Determination of Antioxidative Potential and Cytokines Mediated Immunomodulation due to *in vitro* exposure of *Aegle marmelos* (L.) Corr. in Chicken Lymphocytes Culture System

Thesis

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Pantnagar
August, 2018

(Amandip Kaur)
Authoress

CERTIFICATE

This is to certify that the thesis entitled **"Determination of Antioxidative Potential and Cytokines Mediated Immunomodulation due to *in vitro* exposure of *Aegle marmelos* (L.) Corr. in Chicken Lymphocytes Culture System"** submitted in partial fulfillment of the requirements for the degree of **Master of Science in Agriculture** with major in **Molecular Biology & Biotechnology** of the college of Post Graduate studies, G.B. Pant University of Agriculture & Technology, Pantnagar, is a record of *bona fide* research carried out by **Ms. Amandip Kaur, Id. No. 51168**, under my supervision, and no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation has been duly acknowledged.

Pantnagar
August, 2018

(Sonu Ambwani)
Chairperson
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CERTIFICATE

We, the undersigned, members of Advisory Committee of **Ms. Amandip Kaur, Id. No. 51168**, a candidate for the degree of **Master of Science in Agriculture** with major in **Molecular Biology and Biotechnology** and minor in **Nil,** agree that the thesis entitled **"Determination of Antioxidative Potential and Cytokines Mediated Immunomodulation due to *in vitro* exposure of *Aegle marmelos* (L.) Corr. in Chicken Lymphocytes Culture System"** may be submitted in the partial fulfillment of the requirements for the degree.

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LIST OF ABBREVIATIONS

AME	:	*Aegle marmelos* leaves extract
MNCD	:	Maximum non cytotoxic dose
LPA	:	Lymphocyte proliferation assay
LPS	:	Lipopolysaccharide
Con A	:	Concanavalin A
PHA	:	Phytohaemagglutinin
DEXA	:	Dexamethasone
DPPH	:	1, 1- diphenyl 2-picrylhydrazyl
LPO	:	Lipid peroxidation
GSH	:	Glutathione reduced
SOD	:	Superoxide dismutase
TBARS	:	Thiobarbituric acid-reactive substances
IL-6	:	Interleukin-6
IL-10	:	Interleukin-10
IFN γ	:	Interferon-γ
iNOS	:	Inducible nitric oxide synthase
ROS	:	Reactive oxygen species
RNS	:	Reactive nitrogen species
GAE	:	Gallic acid
RE	:	Rutin
NO	:	Nitric oxide
BSA	:	Bovine serum albumin
DEPC	:	Diethyl pyrocarbonate

Introduction

The Ancient health care system "Ayurveda" has been evolved in India 5000 years ago. Indian Ayurveda system mainly focuses on promoting good health and prevention of diseases by adapting healthy life practices. The Ayurveda system of medicine has its appropriate meaning as "Science of Life". In other parts of the world this health care system is used as the complementary medicine **(Shroff, 2017)**. In rural and tribal regions of India approximately about 7500 plants are used in local human and animal health care system. Our country is bestowed by nature with boundless wealth of medicinal plants, reason to introduce it as "Medicine garden of the world". In traditional health care system, the plant based medicines (herbal medicines) are playing major role which results in worldwide increased demand for the herbal medicines, pharmaceuticals, food supplements, nutraceuticals, etc. **(Kumar, 2016)**. Herbal medicine or phytomedicine is the utilization of plants for their medicinal and therapeutic potential for amelioration of diseases. Plant exhibit their beneficial medicinal effects due to the combinations of secondary metabolites present in them. These secondary products are known as phytochemicals which are active ingredient responsible for therapeutic properties. Besides playing essential role for plant itself these phytochemical are known to have potential medicinal effect in human and animals as well. For example some phytochemicals are involved in plant defence system against various microbial pathogen by exhibiting their cytotoxic effects, and thus can also be used as antimicrobial medicine in human and animals **(Briskin, 2000)**.

Immune system is body's natural defence system that works against a vast variety of diseases. In innate and adaptive immune system there are different types of cells like macrophages, natural killer cells and antigen presenting cells that are part of complex immune system **(Archana, 2011)**. The process of stimulating and suppressing immune system for curing various diseases and disorders is known as Immunomodulation. The substances which are capable of amplifying and suppressing different cells or components of immune system are called immunostimulants and immunosuppressants, respectively **(Sharma *et al.,* 2017)**. Products of immunomodulatory plants target mainly macrophages for the generation of immune

response. The immunosuppressant agents could be used for the control of autoimmune diseases, graft rejection, graft versus host disease, hypersensitivity immune reaction (immediate or delayed type) and selective immunosuppression for prevention of Rh hemolytic disease of the newborn **(Archana *et al.*, 2011; Singh *et al.*, 2016)**.

Synthetic drugs are used for treating immunological disorders like cyclosporine, corticosteroids, azathioprine etc. however, these drugs also show undesirable side effects like nephrotoxicity, anaemia, thrombocytopenia, bone marrow suppression etc **(Majeedi *et al.*, 2015)**. Therapeutic strategy for graft rejection and autoimmune disorder is inhibiting signal transduction for T-cell activation which results in selective immunosuppression **(Devasagayam and Sainis, 2002)**.

Cytokines regulate the innate and adaptive immunity and play an important role in modulating the immune system. Basically, cytokines are the group of extracellular soluble proteins or glycoproteins in the form of interleukins (ILs), interferons, chemokines, etc. Cytokines are acting as signals in mammalian system to trigger the immune response, which could be antibody mediated or cell mediated **(Asif *et al.*, 2004)**. Growing interest in the usefulness of cytokines, alterations in cytokine expression, and targeting their receptors may offer a new approach for using them as therapeutic targets **(Sharma *et al.*, 2017)**. Since, there is similarity between chicken and mammalian immune system the use of Chicken cytokine becomes more feasible and moreover, it can be used as potent adjuvants **(Rahman *et al.*, 2012)**.

In human body due to aerobic metabolism involving various physiological and biochemical processes number of oxygen related free radical and reactive species are produced. Free radical related to oxygen includes superoxide, hydroxyl radicals, hydrogen peroxide, nitric oxide etc **(Halliwal, 1995)**. Biochemical activities of the cells like signal transduction, gene transcription etc have involvement of these oxygen radicals and nitric oxide (NO) which are crucial signalling molecule having regulatory function such as proliferation of vascular smooth muscle cells, leukocytes adhesion, platelets aggregation, angiogenesis etc **(Zheng and Storz, 2000)**. Production of the oxygen related free radicals in excess may cause damage to biomolecules by oxidation. Oxidative damage results in neural damage, diabetics, inflammation, cardiovascular diseases, cancer and vascular oxygen in excess can leads to hypertension and vasospasm **(Uttara *et al.*, 2009)**. To counteract the oxidative damage by free radicals,

antioxidants are acting as defence system. Antioxidant removes the free radicals and inhibits the formation of free radicals by binding to metal ions required for their generation. Phenolics and flavonoids presents in medicinal plants and fruits contain the free radical scavenging molecules. Some of the vitamins like C, E and beta carotene are major antioxidant present in diet. Lipid peroxidation of plasma membrane is prevented by the vitamin E by scavenging the singlet oxygen and neutralizes the reactive oxygen radicals **(Bagchi and Puri, 1998; Ceriello, 2000)**. Medicinal plants have various phytochemicals which are responsible for their antioxidative potential and therapeutic effect. Plant based antioxidants are preferred because of being natural and non-toxic in nature **(Padmanabhan and Jangle, 2012)**. Natural antioxidants present in diet or herbal plants are replacing the synthetic antioxidants. Various medicinal plants are being studied for their antioxidative potential **(Nahak and Sahu, 2010)**.

In poultry industry main emphasis is on growth and production, which can be drastically affected by immune system of birds. Various microbes and pathogen are developing resistant toward antibiotics which lead to immunomodulation in the infected organism/ birds. Many reports are available related to the immunomodulatory effects of herbal plants in poultry which may cause histamine release, modulation of cytokine expression, class switching, lymphocyte proliferation, cellular co-receptor expression etc **(Dhama *et al*., 2015)**. Herbal preparation involved in poultry feed may affect the bird metabolic process by reducing stress and other antimicrobial activities. It also promotes the growth and activity of the beneficial bacteria and inhibiting colonization/ growth of the pathogenic bacteria in gastrointestinal tract **(Lee *et al*., 2003)**.

Aegle marmelos (L.) Corr. commonly known as "bael" in India, belongs to *Rutaceae* family and indigenous to Southeast Asia. The leaves, fruit, bark and root of the plant is having medicinal properties. Leaves of the plant are used mostly in fever, abdominal pain, asthma, hypoglycaemia, dysentery, vomiting and swelling etc **(Baliga *et al.*, 2011)**. Fruit of bael is also a good source of vitamin, minerals, water, protein and fibres. It has been proven experimentally that leaves of this plant is having antimicrobial, anti-inflammatory, antipyretic, analgesic, antidiabetic, anti-fertility cardio protective and antioxidant properties **(Chauhan and Agarwal, 2009)**. In the leaves of *Aegle marmelos* various phytochemicals are present *i.e.* aegelin, lupeol,

cineole, citral, eugenol, skimmianine, marmesinin. Aegelin and lupeol that are potent cardioactive compounds having anti-inflammatory activity **(Maity *et al.*, 2009)**. Active metabolites of the *Aegle marmelos* are responsible for its immunosuppressive activity as shown in various experimental systems **(Gupta *et al.*, 2016)**.

Keeping in view of the above, present study was planned to explore immunomodulatory and antioxidative potential of leaves extracts of *Aegle marmelos* (L.) Corr. in chicken lymphocytes culture system with the following objectives:

- Preparation and phytochemical analyses of Aqueous extract of *Aegle marmelos* (L.) Corr.
- Evaluation of immunomodulatory potential of *Aegle marmelos* extract in chicken lymphocytes culture system.
- Evaluations of antioxidative potential of *Aegle marmelos* extract in chicken lymphocytes culture system.
- Determination of role of different cytokine in *Aegle marmelos* induced immunomodulatory effect in chicken splenocytes.

Review of Literature

Traditional medicine also known as herbal medicines are always been part of a different cultures and civilizations around the world. WHO (World Health Organization) reported that more than three quarter part of the world relies on herbal medicine system for curing the diseases. Around the world India has one of the richest plant based medicinal system. In India, herbal medicine system "Ayurveda" is ancient system and it includes various ethanopharmological activities of medicinal plants. These activities involve immunostimulation, anti-aging, anticancer, antibacterial, neurostimulation etc. Various medicinal plants used in "Rasanyana" (Ayurvedia) can increase the resistance of the body against variety of diseases and these can also delay aging, improve mental functioning, possess good antioxidant activities **(Kumar 2012; Mahima *et al.*, 2012)**. Immunomodulation of immune system is now recognized as alternative to conventional chemotherapies for various diseases **(Singh, 2016)**.

2.1 Immunomodulatory Role of Medicinal Plants

Many medicinal plants and their products that are capable of immunomodulation have been accepted as therapeutics. Medicinal plants possess various phytochemicals like flavonoids, carotenoids, polyphenols, saponins and tannins etc. which are responsible for their immunomodulatory and antioxidant properties (**Zhang *et al.*, 2007)**. The complicated defence system is interaction between nonspecific and specific, cellular and humoral immune response, immunocompetent cells that are stimulated and suppressed. Immunomodulators are the substances that can be of biological origin or synthetic having ability to modulate (stimulates and suppress) immune system **(Nagarathna *et al.*, 2013)**.

In immunostimulation central role is played by macrophages and granulocytes which increase the phagocytosis and activation of both T and B lymphocytes. Immunosuppressants mainly used to restraint the immune response in case of autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis), graft rejection, graft versus host rejection in bone marrow transplant etc. **(Makare *et al.*, 2001)**. Generally, Glucocorticoids are used as immunosuppressive drug in graft rejection and autoimmune diseases. They directly influence the protein

synthesis by altering the gene transcription. In case of inflammation, glucocorticoids restrict the vascular permeability and vasodilation that affects the leukocyte migration at the site of inflammation **(Castiblanco and Foster, 2014)**.

Immunosuppressive drugs are having multiple side effects like nausea, vomiting, diarrhoea, abdominal pain, hyperglycaemia, oedema, bone marrow suppression, bladder toxicity etc. **(Khan and Sewell, 2006)**. There are number of medicinal plants having immunosuppressive effects when used in dose dependent manner. Certain herbal preparations that are able to inhibit the cell mediated and humoral immune response could be useful in treatment of immunological disorders *i.e.* autoimmune diseases. Scientists are investigating the immunosuppressive role of various medicinal plants **(Mirshafiey, 2004)**. Cyclosporin A is fungal derived immunosuppressant which is primarily used in organ transplantation. Cyclosporine suppresses the alloimmune and autoimmune response by inhibiting the signal transmission from T cell receptor to multiple lymphokines genes which are required for T helper, T cytotoxic cells activation without affecting T suppressor cells **(Huang and Gaudio, 2010)**.

Amirghofran *et al.* (2010) reported methanolic extract of *Salvia mirzayanii* in PHA activated cells displayed the suppression of lymphocyte proliferation, reduce level of IL-2 and induced apoptosis resulted in inhibition of immune response. Punicalagin present in fruit of *Punica granatum* suppressed the mixed leukocyte reaction in allogenic cells **(Lee *et al.*, 2008)**. *Artemisia annuas* reported to have immunosuppressive effect on delayed type hypersensitivity against sheep blood capsule in Balb/c mice which is due to Artemisinin, purified compound of the plant **(Noori *et al.*, 2004)**.

Flavonoids (isoflavone and isoflavanonol) in *Campylotropis hirtella* showed very strong immunosuppression activity in Con A and LPS induced T and B lymphocyte **(Xuan *et al.*, 2015)**. Rhizome of *Acorus calamus* is reported to show immunosuppressant activity by inhibiting the proliferation of human peripheral blood mononuclear cells which are induced by mitogen and purified protein derivative **(Mehrotra *et al.*, 2003)**. In *Periploca sepium bge*, pregnane glycoside (Periplocoside) is present which is reported to have immunosuppressive effect as it showed decrease in Con A induced lymphocyte proliferation in dose dependent manner and this can be

exploited for rheumatoid arthritis treatment, delayed type hypersensitivity **(Zhu *et al.*, 2006)**.

2.2 Role of cytokine in immunomodulation

During the innate and adaptive responses, homeostasis, inflammatory response, cell death, angiogenesis and repair process etc, cytokines play crucial role for managing and coordinating the immune system. Cytokines are soluble extracellular protein or glycoproteins regulating the immune response. Based on structural homology of their receptors these are divided into different groups *i.e.* interleukins, interferons, chemokines etc **(Oppenheim, 2001)**. Cytokines are considered as "hormone" of immune and inflammatory responses. Cytokines behave in autocrine, paracrine and endocrine fashion by binding to specific receptor of cells, binding to cell receptors in close vicinity and can also binds to receptor of cells that are present at distance, respectively. Strength and extent of the immune response is regulated through cytokines, they do so by influencing the activation, proliferation and differentiation of cells involved in immune response **(Arai *et al.*, 1990)**.

In the network of immune system, T cells play determinative role as most of the cytokines are secreted by them. According to the pattern of cytokines production differentiated T cells are characterized into T helper 1 and T helper 2 subsets. Type 1 cytokines IL-2, IL-12, IFN-α, IFN-β and IFN-γ are mostly produced by T helper 1 cells that induce cell mediated immune response **(Banchereau and Steinman, 1998)**. Type 2 cytokines IL-4, IL-5, IL-10 are primarily produced by T helper 2 cells which promote humoral immunity by B cell activation, increase in antibody titter, class switching (IgG to IgE). Depending on the antigen, the activation of either T helper 1 or T helper 2 cells occurs which influence the cytokines production **(Chandraker *et al.*, 1998)**. Macrophage and dendritic cells of innate immune response produce proinflammatory cytokines (TNF-α and IL-1), which generates and regulates the adaptive immune response **(Belardelli, 1995)**.

Cytokine binds to specific receptor present on the cell surface which leads to activation of cascade of intracellular signals consequently alternating gene expression mediated by different transcription factors *i.e.* NF kB and AIP-1. Secretion of cytokines in nucleated cell is inducible response due to injurious stimuli. Cytokines are acting as molecular signals for maintaining physiological stability and also a connection between

different organ systems. In avian system numbers of genes in cytokine families are smaller than mammals **(Kaiser, 2010)**. Cytokine antagonist, agonist, inhibition and stimulation of cytokines are major approach that can be exploited in various therapeutic applications. Decrease in the cytokine expression produced by T helper cells result in reduces production of Ig E and eosinophils **(Stirling and Chung, 2000)**. Disorders which are supposed to be irrelevant to immune system can also be treated by exploiting the diverse and pleiotropic effects of the cytokines. Role of various cytokines explored in the study are described here under.

2.2.1 Role of IL-6 in immune response

IL-6 is a small 21 kDa glycoprotein known as cytokine of innate immunity because it is produced by various cells of the innate immune response *i.e.* mast cells, macrophages, dendritic cells, B cells, and to lesser extent T cells. IL-6 modulates the adaptive immune response by inducing the proliferation of B cells into antibody secreting cells. High level of IL-6 is associated with infection, inflammation, malignant and autoimmune diseases (Rheumatoid arthritis). It also enhances the differentiation of Th2 by inducing the IL-4 production by CD_4 T cells. Depending upon the nature and site of inflammation, IL-6 also stimulates the production of acute phase proteins. It also plays important role in transition between acute to chronic inflammations **(Rincon, 2012; Gabay, 2006)**.

2.2.2 Role of IL-10 in immune response

IL-10 regulates the immune response by supressing the immune response through pleiotropic effects. IL-10 is secreted by monocytes and from the mature form of monocytes like Macrophages and myeloid dendritic cells. Natural killer cells, B and T lymphocytes also secrete the IL-10 at low level. IL-10 mainly affects the antigen presenting cells resulting in decreased expression of MHC class II molecules complemented by decrease in antigen presentation. IL-10 promotes the shift of Th1 cells into Th2 cell via reducing the IL-12 and IFN-γ and increasing the IL-4, IL-5 and IL-13. IL-10 prevent the chronic immune responses, it also limits the development of the autoimmune and delayed type hypersensitivity immune responses **(Bijjiga and Martino, 2013)**.

2.2.3 Role of interferon gamma

Interferon's are classified as type I and type II based on the similarities between sequence and specificity of receptors. Type I interferon's include IFN α, IFN β, IFN ω and Type II interferon's includes the IFN γ. IFN γ differs from other interferon on basis of their receptor and encoded by different chromosomal locus. IFN γ is produced by Th1 cells, T cytotoxic cells, natural killer cells, antigen presenting cells etc., in adaptive immune response T lymphocytes are the major producer of the IFN γ. During infection IL-12 and IL-18 act as connection between IFN γ and innate immune response. In infection, macrophages recognize the pathogen and induce secretion of IL-12. Macrophages also release various chemokines which attract natural killer cells (NK cells) at the site of infection. NK cells and IL-12 induces the secretion of IFN γ at site of infection **(Schroder, 2004)**.

2.2.4 Role of inducible nitric oxide synthase

The expression of inducible NOS (iNOS) can be triggered/increased by bacterial lipopolysaccharide. iNOS mainly identified in macrophages but its expression can be stimulated in any cell or tissue. NO has an affinity for protein bound iron, it can inhibit the enzymes having iron in catalytic centres. Induced macrophages produce higher level of NO which can directly interfere with DNA of target cells and can cause fragmentation of DNA strands. Cytokines can induced non-immune cells to secrete higher amount of NO to affect adjacent cells. Elevated level of the NO produced by activated macrophages and neutrophils also leads to inflammatory and autoimmune lesions **(Forstermann and Sessa, 2012)**.

Down regulation of expression of iNOS could be desirable for reducing the inflammatory response. Various derivatives of the flavonoids can inhibit the NO production. Apigenin, Genistein and Kaempferol were reported to inhibit the nuclear transcription factor NF-kB which inhibits the expression of COX-2 involved in inflammation **(Liang, 1999)**.

Medicinal plants modulate the immune system by altering the information molecules *i.e.* cytokine, neurotransmitter, hormones etc. **(Spelman *et al.*, 2006)**. *Periploca sepium bge* reported to suppress the production of Th1 cytokine (IL-2 and IFN-γ) from splenocytes in *in vitro* system. *Fissistigma oldhamii* showed its

immunomodulatory effect by directly blocking cytokine mediated signalling pathway **(Zhu *et al.*, 2006; Mahady *et al.*, 2000)**. Ginsenoside Rb1, a ginsenoside isolated from ginseng reported to have inhibitory effect on LPS induced proinflammatory cytokine TNF-α in macrophage RAW264.7 cell line **(Smolinski, 2003)**.

2.3 Plants as antioxidants

Due to basic metabolic processes in human body number of free radicals and reactive oxygen species (ROS) are generated. In origin of life and biological evolution, free radicals are having key functions. Both enzymatic and non-enzymatic reactions are constantly producing the reactive oxygen species. Respiratory chain, phagocytosis, cytochrome 450 system, prostaglandin synthesis are enzymatic reactions producing free radicals and Ionizing radiations, reaction of oxygen with organic compounds are non-enzymatic reactions for production of ROS **(Bagchi and puri, 1998)**. Due to high concentration of ROS oxidative stress is generated. Because of oxidative stress various cellular constituents are altered and lead to generation of various conditions like rheumatoid arthritis, asthma, autoimmune diseases, diabetics, cardiovascular diseases, cancer, chronic inflammation, neurodegenerative disorders etc. **(Gupta and Sharma, 2006)**.

Rapid reaction of free radicals with polyunsaturated fatty acids of the cell membranes cause the cellular oxidative damage results in death of cells. In neuronal damage ROS attacks on glial cells and neurons which are sensitive to them. Because of the increased oxidative stress, reactive oxygen species also affects the insulin intracellular signalling that leads to progression and development of diabetes. To contradict the effects of elevated level of ROS, antioxidants act as defense system. The substances which at low concentration can quench and inhibit the free radicals to delay the oxidation of lipids, proteins, carbohydrate and DNA to inhibit the cellular damage are known as antioxidants **(Nimse and Pal, 2015)**.

Antioxidants are grouped as enzymatic and non-enzymatic antioxidants based on their mechanism of action, Enzymatic antioxidants remove the free radicals by breaking them and non-enzymatic antioxidants utilizes the multiple steps for converting free radicals into hydrogen peroxide then into water in the presence of various cofactors like Zinc, Iron, Manganese, Copper ions. Superoxide dismutase, catalase, glutathione

reductase are acting as enzymatic antioxidants. Plant polyphenols, flavonoids, Vitamin C, Vitamin E act as non-enzymatic antioxidants by halting the chain reaction of free radicals **(Shahidi and Zhong, 2010)**.

Rhizome of the *Zingiber officinalis* has an active compound 6-gingerol which is reported to have antioxidant and therapeutic activity studied in UV induced skin disorders both *in vitro* and *in vivo* models **(Ghosh *et al.*, 2011)**. **Anand *et al.* (2007)** reported that alkaloid piperine (1 –piperoylpiperidine) present in fruit of the *Piper nigrum* (Black papper) has an antioxidative activity when present in low concentration. It also dislayed synergistic antioxidant activity by increasing the absorption of dietary curcumin 38 present in turmeric.

2.4 Dexamethasone as Immunosuppressor

Dexamethasone is synthetic pregnane glucocorticoid which shows anti-inflammatory and immunosuppressive effect. Glucocorticoids in excess amount in body can cause immune suppression, cell apoptosis and induce oxidative stress. Oxidative stress generated by glucocorticoids can be acute (short term) and chronic (long term) depending upon the duration of stress **(Coutinho and Chapman, 2011)**.

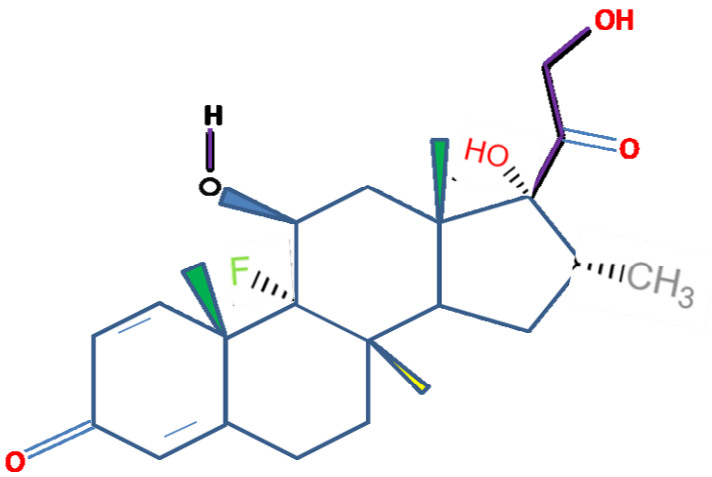


Figure 2.1: 2- dimensional structure of Dexamethasone

Glucocorticoids affect the inflammation by down regulating the expression of genes of pro-inflammation and up regulating the expression of genes of anti-inflammatory response. Leukotrienes and prostaglandins produced by membrane are inhibited by corticosteroids, which results in decrease in swelling **(Mer and Richards, 1998)**.

Dexamethasone act as immunosuppressive mainly by inhibiting the proliferation of lymphocyte and reduce production of lymphocyte cytokines **(Schmidt *et al.*, 1994)**. **Buxant *et al.* (2015)** performed experiment on MCF-7 breast cancer cell line for evaluating the effect of glucocorticoids. Dexamethasone showed antiprolifertaive effect on MCF-7 cell line in a dose dependent manner, higher doses showed inhibitory effect up to 30-35%. Dexamethasone is reported to inhibit various cytokines production in *in vitro* and *in vivo* models. **Wershil *et al.* (1995)** reported that dexamethasone in *in vivo* mouse mast cells inhibited the production of pro inflammatory cytokine TNF-α. **Gessani *et al.* (1988)** reported that human fibroblasts were treated with different concentration of dexamethasone for 2 hours and showed the reduced production of Interferon in dose dependent manner. **Agarwal and Marshal (2001)** reported that dexamethasone at dose of 100µg (similar to plasma corticoids concentration), increased the level of type 2 cytokine including IL-10, IL-4 and decrease the level of IFNγ in human PBMC.

Dexamethasone reported to have the antioxidant effects by altering the level of enzymatic antioxidants i.e. Reduced glutathione, superoxide dismutase, catalase. **Biagiotti *et al.* (2016)** reported that in experiment performed on lympho-blastoid cell lines derived from Ataxia telangiectasia (neurodegenerative disorder), the level of GSH was significantly increased after the dexamethasone exposure for 24 hours. Increase level of GSH helps in reducing the oxidative stress. **Jose *et al.* (1997)** reported that dexamethasone increase the level of antioxidant enzymes in Sprague Dawley rat. The level of glutathione peroxidase and superoxide dismutase was increased after the 3 hour of administration of dexamethasone at dose of (0.2mg/ 100µl).

2.5 Role of medicinal plants in poultry

Since the life emerged, nature has provided the food and medicine to animals in form of various herbs or medicinal plants. Thriving in same environment as the plants, animals utilize the plants to cure their health problems. Medicinal plants are used as herbal therapy in livestock among poor framholders **(Mills and Bone, 2000)**. Around the globe poultry industry has been growing persistently, providing the major source of animal protein in form of eggs and meat. There are various ethanoveterinary practices used quite commonly in poultry industry. For improving the health and poultry production, the herbal extracts (garlic, tulsi, ginger, turmeric, neem, rosemary etc.) are

being explored and reported to exhibit beneficial results **(Sudarshan *et al.*, 2010)**. Increased incidence of bacterial strain resistance against antibiotics is increasing threat for animal and human health. Various antimicrobial substances are synthesized by plants during their secondary metabolites production and essential oils of the plants are used as alternative to antibiotics for controlling microbial infection in poultry. Due to prophylactic action of the essential oils, these are used as feed additives. Thymol and carvacrol are compounds derived from the thyme oil reported to have antimicrobial activity against variety of poultry pathogens **(Levic *et al.*, 2011)**. Herbal plants are used for immunomodulation for enhancing or supressing the immune response which is proven to be an alternative for conventional chemotherapy for several diseases.

In modern allopath medicine system, usually the compounds are designed to mimic the active compound of the plants for defense properties. Various compounds and formulations of plants extract *i.e.* tannins, lectins, flavonoids, polysaccharides are explored in various *in-vitro* models and patented for their immunomodulatory and other therapeutic effects. Presence of Vitamin C, flavonoids, carotenoids and essential oils present in herbs or medicinal plants enhances the immune response in animals **(Stef *et al.*, 2009)**.In chicken infectious anaemia, an immunosuppressive viral disease in chicken, herbal plants (*Tinospora cordifolia, Azadirachta indica, Withana somnifera*) were reported to have stimulating effects for promoting both cellular and humoral immune response and reducing the effect of viral pathogen. Herbal formulation having *Asparagus racemosus* extract was used as immunomodulator in immune compromised broiler chicken **(Latheef *et al.*, 2013)**.

In poultry industry, the phytobiotics present in root, shoot, leaves, tuber of the herbs and medicinal plants have reported to show synergistic growth enhancing effects. Basically, the herbs in poultry diet increase the metabolism by reducing the stress, antimicrobial activity and also promote the growth of beneficial bacteria in gastrointestinal tract of the poultry. Number of medicinal plants like *Emblica officinalis*, *Aloe vera*, *Thymus vulagris, Curcuma longa*, *Allium sativum, Zingiber officinalis* are reported to have growth enhancing effects in poultry. Alfa alfa (*Medicago sativa*), corn flower (*Centaurea cyanus*) are used as feed additive for enhancing the growth **(Khaligh *et al.*, 2011; Kumar *et al.*, 2013)**.

Bharavi *et al.* (2010) reported that Tulsi (*Ocimum sanctum*) and Ashwagandha (*Withania somnifera*) are admirable anti stress agent by reducing the cadmium induced oxidative stress in poultry. Medicinal plants are the potentially used as source of various therapeutic benefits in human and animal.

2.6 *Aegle Marmelos*

Aegle marmelos (L.) Corr commonly known as Bael in Hindi belongs to *Rutaceae* family. It is deciduous tree native to Indian subcontinent and Southeast Asia and also one of the sacred trees of Hindus. Bael also possesses various medicinal properties. Various active compounds are isolated from various part of tree (leaves, bark and fruit) that are explored for medicinal value.

Taxonomical Hierarchy

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Spindales
Family	:	Rutaceae
Sub family	:	Aurantioideae
Genus	:	*Aegle*
Species	:	*Aegle Marmelos*
Binomial name	:	*Aegle marmelos*

Monograph

Common name	:	Bel, Bela, Bael, Adhararutha
Scientific name	:	*Aegle marmelos* (L.) Corr.
Duration	:	Perennial
Growth habit	:	Multi-branched tree

Morphology

Aegle marmelos is aromatic tree of moderate sized it can attain height of 12-15 meter. It is deciduous tree growing wild throughout the forests of India at altitude of

1200 meter in western Himalayas. It can grow in well drain loamy soil or alkaline stony soil having pH from 5-8. It can also grow well even in harsh and dry climates. It can be vegetatively propagated or through planting seedling. Tree of bael have spiny branches and alternative leaves morphology. Flowers of the tree are founds in cluster having fragranance. Fruit of the tree is oval, round in shape having thin or hard wood shell depending upon the variety. Fruit pulp is highly aromatic and pleasant flavour **(Sharma and Dubey, 2005)**.

Traditional Uses

Leaves, fruit, pulp, stem and roots of plants are used in ethno medicine for treating various diseases. Leaves of the tree are used to treat, inflammation, asthma, hypoglycemia, febrifuge, hepatitis and analgesic. Flowers of the tree are used to yield a tonic which is used as anti- dysenteric, antidiabetic and local anaesthetic. Fruit is used in gastric conditions like constipation, stomachic, ulcer, intestinal parasites as well as epilepsy, typhoid, debility, cholera, hemorrhoids, intermittent fever, hypocondria, melancholia, and for heart palpitation. The preparation of stem and root is used for intermittent fever, hypochondriasis **(Roberrs and Tylor, 2002)**.

