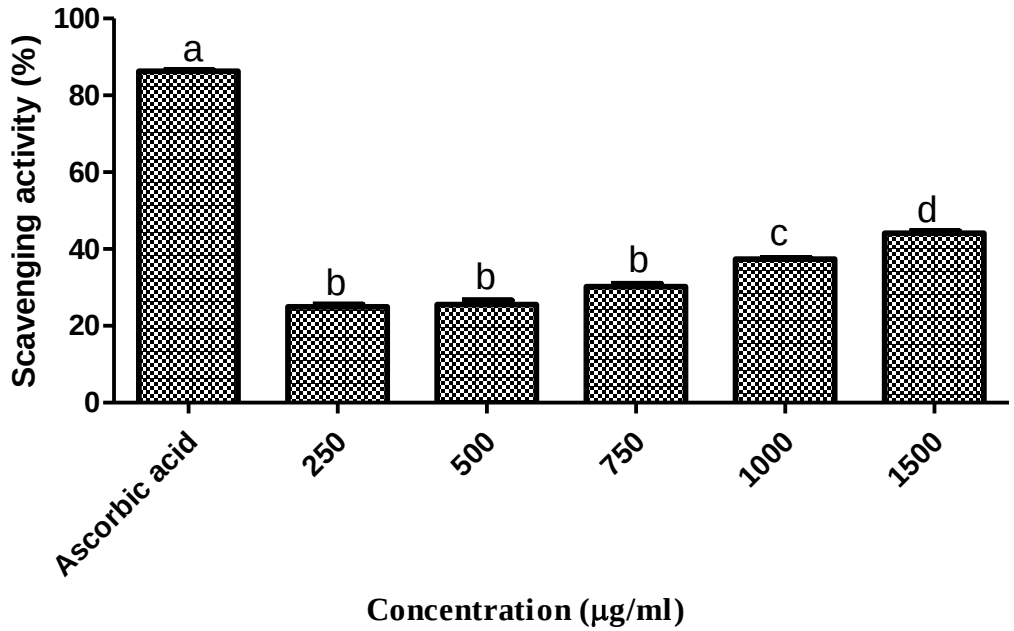


Fig. 20: Free radical scavenging activity of crude water soluble polymeric substances. Bars with the same letters are not significantly different at p>0.05.

Table 18: Free radical scavenging activity of ethyl acetate fraction of polymeric substances

Sample (µg/mL)	Mean ± SEM
Ascorbic acid	86.05 ± 0.5243
250	26.96 ± 0.7294
500	26.6 ± 0.6161
750	22.62 ± 1.819
1000	22.14 ± 0.2099
1500	22.53 ± 0.5813