Phytochemical Profile

Preliminary phytochemical screening of *Aegle marmelos*, showed the occurrence of number of organic molecules *i.e.* terpenoids, flavonoids, alkaloids, tannins, coumarins, polysaccharides and phenylpropanoids. Various active phytochemical are reported to be present in bael i.e. aegeline, aegelenine, aegelinosides, marmelin, marmelosin, methyl ether, xanthotoxol, malondialdehyde (MDA), anhydromarmeline, marmelide, umbelliferone β-D-galactopyranoside, lupeol, halfordinol, butyl p-tolyl sulfide, 6-methyl-4-chromanone, butylated hydroxyanisole, imperatoin, xanthorrhizol, xanthoarnol, 1-hydroxy-5,7-dimethoxy-2-naphthalene-carboxaldehyde, 1-methyl2-(3'-methyl-but-2'-enyloxy) anthraquinone **(Manandhar *et al.*, 2017)**.

Habitat

It is native in India, Pakistan, Sri Lanka, Bangladesh, Myanmar, Egypt Southeast Asia. It is widespread and naturalized in most of the tropics and subtropical countries **(Orwa, 2009).**

Immunomodulating and antioxidative potential of *Aegle marmelos*

The effect of *Aegle marmelo*s aqueous and methanolic leaf extracts on *in vitro* lymphocyte proliferation and humoral and cellular immune responses were studied by **Gupta *et al.* 2016; Arokiyaraj *et al.* 2007**. The aqueous leaves extract showed the decrease in proliferation of different splenocytes. Methanolic leaves extract of the plant also showed inhibition in proliferation in PBMC (peripheral blood mononuclear cells) cells under mitogen stimulated condition. **Lampronti *et al.* (2003)** reported that ethanolic extract of bark of *Aegle marmelos* showed *in vitro* antiproliferative action against various human cell lines like leukemic K562, T-lymphoid Jurkat, Blymphoid Raji, erythroleukemic HEL, melanoma Colo38, and breast cancer MCF7 and MDAMB-231 cell line.

Subramaniam *et al.* (2008) studied that active compound 1-Hydroxy-5,7-Dimethoxy-2-Naphthalene Carboxaldehyde (marmelin) isolated from leaves of *Aegle marmelos* in ethyl acetate fraction showed decrease in proliferation of HCT-116, HT-29 colon, AGS gastric, and HEp-2 alveolar cancer cells. **Rajaram *et al.* (2017)** reported that aqueous extract of roots of *Aegle marmelos* showed decrease in cell proliferation in LPS induced RAW 264.7 cell line. Increase in anti-inflammatory cytokine IL-2 and decrease in pro inflammatory cytokine IL-6 and IL-1β was observed in LPS induced RAW 264.7 cell line. **Rahman *et al.* (2016)** studied antioxidant potential of the chloroform and aqueous extract of *Aegle marmelo*s fruit. Results have shown significant free radical scavenging and lipoxygenase inhibitory activity. Ethanolic leaf extract of *Aegle marmelo*s reported to possess significant antioxidant activity and it has also inhibited the formation of malondialdehyde which is indicative of decrease in lipid peroxidation activity **(Reddy *et al.*, 2013)**. **Ramakrishna *et al.* (2015)** reported that methanolic extract of unripe fruit of *Aegle marmelo*s prevented the decrease in enzymatic and non-enzymatic antioxidants in Sprague-Dawley rat gastric system at various concentrations. **Sabu and Kuttan (2004)** studied the antidiabetic activity of hydro methanolic leaves extract of *Aegle marmelos* in relation to antioxidative potential in wistar rats. They reported that hydromethanolic extract decrease the level of serum glucose and serum lipid peroxidation in hyperglycaemic rat. The level of enzymatic antioxidants GSH, SOD, Catalase was increased in erythrocytes of wistar rat after exposure of the extract.

Materials and Methods

The present study was carried out in the Department of Molecular Biology and Genetic Engineering, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar with the aim to study Antioxidative potential and cytokine mediated Immunomodulation due to *in vitro* exposure of *Aegle marmelos* (L.) Corr. in chicken lymphocytes culture system.

3.1 Materials

3.1.1 Chemicals Used

List of chemicals used in the present study is given in the **Table 3.1.**

Table 3.1: List of chemicals

S.No.	Name of chemicals	Company
1	Di-Sodium hydrogen phosphate anhydrous	Himedia
2	Glutathione reduced (GSH)	Himedia
3	3-(4,5-Dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide(MTT)	Himedia
4	Antibiotic Antimycotic Solution 100X liquid, Endotoxin tested	Himedia
5	Ethanol	SDFCL
6	EDTA disodium salt dehydrate, A.R.	Himedia
7	Rutin	Himedia
8	DMSO (AS089-500ML)	Himedia
9	Hisep (LSM002-100ML)	Himedia
10	Dulbecco's phosphate buffer saline	Himedia
11	Trichloroacetic acid	Himedia
12	Di-sodium hydrogen phosphate (Na_2HPO_4)	Himedia
13	Sodium di-hydrogen phosphate (NaH_2PO_4)	Himedia
14	Sodium bicarbonate	Himedia
15	Sodium chlorite	Himedia
16	HEPES buffer	Himedia
17	RPMI 1640	Himedia
18	Griess reagent	Sigma-Aldrich
19	RNA X-press Reagent	Himedia
20	Revert Aid cDNA synthesis Kit	ThermoScientifics
21	SYBER Green Master Mix	ThermoScientifics
22	Chloroform	Himedia
23	Dexamethasone	Himedia
24	DTNB	Himedia
25	Folin- Ciocalteu reagent	Himedia

3.1.2 Equipments, Glasswares and Plasticwares

Various equipments, glasswares and plasticwares used during the experimentation are mentioned in **Tables 3.2** and **3.3.**

Table 3.2: List of equipments

S.No.	Name of equipment	Company
1	Phase contrast inverted microscope	Nikon
2	CO_2 incubator	New Brunswick Scientific
3	Refrigerated centrifuge	Tommy
4	Digital balance	Precisa
5	pH meter	Lab companion
6	Laminar air flow	Lab companion
7	Incubator cum shaker	Lab companion
8	Water bath	Lab companion
9	Magnetic stirrer	Genie
10	Spectrophotometer	Shimadzu
11	Media filtration assembly	Millipore
12	ELISA reader	Biotek
13	Haemocytometer	New improved Neubauer
14	Micropipettes	Eppendorf
15	Deep freezer (-80°C)	New Brunswick Scientific
16	-20°C Deep freezer	Vestfrost(BSF-345)
17	Autoclave	Labtech
18	Lyophilizer	Shimadzu
19	Rotary evaporator	Shimadzu
20	Real time Thermo cycler PCR	Eppendrof
21	Thermo cycler PCR	Himedia
22	Nano-drop 1000 Spectrophotometer	Thermo scientific
23	Gel documentation system	Alphalmager, AT126SL, USA
24	Multichannel pipette	Thermo scientific

Table 3.3: List of glasswares, plasticwares and disposables

S.No.	Name of instrument	Company
1	Conical tubes (15ml)	Tarson
2	Conical flasks	Borosil
3	Single use disposable syringe	DISPOVAN
4	Reagent bottles (100ml, 250ml, 500ml,1000ml)	Borosil
5	Disposableserological pipettes (1ml, 5ml, 10ml)	Himedia
6	Cryopreservation vials	Tarson
7	Microtips (10µl, 200µl, 1000µl)	Tarson
8	Disposable petriplates	Tarson
9	Centrifuge tubes (1.5ml, 2ml)	Tarson
10	Syringe filters (0.22 µm, 0.45 µm)	Millipore
11	Membrane filter (0.22 µm)	Millipore
12	96 well tissue culture plates	Grenier Bio-one
13	-20^0C mini cooler	Tarson
14	6 well tissue culture plates	Himedia
15	0.2ml PCR tubes	Tarson
16	Real time PCR tubes	Genexy

3.1.3 Biological Materials

S.No.	Name of materials	Company
1	Lipopolysacchride (LPS)	Sigma
2	Concanavalin (Con A)	Himedia
3	Phytohaemagglutinin (PHA)	Himedia
4	Fetal Bovine Serum	Himedia

3.1.4 Collection of plant material

The leaves of *Aegle marmelo*s were collected from Ludhiana, Punjab and authenticated by Dr. D.S. Rawat, Astt. Prof., Biological Sciences, CBSH, GBPUAT, Pantnagar.

3.1.5 Collection of chicken spleens

Chicken spleens were collected from healthy chickens (White Leghorn)ofthe age of 5-7 weeks in sterile Dulbecco's phosphate buffer saline from local slaughterhouse and brought to the laboratory. Due approval for animal experimentation was procured from Institutional Animal Ethics Committee, GBPUAT, Pantnagar (IAEC/CBSH/MBGE/313). Then spleens were further processed immediately to isolate lymphocytes under strict aseptic conditions.

3.2 Methods

3.2.1 Preparation of the plant extract

The following procedure was adopted for the preparation of *Aegle marmelos* extract (AME) as per the method reported by **Roopashree*et al.* (2008)** with few modifications.

Materials required

Autoclaved distilled water, measuring cylinder, conical flasks, incubator cum shaker, muslin cloth, Petri plates, Whatman filter paper No.1, lyophilizer and rotary evaporator.

Procedure

1. The plant material was thoroughly washed with running tap water and then with distilled water and kept for shade drying. After complete drying, plant material was grinded to make fine powder in a grinder.
2. 100 gram shade dried powder of *Aegle marmelos* was added to 1000 ml of autoclaved distilled water and mixed gently with the help of clean glass rod.
3. The solution was homogenized at 37°C for 48 hours in an incubator cum shaker.
4. The mixture was filtered through muslin cloth and then through Whatman filters paper No 1.
5. The aqueous extract was rotary evaporated at 45°C to evaporate the excess solvent and then subjected to freeze drying. Finally the extract was obtained after lyophilization, weighed and stored at -20^{0}C in deep freezer till further use.

6. The percentage yield for the plant extract was determined.

The percent yield of the extract was calculated as:

$$\text{Yield}(\%) = \frac{W1}{W2} \times 100$$

Where,

W1 is the weight of the extract obtained after lyophilisation or complete drying and W2 is the weight of the dried powder initially taken for extract preparation.

3.2.2 Phytochemical analyses of the plant extract

Analysis of the plant extract was carried out for the detection of various important phytochemicals and bioactive compounds. The plant extract was subjected to various qualitative and quantitative analyses.

3.2.2.1 Qualitative analyses

Qualitative tests were carried out to detect the presence of phenolics, flavonoids, tannins, alkaloids, saponins, carbohydrate, protein, amino and phytosterolin the plant extract as per the method described by**Trease and Evans (2002)** and **Tiwari*et al.*(2011).**

A. Phenolics:

- **Ferric Chloride Test** -500 mg of extract was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds

B. Flavonoids:

- **Ammonia Test** - A few drops of 1% ammonia solution was added to the extract of plant sample in a test tube. A yellow coloration shows the presence of flavonoid compounds in the sample.
- **H_2SO_4 Test**- 50 mg of the extract was taken in test tube and was treated with a few drops of sulphuric acid. Change in colour of the solution to orange indicates the presence of several flavanoids in the sample.
- **Alkaline reagent Test** – 50 mg of the plant extract was taken in test tube and a few drops of sodium hydroxide was added to each tube. Formation of yellow

colour, which on addition of dilute acid, becomes colourless, shows the presence of flavanoids in the extract.

C. Tannins:

- **Gelatin Test** - To 20 mg of the plant extract, 1% gelatine solution was added. Formation of white precipitate is an indication for the presence of tannins in the sample.

D. Phytosterols:

- **Salkowski's Test** - Small quantity of the extract was dissolved in 5ml of chloroform separately. To 1ml of above prepared chloroform solution, few drops of concentrated sulphuric acid was added. Presence of brown colourshows the presence of phytosterols in the plant extract**.**

E. Alkaloids:

- Small quantity of the extract was separately treated with few drops of dilute hydrochloric acid and filtered. The filtrates were used for the test with Dragendroffsreagent. The appearance of orange brown precipitate indicates the presence of alkaloids.

F. Saponins:

- **Foam test**- 0.5 gm of the plant extract was taken in 2 ml vials and was dissolved in 2 ml of water. The sample was shaken vigorously for 10-15 seconds. If the foam produced persists for 10 minutes, saponins are present in the sample.

G. Proteins:

- **Xanthoprotic Test**– To the plant extract, a few drops of nitric acid was added. Formation of yellow colour shows the presence of proteins in the plant extract.

H. Amino acids:

- **Ninhydrin Test**–To the plant extract, few drops of 25% ninhydrin solution was added. Then the solution was boiled for few minutes. Change in colour of the solution to blue, indicates the presence of amino acids.

I. Carbohydrates:

The plant extract was dissolved in 5 ml distilled water in test tubes to carry out following tests.

- **Molisch's Test** - To above solutions, 2-3 drops of alcoholic α naphthol solution was added. Formation of violet ring shows the presence of carbohydrates.
- **Benedict's test** – The above solutions of plant extract was treated with Benedict's reagent and heated. Formation of orange precipitate shows the presence of carbohydrates.

3.2.2.2 Quantitative analyses of the plant extract

Qualitative analysis for determination of total phenolic and total flavonoid content present in AME was carried out.

A.Determination of total phenolic content

Plant's total phenolic content was determined by the method describedby**Mansour *et al.* (2011)**.

Materials required

Gallic acid, methanol, double distilled water, Folin-ciocalteu reagent, test tubes, conical flasks, measuring cylinder and pipettes.

Principle

This method is based on the reduction ability of functional group of phenol. An oxidation and reduction reaction of the phenolic ion takes place in basic conditions. Reduction of phosphotungstate-phosphomolybdenum complex (Folin-ciocalteu reagent) by phenolic ions changes the reagent to dark blue. The color becomes darker, absorbing at progressively higher wavelengths as reduction ability increases with the increasing phenolic compounds.

Procedure

1. Stock solution of the plant extract and gallic acid were prepared by dissolving 1 mg of extract or gallic acid in 1 ml of methanol:water mixture (50:50 v/v).
2. 50µl of 40µg/ml of extract was taken in a test tube.

3. 250µl of 50% Folin- Ciocalteu reagent was added to each tube and mixed properly.
4. The mixture was allowed to stand for 10 min followed by addition of 500µl 20% sodium carbonate.
5. The mixture was vortexed and final volume was made up to 5 ml using autoclaved distilled water.
6. After 30 min of incubation the absorbance of the blue coloured complex was measured at 765 nm wavelength in spectrophotometer.
7. The mixture devoid of the extract was taken as blank.
8. Result was calculated from the standard curve of Gallic acid

B. Determination of total flavonoids

Plant's flavonoid content was determined by following the aluminium chloride colorimetric assay described by **Ahmed *et al*.(2012)**.

Materials required

Rutin, methanol, double distilled water, sodium nitrite, aluminium chloride, sodium hydroxide, test tubes, conical flasks, measuring cylinder and pipettes.

Principle

The principle of aluminium chloride colorimetric method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the ortho hydroxyl groups in the A- or B-ring of flavonoids. The yellow coloured complexes so formed can be detected at 510 nm wavelength spectrophotometrically.

Procedure

1. 1 mg/ml of stock solutions were prepared in distilled water for rutin and the plant extract.
2. Different dilutions of standard solution of rutin (5-40µg/ml) were taken in a series of test tubes and the volume was made up to 5 ml with distilled water.
3. 5ml of 100µg/ml of the plant extract was taken in a test tube.

4. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. Then after 5 min, 0.3ml of 10% $AlCl_3$ was added which gave yellow colour.
5. After incubation of 6 min at room temperature, 2 ml of 1M NaOH was added and the total volume was made up to 10ml with the distilled water.
6. The solution was mixed well and the absorbance was measured against a reagent blank devoid of the extract at 510 nm.
7. Total flavonoid content was calculated from the standard curve of rutin.

3.2.3 Evaluation of antioxidant potential of plant extract

3.2.3.1 DPPH radical - scavenging assay

Scavenging of stable DPPH radical was performed to evaluate antioxidative potential of the plant extract*in vitro*. The antioxidant activity of the extract was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical according to the method described by**Shivhare*et al.*** **(2010**).

Principle

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical is widely used for investigating the scavenging activities of several natural compounds such as phenolic or crude mixtures of plant. DPPH radical is scavenged by antioxidants through the donation of a proton forming the reduced DPPH. The scavenging reaction between (DPPH) and an antioxidant (HA) can be written as,

(DPPH) + (H-A) = DPPH-H + (A)

Antioxidants react with DPPH and reduce it to DPPH-H and as a result absorbance decreases due to change occur in colour from purple to yellow. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. The colour changes can be quantified by measuring decrease of absorbance at a wavelength of 517 nm.

Materials required

DPPH (2, 2-diphenyl-1-picrylhydrazyl), methanol, double distilled water, sodium

nitrite, aluminum chloride, sodium hydroxide, test tubes, conical flasks, measuring cylinder and pipettes.

Procedure

1. 100µl of various concentrations ofthe plant extract dissolved in distilled water or methanolranging from 5-100µg/ml were added to the test tubes.
2. Addition of 100µl of0.3mM DPPH solution in methanol started the reaction.
3. The reaction mixture was added to the 96 well plates in triplicate and kept at 30°C for 30 min and the absorbance was measured at 517 nm.
4. Ascorbic acid was used as a positive control and Methanol was used as blank.
5. DPPH solution in the absence of test sample was used as a negative control.
6. Percent DPPH radical scavenging activity was calculated according to decrease in absorbance at 517nm in comparison with negative control. The formula used for calculation is as follows:

$$Percentage(\%)\ DPPH\ scavenging\ = \frac{(Abs\ of\ negative\ control - Abs\ of\ sample)}{Abs\ of\ negative\ control} \times 100$$

7. The IC_{50} values were calculated fromregression curve and statistical analysis was done (2 way ANNOVA) using STPR software.

3.2.3.2. NO radical - scavenging assay

When oxygen and reactive oxygen species reacts with NO, its results in production of free radicals of the Nitric oxide and reactive nitrogen species. Free radicals of nitric oxide have unpaired electron and reacts with various proteins. When Nitric oxide reacts with superoxide radicals, it forms highly reactive oxidantnperoxynitritenanions($ONOO^-$) which on decomposition produce OH and NO and create oxidative damage. The antioxidant activity of the extract was measured on the basis of the scavenging activity of free radical according to the method described by**Jagetia*et al.* (2004).**

Principle

At physiological pH, sodium nitroprusside in aqueous solution spontaneously generates nitric oxide. Nitric oxide on interaction with oxygen generates nitrite ions

which are estimated by using Griess reagent. Nitric oxide scavengers combat with oxygen and reduced production of nitrite ions.The radicals of nitric oxide are scavenged by flavonoids. Diazotization of nitrite with Griess reagent produce a chromophere and absorbance of chromophere was measured at 546 nm on UV-visible spectrometer. IC_{50} value was calculated.

Materials required

Griess reagent, phosphate buffer saline, sodium nitroprusside, double distilled water, 96 well plates, measuring cylinder and pipettes.

Procedure

1. 10mM sodium nitroprusside in phosphate buffer saline was prepared.
2. 200 mg of Griess reagent was dissolved in 5 ml of autoclaved double distilled water.
3. Plant extract at various concentrations ranging from 10-100µg/ml was mixed in sodium nitroprusside to make final volume of 100µl.
4. 100µl of each concentration of plant extract was added to 96 well plates in triplicate.
5. The plate was incubated for 150 minutes at 25^0C in dark.
6. 100µl of Griess solution was added in each well of 96 well plate and absorbance was measured at 546 nm.
7. Ascorbic acid was used as a positive control and Griess solution along with sodium nitroprusside in the absence of test sample was used as a negative control.
8. Percent NO radical scavenging activity was calculated according to the absorbance at 517nm in comparison with negative control. The formula used for calculation as follows:

$$Percentage(\%)\ NO\ scavenging\ = \frac{(Abs\ of\ negative\ control - Abs\ of\ sample)}{Abs\ of\ negative\ control} \times 100$$

9. The IC_{50} values were calculated fromregression curve and statistical analysis was done (2 way ANNOVA) using STPR software.

3.2.4 Chicken lymphocytes culture

Lymphocytes isolated from healthy chicken spleens were cultured in Roswell Park Memorial Institute 1640 media (RPMI-1640) at cell count of $1x10^7$ cells/ml. The cell count was maintained after viable cell counting by dye exclusion method (trypan blue staining) on haemocytometer.

3.2.4.1 RPMI-1640 media preparation

Materials required

1000 ml of autoclaved distilled water, sterile media filtration assembly with 0.22 µm Millipore filter membrane, Vacuum pump,7.5% $NaHCO_3$, 10ml antibiotic and antimycotic solution, Magnetic stirrer, Sterile reagents bottles , pH indicator.

Procedure

1. For the preparation of RPMI -1640 media sufficient amount of autoclaved water was added in a beaker and placed on magnetic stirrer under aseptic sterile condition (laminar air hood).
2. 10.4 g of powder was added to the beaker slowly while mixing (200 rpm) till the powder was completely dissolved. 10 ml of antibiotic and antimycotic solution was added and its pH was adjusted to 7.4 with the help of sterile tissue culture grade sodium bicarbonate (7.5%). Finally the volume was made up to one liter with the ultrapure autoclaved water.
3. Medium was filtersterilized through 0.22 µm Millipore filter membrane in the sterile media filtration assembly. Sterilized media was dispensed in sterile reagent bottles in multiple aliquots. Bottles were kept in incubator at 37^0 C to check the sterility. Finally the media was stored at 4^0 C in refrigerator till further use (**Freshney, 2005)**.

3.2.4.2 Preparation of lymphocytes suspension from spleens

Lymphocytes were isolated from chicken spleens as per the method described by **Janossy and Greaves (1971)**. All the steps were carried out aseptically under laminar air flow.

Material Required

DPBS, Sterile forceps and scissors, Sterile glass slides, Sterile Petri plates, lymphocyte separation media (HiSep, Himedia), conical bottom tubes (15 ml), RPMI-1640 media,10% FBS (Himedia, India).

Procedure

1. Spleens were placed in a 90 mm sterile petri dish containing RPMI-1640 media. The contents of spleens were teased out with the help of sterile forceps and scissors and minced with the help of two sterile glass slides.
2. The cell suspension obtained from these small tissue pieces was passed through muslin cloth (sterilized) and collected in a sterile beaker.
3. Lymphocytes were separated through density gradient centrifugation as per the method described by **Rose and Friedman (1976)**. 3.0 ml of lymphocytes separation media was aseptically transferred to a sterile 15 ml clean conical tube. Lymphocytes separation media was carefully overlayedwith 8 ml of cells suspension.
4. Tubes were centrifuged at 400 x g for 30 minutes at room temperature. Centrifugation leads to sedimentation of erythrocytes, polynuclear leukocytes and mononuclear lymphocytes. Top of the tube was containing plasma layer followed by lymphocyte layer. Pellet of erythrocyte and granulocytes was settled at the bottom of the tube.
5. Plasma layer was withdrawn and lymphocytes layer was aspirated with the help of Pasteur pipette to a fresh sterile 15 ml conical bottom tube. Equal volume of RPMI-1640 medium was added to the aspirated lymphocytes layer and mixed by gentle pipetting.
6. Centrifugation was carried out for 10 minutes at room temperature at low speed at 200 x g. Washing was done with the sterile RPMI 1640 media to remove traces of lymphocytes separation media and to reduce the number of platelets. Cell pellet was suspended in the media containing 10% FBS.

3.2.4.3 Viable cell counting

Cell count was adjusted to 1×10^7 cells/ml using Trypan blue dye exclusion method as described by **Freshney (2005)**.

Material required

Haemocytometer, 70% ethanol, 0.4% trypan blue dye, cell suspension, micropipettes.

Procedure

1. Cells were dispensed in RPMI-1640 and mixed thoroughly to disperse any clumps. Slides and cover slips were prepared by wiping the surface with 70% alcohol.
2. Cells suspension was mixed properly and 10 µl of the cells suspension was taken and mixed with 10 µl (0.4%) trypan blue dye in 1:1 ratio.
3. Cells suspension was transferred to one edge of haemocytometer, pipette was reloaded and transferred to the second chamber of haemocytometer.
4. Five squares bounded by three lines were counted. The average of two counts was calculated and concentration was derived by the following formula:

$$C = n \times 10^4 \times 5$$

Where,

C= cell concentration (cells/ml)

n= the number of cells counted

3.2.5 Determination of maximum non cytotoxic dose of the plant extract and dexamethasone in chicken lymphocytes

Principle

Various concentration from 1µg/ml to 1500µg/ml, of AME, was used to determine the maximum non cytotoxic dose (MNCD) of the plant extract using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] dye reduction assay. The yellow tetrazolium MTT is reduced by metabolically active cells, by the action of mitochondrial dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. These formazan crystals are largely impermeable to cell membranes and, therefore accumulate within healthy cells. The concentration of formazan product formed is a direct representative of the number of surviving cells.

The resulting intracellular purple formazan can be quantified by spectrophotometric means by taking absorbance at 570 nm.

Materials required

MTT dye solution (5 mg/ml in Phosphate Buffer Saline (PBS)), RPMI-1640 media, FBS, 96 well plates, syringe filters (0.22µm), disposable sterile pipettes, multichannel pipette, petri plates, cell culture tested Dexamethasone (Himedia, India, $C_{22}H_{29}FO_5$, Mol. Wt.-392.46, CAS NO.- 50-20-2)

Procedure

1. 1mg of the extract was taken and dissolved in 1ml of the media to be used as stock solution. The extract was dissolved completely with the help of vortexing and filter sterilized through 0.22µm syringe filter under laminar air hood to maintain aseptic sterile condition.
2. 1mg of the Dexamethasone was taken and dissolved in 1ml of the DPBS to be used as stock solution. The Dexamethasone was dissolved completely with the help of vortexing and filter sterilized through 0.22µm syringe filter under laminar air hood to maintain aseptic sterile condition
3. Chicken lymphocyte cells were seeded in 96 well plates at 1 X 10^7 cells per ml. First and last column were not seeded, they later on filled with RPMI -1640 media.
4. Various dilutions of the plant extract and Dexamethasone were prepared in media ranging from 1µg/ml to 1500 µg/ml and 1ng/ml to 200ng/ml, respectively.
5. Cells were exposed to various dilutions of the plant extract in triplicate and incubated for 68 hours at 37°C in a CO_2 incubator at 5% CO_2.
6. The range of concentrations for both the extract and dexamethasone used for cytotoxicity evaluation is given in **Table 3.4.**

Table 3.4: Range of concentrations used for determination of maximum non cytotoxic dose by MTT assay

S. No.	Treatments	Range of concentrations
1.	*Aegle marmelos* aqueous leaves extract	1µg/ml - 1500 µg/ml
2.	Dexamethasone	1ng/ml - 200 ng/ml

7. As incubation period was over, the media was removed. All the wells were fed with 150 µl of fresh serum free medium and 20 µl of MTT (5mg/ml in DPBS).
8. The plate was covered with aluminium foil, and incubated for 4 hours in humidified atmosphere at 37^0C in a CO_2 incubator.
9. After incubation, the media containing MTT was removed and all the wells were fed with 150 µl of DMSO and incubated for 15 minutes.
10. After 15 min incubation plate was then read immediately in a 96 well plate reader at 570 nm absorbance.

Calculation

The percent viability was calculated as per the following formula:

$$\%\ viability = \frac{Abs\ of\ treated\ sample - Abs\ of\ blank}{Abs\ of\ control - Abs\ of\ blank} \times 100$$

The percent cytotoxicity was calculated by

$$\%\ cytotoxicity = (100 - \%cellviability)$$

3.2.6 Evaluation of immunomodulatory potential of the plant extract through Lymphocyte Proliferation Assay (LPA)

Immunomodulatory properties of the plant extract was evaluated through LPA by exposing cells to maximum non-cytotoxic dose of the plant extract in presence of various mitogens, viz. Concanavalin A (Con A), phytohaemagglutinin (PHA) and lipopolysaccharide (LPS). Dexamethasone (Dexa) was taken as control as a known immunosuppressive glucocorticoid.

Principle

It is a proliferation assay, simple method of non-specifically measuring lymphocytes activation and proliferation ability. When B cells encounter their specific antigens, with the help of T cells, B cells gets stimulated to undergo proliferation. When T cells are activated by antigen-presenting cells and cytokines, T cells undergo proliferation. The proliferation of B and T cells leads to clonal expansion and the initiation of the specific immune responses. Concanavalin A, phytohaemaggludnin(PHA), lentil mitogen (LM) are required which are selectively

activate T cells**,** whereas LPS mitogen activates B cells**(Janossy and Greaves, 1971)**.The lymphocytes can be artificially stimulated using the following mitogens:

Phytohemagglutinin (PHA): T-cell activation

Concanavalin (Con A): T-cell activation

Lipopolysaccharide (LPS): B-cell activation

Lymphocyte proliferation assay (LPA) was carried out as per the method described by **Creed *et al.* (2009)**.

Material Required

Flat bottom 96 well plate, concanavalin A, phytohemagglutinin, lipopolysaccharides, phosphate buffer saline (PBS), eppendorf tube (2ml), 10 % FBS

MitogensRequired

Stock of 100µg/ml was prepared for each mitogen [ConcanavalinA, Phytohaemagglutinin (Himedia) and lipopolysaccharide (Sigma)] in phosphate buffer saline (pH 7.4). Working concentration used for mitogenic stimulation was kept as 5µg/ml.

Procedure

1. The isolated lymphocytes were suspended in RPMI 1640 media supplemented with 10% FBS and the cell density was adjusted to 1×10^7 cells/ml after viable cell counting.
2. Each well of 96 well was seeded with 100 µl of lymphocytes suspension. Three wells containing cells with media were left as controls.
3. Cells were exposed to the maximum non-cytotoxic dose of the plant extract and Dexamethasone in presence of 7.5µl of different mitogens, *viz.* LPS, Con A and PHA which were used to stimulate B and T cells, respectively. The plate was incubated for 68 hours in a CO_2 incubator at 37^0 C.
4. As incubation period was over, the media was removed. All the wells were fed with 150 µl of fresh serum free medium and 20 µl of MTT (5mg/ml in DPBS).
5. The plate was covered withaluminium foil, and incubated for 4 hours in humidified atmosphere at 37^0C in a CO_2 incubator.

6. After incubation, the media containing MTT was removed and all the wells were fed with 150 µl of DMSO, mixed and incubated for 15 minutes.
7. After 15 min incubation plate was then read immediately in a 96 well plate reader at 570 nm absorbance.

Calculation

The percent viability was calculated as per the following formula:

$$\%\ viability = \frac{Abs\ of\ treated\ sample - Abs\ of\ blank}{Abs\ of\ control - Abs\ of\ blank} \times 100$$

The percent cytotoxicity was calculated by

$$\%\ cytotoxicity = (100 - \%cellviability)$$

3.2.7 Evaluation of antioxidative potential of the plant extract in chicken lymphocytes

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols **(Sies et *al.*, 1979)**.

The chicken lymphocytes were treated with maximum non cytotoxic dose of 75µg/ml of AME and incubated for 68 hours in CO_2 incubator chamber. After incubation the treated cells were harvested to prepare the cell lysate.

Preparation of cell lysate

The cell lysates were prepared for AME treated lymphocytes cells to determine the antioxidative status.

Composition of cell lysis buffer

500mM NaCl, 100mM HEPES buffer, 0.1% triton X.

Procedure

1. The plant extract treated cells were trypsinized and removed from the culture plates.
2. The cell suspension was transferred into a centrifuge tube and pellet was obtained by centrifugation at 3000 rpm for 5 min at room temperature.
3. The supernatant was discarded and the pellet was washed twice with PBS. The pellet was then suspended in 500 µl of lysis buffer and mixed properly.
4. The resulting mixture was centrifuged at 2300 rpm for 15 min at 4°C.
5. The supernatant was transferred to a fresh tube and stored at -80°C till further analysis.

3.2.7.1. Membrane lipid peroxidation (LPO)

The extent of membrane LPO was estimated by measuring the formation of malondialdehyde (MDA) using the method of **Ohkawa et *al.* (1979)**. MDA is one of the end products of membrane LPO. It is used as an indicator of oxidative stress in cells and tissues.

Principle

Malondialdehyde (MDA), produced during peroxidation of lipids, served as an index of lipid peroxidation. In this method MDA reacts with thiobarbituric acid and generate a coloured product, which is measured by taking absorbance at 532 nm wavelength.

Materials required

0.1 M sodium phosphate buffer (pH 7.4), 5% TCA, 1% TBA, test tubes, centrifuge tubes.

Procedure

1. Cell lysate of untreated control cells, plant extract treated cells and ascorbic acid treated cells were prepared as described in section 3.2.7.
2. Briefly, a mixture of 0.1 ml cell lysate and 1.9 ml of 0.1 M sodium phosphate buffer (pH 7.4) was incubated at 37^0 C for 1 hour.