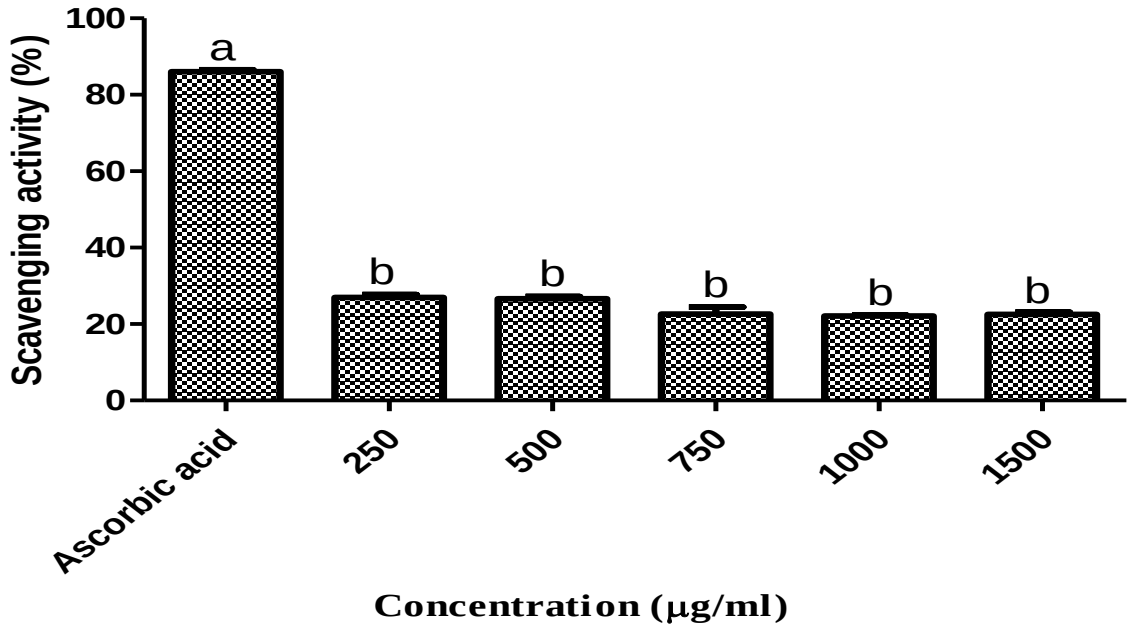


Fig. 21: Free radical scavenging activity of ethyl acetate fraction of water soluble polymeric substances. Bars with the same letters are not significantly different at p>0.05.

CHAPTER 6: DISCUSSION

Spices have been used since centuries to cure various illness and it comes under the category of traditional medicines. Spices are known to possess properties such as anti-bacterial, anti-oxidant, anti-tumour, anti-inflammatory, anti-viral, and anti-microbial. In contrast to the modern medicines, herbal medicines are natural having less side effects. *Cinnamomum zeylanicum* is one of the oldest spices which is acknowledged as herbal medicine described in the Chinese literature due to its remarkable properties. It is known to possess antimicrobial, anti-inflammatory, immune-suppressant and immune-stimulant properties (Rao, 2014).

In the present study, water soluble extract of *C. zeylanicum* was obtained after performing Soxhlet extraction with four different solvents in which sugar content was found to be present. Phytochemical chemical analysis and bradford assay revealed that it does not have any phytochemicals and proteins. Polysaccharides isolated from natural sources are reported to possess diverse biological properties such as antioxidant, anti-cancer and immunomodulating activity (Li et al 2015). In the earlier study, *Cinnamonum* was found to inhibit nuclear transcription factor kappa B (NFκB) and Activator protein 1 (AP1) genes thus enhancing the proapoptotic activity (Kwon et al., 2010). It has also been reported that cinnamaldehyde and its derivatives have cytotoxic effect on various cancers (Lee et al., 1999, Singh et al., 2009). In the present study, water soluble polysaccharides were purified with ethyl acetate and crude and purified ethyl acetate polysaccharides was found to inhibit the growth of murine macrophages (RAW 246.7) cell lines and the inhibition is increased with the concentration. The RAW cells were further analysed for the nitric oxide release in crude water soluble polymeric extract and it was observed that there was a profound release of nitric oxide at a concentration of 1500 µg/mL.

Cinnamon was reported to possess different immunological properties when studies in human monocytic cell line (THP1) and PBMC (Walter et al 2014). Current study has shown increase in cell proliferation of PBMC in both crude and ethyl acetate fraction on PBMC which is indicator of immunostimulatory potential. Further, immuno-stimulant qualities of ethyl acetate fraction were investigated for its effect on cytokine (IFN -γ) secretion against PBMC and it was observed that the cytokine production increased with the increase in concentration.

Various enzymatic reactions occurring in cells are responsible for the generation of free radicals (Alam et al., 2013). Generation of free radicals has impact on health ailments such as cancer, cardiovascular disorder, and inflammatory disease. Hence, researchers are working on

the herbal formulations or food adjunct as source of antioxidants. Free radical scavenging activity was performed to evaluate the antioxidant effect of *Cinnamomum* extract of water soluble polysaccharides. Both crude and ethyl acetate fraction was found to possess antioxidant effect. In earlier study also, polysaccharides isolated from *Cinnamomum* reported to possess antioxidant effect (Ghosh et al 2014).

CHAPTER 7: CONCLUSION

Cinnamon oil has already been commercialised with several therapeutic purposes. Present study has shown that the presence of polysaccharides in water soluble extract from *Cinnamomum zeylanicum*is associated with cytotoxic, Immunostimulant, and antioxidant effect. Further, research work is required to explore the potential bioactive compounds present in it and understand the underlying molecular mechanisms behind the biological effects.

APPENDIX I

APPENDIX II

Fig. 21: Free radical scavenging activity of ethyl acetate fraction of water soluble polymeric substances

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