3. After the incubation mixture was precipitated with 5% TCA and centrifuged at 2300 x g for 15 min at room temperature. Supernatant was transferred in fresh tube.
4. Then 1.0 ml of 1% TBA was added to the supernatant and placed in the boiling water for 15 min.
5. After cooling to room temperature absorbance of the mixture was taken at 532 nm and was converted to MDA and expressed in nmol MDA/mg protein using molar extinction coefficient of $1.56\times10^{8}\ M^{-1}\ cm^{-1}$.
6. A reaction mixture devoid of cell lysate served as blank.

Calculation

Calculation was done by using the molar extinction coefficient (EC) of MDA-TBA complex, 1.56 X $10^{8}\ M^{-1}cm^{-1}$. The amount of LPO was expressed as nM MDA formed per ml of lymphocyte cells.

$\times$ DF $\times$ IT

Where,

DF = Dilution factor *i.e.* 10

IT= Incubation time *i.e.* 1 hours

OD= Optical density

3.2.7.2 Reduced Glutathione

GSH level in the cell lysate treated with plant extract was quantified using **Ellman's method (1959)**.

Principle

Reduced glutathione (GSH) is measured by its reaction with DTNB (5,5′-dithiobis-2-nitrobenzoic acid) (Ellman's reaction) to give a yellow coloured product that absorbs at 412 nm. The general thiol reagent, DTNB also calledEllman's Reagent, reacts with GSH to form chromophore, 5-thio nitrobenzoic acid (TNB) and GS-TNB whose absorbance can be read at 412 nm. The GS-TNB is subsequently reduced by

glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADPH), releasing a second TNB molecule and recycling the GSH; thus amplifying the response. Any oxidized GSH (GSSG) initially present in the reaction mixture or formed from the mixed disulfide reaction of GSH with GS-TNB is rapidly reduced to GSH **(Ithayaraja, 2011)**.

Materials required

5% TCA (Tri Carboxylic Acid), 0.01% DTNB, GSH (reduced Glutathione)

Procedure

1. Cell lysate of untreated control cells, plant extract treated cells and ascorbic acid treated cells were prepared as described in section 3.2.7.
2. Briefly, a mixture of 0.1 ml of cell lysate and 0.9 ml of 5% TCA was centrifuged at 2300 x g for 15 min at 4^0 C.
3. Then 0.5 ml of supernatant was added into 1.5 ml of 0.01% DTNB and the absorbance was taken at 412 nm.

Calculation

The concentration of GSH was calculated by using the formula given below and results were expressed in mM of GSH per ml of sample

$$\text{GSH (mM/gram)} = \frac{\text{OD} \times \text{total volume of reaction mixture} \times 1000 \times \text{DF}}{\text{EC} \times \text{volume of sample taken}}$$

OD = Optical density at 412 nm

EC = 1.3×10^4

DF = Dilution factor, i.e, 20

3.2.7.3 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) was estimated as per the method described by **Madesh and Balasubramanian (1998)**.

Principle

Superoxide dismutase (SOD) acts as a primary defense against reactive oxygen species (ROS) by converting O_2- to O_2 and H_2O_2. Members of this enzyme family

include CuZnSOD, MnSOD and FeSOD. Most eukaryotes contain CuZnSOD and MnSOD. FeSOD is found in plants and prokaryotes. It involved generation of superoxide by pyrogallol auto oxidation and the inhibition of superoxide dependent reduction of the tetrazolium dye MTT [3-(4-5 dimethylthiazol 2-xl) 2, 5-diphenyl tetrazolium bromide] to its formazan, which was measured at 570 nm. The reaction was terminated by the addition of dimethyl sulfoxide (DMSO), which helped to solubilize formazan formed during the reaction. The colour evolved was stable for many hours and was expressed as SOD Units (one unit of SOD was the amount (mg) of protein required to inhibit the MTT reduction by 50%).

Reagents

1) Pyrogallol (100 µM): 0.12 mg of pyrogallol was dissolved in 10 ml of distilled water. One ml from this solution was diluted to 100ml with distilled water.
2) MTT (1.25 mM): 2.58 mg MTT was dissolved in 5 ml of distilled water.
3) Phosphate buffer saline (pH 7.4).

Procedure

The reagents were added in the sample, control and the blank as shown below in **Table 3.5**. The absorbance was read at 570 nm against distilled water (blank).

Table 3.5: Different reagents used in superoxide dismutase assay

	Sample	**Control**	**Blank (Duplicate)**
PBS	0.65 ml	0.65 ml	0. 65 ml
MTT	30 µl	30 µl	30 µl
Cell lysate	10 µl	-	-
Pyragallol	75 µl	75 µl	75 µl

The sample, control and blank were incubated for 5 min at room temperature

DMSO	0.75 ml	0.75 ml	0.75 ml
Cell lysate	-	10 µl	-

Calculation

SOD was expressed as SOD units/ mg of protein

$$Y\% = \frac{\text{OD of test}}{\text{OD of control}} \times 100$$

$$\text{SOD (U/mg of protein)} = \frac{\text{mg of protein in 0.01 ml of cell lysate} \times 50 \times \text{DF}}{Y\%}$$

Where,

Y% = % inhibition of MTT reduction by SOD protein

DF = Dilution Factor, *i.e,* 150

A. Protein estimation by Bradford dye method

Protein estimation by Bradford dye method was carried out as per the method described by **He(2011)**.

Principle

To measure the concentration of total protein in a sample Bradford protein assay is one of the most reliable methods. The principle of this assay is that the binding of protein molecules to Coomassie dye under acidic conditions results in a colour change from brown to blue. This method actually measures the presence of the basic amino acid residues, which contributes to formation of the protein-dye complex.

Reagent

Bovine Serum Albumin (BSA), Coomassie Brilliant Blue G-250, Methanol, Phosphoric acid, Bradford reagent.

Bradford reagent preparation

1. 50mg of CBB G-250 wasdissolve in 50 ml of methanol and add 100 ml 85% phosphoric acid.
2. Add acid solution into 850 ml of water and let the dye dissolve completely.
3. Dye solution was filtered with help of Whatman filter paper No.1.
4. Kept Bradford reagent in a dark bottle.

Procedure

1. BSA standard solution with a range of 5 to 100 µg protein was prepared.
2. 30µl each of BSA was added tolabeled test tube.

3. 30µlwas added water instead of BSA standard solution for preparation of blanks
4. 1.5 ml of Bradford reagent was added to each tube and mix well.
5. The components were incubated at room temperaturefor about 15 min.
6. Absorbance wasmeasure at 595 nm.

3.2.7.4 Catalase

Catalase was estimated in chicken lymphocytes by spectrophotometric method as described by **Aebi (1984)**.

Principle

Catalase catalyses the decomposition of H_2O_2. In the ultraviolet range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in extinction at 240 nm.

Reagents

1. Phosphate buffer (50 mM, pH 7.0)
   a. 50 mM KH_2PO_4 – 1.37 g/ 200 ml
   b. 50 mM Na_2HPO_4 – 1.42 g/ 200 ml. Solution a and b were mixed in 1:1.5 (V/V) and the pH was adjusted to 7.0.
2. H_2O_2 (10 mM): 0.1 ml of 30 % H_2O_2 was diluted to 100 ml distilled water.

Procedure

2 ml of phosphate buffer and 10 µl of cell lysate (1:10 dilution in PBS) was added, the contents were transferred to the cuvette. Adding 1 ml of H_2O_2 directly into the cuvette, the reaction was started and the OD was measured at every 30 seconds for 3 min at 240 nm wavelength (UV-VIS Spectrophotometer) against water blank.

Calculation

The activity of catalase was expressed as mM H_2O_2 utilized $Min^{-1}mg^{-1}$cell lysate and calculated using the formula.

$$\frac{\Delta OD/time \times total\ volume\ of\ reaction\ mixture}{0.067 \times amount\ of\ sample\ taken \times mg\ protein\ in\ 0.01\ ml\ of\ cell\ lysate}$$

Protein estimation is done with help of the Bradford dye method using BSA as a standard. The procedure is described previously.

3.2.6.5Nitric Oxide assay

Nitric oxide (NO) production by macrophages in the medium was measured by microplate assay method **(Stuehrand Nathan., 1989**). Macrophage rich cells were obtained as per the protocol of **Lavelle *et al.*(1972)**.Cells isolated from chicken spleens after density gradient centrifugation, were plated as described earlier and incubated for 2 hr at 37°C in a CO_2 incubator. Then media was aspirated and the plates were washed twice with RPMI-1640 to remove non-adherent cells. 1333 µl of fresh growth media along with 667 µl of extract prepared in PBS (1 mg/ml stock) was added.Plate was kept for incubation at 37□C in CO_2 incubator for 68 hours.100µl aliquots of supernatant fluid was removed from each well in an ELISA plate and incubated with an equal volume of Griess reagent (Sigma, USA) at room temperature for 30 min in dark. The absorbance at 548 nm wavelengthwas determined in Biotek ELISA plate reader. NOwas determined by using sodium nitrite ($NaNO_2$) as a standard and values were determined from the standard curve. The standard curve to calculate the NO production was prepared using different dilutions of $NaNO_2$.

Principle

Integrated nitric oxide production can be estimated from determining the concentrations of nitrite and nitrate end products. The measurement of nitrate/nitrite concentration or of total nitrate and nitrite concentration is routinely used as an index of NO production.

Materials required

Griess reagents, Sodium nitrite, 96 well plate, 200ul pipettes,RPMI 1640 medi**a**

Procedure

1. Different dilutions of standard solution of sodium nitrite (10-200µg/ml) were taken in different wells of 96 well plates.
2. 100 µl of griess reagents was added in all the wells and the plate was incubated it for 30 min in the dark.

3. Absorbance was taken at 548 nm and standard curve was plotted to determine total NO concentration.

3.2.7. Expression profiling of the genes due to *in vitro* exposure of AME

Expression analysis of various cytokines modulated after the treatment with extract was carried out for two cytokines (IL-6 and IL-10) and iNOS. Isolated lymphocytes were treated with maximum non-cytotoxic dose of the AME. Cells were incubated at37°C for24 hoursin a CO_2 incubator. The total RNA from cells was isolated by using RNA –Xpress reagent (Himedia). The total RNA was treated with DNase to remove the DNAcontamination. RNA was then quantified using nano drop. cDNA was synthesizedfrom the total RNA by following the thermo scientific revert aid cDNA synthesis Kit. cDNA was then checked with housekeeping gene in semi quantitative PCR. Real time quantitative PCR was used for the expression analysis of the cytokines.

3.2.8.1Retrieval of cytokine sequence and designing of gene specific primers

The CDS sequence of IL-6, IL-10 and iNOS were retrieved from NCBI database. The sequences were then fed into primer BLAST for designing of primers by adjusting all the parameters. The sequences of primers used in the study are described in **Table 3.6**.

Table 3.6: Details of primer along with their Tm and product size

Primer Name	Sequence	Product size	Tm (ºC)	Annealing temperature(ºC)
iNOS-2 f iNOS-2 r	CGTGTTCCACCAGGAGATGT ATGACGCCAAGAGTACAGCC	158 bp	59.4(ºC) 59.4 (ºC)	53(ºC)
IL-10 f IL-10 r	TGTACTGCAAGATCTCCCTGG ACATCCTCCCTATCGCTCTGA	179 bp	59.8 (ºC) 59.8 (ºC)	54(ºC)
IL-6 f IL-6 r	CGCCTTTCAGACCTACCTGG CTTCAGATTGGCAGGAGGG	181 bp	61.4 (ºC) 61.4 (ºC)	55(ºC)
Actin f Actin r	TGACTGACCGCGTTACTCC TAGATGGGAACACAGCAC	130bp	58.8 (ºC) 59.4(ºC)	54(ºC)

3.2.8.2 Combinations of the plant extract (AME), dexamethasone and mitogens used for cytokine expression analysis

Maximum non cytotoxic dose of AME and dexamethasone was used for *invitro* exposure of the cells. Different combination of AME, DEXA and mitogens used for giving *in vitro* exposure to the cells for evaluating of modulation in given expression are described in **Table 3.7.**

Table 3.7: Various treatments of extract, dexamethasone and mitogens used for expression analysis

Treatment groups	Mitogens	Treatments	Concentration (µg/ml)
Group A	---	---	---
		AME	75
		DEXA	0.030
Group B	LPS stimulated (5µg/ml)	---	---
		AME	75
		DEXA	0.030
Group C	Con A stimulated (5µg/ml)	---	---
		AME	75
		DEXA	0.030
Group D (in case of iNOS) macrophages enriched cells	---	---	---
		AME	75
		DEXA	0.030

3.2.8.3 Isolation of the total RNA

Total RNA from lymphocytes was isolated by following the method recommended by manufacturer of RNA-Xpress reagent (Himedia) as described by **Chomczynski (1993)** with slight modification.

Principle

RNA Xpress reagent is mixture of guanidine thiocyanate (strong protein denaturant) and phenol in mono-phase solution which dissolve the RNA very effectively. Guanidine thiocyanate is chaotropic agent, which degrade the protein that interferes with RNA.Chloroform addition followed by centrifugation separate the

solution into different phases. An aqueous phase have the RNA, other phases carried the DNA and protein.

Materials required

Chloroform, Isopropyl alcohol, 75% ethanol in DEPC treated water, DEPC treated micro tips, DEPC treated 2 ml micro centrifuge tubes, centrifuge, micropipettes, RNA X-press reagentand molecular grade water, micropipettes.

Procedure

1. Cells were seeded in the six well tissue culture plates after maintaining the cell count of 1x 10^7per ml and treatments of AME, DEXA and mitogens were given as mentioned above in **Table 3.7**. Overnight incubation was done for AME and 4h incubation for dexamethasone at 37□C in CO_2 incubator. The cells were harvested and pelleted down. Then cells were pelleted by centrifugation at 4^{o}C for 5 minutes at 1500 rpm in 2 ml micro centrifuge tube. 700µl of RNA X-press was used for lysis of the cells and mixed by repeated pipetting.
2. Samples were incubated at 25^{o}C for 5 minutes for complete dissociation of nucleoprotein complexes.
3. 200µl of chloroform was added and the micro centrifuge tube was shaken vigorously for 15 seconds.
4. Incubation for 5 minutes was done at room temperature followed by centrifugation at 13,000 rpm for 10 minutes at 4^{o}C.
5. After centrifugation, the mixture was separated into three different layers. Organic phase containing protein, interphase containing DNA and aqueous phase containing RNA.
6. Upper aqueous phase containing RNA was transferred into fresh micro centrifuge and 100 µl of isopropyl alcohol was added.
7. The tube was incubation at room temperature for 10 minutes followed by centrifugation at 12,000 rpm for 10 minutes at 4^{o}C.
8. Isopropyl alcohol precipitate the RNA, upon centrifugation of RNA was pelleted.

9. The supernatant was discarded and pellet was washed with 100µl of 75% ethanol. Centrifugation at 10,500 rpm for 1 minute at 4^{o}C was carried out.
10. Supernatant was removed and pellet was air dried.
11. Pellet was dissolved in 25µl of RNase-free molecular grade water and stored at -80^{o}C till further use.

3.2.8.4DNase treatment for purification of RNA

Isolated RNA samples were treated with DNase for degradation of DNA. DNase is endonuclease that breaks the phosphodiester bond in DNA strands. DNase should be removed or inactivated after the treatment; otherwise it will break the newly formed amplified DNA in PCR. DNase was inactivated by heat inactivation method.

Procedure

1. Following component were added in micro centrifuge tube:

RNA sample	8µl
DNase	1µl
10X DNase Buffer	1µl

2. Incubation at 37^{o}C for 30 minutes was done.
3. 1.25µl of 50mM EDTA was added.
4. Incubation for 5 minutes at 65^{o}C was done.
5. Samples were further utilized for cDNA synthesis after quantification.

3.8.3.5 Agarose gel electrophoresis of RNA

Agarose gel electrophoresis was done to check the quality of RNA. The method was very simple, quick and easy to perform which could easily resolve the RNA bands. Electrophoresis was done in 1x TAE buffer.

Solutions required

i. **Electrophoresis buffer**: 50x TAE, 1lt.

Tris base - 242g

Glacial acetic acid - 57.1 ml

0.5 M EDTA (pH 8.0) – 100 ml

Final volume was made up to 1000 ml with double distilled water, autoclaved and stored at room temperature.

ii. **6xGel loading buffer**: - (40% (w/v) glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol was prepared in working concentration (1x) of TAE, filter sterilized and stored at 4^0C.

iii. **Ethidium bromide solution (10mg/ml)** – 10 mg was dissolved in 1.0 ml of double distilled water. The working concentration of ethidium bromide was 0.5 ug/ml. It was stored in dark at 4^0C.

Procedure

a) Prior to preparation of gel, electrophoresis tank, comb and casting trays were thoroughly washed with autoclaved DEPC treated water.

b) Agarose gel (1.2%) was prepared in 1x TAE, DEPC buffer - to prepare 1.2% agarose gel, 1.2 g of agarose was weighed and dissolved in 100 ml 1x TAE by heating to melt the agarose until a clear solution was obtained.After cooling the melted gel a bit,ethidium bromide was added to it at a concentration of 0.5µg/ml.

c) Electrophoresis was done and the gel was visualized under UV light.

3.2.8.6 Quantification of RNA by Nanodrop spectrometer method

Nitrogenous bases present in DNA, RNA can absorb the UV light and give the maximum absorbance at wavelength of 260nm in UV light. Purity of RNA was assessed by calculating ratio of absorbance at 260nm and 280nm in Nano drop spectrometer. The concentration of RNA was determined by using 1µl of RNA sample. Generally, for pure RNA sample ratio of 260/280nm should be higher than 1.8.

3.2.8.7cDNA synthesis

RNA in presence of reverse transcriptase, primer and dNTPs converted into double stranded molecule. Reverse transcriptase enzyme has two activities*viz.* polymerase and ribonuclease H activity (exonuclease). Polymerase activity convert the single stranded RNA molecule in presence of oligo dT primer into double

stranded DNA. Ribonuclease H activity degrades the RNA in DNA-RNA hybrid.RNA isolated was utilized for synthesis of cDNA by using the thermo scientific Revert aid cDNA synthesis kit.

Procedure

1. Reagents provided in kit was added in micro centrifuge tube as following:

Template RNA	1µg
Oligo (dT) primer	1µl
Nuclease-free water	upto 12µl
Total volume	12µl

2. The above components were incubation at 65^{o}C for 5 minutes
3. Then, the following components were added:

5X reaction buffer	4µl
RiboLock RNase inhibitor	1µl
10mM dNTP Mix	2µl
RevertAid M-Mul V RT	1µl

4. After the addition of the component listed above, total volume of reaction was 20µl.
5. The components were mixed gently and a quick spin was given. Then, the components were incubated at 42^{o}C for 60 minutes.
6. Reaction was terminated by incubating the tubes at 70^{o}C for 5 minutes.
7. Product of the reaction was stored at -20^{o}C and further used for Real time PCR analysis.
8. cDNA samples were checked with housekeeping gene.

3.2.8.8 Confirmation of cDNA by amplifying actin housekeeping gene

The synthesized cDNA is used as template and checked by amplifying housekeeping gene. Actin was used as housekeeping gene. 1.2% agarose gel

electrophoresis was performed after conducting a PCR reaction with the housekeeping gene. cDNA was confirmed by the presence of band of size approximatelyaround 150 bp, that was observed under UV light after electrophoresis.

Procedure

1. Components used in Confirmation of cDNA by amplifying actin housekeeping gene was given below:

Buffer	1.25 µl
Primer Forward	0.5 µl
Primer Reverse	0.5 µl
dNTPs	0.5 µl
Taq Polymerase	0.5 µl
Template	1 µl
Double distilled water	8.25 µl

2. Total reaction volume was 12µl. The above master mix was mixed and dispensed equally in PCR tubes. Short spin was given to facilitate mixing.

3. Following reaction conditions were used in the experiment:

Step	Temperature	Time
1. Initial denaturation	94^0C	3 minute
2. Denaturation	94^0C	30 seconds
3. Annealing	54^0C	40 seconds
4. Extension	72^0C	1 minute
5. Final Extension	72^0C	7 minute

4. Step 2 to 4 was repeated for 35 cycles.

5. After PCR reaction was over, 10 µl of amplicons were loaded onto 1.2%agarose gel along with 2.0 µl of gel loading dye and the bands were analysed under UV transilluminator.

3.2.8.9 Standardization of annealing temperatures of the primerswith the help of gradient PCR

In order to determine the annealing temperature for primers of different genes, gradient PCR was performed. The PCR profile and temperature range for primers of the genes is depicted in **table 3.8.**

Table 3.8: Gradient PCR programme for amplification of Actin, IL-6, IL-10 and iNOS with their annealing temperature

Parameters	Time	Temperature
1.Intial denaturation	5 min	94 ºC
2.Denaturation	30 sec	94 ºC
3.Annealing	40 sec	Actin- 54-57 ºC IL-6- 50-55 ºC IL-10- 50-55 ºC iNOS- 50-55 ºC
4.Extension	2 min	72 ºC
5.Final extension	7min	72 ºC
Numberof cycle	35 (from step 2 to 4)	

3.2.8.10Relativeexpression analysis of cytokines by quantitative Real time PCR

The amplification of DNA sample at each step can be monitored in real time PCR. Detection of amplification was done by utilizing the non-specific fluorescent dye which intercalates in between the bases of double stranded DNA. As the DNA product increase with each proceeding step of PCR reaction, increases the fluorescent also occur which can be measured at each cycle. Mostly, SYBR Green is utilized as dsDNA binding dye in real time PCR, which binds to non-specific PCR products like primer dimers. Insertion of melting curve at end of the PCR profile will provide aid for removal of non-specific fluorescence.

Procedure

1. PCR reaction mixture was prepared by adding the following components:

Template cDNA	1µl
10µM Forward primer	0.5µl
10µM Reverse primer	0.5µl
SYBR green qPCR master mix (2X)	6µl
Nuclease-free water	4.5µl
Total volume	12.5µl

2. Contents of the PCR reaction were mixed gently.
3. Following reaction conditions were usedfor the experiment:

Step	**Temperature**	**Time**
1. Initial denaturation	94^0C	3 minute
2. Denaturation	94^0C	30 seconds
3. Annealing	(According to different primer)	40 seconds
4. Extension	72^0C	1 minute
5. Final Extension	72^0C	7 minute

4. Step 2 to 4 was repeated for 40 cycles.
5. Melting curve was added at last step for prevention of primer dimers.
6. After reaction was over, analysis was done for detection of Ct value of each sample.
7. From obtained Ct values, the fold expression of respected gene was calculated.
8. After PCR reaction was over, 10 µl of amplicons were loaded onto 1.2% gel along with 2.0 µl of gel loading dye and the bands were analysed.

Statistical analysis

For analysis of all kinds of data including biochemical and molecular, the observations were taken as triplicates followed by taking their mean ±SE value for statistical analysis. To analyse the results, two factorial Completely Randomised Design was used with the help of STPR software. Valid conclusions were made by analysis of variance. To evaluate significance of difference between the means of treatments, critical difference was calculated at 5% level of significance using the following formula:

$$CD = (2\times EMS/r)^{1/2}\times 1/t$$

Where,

CD = critical difference

EMS = Error Mean Square

t = table value of t at 5% level of significance at error degree of freedom

Results and Discussion

The present study was accomplished to analyze the antioxidative potential and cytokine mediated immunomodulation of medicinal plant *Aegle marmelos* (L.) Corr. Result of various experiments were conducted during the course of the study is presented in this chapter and under the light of scientific evidences all the results are discussed.

4.1 Preparation of aqueous extracts from *Aegle marmelos*

Plant material was collected and authenticated. It was thoroughly washed, shade dried and crushed into powder **(Figures 4.1, 4.2)** and utilized for extract preparation. 2 kg leaves of *Aegle marmelos* were collected. The weight of grinded powder obtained from *Aegle marmelos* leaves was 500 gms. Aqueous extract was prepared from the dried powder of *Aegle marmelos* leaves. Dried form of aqueous extract of *Aegle marmelos* (AME) was obtained after lyophilisation and stored at -20^{0}C till further use **(Figures 4.3** and **4.4)**.The extraction yield of aqueous leaves extract of *Aegle marmelos* was found to be 14.01% **(Table 4.1**).

Table 4.1: Percentage yield of plant extract

S. No.	Plant name	Dry weight of leaves (g)	Weight of the extract obtained (g)	Percent yield
1	*Aegle marmelos*	500	14.01	14.01%

Based on the method adopted for the extract preparation, phytochemical composition of the extract is influenced. Bioactive components of the plant are separated from other components of plant during plant extract preparation using suitable solvents. Extracted components are mixture of various metabolites. Solid plant components are diffused into solvent and according to polarity of the solvent these get solubilized **(Ncube *et al.,* 2008)**. Water is the most commonly used solvent for extraction of plant metabolites. Phytochemicals extracted from aqueous extract showed antioxidative and antimicrobial activity **(Das *et al.,* 2010).**

Kesari *et al.* (2006) used 500 gram of dried seed powder of *Aegle marmelos* fruit for aqueous extract preparation with extraction yield of 12.8%. Yield of absolute ethanol extract of stem bark of *Aegle marmelos* was reported to be 15.31% and extract exposure showed antiproliferative effects on human leukemic K562 cells at 100 µg/ ml concentration **(Lampronti *et al.*, 2003)**. **Upadhya *et al.* (2004)** reported that aqueous extract of *Aegle marmelos* leaves showed antioxidative and hypoglycemic activity in male diabetic albino rats.

4.2 Phytochemical analyses of aqueous extract of *Aegle marmelos* (AME)

Different qualitative and quantitative phytochemical analyses of aqueous extract of *Aegle marmelos* were conducted for determination of presence of various phytochemical in AME.

4.2.1 Qualitative analyses of the plant extract

Bioactive non-nutrient compounds derived from plants are known as phytochemicals. These phytochemicals are heterogeneous group of bio active compounds that includes alkaloids, polyphenols, carotenoids and nitrogen compounds **(Mehta and Shike, 2014)**. Various reports are there disclosing different beneficial effects of the phytochemicals. These phytochemicals can influence the proliferation of cells, regulation of cell cycle and signaling pathways **(Lee *et al.*, 2011)**. Phenols and flavonoids are two most explored polyphenols from plants. These polyphenols are synthesized as secondary metabolites and acting as potent antioxidants. Phenolic compounds of the plants are intermediate of phenylalanine. Mostly the conjugated form of the phenolic compounds exists in nature. Sugar residue of the phenols is linked with hydroxyl groups or direct linkage with aromatic carbons **(Kondratyuk and Pezzuto, 2004).**

The aqueous leaves extract of the *Aegle marmelos* was subjected to biochemical assays for qualitative analysis to detect presence of various phytochemicals. The AME showed presence of phenols, flavonoids, tannins, saponins, proteins and phytosterols however amino acids, reducing sugars were absent in the extract.

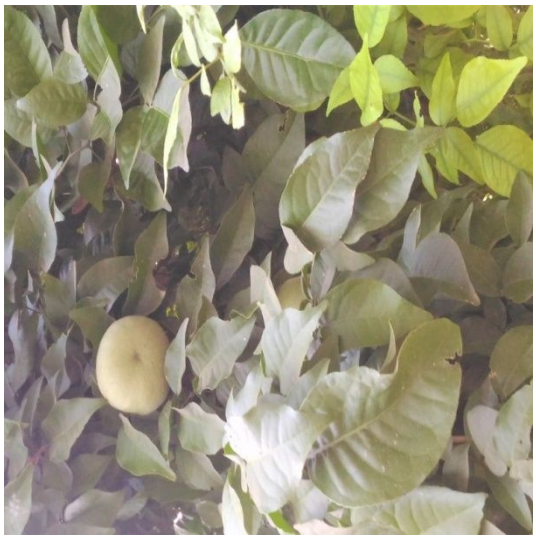


Figure: 4.1 *Aegle marmelos*plant

Figure: 4.2 *Aegle marmelos*leaves powder

Figure: 4.3Aqueousextract of*Aegle marmelos*

Figure: 4.4Aqueous lyophilized extract of*Aegle marmelos*

Table 4.2: Qualitative test of the plant extract (AME)

S. No.	Phytochemical Analysis	AME
1	Phenolics	+
2	Flavonoids	+
3	Tannins	+
4	Alkaloids	+
5	Saponins	+
6	Reducing sugars	-
7	Protein	+
8	Amino acids	-
9	Phytosterols	+

Sharma *et al.* (2011) reported that aqueous seed extract of *Aegle marmelos* showed presence of alkaloids, phenolics, flavonoids, proteins, steroids qualitatively but amino acids, reducing sugars were not present. One of study reported that aqueous leaf extract of *Aegle marmelos* possesses various phytochemicals *viz*. alkaloids, flavonoids, phenolics, saponins and tannin etc. The presence of these phytochemicals play important role in medicinal properties of the plant **(Mujeeb *et al.*, 2014)**.

4.2.2 Quantitative analyses of the plant extract

4.2.2.1 Determination of total phenolics content in AME

Secondary metabolite of the plants, having benzene ring with one or more hydroxyl substituents are known as phenolic compounds. They are produced during shikimic acid and pentose phosphate pathway of the plants. Phenolic compounds plays major role in plant defense response against herbivores as well for the antibacterial, antifungal activities **(Edreva *et al.*, 2008)**. Phenolic compounds can also alter the auto oxidation of organic molecules by suppressing or delaying it. Due to this property phenolic compounds are excellent antioxidants **(Foti, 2007).**

The total phenolic content of the plant extract was determined from stranded curve of Gallic acid ($y=0.0075x$, $R^2 = 0.9898$) as presented in **Table 4.3** and **Figure 4.5**. Total phenolic content of plant extract was estimated to be 480 mg GAE/g as presented in **Table 4.4.**

Table 4.3: Optical density of different concentrations of Gallic acid

S. No.	Concentrations (µg/ml)	Optical density (765 nm)
1.	1	0.002
2.	2.5	0.004
3.	5	0.006
4.	7.5	0.009
5.	10	0.011
6.	15	0.023
7.	20	0.029
8.	25	0.032
9.	30	0.046
10.	35	0.053
11.	40	0.060

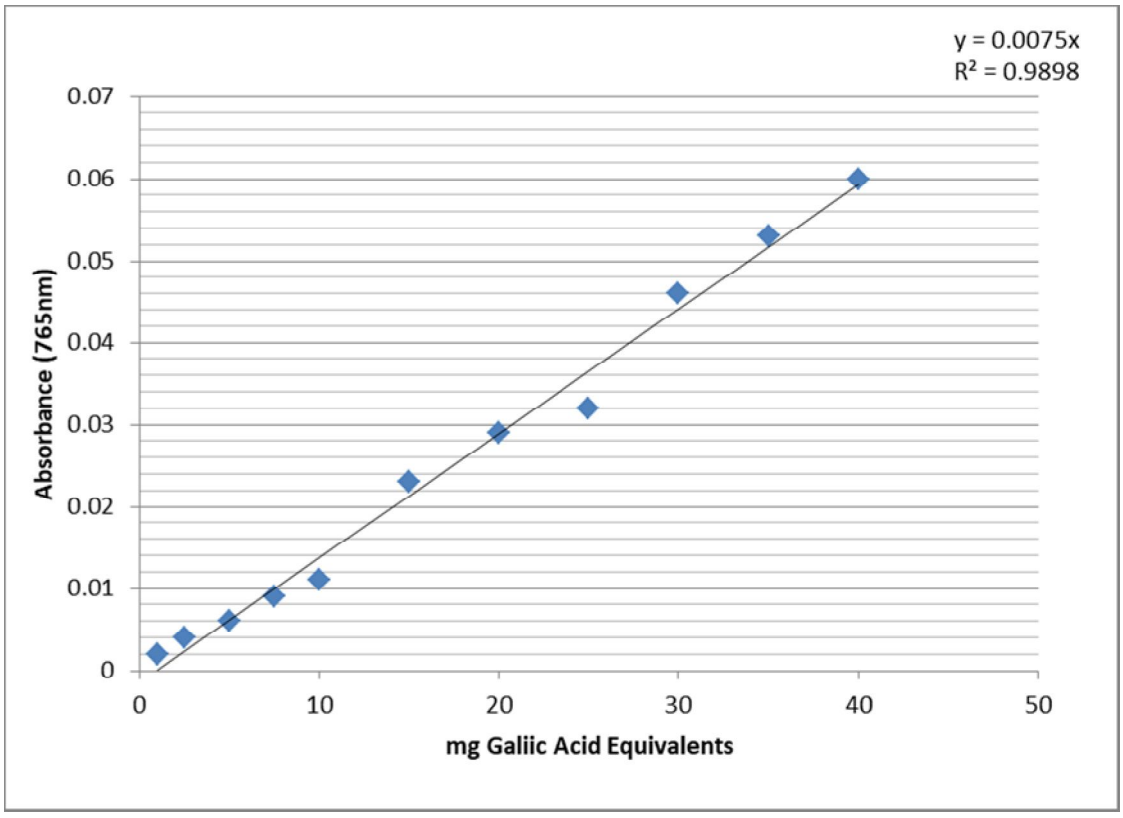


Figure 4.5: Gallic acid standard curve

Phenolic compounds are better antioxidants then other polyphenol due to presence of the hydroxyl group in their structure. The phenolic content of hydromethanolic leaves and stem extract of *Aegle marmelos* was 9.83 mg/kg and 7.46 mg/kg respectively as reported by **Siddique *et al.* (2010)**. Leaves of *Aegle marmelos* have the higher content of phenolic in comparison to stem bark. **Vardhini *et al.* (2018)** reported the total phenolic content in aqueous fruit pulp extract of *Aegle marmelos* was 343.00±1.33 µg/mg of GAE. Higher content of phenolic in extract reflects high antioxidative potential of the plant.

Table 4.4: Total phenolics content in AME

S. No.	Plant extract	Concentration of extract (µg/ml)	O.D. (765nm)	Total phenolics content (µg/µg extract)	Total phenolics content (mg GAE/g extract)
1.	*Aegle marmelos* (AME)	5µg	0.045	0.480	480

4.2.2.2 Determination of total Flavonoids Content in AME

Flavonoids are polyphenols that are produced as secondary metabolites by plants. In flavonoids synthesis, phenylpropanoid pathway is involved, which consist of the transformation of phenylalanine into 4-coumaroylCoA and enter the flavonoids synthesis pathway **(Martens *et al.*, 2010)**. Flavonoid is major group consisting of flavonols (quercetin), flavones, flavanols (catechins), anthocyanidins, flavanones and isoflavones (genistein and daidzein). Flavonoids are explored by various scientists for their antimicrobial, antioxidative and anti infammatory activity **(Crozier *et al.*, 2009)**.

Flavonoids are reported to modulate the immune system by inhibiting the Th1 cells and promoting Th2 cells. Flavonoids are having ability to reduce the production of reactive oxygen species and inhibit the oxidative burst which results in cytoprotective effects and prevention of cell apoptosis **(Greenrod and Fenech, 2003)**.The total flavonoid content of the AME was calculated from the Rutin stranded curve ($y = 0.0074x+0.0003$, $R^2 = 0.9595$) and represented as mg Rutin equivalent (RE) per g of extract which is presented in **Table 4.5** and **Figure 4.6.** Estimated total flavonoid content in AME was 342.70mg RE/g as presented in **Table 4.6.**

Table 4.5: Optical density of different concentrations of Rutin

S. No.	Concentrations (µg/ml)	Optical density (510 nm)
1.	0.1	0.001
2.	0.25	0.008
3.	0.5	0.016
4.	1	0.017
5.	2.5	0.024
6.	5	0.047
7.	10	0.060
8.	15	0.071
9.	20	0.126
10.	25	0.175
11.	30	0.257
12.	35	0.266

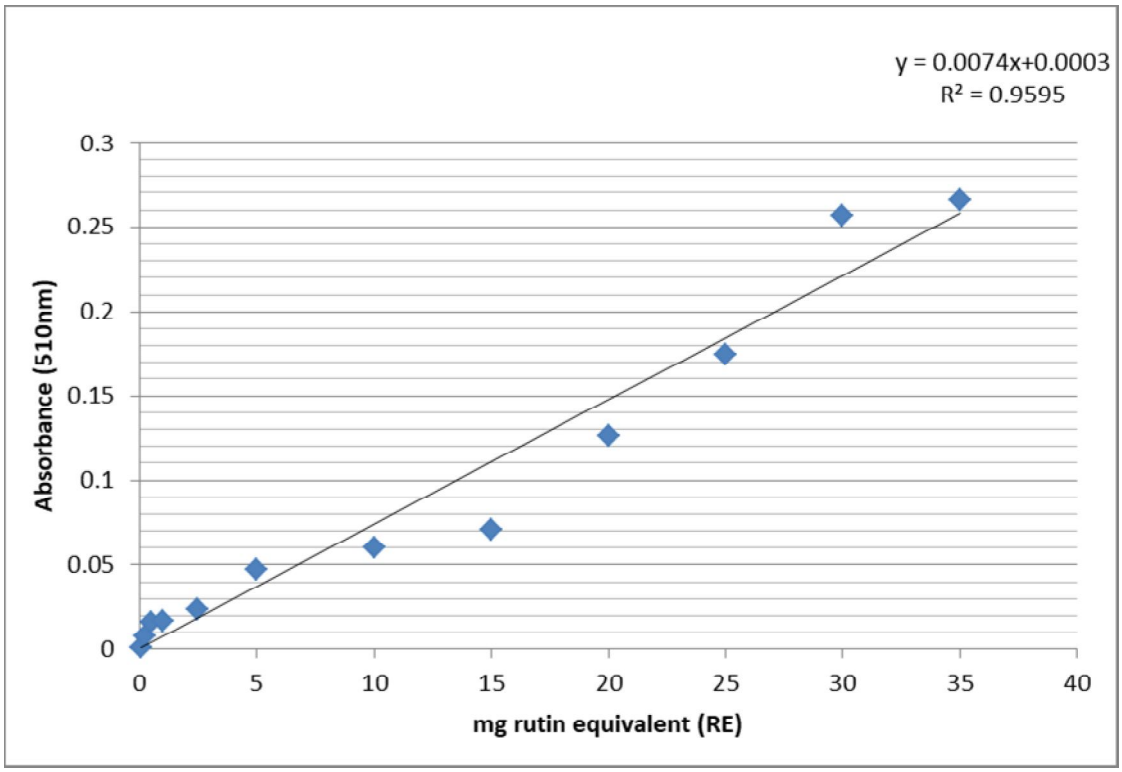


Figure 4.6: Rutin standard curve

Table 4.6: Total flavanoids content in AME

S. No.	Plant extract	Concentration of extract (µg/ml)	Mean O.D. (510 nm)	Total flavanoids content (µg/µg extract)	Total flavanoids content (mg RE/g extract)
1.	*Aegle marmelos* (AME)	5µg	0.032	0.342	342.70

Siddique *et al.* (2010) reported the flavonoid content in hydromethanolic extract of leaves and stem of *Aegle marmelos* to be 8.24 mg/kg and 1.40 mg/kg respectively. The flavonoid content was reported to be higher in leaves than stem. **Vardhini *et al.* (2018)** estimated the flavonoid content of aqueous extract of the pulp of *Aegle marmelos* fruit to be 21.92 µg/mg of QE (Quercetin). The ethanolic extract of the leaves of *Aegle marmelos* was reported to have 66.48 mg QE/g of total flavonoid content **(Raja and Khan, 2017)**. The higher content of flavonoids signifies the antioxidative activity of the plant extract.

In qualitative analyses, the total phenolic content was higher in plant leaves extract as comparison to total flavonoid content.

4.3 Determination of antioxidative potential of AME

Plants possess various phytochemicals *i.e.* flavonoids, phenolic compounds, carotenoids, sterols, glucosinolates and other sulphur-containing compound that are reported to have very good antioxidative potential. Due to various metabolic processes and environmental factors, oxidative stress is generated in human beings/ birds etc in the form of ROS/ RNS. The plant based antioxidant can be beneficial to counter balance these ROS **(Embuscado, 2015)**.

In view of the above stated fact, antioxidant activity was determined for the aqueous leaf extract of *Aegle marmelos.*

4.3.1 DPPH free radical scavenging assay

Aegle marmelos extract showed significant antioxidant activity in DPPH free radicals scavenging assay (**Figure 4.7**). Dose dependent relationship was observed in DPPH assay i.e. scavenging activity was increased as the concentration of extract was increased as shown in **Table 4.7.** Antioxidative potential of the extract was due to the presence of various phytochemicals and higher concentration of phenolics and flavonoids.

Table 4.7: Percent scavenging of AME and ascorbic acid in DPPH assay

S. No.	Concentrations used (µg/ml)	O.D. of AME (517nm)			Mean value	O.D. of Ascorbic acid (517nm)			Mean value	Percent Scavenging of AME± SE	Percent Scavenging of Ascorbic acid± SE
		I	II	III		I	II	III			
1	1	0.783	0.742	0.816	0.780	0.483	0.342	0.644	0.489	19.33±0.021	49.43±0.087
2	5	0.790	0.740	0.755	0.761	0.534	0.389	0.440	0.454	21.30±0.014	53.05±0.042
3	10	0.763	0.716	0.797	0.758	0.460	0.506	0.302	0.422	21.61±0.023	56.35±0.061
4	20	0.658	0.707	0.757	0.707	0.500	0.392	0.252	0.381	26.80±0.028	60.59±0.071
5	40	0.691	0.695	0.711	0.699	0.427	0.225	0.380	0.344	27.71±0.006	64.42±0.061
6	60	0.741	0.680	0.694	0.696	0.283	0.412	0.344	0.256	28.02±0.009	73.52±0.059
7	80	0.688	0.674	0.707	0.689	0.160	0.107	0.102	0.123	28.74±0.009	87.28±0.018
8	100	0.619	0.641	0.642	0.634	0.121	0.146	0.063	0.110	34.43±0.007	88.62±0.024
9	Control	0.941	0.963	0.997	0.967						
CD (5%)						**SE(m)±**					
0.122						0.042					

IC_{50} value of the AME was calculated from the regression equation prepared from various concentration of the extract and percentage inhibition of formation of free radicals. IC_{50} value is the concentration of sample that is required to scavenge 50% of free radicals. The IC_{50} value for AME was found to be 233.11µg/ml and for Ascorbic acid IC_{50} value was determined to be 1.66 µg/ml. Lower value of IC_{50} represent higher antioxidative potential of the plant. In statistical analysis, both ascorbic acid and AME showed significant scavenging activity.

Raja and Khan, (2017) evaluated various solvent extracts (viz. ethanol, methanol, aqueous) of leaves of *Aegle marmelos* for their antioxidative potential. In their study aqueous leaves extract of *Aegle marmelos* showed higher IC_{50} value ***i.e.*** 125µg/ml identicating higher antioxidative potential as compared to ethanolic (IC_{50} 150 µg/ml) and methanolic (IC_{50} 185µg/ml) extracts.

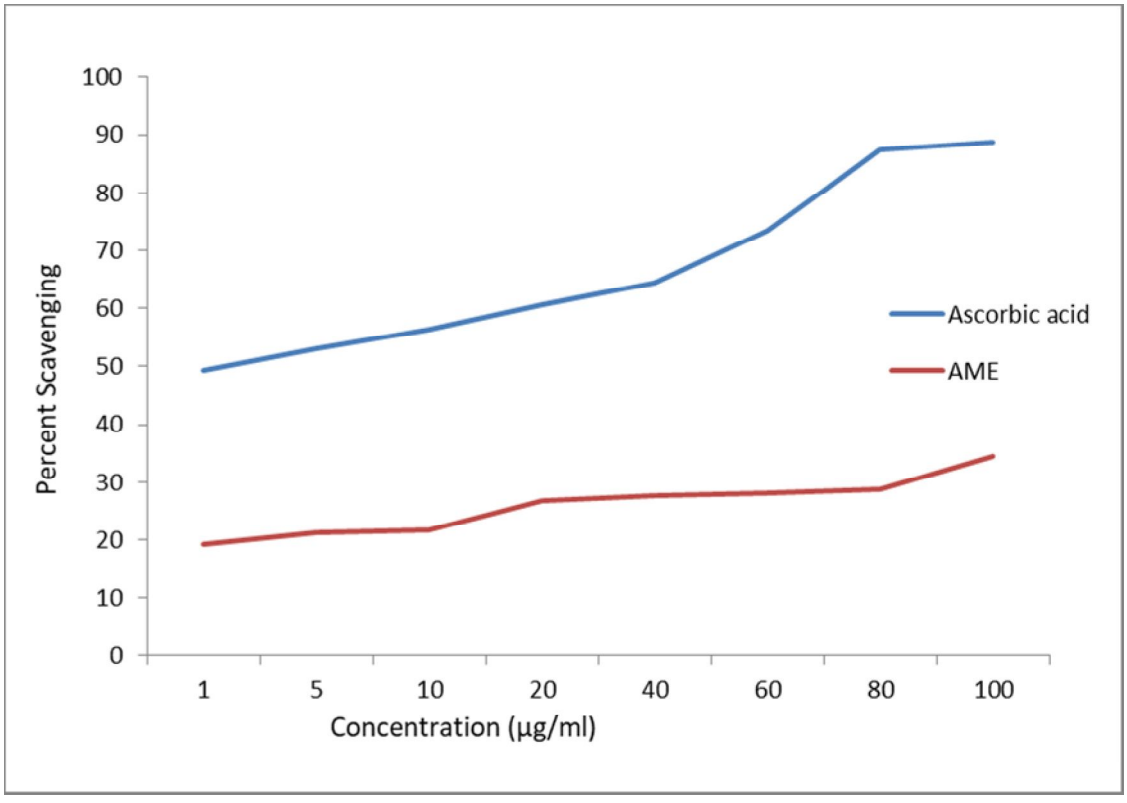


Figure 4.7: Percent Scavenging of AME in DPPH assay

Kumar *et al.* (2016) reported that ethanolic leaves extract of *Aegle marmelos* possess antioxidative potential and showed good reducing power for DPPH radicals. The IC_{50} value of the ethanolic extract was reported to be 160.47µg/ml.

Due to presence of various antioxidant components *i.e.* β-carotene, glutathione, sitosterol, aegelin, lupcol, rutin, marmesinin, eugenol, β- sitosterol, flavon, glycoside, montanine, o-isopentenyl halfordiol marmelin α-tocopherol, total polyphenols and flavonoids etc in the of leaves of *Aegle marmelos*, possess significant antioxidative potential. In another study, the methanolic and aqueous extract of leaves of *Aegle marmelos* showed 87 % and 93% DPPH radicals scavenging activity, respectively (**Reddy and Urooj, 2013**).

4.3.2 Nitric oxide radical scavenging assay

Nitric oxide having unpaired electron making it NO radicals which is a colourless gas having solubility in water and organic solvents. Nitric oxide is very short lived, lipophilic in nature and can diffuse easily in between cells. It is produces by various NAPDH dependent enzymes (NO synthases) from terminal guanido nitrogen atom of L-arginine **(Nathan, 1992)**. Nitric oxide acts as bioreguatory molecule essential for various physiological processes like neural signal transmission, immune response, control vasodilation and control of blood pressure. But the excessive of NO radicals are associated with various pathological conditions such as cancer **(Gold *et al.*, 1990)**.

Nitric oxide reacts with oxygen under aerobic conditions and produces various intermediates such NO_2, N_2O_4, N_3O_4 and nitrate, nitrite, peroxynitrite that are reacts with superoxide. These intermediates react with various DNA repair enzymes which are required for maintaining the genome integrity. These enzymes include DNA alkyl transferase, formamido pyrimidine-DNA glycosylase and DNA ligase **(Marcocci *et al.*, 1994)**.

Aegle marmelos extract showed significant antioxidant activity in NO radical scavenging assay (**Figure 4.8**). Dose dependent relationship was observed in NO radical scavenging assay i.e. scavenging activity was increased as the concentration of extract was increased as shown in **Table 4.8.** IC_{50} value of the AME was calculated from the regression equation prepared from various concentrations of the extract and percentage inhibition of formation of free radicals. IC_{50} value is the concentration of sample is required to scavenge the 50% of free radicals. The AME was found to have IC_{50} value of 113.80µg/ml and Ascorbic acid has IC_{50} value the 131.93µg/ml. Lower value of IC_{50} represent the higher antioxidative potential of the plant. Statistically, AME shows higher NO radical scavenging activity then ascorbic acid.

Table 4.8: Percent scavenging of AME and ascorbic acid in NO scavenging assay

S. No.	Concentrations used (µg/ml)	O.D. of AME (546nm)			Mean value	O.D. of Ascorbic acid (546 nm)			Mean value	Percent Scavenging of AME± SE	Percent Scavenging of Ascorbic acid± SE
		I	II	III		I	II	III			
1	10	0.407	0.439	0.446	0.430	0.437	0.397	0.492	0.442	14.71±0.012	11.77±0.027
2	20	0.339	0.398	0.367	0.368	0.482	0.398	0.418	0.432	26.54±0.017	13.75±0.025
3	40	0.353	0.279	0.374	0.335	0.379	0.349	0.497	0.408	30.13±0.028	18.60±0.045
4	60	0.317	0.356	0.314	0.329	0.351	0.319	0.469	0.379	34.33±0.013	24.31±0.045
5	80	0.243	0.273	0.361	0.292	0.298	0.307	0.392	0.332	41.71±0.035	33.75±0.029
6	100	0.290	0.242	0.317	0.283	0.268	0.323	0.301	0.297	43.51±0.021	40.73±0.015
7	Control	0.499	0.501	0.505	0.501						
CD (5%)						**SE(m)±**					
0.083						0.028					

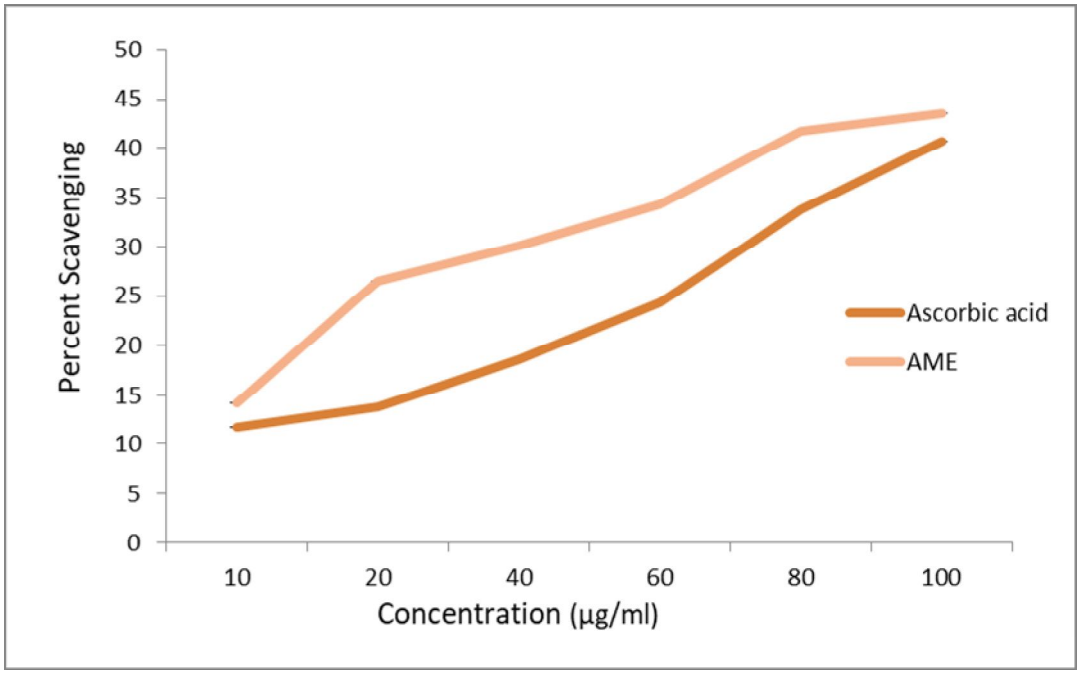


Figure 4.8: Percent Scavenging of AME in NO scavenging assay

Bristy *et al.* (2017) studied the NO radical scavenging activity of various plant parts of *Aegle marmelos* including ripe fruit, seed and leaves. Methanolic extract of *Aegle marmelos* fruit showed highest NO radical scavenging activity with IC_{50} value of 46.36µg/ml. Aqueous extract of unripe fruit of *Aegle marmelos* showed increase in NO scavenging activity in dose dependent manner as reported by **Krushna *et al.* (2011)**. At higher dose the NO scavenging activity increases. **Jagetia *et al.* (2003)** reported that hydromethanolic leaves extract of *Aegle marmelos* showed significant NO scavenging in dose dependent manner.

Extract till dose of 400 µg/ml showed increase in NO scavenging after this dose, the increase was steady.

4.5 Immomodulatory effect of aqueous leaves extract of *Aegle marmelos* in chicken lymphocytes culture system

Immunomodulatory effects of the AME were studied on chicken lymphocytes culture system by employing lymphocytes proliferation assay. Immunomodulatory effects in mitogen stimulated T and B cells were evaluated by giving exposure of maximum non cytotoxic dose of AME to chicken lymphocyte in *in vitro* system. Various dilutions of AME were used to give exposure to chicken lymphocytes for determination of maximum non cytotoxic dose (MNCD) through MTT assay.

4.5.1 Evaluation of maximum non cytotoxic dose of AME in chicken lymphocytes

Various dilutions of AME for determination of maximum non cytotoxic dose at which chicken lymphocytes were exposed are presented in **Table 4.9 and Figure 4.11**. Treated lymphocytes were observed under microscope at different concentration **(Figure 4.12)**. Percent viability of the cells at various dilutions is presented in **Figure 4.10**.

Table 4.9: Percent cell viability in chicken lymphocytes due to *in vitro* exposure of *Aegle marmelos* extract

S. No.	Concentration (µg/ml)	(O.D. at 570nm)			Means ± SE	Percent cell viability	Percent Cytotoxicity
		I	II	III			
1	Control	0.432	0.445	0.423	0.433±0.006	100	-
2	1	0.440	0.459	0.449	0.449±0.005	100	-
3	5	0.442	0.471	0.450	0.454±0.008	100	-
4	10	0.463	0.517	0.478	0.486±0.016	100	-
5	25	0.471	0.490	0.513	0.491±0.012	100	-
6	50	0.527	0.494	0.514	0.511±0.009	100	-
7	75	0.442	0.492	0.478	0.470±0.014	100	-
8	100	0.413	0.411	0.453	0.425±0.013*	97.11	2.88
9	150	0.415	0.390	0.385	0.396±0.009*	86.34	13.65
10	200	0.373	0.404	0.395	0.390±0.009*	84.08	15.91
11	250	0.363	0.398	0.407	0.389±0.013*	83.58	16.41
12	300	0.395	0.386	0.375	0.385±0.005*	82.08	17.91
13	350	0.372	0.392	0.380	0.381±0.005*	80.45	19.54
14	400	0.398	0.367	0.362	0.375±0.011*	78.44	21.55
15	450	0.341	0.358	0.397	0.365±0.016*	74.56	25.43
16	500	0.371	0.340	0.363	0.358±0.009*	71.80	28.19
17	550	0.358	0.324	0.340	0.340±0.009*	65.28	34.71
18	600	0.338	0.331	0.330	0.333±0.002*	62.40	37.59
19	650	0.339	0.317	0.327	0.327±0.006*	60.40	39.59
20	700	0.328	0.305	0.316	0.316±0.006*	56.14	43.85
21	800	0.312	0.307	0.295	0.304±0.005*	51.75	48.24
22	900	0.290	0.245	0.301	0.278±0.017*	41.97	58.02
23	1000	0.274	0.291	0.252	0.272±0.011*	39.47	60.52
24	1250	0.249	0.256	0.279	0.261±0.009*	35.46	64.53
25	1500	0.238	0.271	0.255	0.254±0.009*	32.83	67.16

CD		SEM
1%	5%	0.0105
0.0398	0.0298	

* **Significant at P<0.05**

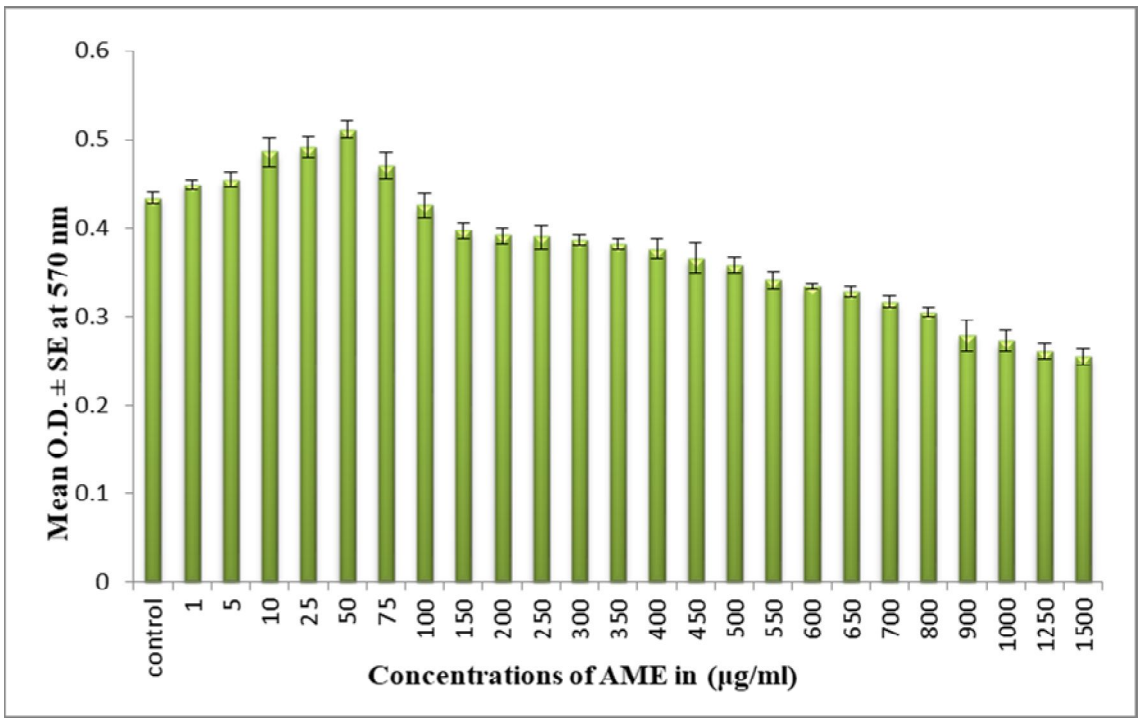


Figure 4.9: Influence on chicken lymphocytes proliferation due to exposure of various concentrations of AME

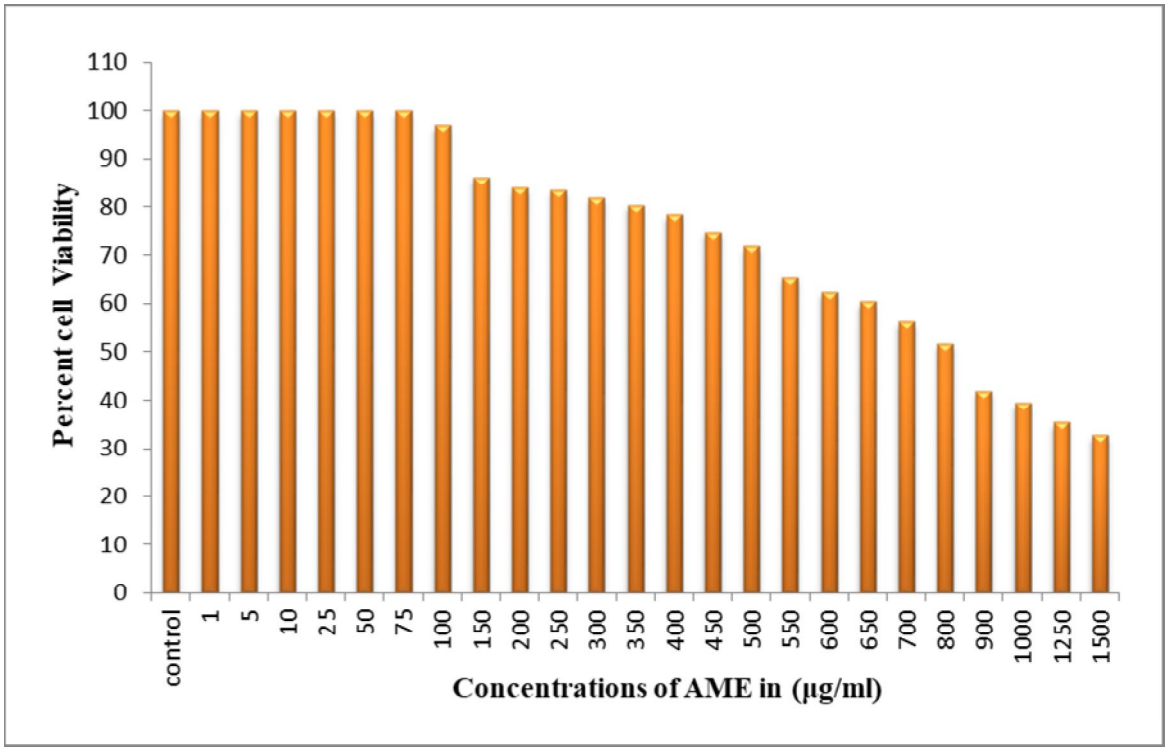


Figure 4.10: Percent viability of chicken lymphocytes in presence of different concentrations of AME

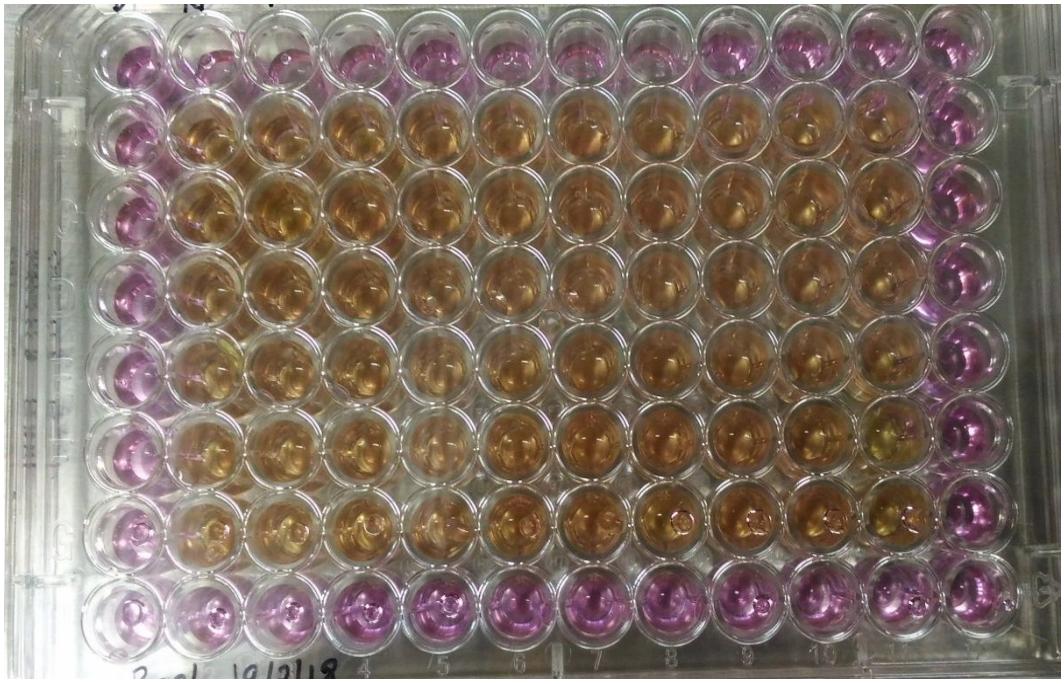


Figure 4.11: MTT assay for evaluation of maximum non-cytotoxic dose of AME in chicken lymphocytes

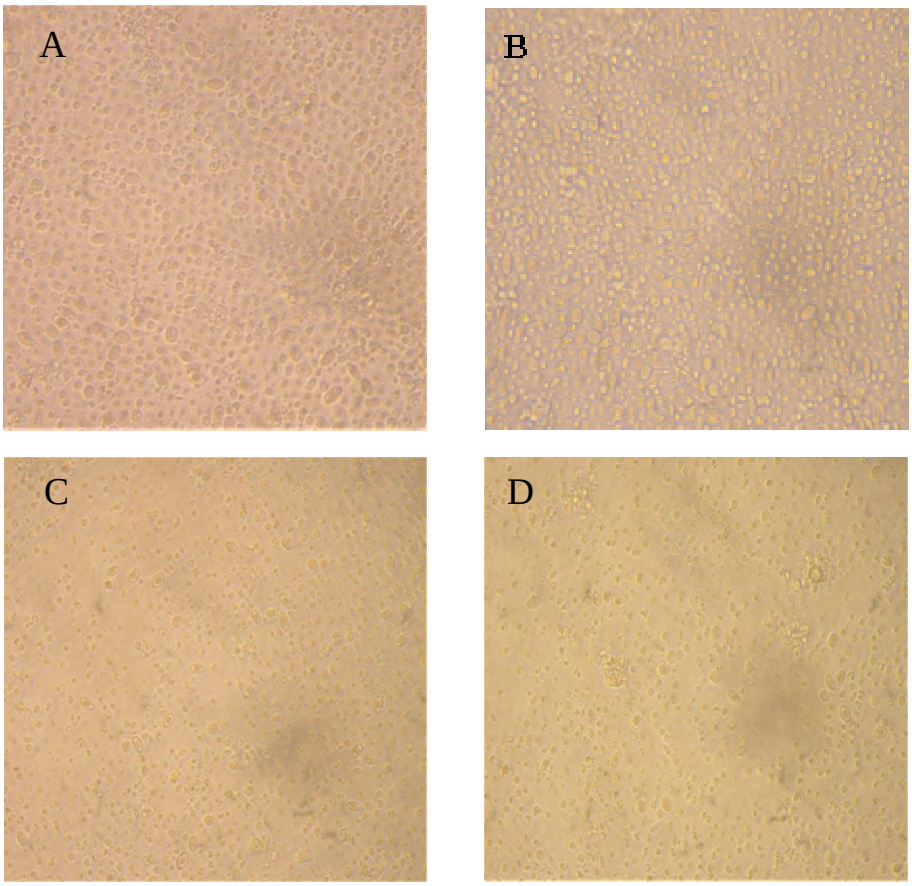


Figure 4.12: Influence on chicken lymphocytes due to *in vitro* exposure ofAMEafter 48 hrs. (A. control, B. 75 µg/ml, C. 150 µg/ml, D. 500 µg/ml) at 40x

Hundred percent viability of the cells was observed up to 75µg/ml AME exposure. AME exposure higher than 75µg/ml showed dose dependent decrease in cell viability and started showing increase in granularity, clump formation and cell detachment. Maximum non cytotoxic dose was determined to be 75µg/ml which was further used for immunomodulatory and antioxidative analysis.

4.5.2 Evaluation of maximum non cytotoxic dose of Dexamethasone in chicken Lymphocytes

Dexamethasone (DEXA) is a glucocorticoids drug having various therapeutic applications. Dexamethasone is one of the immunosuppressive drugs. Dexamethasone (DEXA) was utilized as negative control in determination of immunomodulatory effects of AME in mitogen stimulated T cell and B cells in chicken lymphocytes culture system. Minimum cytotoxic dose of the DEXA was determined by using MTT assay.

Various dilutions of DEXA for determination of minimum cytotoxic dose at which chicken lymphocytes were exposed are presented in **Table 4.10** and **Figure 4.15**. Treated lymphocytes were observed under microscope at different concentration **(Figure 4.16)**. Percent viability of the cells at various dilutions was represented in **Figure 4.14**. Hundred percent cell viability was observed up to 30ng/ml dose of DEXA and at doses higher than 30ng/ml cells started showing increase in granularity, clump formation and detachment in dose dependent manner. Maximum non cytotoxic dose was determined to be 30ng/ml in chicken lymphocytes culture system which was further used for immunomodulatory and antioxidative analysis.

Table 4.10: Percent cell viability in chicken lymphocytes cells due to *in vitro* exposure of dexamethasone

S. No.	Concentration (µg/ml)	(O.D. at 570nm)			Means ± SE	Percent cell viability	Percent Cytotoxicity
		I	II	III			
1	Control	0.425	0.438	0.459	0.440±0.009	100	-
2	1	0.466	0.456	0.426	0.449±0.012	100	-
3	5	0.458	0.473	0.443	0.458±0.008	100	-
4	10	0.463	0.437	0.448	0.449±0.007	100	-
5	15	0.443	0.445	0.468	0.452±0.008	100	-
6	20	0.441	0.455	0.431	0.442±0.006	100	-
7	25	0.418	0.449	0.474	0.447±0.016	100	-
8	30	0.438	0.429	0.455	0.440±0.007	100	-
9	35	0.415	0.432	0.424	0.423±0.004*	95.31	4.69
10	40	0.421	0.389	0.431	0.413±0.012*	88.38	11.62
11	50	0.399	0.419	0.382	0.400±0.010*	83.81	16.19
12	60	0.388	0.376	0.409	0.391±0.009*	80.70	19.30
13	70	0.382	0.404	0.375	0.387±0.008*	79.49	20.51
14	80	0.368	0.378	0.412	0.386±0.013*	77.31	22.69
15	90	0.368	0.380	0.379	0.375±0.003*	76.98	23.02
16	100	0.3799	0.368	0.375	0.374±0.003*	76.57	23.43
17	150	0.376	0.373	0.365	0.371±0.003*	75.50	24.50
18	200	0.357	0.383	0.368	0.369±0.007*	73.06	26.94
CD					**SEM**		
1%		5%			0.009		
0.035		0.026					

* **Significant at P<0.05**

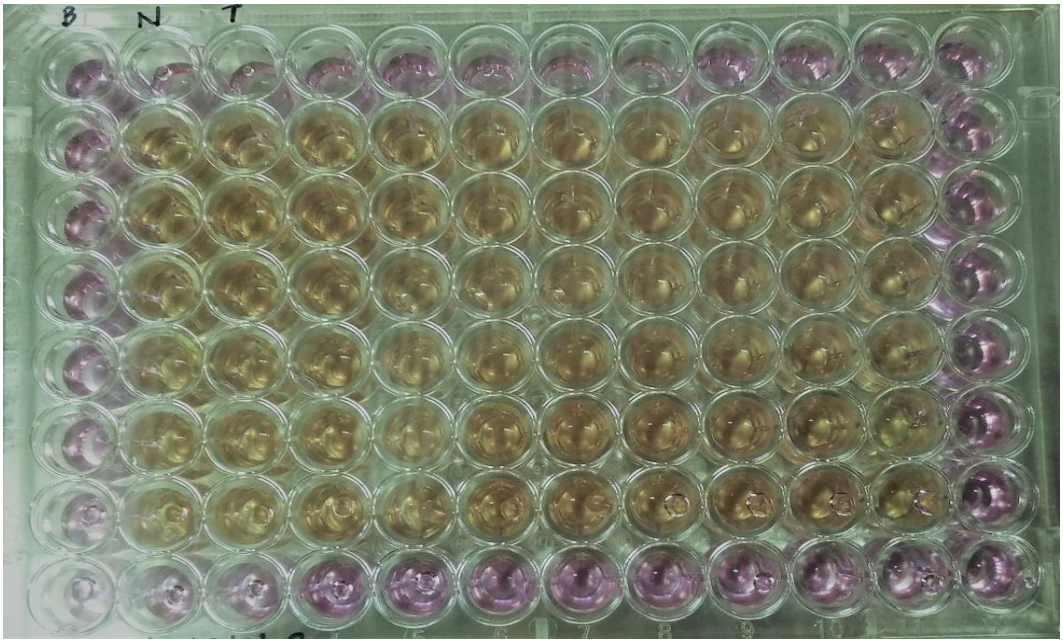


Figure 4.15: MTT assay for evaluation of maximum non-cytotoxic dose of DEXA in chicken lymphocytes

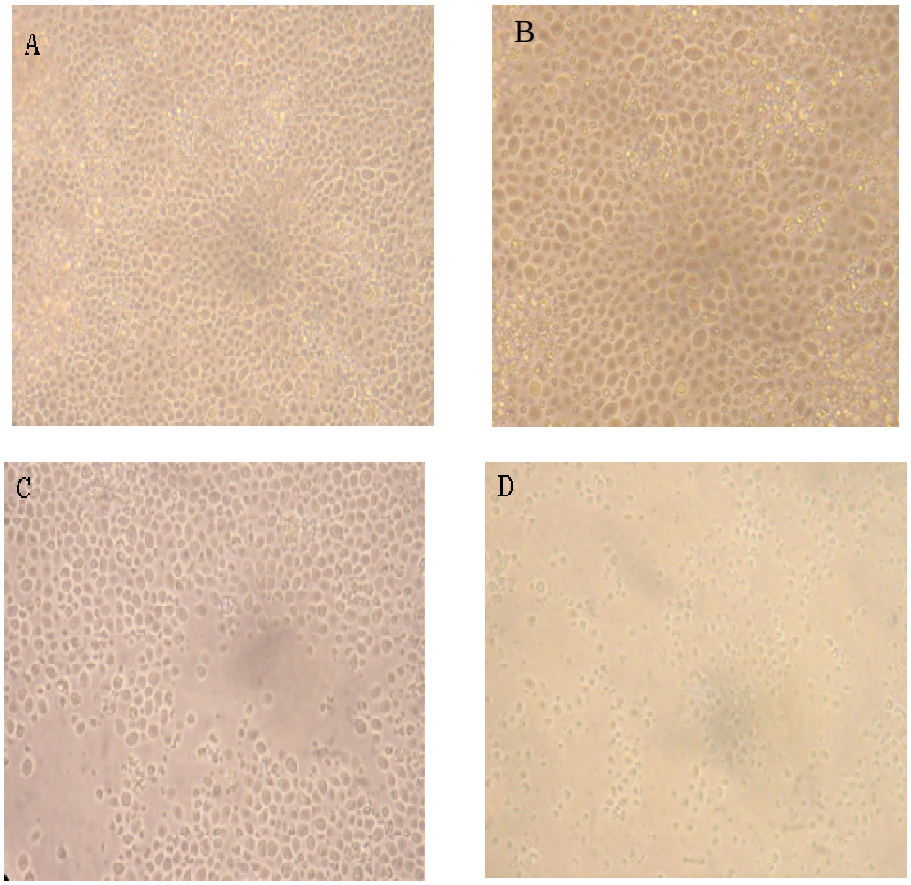


Figure 4.16: Influence on chicken lymphocytes due to *in vitro* exposure ofDEXA after 48 hrs. (A. control, B. 30 ng/ml, C. 100 ng/ml, D. 150 ng/ml) at 40X

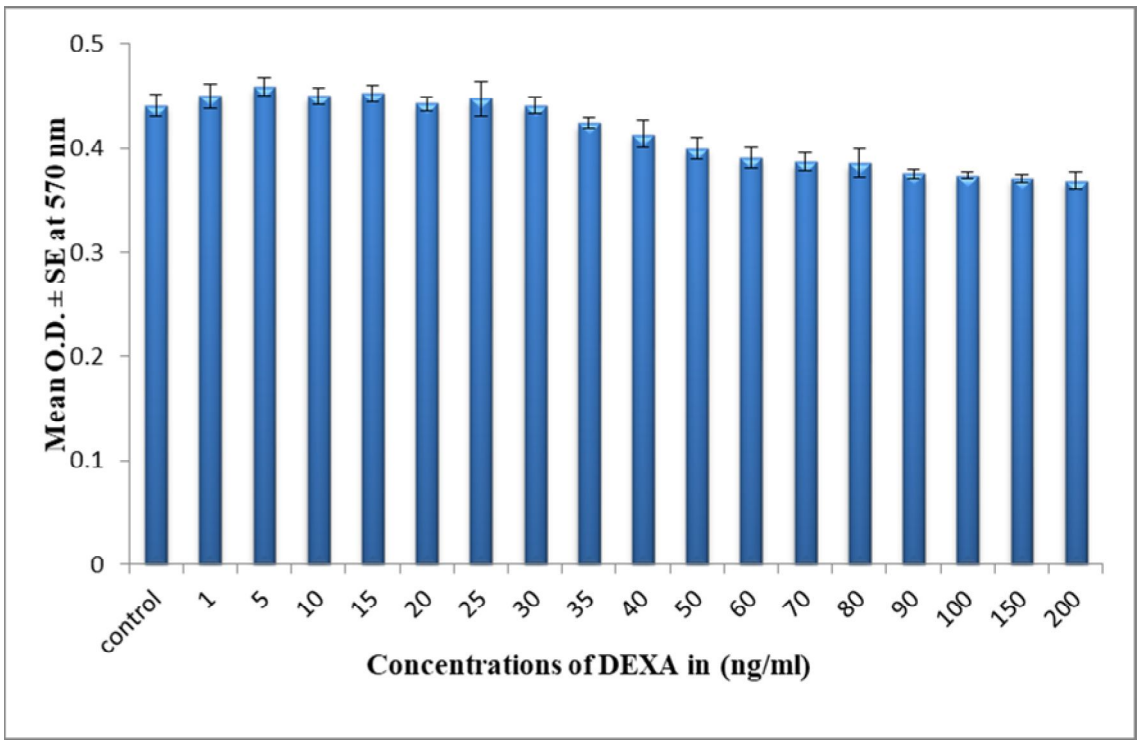


Figure 4.13: Influence on chicken lymphocytes proliferation due to exposure of various concentrations of Dexamethasone

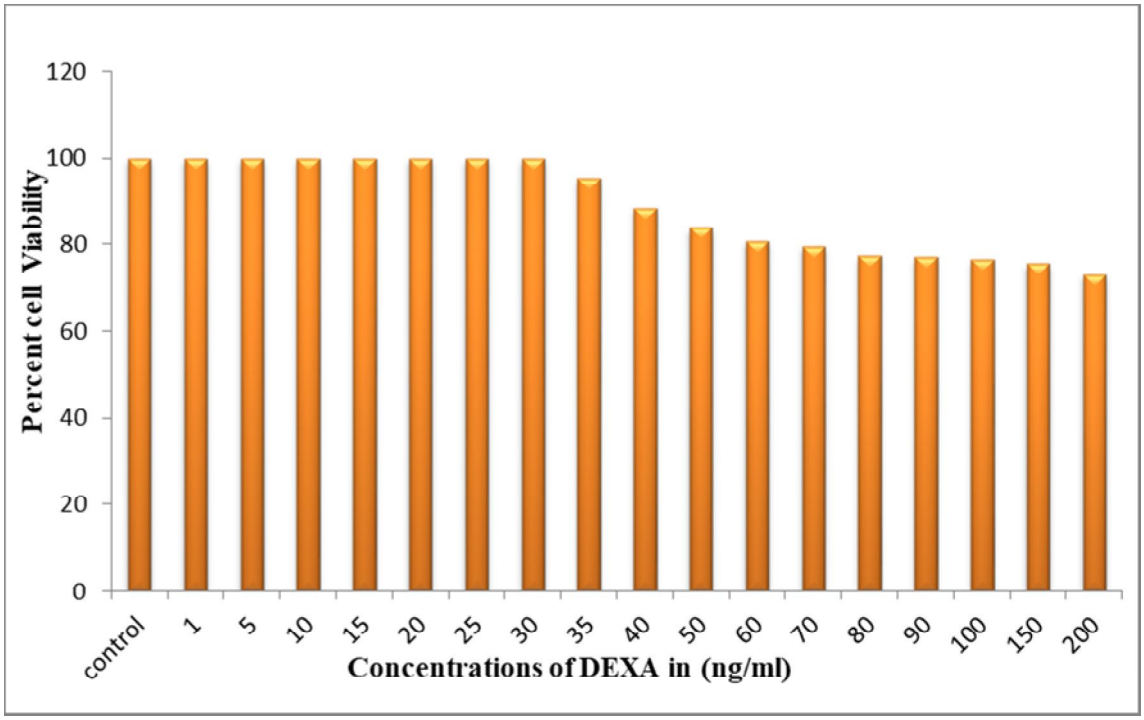


Figure 4.14: Percent viability of chicken lymphocytes in presence of different concentrations of Dexamethasone

4.6 Effect of AME exposure on lymphocytes proliferation

In presence of maximum no-cytotoxic dose of the AME, the lymphocyte proliferation was compared with AME untreated control cells for determination of influence of AME on lymphocyte proliferation. The results are depicted in **Table 4.11** and **Figure 4.17**. There was minute decrease in lymphocyte proliferation as compared to control as well as dexamethasone. Dexamethasone also showed decrease in lymphocyte proliferation. Decrease due to AME exposure was not higher than the decrease by DEXA exposure.

Table 4.11: Effect of AME exposure on lymphocytes proliferation

Treatment Groups	OD at 570 nm				% cell proliferation	% change in cell proliferation
	I	II	III	Mean±SE		
Control	0.302	0.305	0.308	0.305±0.0017	100	-
AME	0.306	0.299	0.309	0.304±0.002	99.67	- 0.327
DEXA	0.302	0.298	0.304	0.301±0.0017	98.68	-1.311
CD (5%)					**SE(m)±**	
0.0076					0.0022	

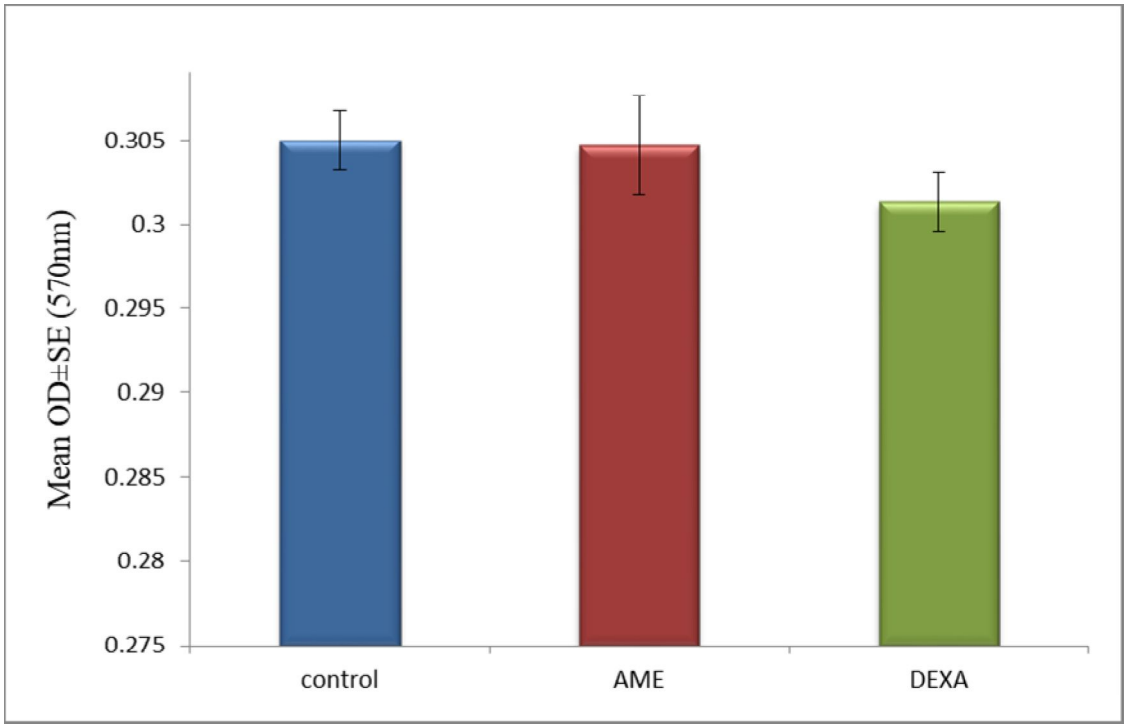


Figure 4.17: Effect of AME exposure on lymphocytes proliferation

4.6.1 Effect of AME exposure on T cells proliferation

Two mitogens i.e. phytohaemagglutinin (PHA) and Concanavalin A (Con A) were employed to study the T cell proliferation. Maximum non cytotoxic dose of the plant extract (AME) and minimum cytotoxic dose of DEXA was used in mitogen stimulated T cells for detection of AME influence on T cell proliferation.

Effect of AME on T lymphocyte proliferation in presence of Con A is depicted in **Table 4.12** and **Figure 4.18**. There was significant decrease in lymphocyte proliferation in AME and DEXA treated cells as compared to control. The Percent inhibition in case of AME was found to be 6.20 %. Dexamethasone was utilized as negative control showing the percent decrease of 11.36% in ConA stimulated T cell proliferation. The Percent decrease of DEXA treated cells was higher in comparison to AME treated cells.

Effect of AME on T lymphocyte proliferation in presence of PHA is depicted in **Table 4.13** and **Figure 4.19**. There was significant decrease in lymphocyte proliferation in AME treated cells as compared to control untreated cells. The Percent inhibition in case of PHA was found to be 15.5%. Dexamethasone was utilized as negative control showing the percent decrease of 18.5% in PHA stimulated T cell proliferation.

Table 4.12: Effect of AME exposure on Con A stimulated T cells proliferation

Treatment Groups	OD at 570 nm				% cell proliferation	% change in cell proliferation
	I	II	III	Mean±SE		
Control	0.386	0.365	0.412	0.387±0.013	100	
AME	0.369	0.372	0.348	0.363±0.007	93.79	-6.20*
DEXA	0.332	0.352	0.346	0.343±0.005	88.64	-11.36*
CD (5%)					**SE(m)±**	
0.033					0.009	

* **Significant at P<0.05**

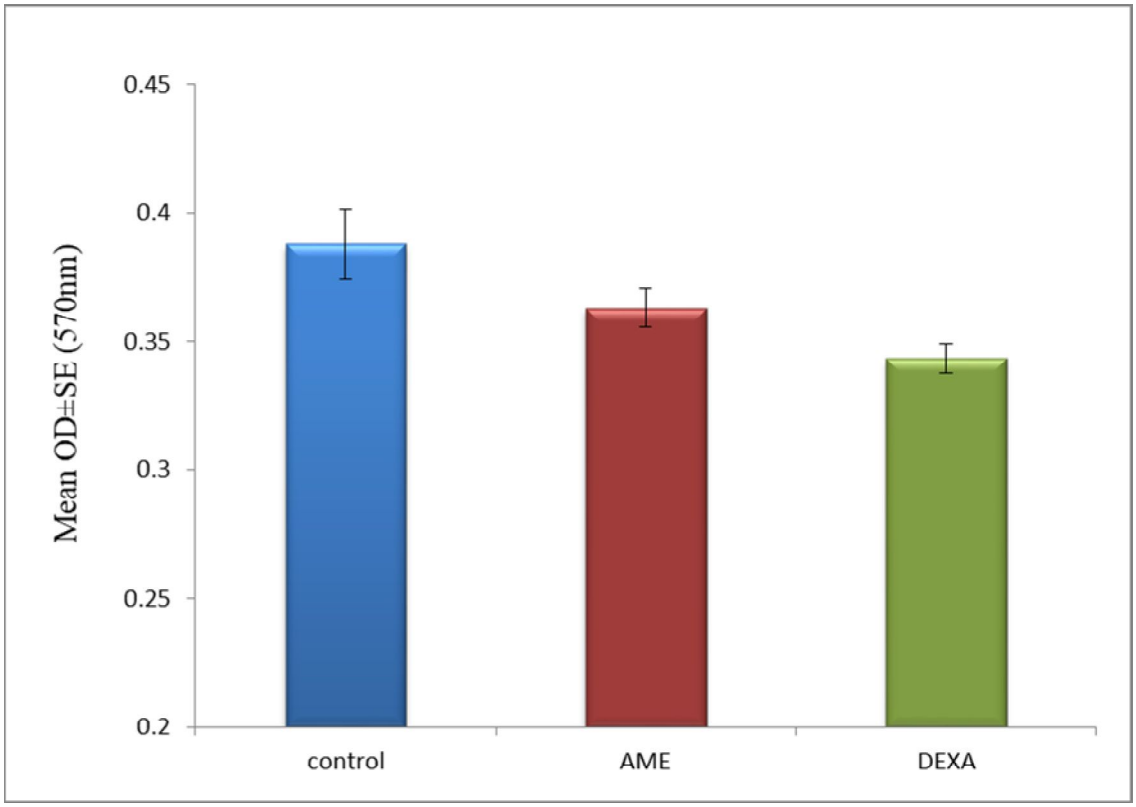


Figure 4.18: Effect of AME on Con A stimulated T cells proliferation

Table 4.13: Effect of AME exposure on PHA stimulated T cells proliferation

Treatment Groups	OD at 570 nm				% cell proliferation	% change in cell proliferation
	I	II	III	Mean±SE		
Control	0.382	0.429	0.391	0.400±0.014	100	-
AME	0.354	0.311	0.349	0.338±0.013	84.5	-15.5*
DEXA	0.347	0.288	0.343	0.326±0.019	81.5	-18.5*
CD (5%)					**SE(m)±**	
0.054					0.015	

* **Significant at P<0.05**

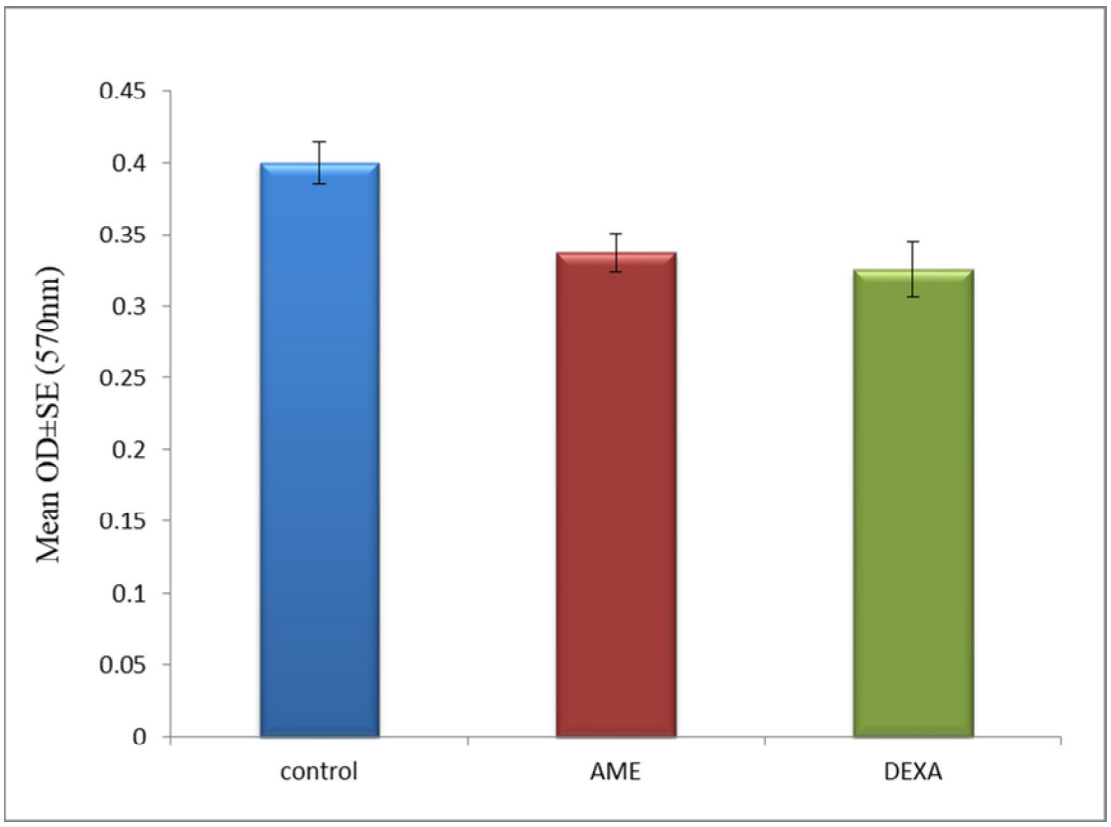


Figure 4.19: Effect of AME on PHA stimulated T cells proliferation

4.6.2 Effect of AME extract on B cells proliferation

In the presence of mitogen Lipopolysaccharide (LPS), the proliferation of B cell was studied. The maximum non cytotoxic dose of AME and DEXA were employed for giving *in vitro* exposure to LPS stimulated B cells. Effect of AME and DEXA on B cells is represented in **Table 4.14** and **Figure 4.20**. There was significant decrease of 9.23% in B cells proliferation due to exposure of maximum non cytotoxic dose of AME. Dexamethasone also showed the decrease of 12.3% on B cells proliferation. Dexamethasone exposure showed more decrease in B cell proliferation than AME. Overall comparatively more decrease in proliferation of T lymphocytes was observed as compared to B lymphocytes proliferation due to *in vitro* exposure of AME in chicken lymphocytes.

Table 4.14: Effect of AME on LPS stimulated B cells proliferation

Treatment Groups	OD at 570 nm				% cell proliferation	% change in cell proliferation
	I	II	III	Mean±SE		
Control	0.304	0.326	0.337	0.325±0.009	100	
AME	0.301	0.295	0.291	0.295±0.002	90.76	-9.23*
DEXA	0.292	0.284	0.281	0.285±0.003	87.69	-12.3*
CD (5%)					SE(m)±	
0.021					0.006	

* **Significant at P<0.05**

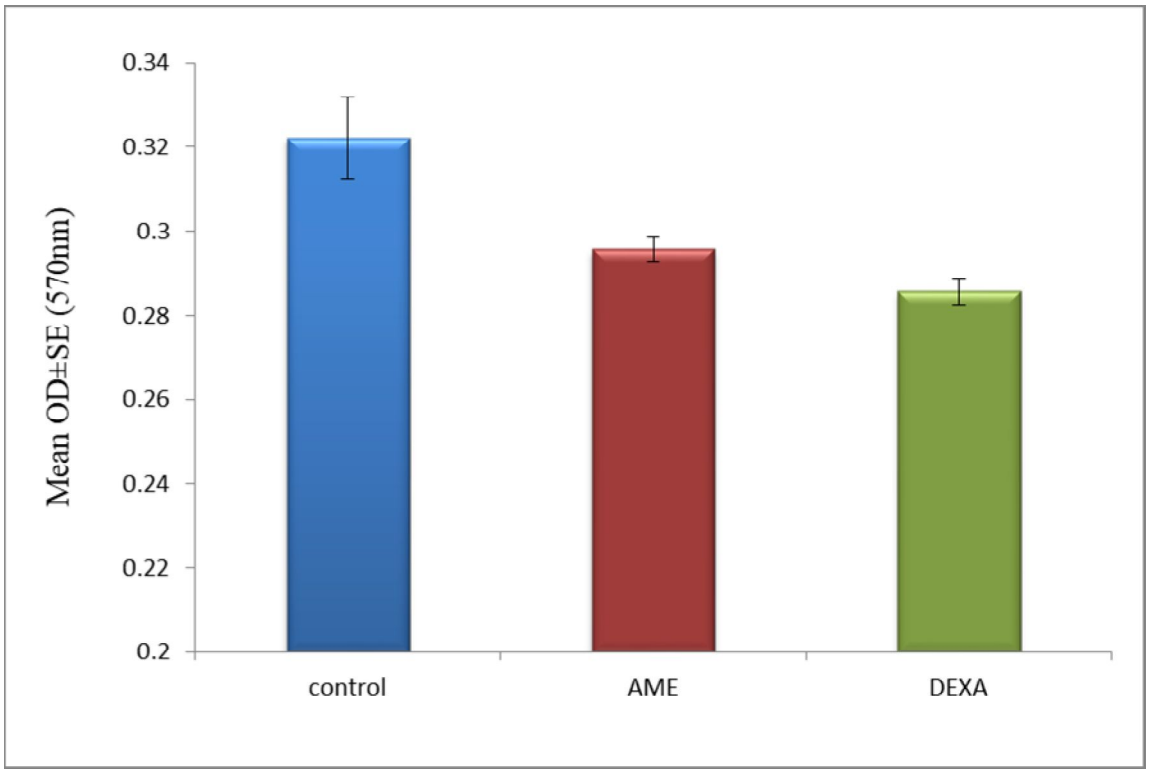


Figure 4.20: Effect of AME on LPS stimulated B cells proliferation

From the above described results it can be concluded that the aqueous extract of *Aegle marmelos* showed immunosuppressive effects in chicken lymphocytes culture system. Decrease in T lymphocytes proliferation was higher than the B lymphocyte in case of *in vitro* exposure of AME. Immunomodulatory effect of the *Aegle marmelos* is due to presence of various phytoconsistuents *i.e.* Noscapin (which is opioid alkoid,

used as tubulin binding anti-tumour agent), α-Erythroidine, amorolfine, marmelin, marmelosin and embelin (which is known as anti-tumour agent having antiprolifertaive activity) **(Rahman and Parvin, 2014)**.

Lampronti *et al.* (2003) reported that ethanolic extract of *Aegle marmelos* stem bark showed antiprolifertaive effects on growth of various tumour cell lines *viz.* T lymphoid Jurkat, erythroleukemic HEL, melanoma Colo38, and breast cancer MCF7. Derivative of *Aegle marmelos* extract including butyl p-tolyl sulfide, 6- methyl-4-chromanone and butylated hydroxyanisole showed strongest antiprolifertaive effects on leukemic K562 cell line. **Gupta and Chaphalkar (2016)** reported that flavonoids isolated from *Aegle marmelos* inhibited in proliferation of HBsAg at higher dose of 25mg/ml as compared to control. **Shaikh *et al.* (2016)** reported that aqueous leaves extract of *Aegle marmelos* showed decline in proliferation of HBsAg (hepatitis B vaccine containing surface antigen) which was assessed through splenocytes proliferation assay in swiss mice model. Aqueous leaves extract of *Aegle marmelos* also down regulate the production of proinflammatory cytokines such as IFN γ and TNF α.

4.7 Antioxidative status of chicken lymphocytes due to AME exposure

Due to continuous on going endogenous metabolic process in human body, the reactive oxygen species (ROS) and free radicals are generated by enzymatic and non-enzymatic reactions. Excessive production of these free radicals can cause oxidative stress. These free radicals can attack on various macromolecules like lipid, proteins, nucleic acid, carbohydrates **(Inoue *et al.*, 2003)**. To prevent the excessive formation of free radicals and reactive oxygen species, antioxidants are required. Antioxidants act by scavenging the free radicals and making them less reactive toward various macromolecules.

After *in vitro* exposure of AME, antioxidative status of chicken lymphocytes was determined through various antioxidative assays *viz* membrane lipid peroxidation, glutathione reductase, superoxide dismutase, catalase and nitric oxide estimation. Ascorbic acid was used as positive control in each assay.

4.7.1 Membrane Lipid peroxidation

In biological system, the free radical oxidation of polyunsaturated fatty acids (PUFA-long chain fatty acids) present in cell membrane, is known as lipid peroxidation. Initation of lipid peroxidation is triggered by any free radical having ability to abstract hydrogen atom from methylene group and leaving unpaired electron on carbon atom. Further reactive oxygen species react with unpaired electron to amplify the chain reaction and produce the peroxidation product malondialdehyde (MDA) which cause damage of the DNA **(Gaschler and Stockwell, 2017)**. Lipids in cell membrane are required for maintaining the cellular integrity. Excessive lipid peroxidation affects the composition, structure of the cell membrane cause its leaking that leads to loss of cell integrity and all the cellular material come out of the cell which result in cell death **(Ricciotti and FitzGerald, 2011)**.

In chicken lymphocytes, the membrane lipid peroxidation was measured after the exposure of AME. Thiobarbituric acid-reactive substances (TBARS) assay was used to determine the degree of lipid peroxidation. More the antioxidative potential of the plant extract lower will be the activity of TBARS **(Halliwell and Chirico, 1993)**.

Aqueous extract of *Aegle marmelos* leaves was used to determine inhibition of lipid peroxidation. The AME show significant decrease in lipid peroxidation as presented in **Table 4.15** and **Figure 4.21.** Ascorbic acid was used as positive control and cells without extract treatment was taken as normal control.

Table 4.15: Lipid peroxidation in chicken lymphocytes treated with AME

S. No.	Treatment groups	Concentration used (µg/ml)	LPO nM mda/g			Mean LPO nM mda/g±SE
			I	II	III	
1.	Control	-	142.93	144	146.13	144.35±0.940
2.	AME	75	109.86	112	105.6	109.15±1.881*
3.	DEXA	0.03	43.733	48	41.6	44.44±1.881*
4.	Ascorbic acid	75	126.93	134.4	129.06	130.13±2.221*
CD (5%)			**SE(m)±**			
5.852			1.795			

* **Significant at P<0.05**

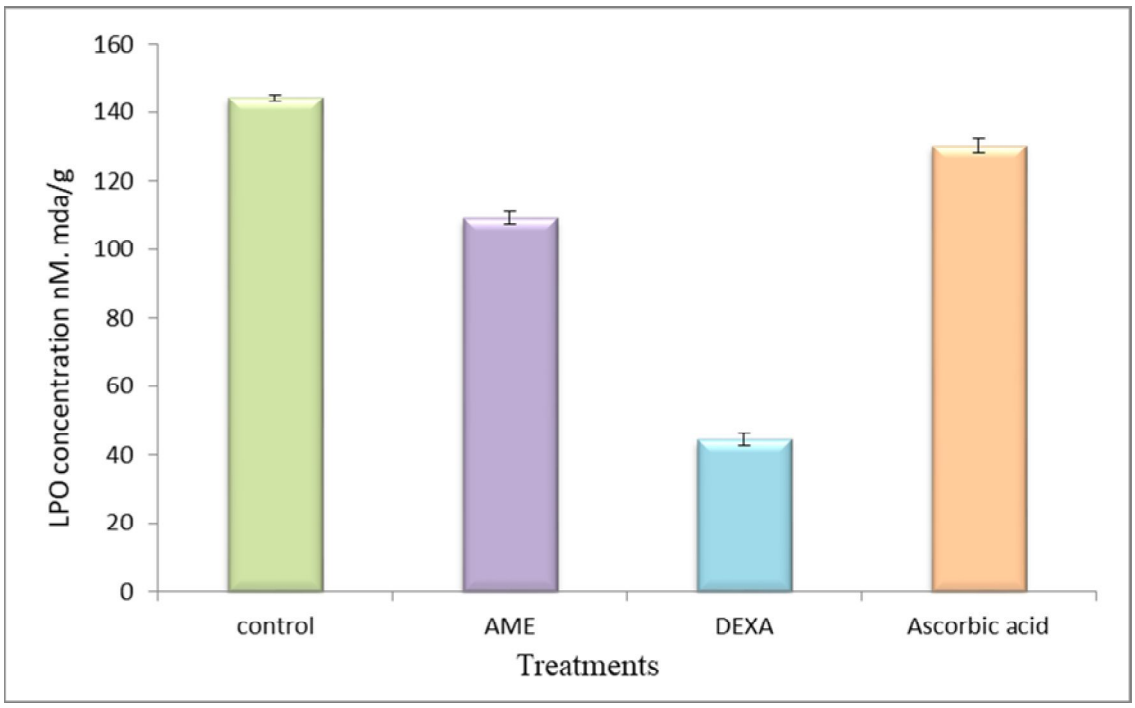


Figure 4.21: Lipid peroxidation in chicken lymphocytes treated with AME

The decrease in lipid peroxidation due to the plant extract exposure was higher than ascorbic acid which reflects better antioxidative potential of the plant. Dexamethasone also displayed marked reduction in LPO as compared to control.

Suriyamoorthy *et al.* (2014) reported that the aqueous extract of leaves of *Aegle marmelos* showed decrease in LPO level in dyslipidemic rat. The percentage decrease in TBARS level was 33.22%. In hepatocellular damage rat liver induced by CCl_4, the level of LPO was decrease on administration of aqueous and ethanolic root extract of *Aegle marmelos* **(Mounika *et al.*, 2017)**. **Dinis-Oliveira *et al.* (2006)** reported that dexamethasone decreased the level of LPO in liver of wister rats at dose of 100mg/kg.

4.7.2 Reduced glutathione (GSH)

γ-glutamylcysteinylglycine (glutathione) is non-protein thiol abundantly present in cells. It protects the cells against reactive oxygen (ROS) and reactive nitrogen species (RNS). These radicals are removed by reduction with GSH which is non-enzymatic reaction. Hydroperoxides are removed by enzyme glutathione peroxidase. Enzymatic and non-enzymatic removal leads to production of glutathione disulfide (GSSG or oxidized glutathione). Glutathione reductase using NADPH from pentose phosphate shunt pathway, convert the glutathione disulfide into reduced glutathione **(Dickinson and Forman, 2002)**.

Reduced glutathione is one of the major cellular antioxidant. Higher level of reduced glutathione is indicative of antioxidative activity. After the exposure of AME to chicken lymphocytes, the level of reduced glutathione was determined. The level of reduced glutathione was increased after the exposure of AME as presented in **Table 4.16** and **Figure 4.22.** Ascorbic acid was used as positive control and untreated cells were taken as normal control.

Table 4.16: Reduced glutathione Content in chicken lymphocytes cells treated with AME

S. No.	Treatment groups	Concentration used (µg/ml)	GSH content (mM/ml)			Mean GSH content (mM/ml) ± SE
			I	II	III	
1	Control	-	3.9	4.03	3.84	3.92±0.056
2	AME	75	4.18	4.30	4.00	4.16±0.081
3	DEXA	0.30	5.29	4.61	5.0	4.99±0.200
4	Ascorbic acid	75	6.30	5.41	5.16	5.62±0.345
CD (5%)			**SE(m)±**			
0.673			0.206			

* **Significant at P<0.05**

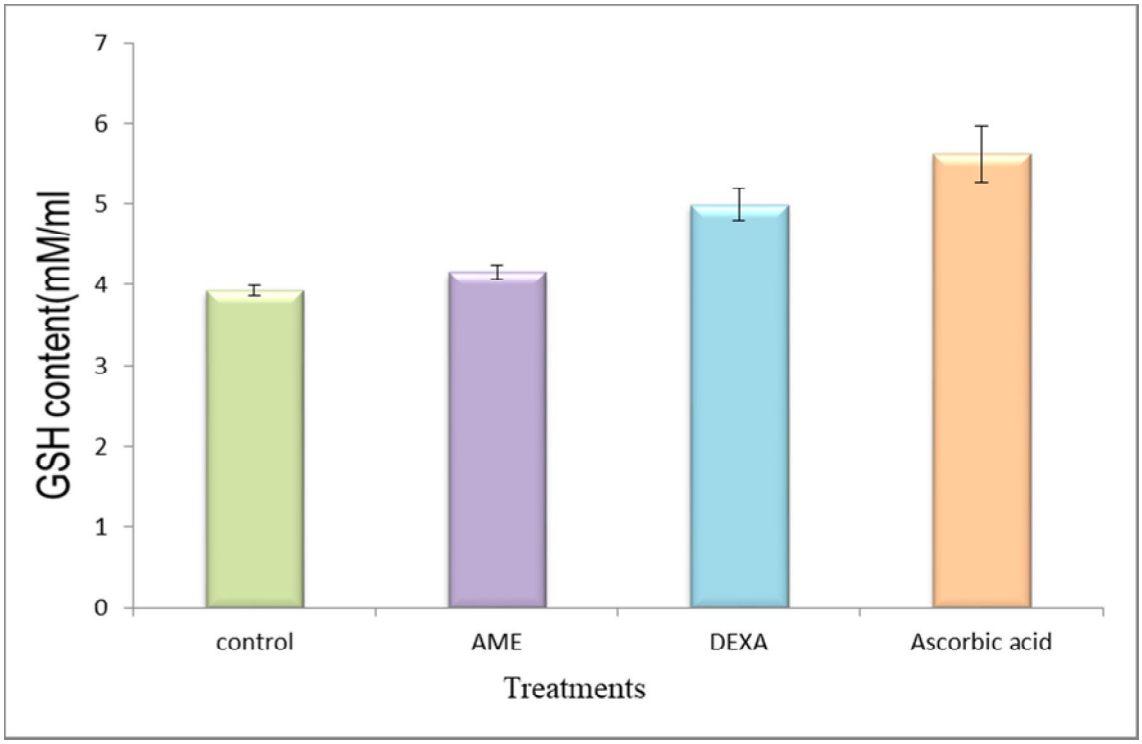


Figure 4.22: GSH content in chicken lymphocytes treated with AME

Ascorbic acid showed the maximum level of reduced glutathione as compare to AME. AME exposure leads to enhanced level of GSH as compared to untreated control cells. **Singh *et al.* (2000)** reported that on administration of hydroalcholic leaves extract of *Aegle marmelos* the level of reduced glutathione was increased in dose dependent manner in swiss albino male mice. The level of reduced glutathione increased by 1.34 and 1.41 fold as the concentration of the extract increased from 50mg/kg to 100 mg/kg. **Upadhya *et al.* (2004)** reported that administration of aqueous extract of leaves of *Aegle marmelos* increased the level of reduced glutathione in erythrocytes of alloxan induced diabetic male albino rats.

4.7.3 Superoxide dismutase (SOD)

In mitochondrial respiration and autooxidation, superoxide radicals are produced. Various enzymes are having transition metals as their prosthetic groups. Superoxide radicals react with transition metals and make the enzyme inactive by losing their active site. Superoxide radicals are not dangerous unless they react with other radicals to make powerful reactive oxygen and nitrogen species. Superoxide's are beneficial when produced by phagocytes and polymorph nuclear leukocytes for antibacterial activity but harmful when present in excess amount **(Halliwell, 2012)**. In cellular antioxidative defense system, superoxide dismutase acts as first line of defense system. Superoxide radicals are dismutated by SOD and hydrogen peroxide is formed which is further resolved into water by catalase and reduced glutathione **(Surai, 2016)**.

Table 4.17: SOD Content in chicken lymphocytes treated with AME

S. No.	Treatment groups	Concentration used (µg/ml)	SOD units/mg of protein			Mean SOD units/mg of protein±SE
			I	II	III	
1	Control	-	66.89	66.09	66.19	66.52±0.202*
2	AME	75	117.47	116.02	112.37	115.28±1.51*
3	DEXA	0.30	73.23	75.70	73.52	74.18±0.766*
4	Ascorbic acid	75	196.5	190.6	204.95	197.35±4.164*
CD (5%)			**SE(m)±**			
7.33			2.251			

* **Significant at P<0.05**

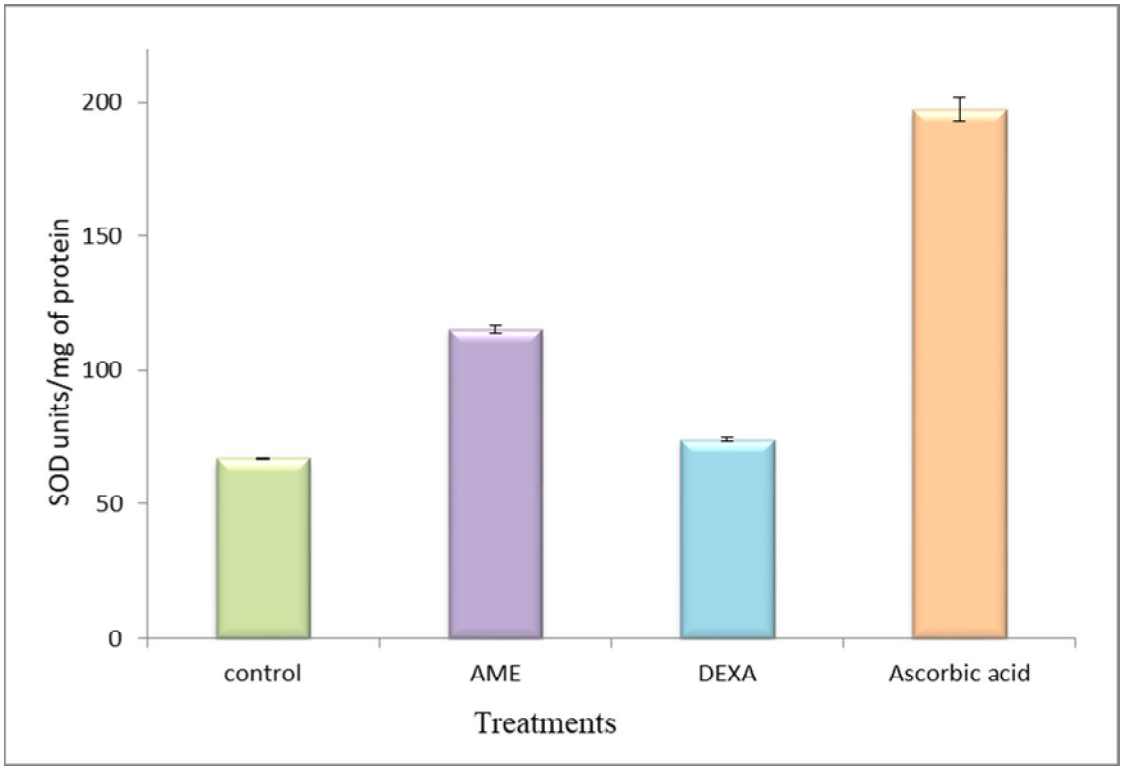


Figure 4.23: SOD content in chicken lymphocytes treated with AME

SOD content in AME treated chicken lymphocytes is presented in **Table 4.17** and **Figure 4.23.** Ascorbic acid was taken as positive control and untreated cells were taken as normal control. The level of SOD was increased in comparison to normal control. The ascorbic acid displayed higher level of SOD. **Mounika *et al.* (2017)** reported that the aqueous and hydroalcholic leaves extract of *Aegle marmelos* at higher doses, increased the level of cellular SOD in CCl_4 induced hepatotoxic wistar albino rats. **Suriyamoorthy *et al.* (2014)** reported that in dyslipidemic rats the level of SOD was increased on administration of aqueous leaves extract of *Aegle marmelos*.

4.7.4 Catalase

In aerobic organism, catalase is present in various tissues but highly active in liver and erythrocytes. Due to presence of various H_2O_2 producing enzymes in peroxisomes the level of catalase is also high as in membrane bound or soluble form. Catalase acts as first line of defense against free radicals in erythrocytes. Catalase breaks down the H_2O_2 into water and oxygen. It also utilizes the hydrogen peroxide to oxidize other toxic compounds like alcohols and phenols **(Djordjevic, 2004)**.

The level of catalase was determined in chicken lymphocytes after the treatment of the AME at maximum non cytotoxic dose. Increase in catalase activity was observed after the AME treatment as presented in **Table 4.18** and **Figure 4.24.** Ascorbic acid treated cells are positive control and untreated cells are taken as normal control.

Table 4.18: Catalase activity in chicken lymphocytes cells treated with AME

S. No.	Treatment groups	Concentration used (µg/ml)	H_2O_2 utilized mM/min/mg of protein			Mean H_2O_2 utilized mM/min/mg of protein±SE
			I	II	III	
1	Control	-	110.68	150.92	140.86	134.15±12.09
2	AME	75	137.52	160.44	142.11	146.69±7.005
3	DEXA	0.30	115.19	138.23	120.95	124.74±6.922
4	Ascorbic acid	75	222.67	231.60	217.65	223.97±4.075
CD (5%)				**SE(m)±**		
26.265				8.058		

* **Significant at P<0.05**

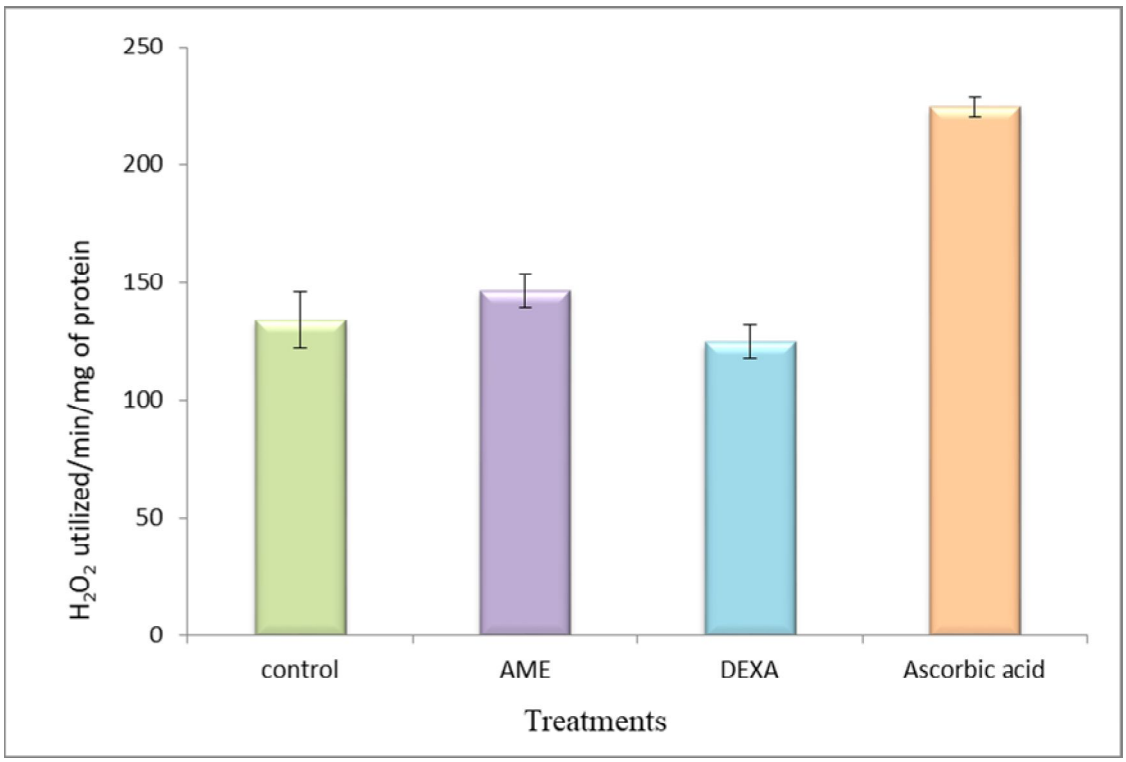


Figure 4.24: Catalase activity in chicken lymphocytes cells treated with AME

Kamalakkannan and Prince (2004) reported that level of catalase was increased in hepatic and renal tissue of Streptozotocin-Induced Diabetic Rats after the treatment of aqueous fruit extract of *Aegle marmelos.* **Mounika *et al.* (2017)** reported that aqueous and hydroalcholic leaves extract of *Aegle marmelos* at higher doses, increase the level of cellular catalase in CCl_4 induced hepatotoxic wistar albino rats.

4.7.5 Nitric Oxide Estimation

Nitric oxide is produced endogenously by enzyme nitric oxide synthase (NOS). NOS has three different isoforms known as eNOS (endotheial NOS), nNOS(neuronal NOS) and iNOS (inducible NOS). Inducible NOS is produced by various immune cells for initiation of immune response. Nitric oxide acts as paracrine signalling molecule. Being small and hydrophobic in nature it can easily diffuse from its site of synthesis. At higher concentration nitric oxide reacts with oxygen and produces reactive nitrogen species. NO and oxygen accumulate in lipid membrane and autoxidation of membrane occurs **(Murphy, 1999)**.

Nitric oxide content is calculated from the sodium nitrite standard curve ($y = 0.0035x$, $R^2 = 0.9702$) as presented in **Table 4.19** and **Figure 4.25**. Nitric oxide content determined after the exposure of AME in macrophage rich cells as presented in **Table 4.20** and **Figure 4.26.** Ascorbic acid was taken as positive control and untreated cells taken as normal control.

Table 4.19: Optical density of different concentrations of Sodium Nitrite

S. No.	Concentration (µM)	Optical density at 548 nm			Mean OD ±SE
		I	II	III	
1.	10	0.136	0.075	0.099	0.103±0.017
2.	20	0.349	0.199	0.206	0.251±0.048
3.	40	0.422	0.264	0.32	0.335±0.046
4.	50	0.576	0.458	0.332	0.455±0.070
5.	80	0.729	0.596	0.437	0.587±0.084
6.	100	0.801	0.642	0.584	0.675±0.064
7.	150	0.901	0.811	0.626	0.779±0.080
8.	200	0.922	0.802	0.751	0.825±0.050

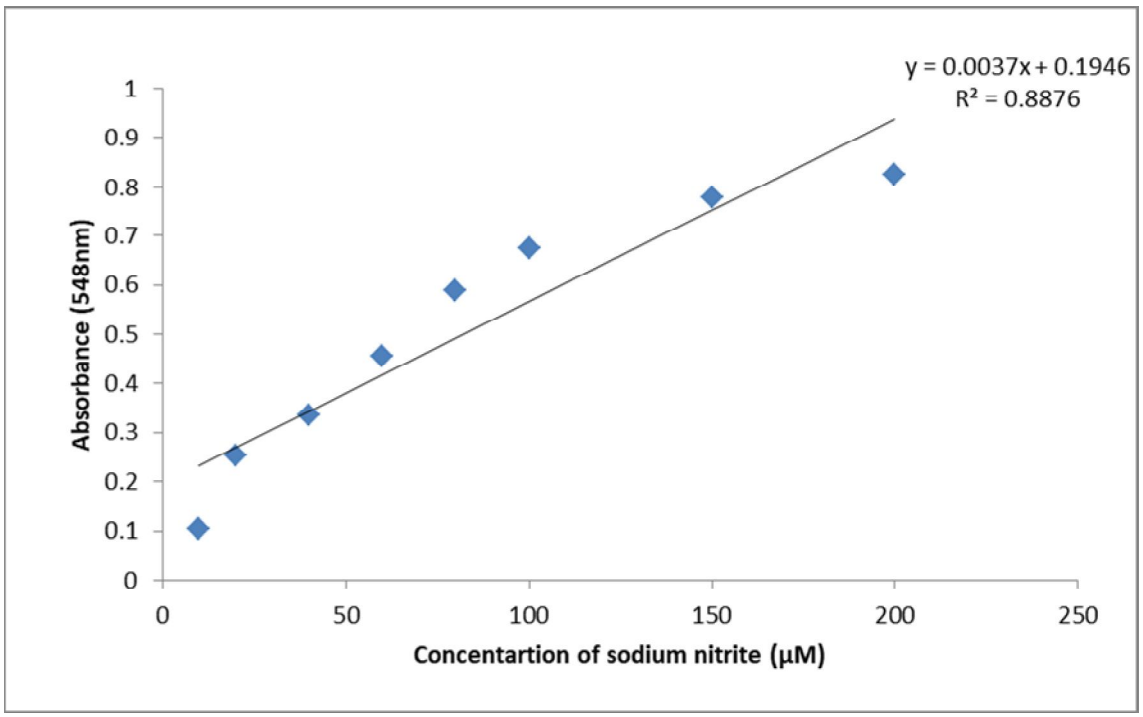


Figure 4.25: Standard curve of sodium nitrite

Table 4.20: Nitric Oxide estimation in AME treated macrophages rich cells

S. No.	Treatment groups	Concentration used (µg/ml)	Total Nitric Oxide content (µM)			Mean Total Nitric Oxide content (µM) ±SE
			I	II	III	
1	Control	-	60.91	57.94	59.83	59.56±0.867
2	AME	75	45.24	51.45	46.54	47.76±1.885
3	DEXA	0.03	27.13	29.02	30.108	28.57±0.870
4	Ascorbic acid	75	39.56	36.59	29.02	35.05±3.137
CD (5%)						**SE(m)±**
6.293						1.930

* **Significant at P<0.05**

Concentration of nitric oxide was decreased after the treatment with AME as compared to untreated cells. The ascorbic acid being good antioxidant showed significant decrease in concentration of nitric acid. **Kumari *et al.* (2014)** reported that aqueous extract of *Aegle marmelos* flower caused significant reduction in the level of nitric oxide in peritoneal cells of wister rat. The flower extract of *Aegle marmelos* showed decrease in nitric oxide content in dose dependent manner from 7.8 to

62.5µg/ml. **Patil *et al.* (2017)** reported that dexamethasone showed decrease in concentration of nitric oxide in dose dependent manner in A549 Human lung adenocarcinoma cell line.

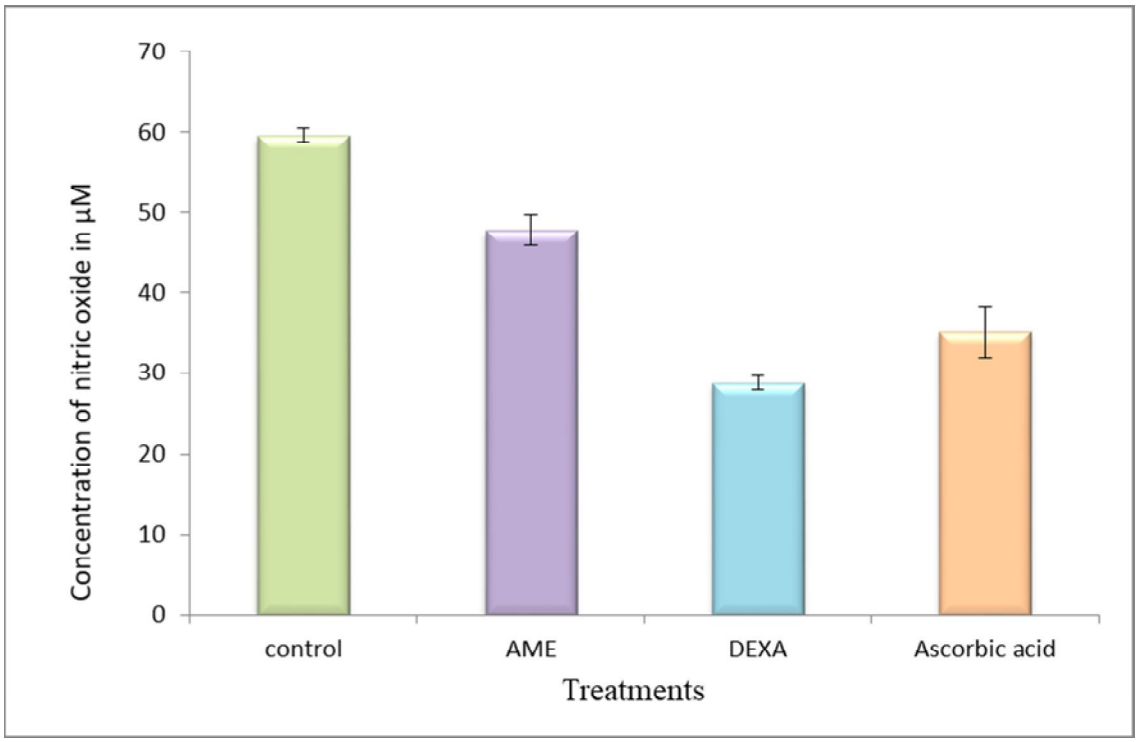


Figure 4.26: Nitric oxide estimation in AME treated cells

4.8 Expression profiling of cytokines genes using Real Time PCR

Expression analysis of various cytokines was carried out to evaluate the immunomodulatory effects of the extract at molecular level. Expression analysis of IL-6, IL-10 and iNOS (inducible NOS) was performed by exposing the lymphocytes at maximum non cytotoxic dose of AME to correlate the expression pattern of these cytokines in response to AME exposure. By using the cDNA of lymphocytes as template exact quantitative expression of genes was carried out through real time quantitative PCR. Variation in quality and quantity of starting cDNA template was standardized by using actin gene. Expression analysis of studied genes is as follows:

4.8.1 Isolation of total RNA from chicken lymphocytes/ macrophage rich cells after the exposure of AME

Total RNA was isolated from chicken lymphocytes/ macrophage rich cells after the exposure of AME. Mitogen stimulated T and B cells were used as control.

Cells without any treatment were used as normal control. Dexamethasone at maximum non cytotoxic dose was used as positive control for expression analysis of various cytokines. Total RNA of good quality and yield was isolated by following the manufacturer's instructions of HiMedia RNA X-press reagent.

Table 4.21: Quantitative study of total RNA from chicken lymphocytes/ macrophages rich cells

	Treatments	Absorbance at 260/280	Concentration ng/µl
Group A	Untreated cells	2.04	1683.4
	DEXA treated cells	1.98	1210.4
	AME treated cells	1.93	1315.7
Group B (LPS stimulated cells)	Untreated cells	1.94	1984.3
	DEXA treated cells	2.01	1675.9
	AME treated cells	2.02	1507.5
Group C (Con A stimulated cells)	Untreated cells	1.95	1804.0
	DEXA treated cells	1.96	1031.2
	AME treated cells	2.01	1452.6
Group D (macrophages enriched cells)	Untreated cells	1.92	1415.3
	DEXA treated cells	1.96	1233.4
	AME treated cells	1.94	1732.6

Treatment with DNase was performed to eliminate the contamination of DNA. Nanodrop spectrophotometry was used to check the quantity of the extracted RNA isolated form chicken lymphocytes as represented in **Table 4.21.**

4.8.2 Synthesis of cDNA from RNA samples isolated from different treatment groups

The DNase treated RNA was used for cDNA synthesis to use it further for expression analysis study. The cDNA was synthesized using cDNA synthesis kit and then confirmed by semi quantitative PCR analysis by using Actin housekeeping gene as internal control. **Figure 4.27** shows the confirmation of cDNA synthesis.

4.8.3 Standardization of annealing temperature of primers of various genes using gradient PCR

Gradient PCR analysis was conducted to confirm the temperature for amplification of transcript of Actin, interleukin-6, interleukin-10 and inducible nitric oxide synthase in chicken lymphocytes. The 130 bp, 181bp, 179bp, 158bp sequence was confirmed to be present at temperature of 54^0C, 55^0C, 54^0C, 53^0C in chicken lymphocyte for actin, IL-6, IL-10, iNOS, respectively. **Figure 4.28** represents the gradient results of different genes.

4.8.4 Quantification of expression level of IL-6 gene transcript in chicken lymphocytes after AME exposure through real time PCR.

Various cells of immune system produce cytokines. These cytokines on the basis of type of cells producing it and developmental state of cell affects the immune response by regulating proliferation and differentiation of different cells. IL-6 is glycoprotein of 21kDa size belongs to family of leukemia inhibitory factor, carditropin-1 and ciliary inhibitory factor and known as cytokine of innate immune response. Macrophage, dendritic cell, mast cell, B cell, T cell and non-leukocytes cell (endothelial cell, fibroblasts) are producing IL-6 cytokine. IL-6 consider as pro inflammatory (chronic inflammation) and under some condition as anti-inflammatory cytokine (acute inflammation) **(Müllberg *et al.*, 2000)**.

In inflammation, the leukocytes migrate through vascular system at site of damaged tissue and destroy the causative agent but sometime it can also cause injury to the tissue. During chronic inflammation, macrophages and lymphocytes are involved. The level of IL-6 is increased in chronic inflammation which leads to various conditions such as rheumatoid arthritis (autoimmune disease) and crohn's disease **(Gabay, 2006)**. The expression pattern of IL-6 was analysed after the exposure of AME to chicken lymphocytes as presented in **Figure 4.29**.

The expression pattern of IL-6 was analysed after the exposure of AME to chicken lymphocytes as presented in **Table 4.22** and **Figure 4.30**. Cells without any treatment were taken as normal control and treatment with Dexamethasone at maximum non cytotoxic dose was taken as positive control. In control cells expression was taken as1 fold in comparison. Positive control showed decrease in expression as compared to control.

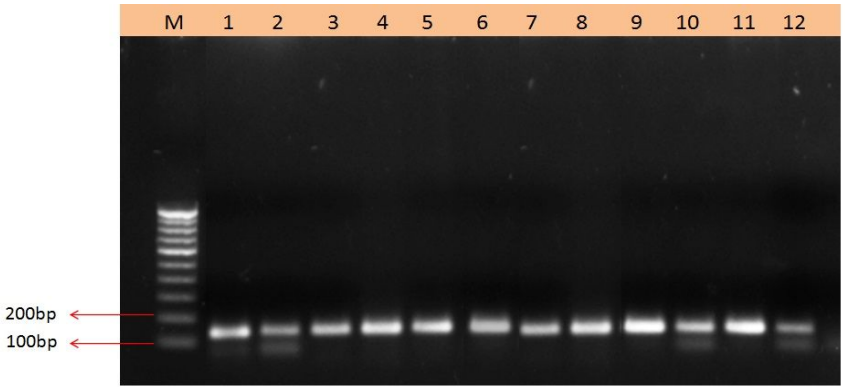
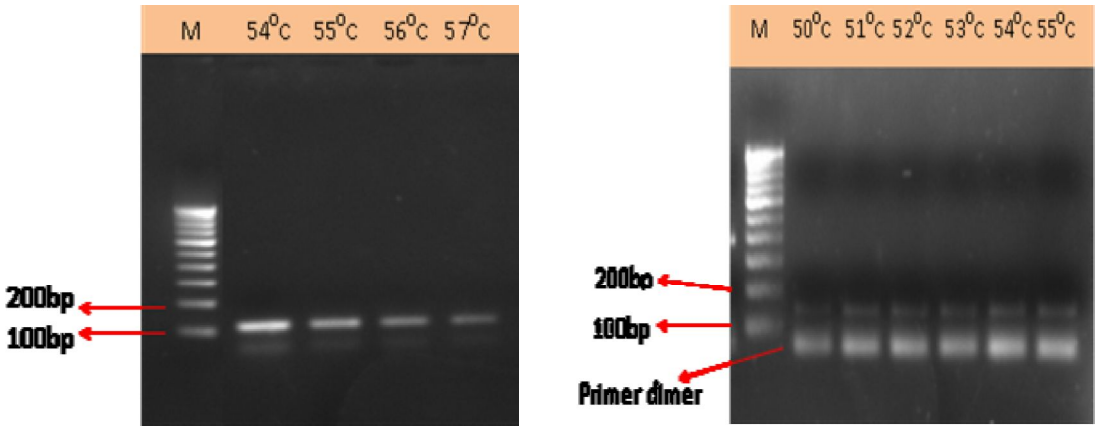
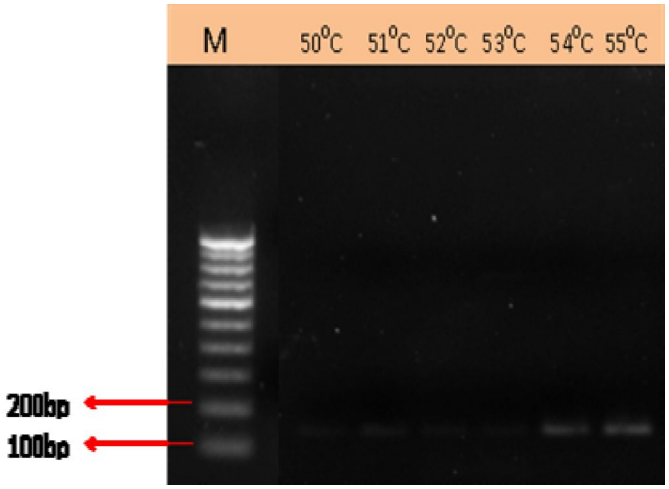


Figure 4.27: Amplification of actin in cDNA synthesized from isolated mRNA from treated lymphocytes (M: marker of 100bp, Lane 1 : control, Lane 2: DEXA, Lane 3: AME, Lane 4 to 6: LPS stimulated B cells (Lane 4: control, Lane 5: DEXA, Lane 6: AME), Lane 7 to 9: Con A stimulated cells (Lane 7: control, Lane 8: DEXA, Lane 9: AME), Lane 10 to 12: Macrophages enriched cells (Lane 10: control, Lane 11: DEXA, Lane 12: AME))



(A) **(B)**



(C)

Figure 4.28: Confirmation of annealing temperature of primers of various genes in gradient PCR (A. gradient PCR of Actin, B. gradient PCR of IL-10, C. gradient PCR of iNOS (M: marker of 100 bp ladder))

Table 4.22: Calculation with the threshold cycle (C_t) values for determining expression of IL-6 after treatment of AME in chicken lymphocytes

Treatments	C_t value of Actin	C_t value of IL-6	ΔC_t value (C_t IL-6- C_t actin)	$\Delta\Delta C_t$ (ΔC_t treatment- ΔC_t Control)	Fold expression $2^{-\Delta\Delta Ct}$ relative to expression of control
Control	26.46	32.12	5.66	0	1
DEXA	26.17	32.47	6.31	0.64	0.641*
AME	23.38	32.03	8.65	2.99	0.125*

CD (1%)	CD (5%)	SE(m)±
0.104	0.069	0.020

* **Significant at P<0.05**

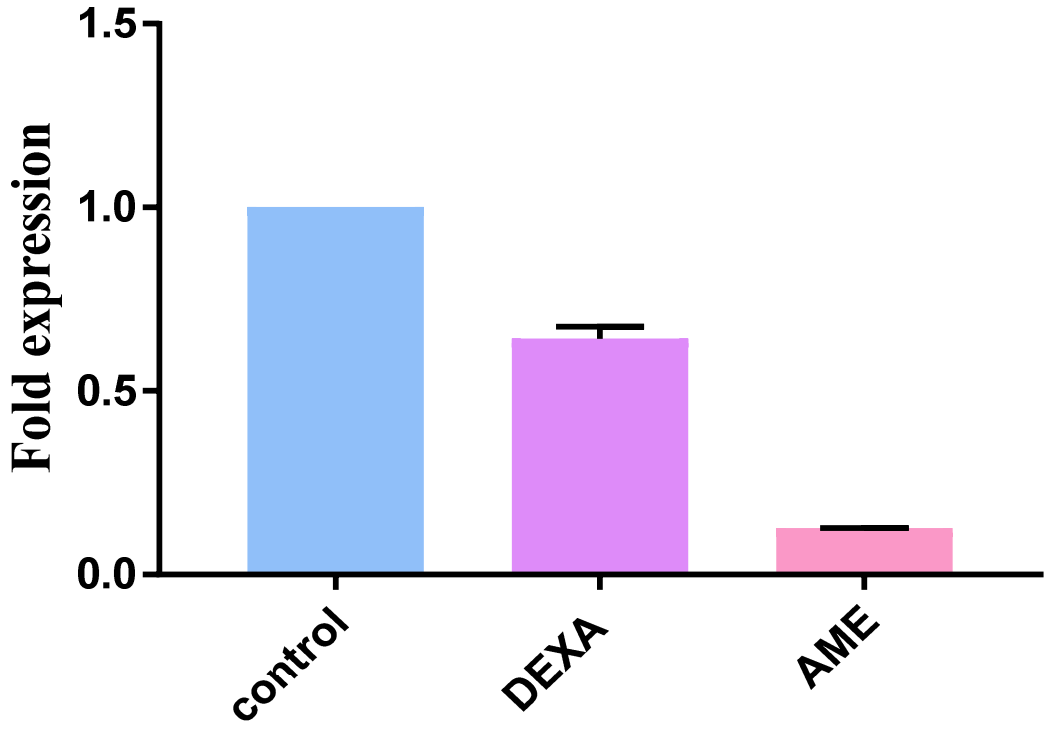


Figure 4.30: Expression analysis of IL-6 after AME treatment in chicken lymphocytes

The treatment of AME at maximum non cytotoxic dose showed significant decrease in the expression level of IL-6 cytokine in comparison to normal and positive control.

As observed through lymphocyte proliferation assay, AME exposure decreased the proliferation of B and T cells. LPS stimulated B and Con A stimulated T cells when treated with AME and DEXA showed decrease in expression level of IL-6 as compared to respective controls. **Table 4.23** and **Figure 4.31** represents expression pattern of IL-6 in LPS stimulated B cells. LPS stimulated B cells were taken as control and effect of AME and DEXA treatment was determined. The AME and DEXA showed significant decrease in expression of IL-6.

In Con A stimulated T cells expression pattern of IL-6 is represented in **Table 4.24** and **Figure 4.32**. Con A stimulated T cells were taken as control and effect of AME and DEXA treatments on AME exposure was determined. The AME and DEXA showed decrease in expression of IL-6. Overall, there was significant decrease in expression of IL-6 due to AME exposure in various groups.

Table 4.23: Calculation with the threshold cycle (C_t) values for determining expression of IL-6 in LPS stimulated B lymphocytes after treatment of AME

Treatments	**C_t value of Actin**	**C_t value of IL-6**	**ΔC_t value(C_t IL-6- C_t actin)**	**$\Delta\Delta C_t$ (ΔC_t treatment- ΔC_t Control)**	**Fold expression $2^{-\Delta\Delta Ct}$ relative to expression of control**
Control	22.72	29.41	6.69	0	1
DEXA	27.45	34.22	6.77	0.08	1.1306
AME	20.62	28.41	7.81	1.12	0.4763
CD (1%)		CD (5%)	SE(m)±		
1.405		0.930	0.296		

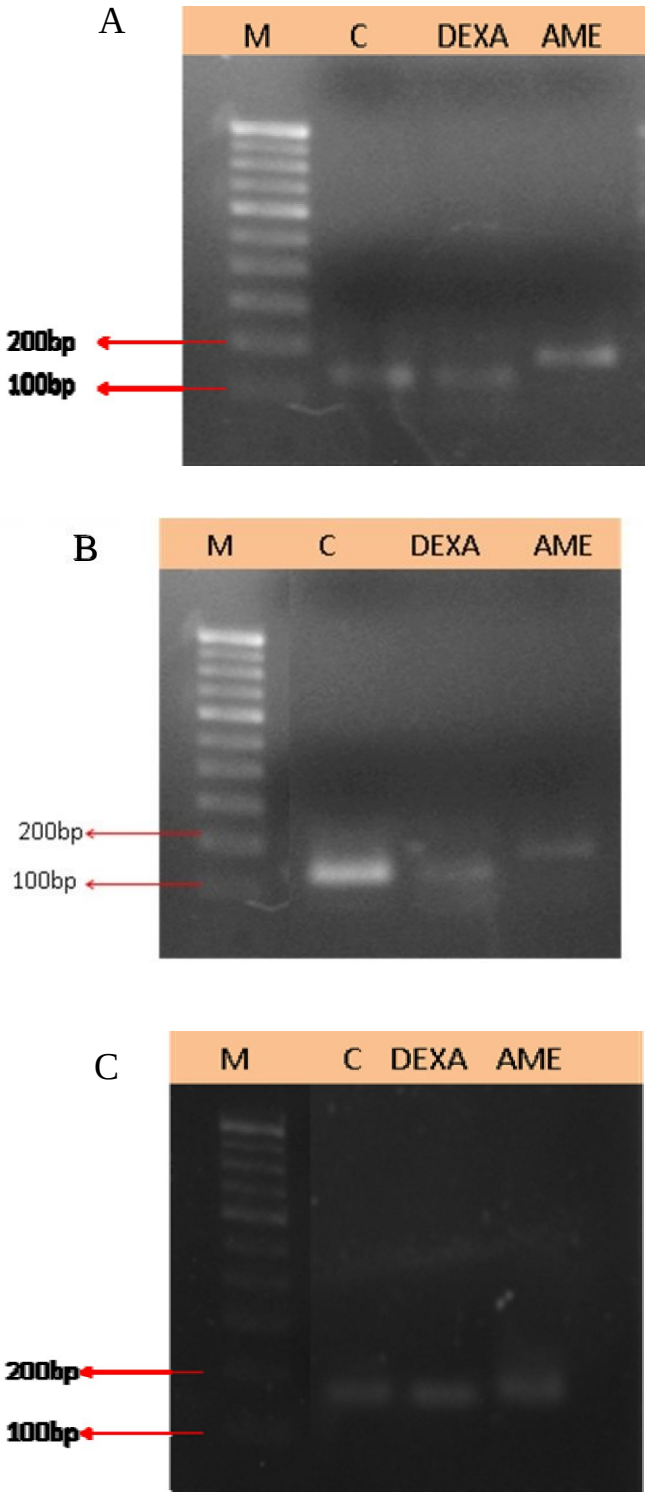


Figure 4.29: Expression analysis of IL-6 in AME treated chicken lymphocytes

A. Lane 1: marker 100 bp ladder, Lane 2: untreated cell as control, Lane 3: DEXA, Lane 4: AME

B. expression analysis of IL-6 in LPS stimulated B lymphocytes after AME treatment Lane 1: marker 100 bp ladder, Lane 2: control, Lane 3: DEXA, Lane 4: AME

C. expression analysis of IL-6 in Con A stimulated T lymphocytes after AME treatment Lane 1: marker 100 bp ladder, Lane 2: control, Lane 3: DEXA, Lane 4: AME

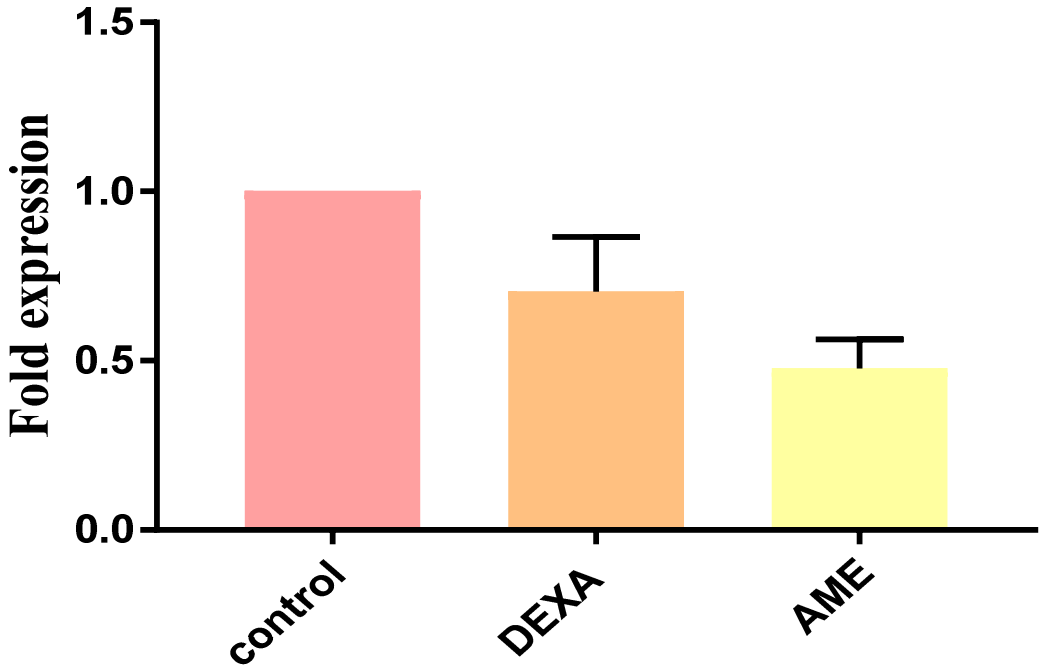


Figure 4.31: Expression analysis of IL-6 in LPS stimulated B lymphocytes after AME treatment

Rajaram *et al.* (2017) reported that ethanolic and aqueous extracts of roots, stem and leaves of *Aegle marmelos* collected from different parts of India, showed decrease in expression level of IL-6 cytokine in LPS induced RAW 264.7 cell line. Root extract of *Aegle marmelos* showed more down regulation of expression level of IL-6 in comparison to stem extract. Leaf and root extracts of *Aegle marmelos* showed decrease in IL-6 expression which could be co related with the anti-inflammatory effect of the plant due to presence of phytoconstituents like lupeol, lupeol linoleate in leaves and Marmin, marmesin, umbelliferine, skimmianine in roots.

Verma *et al.* (2013) reported the hydromethanolic leaves extract of *Aegle marmelos* showed 25% decrease in expression level of IL-6 in N-methyl N-nitrosourea-induced hepatocarcinogensis in Balb/c mice. **Kasinathan *et al.* (2014)** reported that the aqueous extract of fruit of *Aegle marmelos* was able to decrease the expression level of pro-inflammatory cytokine IL-6 in dextran sodium sulfate induced acute colitis in mice.

Table 4.24: Calculation with the threshold cycle (C_t) values for determining expression of IL-6 in Con A stimulated T lymphocytes after treatment of AME

Treatments	C_t value of Actin	C_t value of IL-6	ΔC_t value(C_t IL-6- C_t actin)	$\Delta\Delta C_t$ (ΔC_t treatment- ΔC_t Control)	Fold expression $2^{-\Delta\Delta Ct}$ relative to expression of control
Control	23.43	28.90	5.47	0	1
DEXA	22.04	29.85	7.81	2.34	0.2227*
AME	23.32	31.12	7.80	2.33	0.2001*
CD (1%)		CD (5%)		SE(m)±	
0.231		0.153		0.0442	

* **Significant at P<0.05**

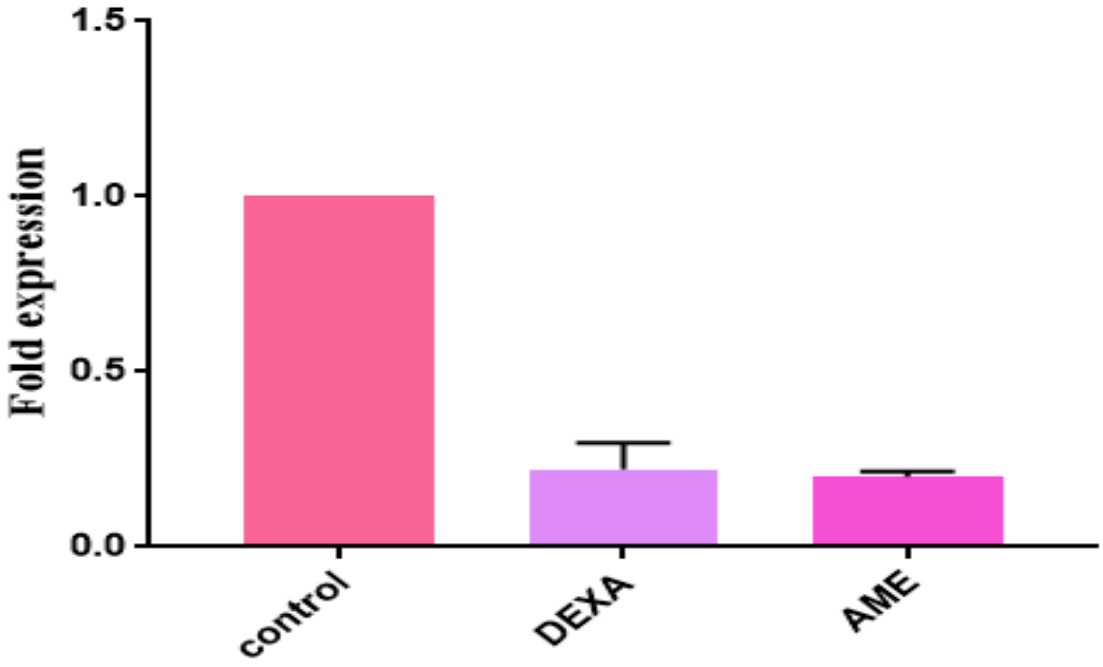


Figure 4.32: Expression analysis of IL-6 in Con A stimulated T lymphocytes after AME treatment

4.8.5 Quantification of expression level of IL-10 gene transcript in chicken lymphocytes after AME exposure through real time PCR.

Interleukin 10 was also known as cytokine synthesis inhibiting factor because it inhibits the synthesis of IFNγ form Th1 cells. Various cells of immune response including macrophages, monocytes, T cell subset (Th1, Th2, Treg cells) can produce

IL-10. IL-10 is known as anti-inflammatory cytokine because it inhibits the pro inflammatory cytokines, chemokines and antigen presenting cells which are involved in inflammation. IL-10 shows anti-inflammatory response by affecting the transcription factor NF-kB. It blocks the nuclear translocation or binding of factor to DNA **(Clarke *et al.*, 1998)**.

Interleukin 10 mainly affects the lymphocytes and antigen presenting cells. It regulates the balance between Th1 and Th2 cells. IL-10 suppresses the cellular immunity by decreasing the antigen presentation, proliferation of T cells and expression level of IL-2, IL-4 produced by T cells. It weakly affects the B cells by enhancing the differentiation of B cell into plasma cells and involves in class switching. IL-10 and IL-4 together inhibits the production of IgE but induces the production of IgG **(Asadullah *et al.*, 2003)**. The expression pattern of IL-10 was analysed after the treatment of AME to chicken lymphocytes as presented in **Figure 4.33**.

The expression pattern of IL-10 was analysed after the treatment of AME to chicken lymphocytes as presented in **Table 4.25** and **Figure 4.34**. Cells without any treatment were taken as normal control and treatment with Dexamethasone at maximum non cytotoxic dose was as positive control. In control cells expression was taken as 1 fold. Dexamethasone treated cells showed significant increase in the expression of IL-10 as compared to normal control and AME treated cells.

Table 4.25: Calculation with the threshold cycle (C_t) values for determining expression of IL-10 after treatment of AME in chicken lymphocytes

Treatments	C_t value of Actin	C_t value of IL-10	ΔC_t value(C_t IL-10 - C_t actin)	$\Delta\Delta C_t$ (ΔC_t treatment- ΔC_t Control)	Fold expression $2^{-\Delta\Delta Ct}$ relative to expression at control
Control	22.52	35.42	12.90	0	1
DEXA	23.82	25.53	1.71	-11.20	2347.30*
AME	22.54	24.51	1.97	-10.94	1960.20*
CD (1%)		**CD (5%)**		**SE(m)±**	
84.743		56.108		16.236	

* **Significant at P<0.05**

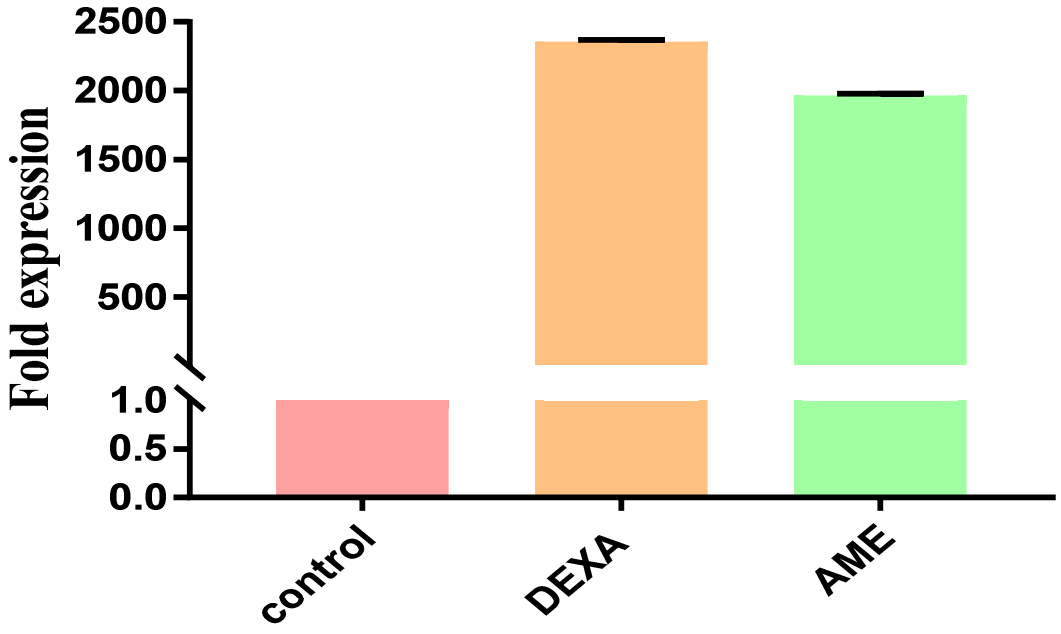


Figure 4.34: Expression analysis of IL-10 after AME treatment in chicken lymphocytes

LPS stimulated B and Con A stimulated T cells when treated with AME and DEXA showed increase in expression level of IL-10 as compared to respective controls. **Table 4.26** and **Figure 4.35** represents expression pattern of IL-10 in LPS stimulated B cells. LPS stimulated B cells were taken as control and effect of AME and DEXA treatments was determined by comparing the expression of IL-10 among group. Both AME and DEXA treated cells showed increase in expression of IL-10.

Table 4.26: Calculation with the threshold cycle (C_t) values for determining expression of IL-10 in LPS stimulated B lymphocytes after treatment of AME

Treatments	C_t value of Actin	C_t value of IL-10	ΔC_t value(C_t IL-10 - C_t actin)	$\Delta\Delta C_t$ (ΔC_t treatment- ΔC_t Control)	Fold expression $2^{-\Delta\Delta Ct}$ relative to expression of control
Control	25.77	34.15	8.38	0	1
DEXA	24.56	26.11	1.55	-6.83	115.204*
AME	23.15	25.61	2.46	-5.92	62.188*
CD (1%)		CD (5%)		SE(m)±	
49.253		32.610		9.436	

* **Significant at P<0.05**

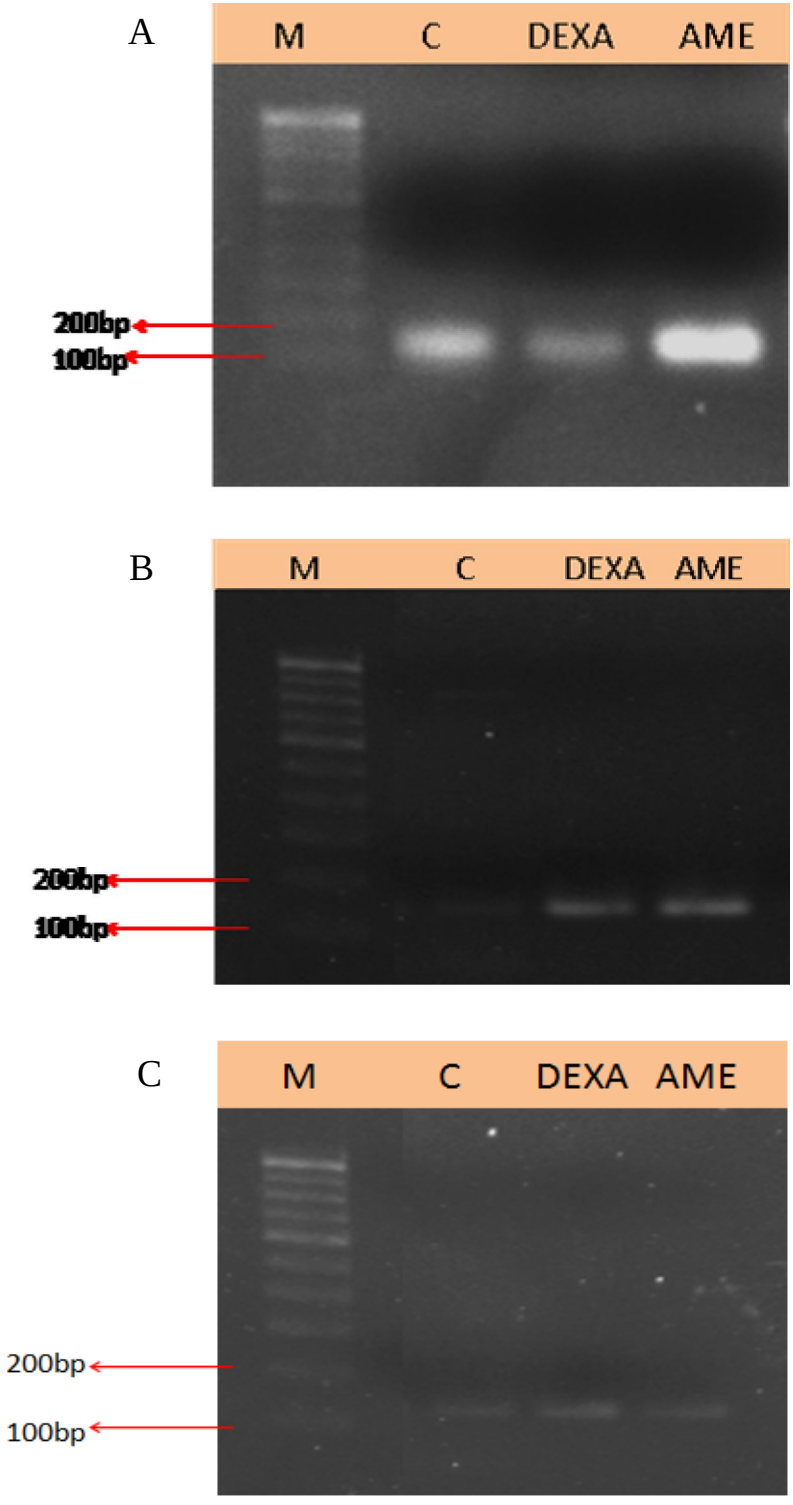


Figure 4.33: Expression analysis of IL-10 of AME treated chicken lymphocytes

A. Lane 1: marker 100 bp ladder, Lane 2: untreated cell as control, Lane 3: DEXA, Lane 4: AME

B. expression analysis of IL-10 in LPS stimulated B lymphocytes after AME treatment Lane 1: marker 100 bp ladder, Lane 2: control, Lane 3: DEXA, Lane 4: AME

C. expression analysis of IL-10 in Con A stimulated T lymphocytes after AME treatment Lane 1: marker 100 bp ladder, Lane 2: control, Lane 3: DEXA, Lane 4: AME

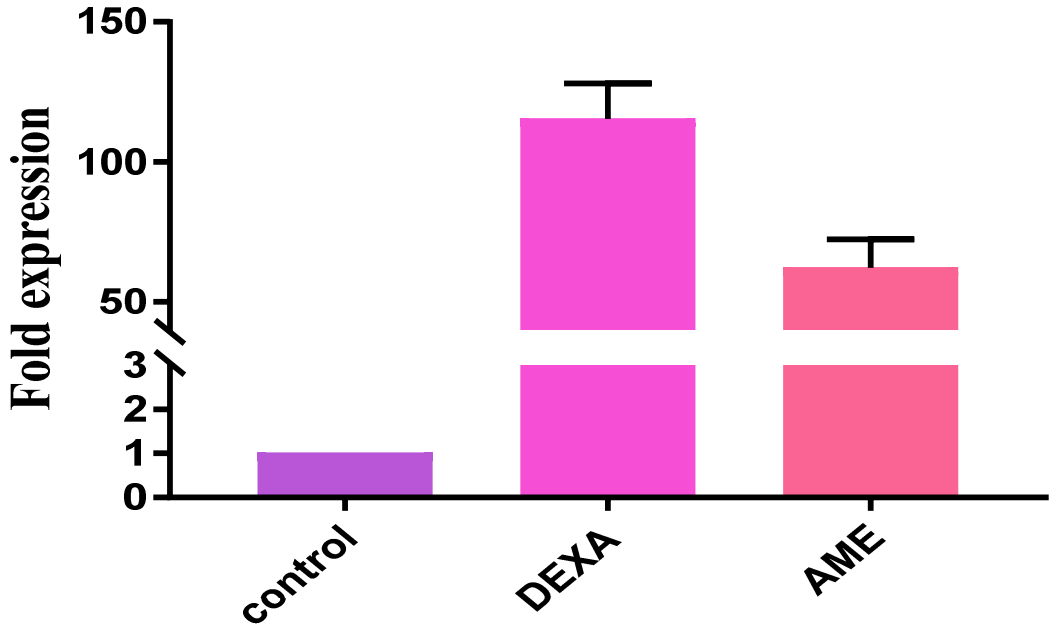


Figure 4.35: Expression analysis of IL-10 in LPS stimulated B lymphocytes after AME treatment

In Con A stimulated T cells expression pattern of IL-10 was represented in **Table 4.27** and **Figure 4.36**. Con A stimulated T cells were taken as control and effect of AME and DEXA treatment was determined.

Table 4.27: Calculation with the threshold cycle (C_t) values for determining expression of IL-10 in Con A stimulated T lymphocytes after treatment of AME

Treatments	C_t value of Actin	C_t value of IL-10	ΔC_t value(C_t IL-10 - C_t actin)	$\Delta\Delta C_t$ (ΔC_t treatment- ΔC_t Control)	Fold expression $2^{-\Delta\Delta Ct}$ relative to expression of control
Control	20.49	27.99	7.17	0	1
DEXA	22.23	24.23	2.16	-5.12	35.918*
AME	23.90	26.97	3.17	-4.10	17.349*
CD (1%)		CD (5%)		SE(m)±	
21.066		13.947		4.036	

* **Significant at P<0.05**

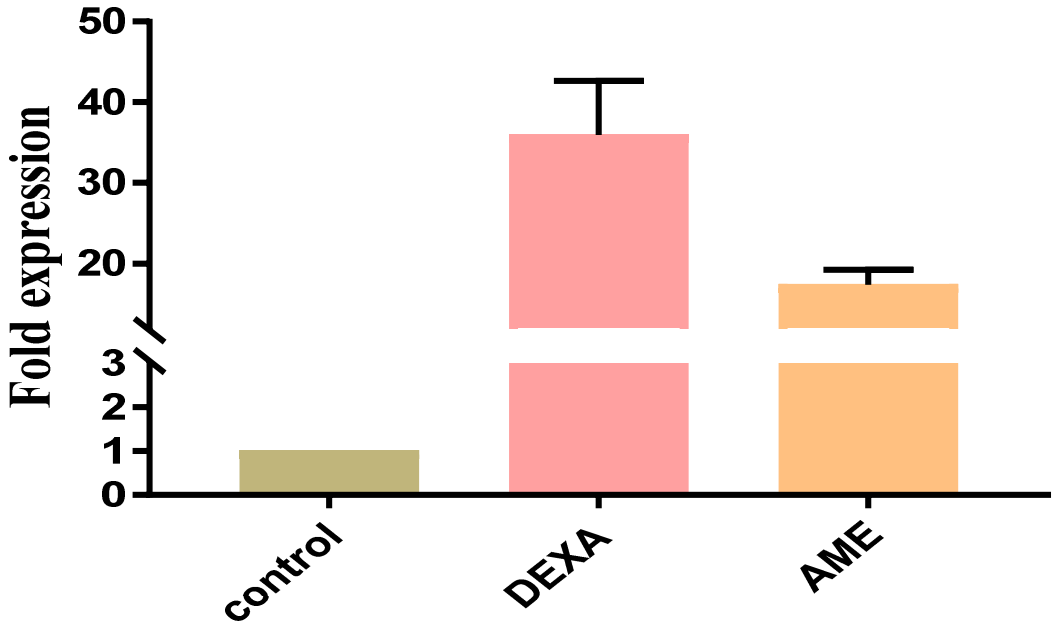


Figure 4.36: Expression analysis of IL-10 in Con A stimulated T lymphocytes after AME treatment

AME and DEXA treated cells showed significant increase in expression of IL-10 in comparison to control. As observed through LPA, AME showed decrease in proliferation of T cell. Overall increase in expression of IL-10 was observed after treatment of AME in all three groups. **Rathee *et al.* (2017)** reported in their study that hydromethanolic extract of leaves of *Aegle marmelos* at 100mg/kg showed significant increase in level of Interleukin-10 in wistar albino rats.

4.8.6 Quantification of expression level of iNOS gene transcript in chicken macrophages enriched cells after AME treatment through real time PCR.

Nitric oxide (NO) is produced by induced macrophages at high levels. The enzyme involved in (NO) production is inducible nitric oxide synthase (iNOS). Macrophages show their cytotoxicity by nitric oxide production. Nitric oxide act as free radical and interact with other radicals to show its effects. Nitric oxide inhibits various enzymes by binding with metal ion (iron) present at catalytic part of enzyme. At higher concentration, nitric oxide directly interacts with DNA of target cell and causes its fragmentation **(Forstermann and Sessa 2011)**. The expression pattern of iNOS was analysed after the treatment of AME in macrophages rich cells as presented in **Figure 4.37**.

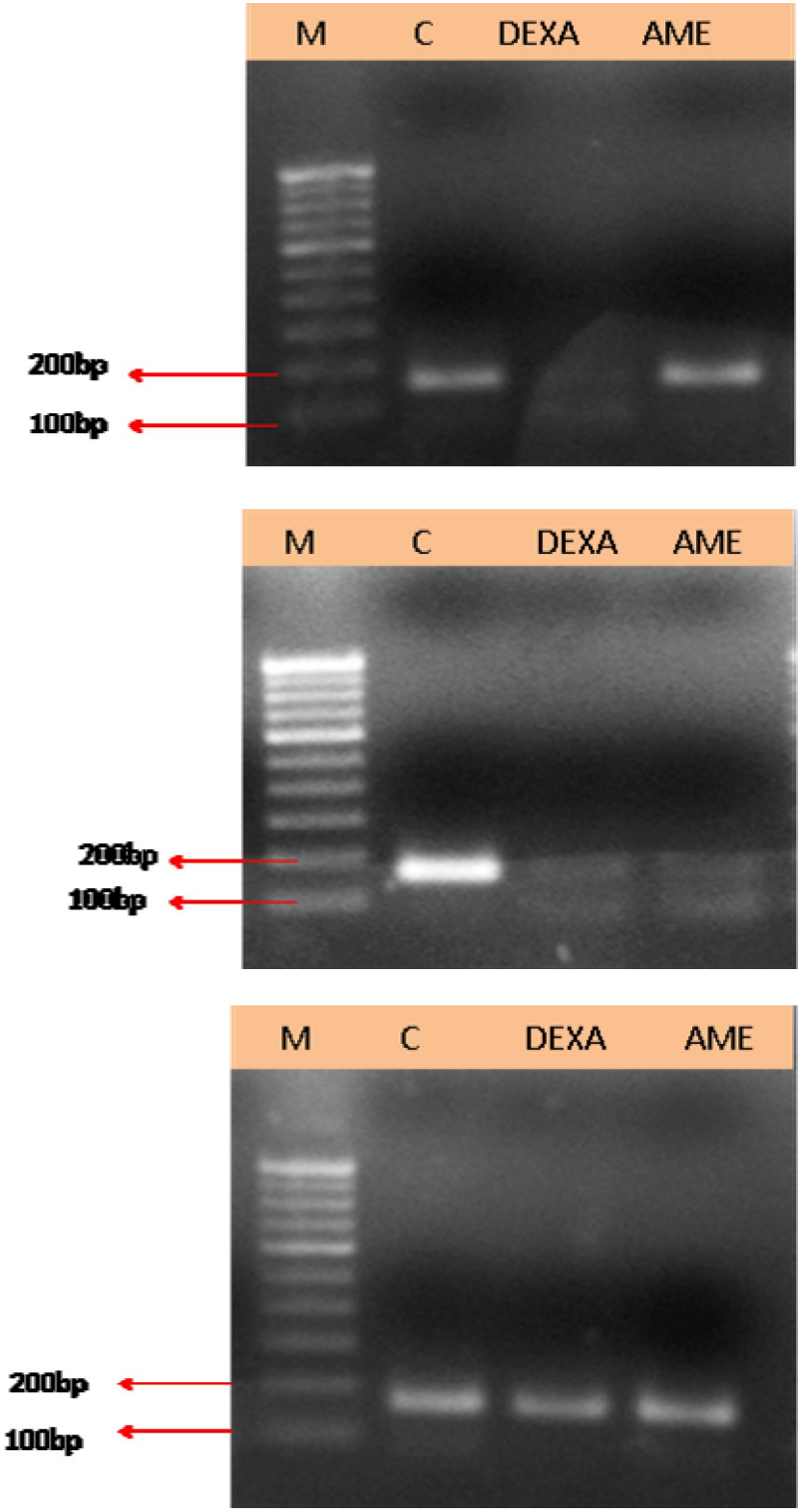


Figure 4.37: Expression analysis of iNOS of AME treated chicken macrophages

A. Lane 1: marker 100 bp ladder, Lane 2: untreated macrophages enriched cell as control, Lane 3: DEXA, Lane 4: AME

B. expression analysis of iNOS in LPS stimulated B lymphocytes after AME treatment Lane 1: marker 100 bp ladder, Lane 2: control, Lane 3: DEXA, Lane 4: AME

C. expression analysis of iNOS in Con A stimulated T lymphocytes after AME treatment Lane 1: marker 100 bp ladder, Lane 2: control, Lane 3: DEXA, Lane 4: AME

Table 4.28: Calculation with the threshold cycle (C_t) values for determining expression of iNOS after treatment of AME in chicken macrophages enriched cells

Treatments	C_t value of Actin	C_t value of iNOS	ΔC_t value(C_t iNOS- C_t actin)	$\Delta\Delta C_t$ (ΔC_t treatment- ΔC_t Control)	Fold expression $2^{-\Delta\Delta Ct}$ relative to expression at control
Control	22.83	31.57	8.74	0	1
DEXA	21.43	32.70	11.27	2.52	0.174*
AME	22.17	33.52	11.34	2.6	0.165*

CD (1%)	CD (5%)	SE(m)±
0.0428	0.0283	0.008

* **Significant at P<0.05**

The expression pattern of iNOS was analysed after the treatment of AME to chicken lymphocytes as presented in **Table 4.28** and **Figure 4.38**. Macrophage rich population without any treatment was taken normal control and treatment with Dexamethasone at maximum non cytotoxic dose was positive control. In control cells expression was taken as1 fold. Positive control showed significant decrease in expression as compared to control, AME treated cells also showed significant decrease in expression in comparison to control.

LPS stimulated B and Con A stimulated T cells rich population when treated with AME and DEXA showed decrease in expression level of iNOS as compared to their respective controls. **Table 4.29** and **Figure 4.39** represents expression pattern of iNOS in LPS stimulated B cells. LPS stimulated B cells were taken as control and effect of AME and DEXA treatment was determined. The AME and DEXA showed significant decrease in expression of iNOS in comparison to control. AME treated cells showed mere decrease in expression of iNOS in comparison to DEXA. In Con A stimulated T cells expression pattern of iNOS is represented in **Table 4.30** and **Figure 4.40**. Con A stimulated T cells were taken as control and effect of AME and DEXA treatment was determined. The DEXA and AME treated cells showed significant decrease in expression of iNOS in comparison to control cells.

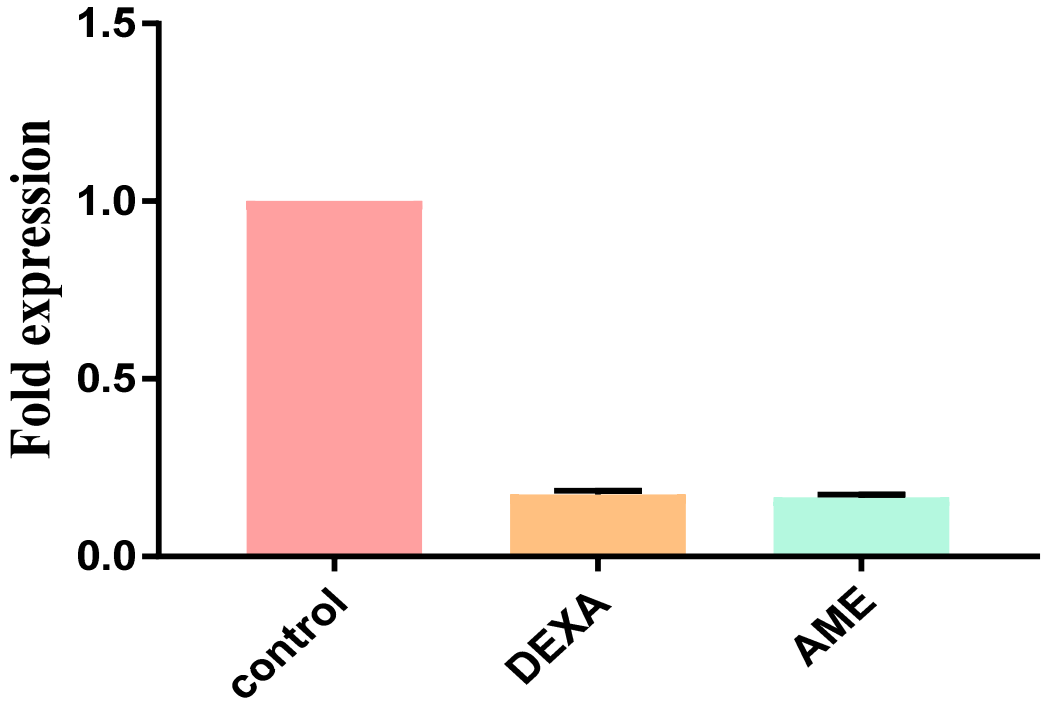


Figure 4.38: Expression analysis of iNOS in macrophages enriched cells after AME treatment

Table 4.29: Calculation with the threshold cycle (C_t) values for determining expression of iNOS in LPS stimulated B lymphocytes after treatment of AME

Treatments	C_t value of Actin	C_t value of iNOS	ΔC_t value(C_t iNOS - C_t actin)	$\Delta\Delta C_t$ (ΔC_t treatment- ΔC_t Control)	Fold expression $2^{-\Delta\Delta Ct}$ relative to expression of control
Control	21.17	29.37	8.19	0	1
DEXA	22.01	34.28	12.26	0.407	0.062*
AME	23.09	36.21	13.12	4.925	0.034*

CD (1%)	CD (5%)	SE(m)±
0.047	0.031	0.009

* **Significant at P<0.05**

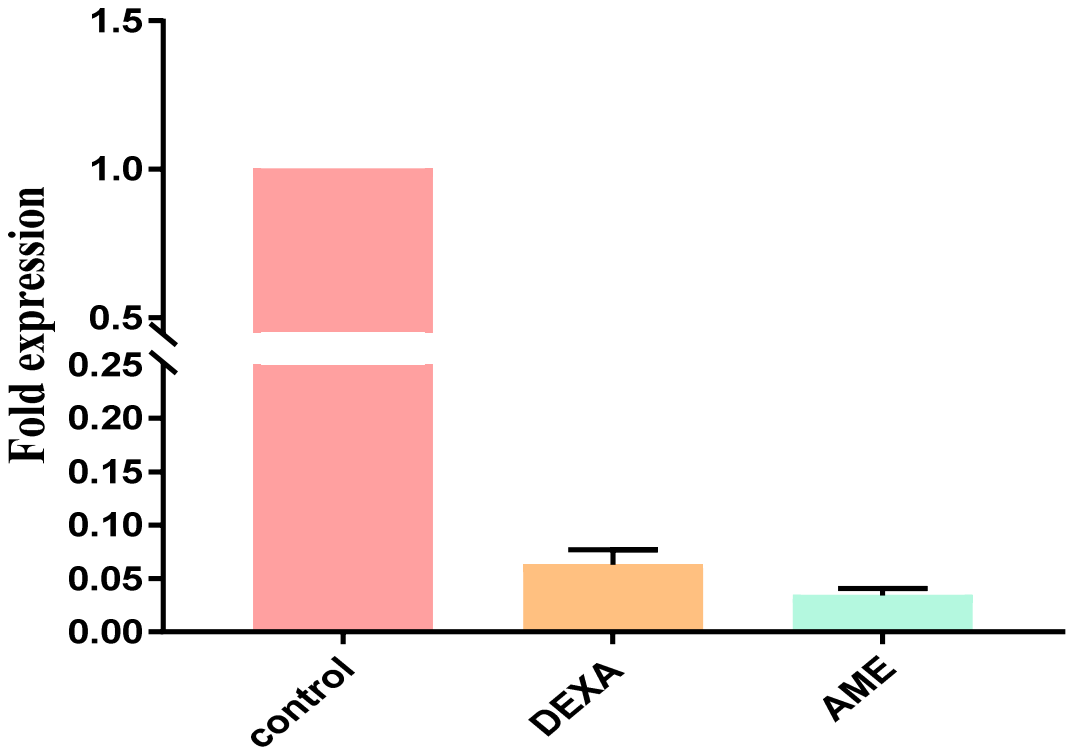


Figure 4.39: Expression analysis of iNOS in LPS stimulated B lymphocytes after AME treatment

Table 4.30: Calculation with the threshold cycle (C_t) values for determining expression of iNOS in Con A stimulated T lymphocytes after treatment of AME

Treatments	C_t value of Actin	C_t value of iNOS	ΔC_t value(C_t iNOS- C_t actin)	$\Delta\Delta C_t$ (ΔC_t treatment- ΔC_t Control)	Fold expression $2^{-\Delta\Delta Ct}$ relative to expression of control
Control	20.91	29.31	8.39	0	1
DEXA	21.80	32.00	10.23	1.83	0.2834*
AME	22.36	34.24	11.58	3.19	0.1103*

CD (1%)	CD (5%)	SE(m)±
0.101	0.067	0.0194

* **Significant at P<0.05**

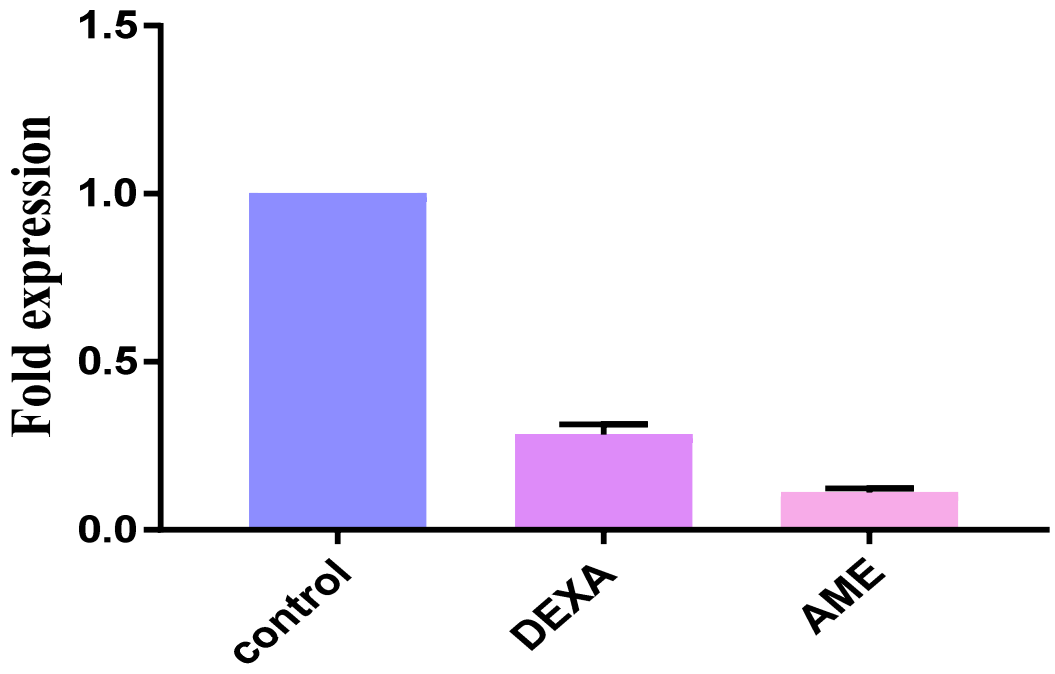


Figure 4.40: Expression analysis of iNOS in Con A stimulated T lymphocytes after AME treatment

Overall significant decrease in the expression of iNOS in AME treated cells was observed in comparison to control was observed. It was also observed in Nitric oxide estimation assay, the treatment with AME showed decrease in content of NO which can be due to decrease in expression of iNOS. Various flavonoids present in extract could be a reason for decrease in expression of iNOS. **Kim *et al.* (2004)** reported that various flavonoids (Genistein, derivative of flavonoids apigenin, quercetin, and morin) can inhibit the production NO by reducing the expression of iNOS in LPS induced condition.

Ozaki *et al.* (2010) reported that dexamethasone showed decrease in expression of iNOS in hepatocytes of Wister rats. The decrease in the expression iNOS was due to dexamethasone induced decrease in stabilization of mRNA of iNOS. Inspite of best efforts none of the reports were found in the searched litrature on inducible NOS expression analysis in chicken splenocytes after treatment of *Aegle marmelos* extract.

Overall study showed that AME have the significant immunosuppressive and anti-inflammatory activity in chicken lymphocytes. AME down regulate the expression

of proinflammatory cytokine IL-6 and inducible NOS and significantly up regulate the expression of anti-inflammatory cytokine IL-10 in chicken lymphocytes. **Al-Harbi *et al.* (2016)** reported that dexamethasone showed dose dependent relationship with expression of proinflammatory and anti-inflammatory cytokines. Decreased in the expression of IL-6 and inducible NOS in LPS induced lung injury in mice and increase in expression of anti-inflammatory cytokine IL-10 was also observed after dexamethasone treatment.

Summary and Conclusion

Chapter 5 SUMMARY AND CONCLUSION

Aegle marmelos (L.) Corr. belongs to *Rutaceae* family, commonly known as bael. Being native to Indian subcontinent and Southeast Asia, it is found in every part of India. It is one of sacred trees of the Hindus. Every part of the tree including leaves, bark, stem, root, fruit and flower is used for their medicinal value. Traditionally leaves of the tree were used to treat inflammation, asthma, hypoglycemia etc.

Leaves of *Aegle marmelos* was collected and authenticated. Aqueous extract of leaves of the plant (AME) was prepared. AME was analyzed for presence of various phytochemicals, both qualitatively and quantitatively. Extract was tested for its antioxidative potential through DPPH assay as well as NO radical scavenging assay. Immunomodulatory potential of the aqueous extract was evaluated in chicken lymphocytes primary cell culture system. AME treated chicken lymphocytes were evaluated for their antioxidative status in comparison to control cell through various assays *viz.* Membrane Lipid peroxidation (LPO), GSH (Reduced Glutathione), SOD (Superoxide dismutase), CAT (Catalase) assays and Nitric oxide (NO) estimation. Cytokine modulations (IL-6, Il-10) and iNOS expression of chicken lymphocytes due to exposure of AME were assessed through real time PCR.

The extraction yield of the aqueous extract of *Aegle marmelos* leaves was found to be 14.01%. Presence of various phytochemicals was revealed in extract which includes phenols, flavonoids, tannins, saponins, phytosterols and alkoids etc. Phenolics and flavonoids content in the extract was found to be 480 GAE mg/g and 342.70 RE mg/g respectively. Higher phenolic and flavonoids content was present in the extract which represent the higher antioxidative activity of the extract.

Maximum non cytotoxic dose (MNCD) of AME and dexamethasone (DEXA) was determined in chicken lymphocytes. Dose dependent reduction in cell viability was observed in the extract and dexamethasone at higher concentration then MNCD *i.e.* 75µg/ml and 30ng/ml, respectively. MTT assay revealed that AME and DEXA showed cytotoxic activity at 100µg/ml and 35ng/ml, respectively which is determined as minimum cytotoxic dose (MCD). Maximum non cytotoxic dose of 75µg/ml and 30ng/ml for AME and DEXA were chosen to explore the immunomodulatory and antioxidative effect in chicken lymphocytes.

AME displayed decrease of 6.20% and 15.5% % in T cell proliferation in case of Con A and PHA stimulated cells respectively. There was 9.23% decrease in B cell proliferation in case of LPS stimulated cells as compared to control untreated cells. Dexamethasone also shows decrease in T and B lymphocytes. DEXA displayed decrease of 11.36% and 18.50% in T cell proliferation in case of Con A and PHA stimulated cells, respectively. There was 12.30% decrease in B cell proliferation in case of LPS stimulated cells in comparison to control and AME.

AME displayed antioxidative activity in DPPH and NO radical scavenging assay. IC_{50} value of extract was calculated to be 233.11 µg/ml and 113.80µg/ml in DPPH and NO radical scavenging assay respectively. Lower IC_{50} value represents the higher antioxidative potential. After the *in-vitro* exposure of MNCD of AME in chicken lymphocytes antioxidative status was determined through Membrane lipid peroxidation, intracellular GSH levels, SOD levels, Catalase activity and nitric oxide content. The level of membrane lipid peroxidation and NO was significantly decreased after AME treatment but there was significant increase level of intracellular GSH, SOD and catalase activity. Increase in level of GSH, SOD and decrease in LPO, NO was considered to be indicative of antioxidative potential of the extract. All the assays conducted confirmed that AME have significant antioxidative potential.

AME exposed chicken lymphocytes were used for RNA isolation. RNA of good quality and quantity was utilized for cDNA preparation. cDNA used as template for expression analysis of cytokines and iNOS in real time PCR. Expression of IL-6, IL-10 and iNOS was evaluated after AME treatment at maximum non cytotoxic dose. AME significantly decreased the expression of proinflammatory cytokine IL-6 and inducible NOS which are involved in inflammatory response. Expression of IL-10 was significantly increased which is an anti-inflammatory and immunosuppressive cytokine. This could be the reason for immunosuppressive attribute of AME.

From above finding it can be concluded that:

- The aqueous leaves extract of *Aegle marmelos* have various phytochemicals present in it, which are responsible for the antioxidative activity specially presence of high phenolics and flavonoids contents in AME.
- AME showed immunosuppressive activity by significantly suppressing the proliferation of T and B lymphocytes. *In vitro* lymphocytes culture system is a quick, convenient and ethical system for such preliminary screening.

- The extract of *Aegle marmelos* showed significant antioxidative potential as observed through DPPH and NO radical scavenging assay as well as in LPO, GSH, SOD, Catalase assays and Nitric Oxide estimation carried out in AME treated chicken lymphocytes in comparison to control untreated cells.
- Expression analysis of cytokines was carried out in the MNCD of AME treated chicken lymphocytes. AME treated cells showed significant decrease in the expression of pro inflammatory cytokine (IL-6 and iNOS) and increase in the expression of anti-inflammatory cytokine (IL-10) in comparison to untreated cells

In consideration to fact that extract shows immunosuppressive, anti-inflammatory and antioxidative activity, it should be explored more for development of plant based preventive and therapeutic drugs. However plant should be evaluated for further pharmacological effects in suitable *in vivo* and *in vitro* system. Similar analysis could be used for delineating the molecular mechanism responsible for immunomodulatory properties of plant and validating the immunosuppressive and anti-inflammatory properties of AME.

Literature Cited

LITERATURE CITED

Aebi H. (1984). Catalase in vitro. In *Methods in enzymology* 105(2):121-126.

Agarwal S. K. and Marshall Jr, G. D. (2001). Dexamethasone promotes type 2 cytokine production primarily through inhibition of type 1 cytokines. *Journal of Interferon & Cytokine Research*, 21(3): 147-155.

Ahmed A. S., Elgorashi E. E., Moodley N., McGaw L. J., Naidoo V. and Eloff, J. N. (2012). The antimicrobial, antioxidative, anti-inflammatory activity and cytotoxicity of different fractions of four South African Bauhinia species used traditionally to treat diarrhoea. *Journal of ethnopharmacology*, 143(3): 826-839.

Alam M. B., Hossain M. S., Chowdhury N. S., Asadujjaman M., Zahan R., Islam M. M., Mazumder M. E. H., Haque M. E. and Islam A.(2011). Antioxidant, Anti-inflammatory and Anti-pyretic Activities of *Trichosanthes dioica Roxb*. Fruits. *Journal of Pharmacology and Toxicology*, 6 (5): 440-453.

Al-Harbi N. O., Imam F., Al-Harbi M. M., Ansari M. A., Zoheir K. M., Korashy H. M., Sayed-Ahmed M.M., Attia S.M., Shabanah O.A. and Ahmad S. F. (2016). Dexamethasone attenuates LPS-induced acute lung injury through inhibition of NF-κB, COX-2, and pro-inflammatory mediators. *Immunological investigations*, 45(4): 349-369.

Amirghofran Z, Bahmani M, Azadmehr A, Javidnia K, Ramazani M and Ziaei A(2010). Effect of *Salvia mirzayanii* on the immune system and induction of apoptosis in peripheral blood lymphocytes. *Natural Product Research*, 24(6): 500-508.

Anand P., Kunnumakkara A. B., Newman R. A. and Aggarwal B. B. (2007). Bioavailability of curcumin: problems and promises. *Molecular pharmaceutics*, 4(6): 807-818.

Arai K., Lee F., Miyajima A. (1990). Cytokines: Coordinators of immune and in inflammatory responses. *Annual Review of Biochemistry*, 59:783-836.

Archana J. S., Paul R. and Tiwari A. (2011). Indian Medicinal Plants: A rich source of natural immuno modulator. *International Journal of Pharmacology*, 7(2): 198-205.

Arokiyaraj S., Perinbam K., Agastian P. and Balaraju K. (2007). Immunosuppressive effect of medicinal plants of Kolli hills on mitogen-stimulated proliferation of the human peripheral blood mononuclear cells in vitro. *Indian Journal of Pharmacology*, 39(4): 180-183.

Asadullah K., Sterry W. and Volk H. D.(2003). Interleukin-10 therapy-review of a new approach. *Pharmacological reviews*, 55(2): 241-269.

Asif M., Jenkins K. A, Hilton L. S., Kimpton W. G, Bean A. G.D. and Lowenthal J. W.(2004). Cytokines as adjuvants for avian vaccines. *Immunology and Cell Biology*, 82: 638–643.

Bagchi K. and Puri S. (1998). Free radicals and Antioxidants in health and disease. *La Revue de Sante de la mediterranee orientale*, 4(2): 350-360.

Baliga M.S., Bhat H.P., Joseph N., Fazal F.(2011). Phytochemistry and medicinal uses of bael fruit (*Aegle Marmelos Correa*): A concise review. *Food research International*, 44 :1768-1775.

Banchereau J. and Steinman R.M. (1998). Dendritic cells and the control of immunity. *Nature*, *392*(6673): *245*-252.

Belardelli F. (1995). Role of interferons and other cytokines in the regulation of the immune response. APMIS 103: 161–179.

Bharavi K., Reddy A.G., Rao G. S., Reddy A.R., Rao S. V. (2010). Reversal of cadmium induced oxidative stress in chicken by herbal adptogen *Withania somnifera* and *Ocimun sanctum*, *International Journal of Toxicology*, 17(2): 159-163.

Biagiotti S., Menotta M., Orazi S., Spapperi C., Brundu S., Fraternale A., Bianchi M., Rossi L., Chessa L. and Magnani M. (2016). Dexamethasone improves redox state in ataxia telangiectasia cells by promoting an NRF2 mediated antioxidant response. *The FEBS journal*, 283(21): 3962-3978.

Bijjiga E. and Martino A. T. (2013). Interleukin 10 (IL-10) Regulatory Cytokine and its Clinical Consequences. *Journal of Clinical & Cellular Immunology.*

Briskin D. P. (2000). Medicinal Plants and Phytomedicines. Linking Plant Biochemistry and Physiology to Human Health. *Plant Physiology*, 124: 507-514.

Bristy N. J., Hasan A. N., Alam M. N., Wahed T. B., Roy P. and Alam K. K. (2017). Characterization of antioxidant and cytotoxic potential of methanolic extracts of different parts of *Aegle marmelos* (L.). *International Journal of Pharmaceutical Sciences and Research*, 8(3):1476-1484.

Castiblanco C., Foster C. S. (2014). Review of Systemic Immunosuppression for Autoimmune Uveitis. *Ophthalmol Therapy*, 3(2):17–36.

Ceriello A. (2000). Oxidative Stress and Glycemic Regulation. *Metabolism*, 49(2): 27-29.

Chandraker A., Azuma H., Nadeau K., Carpenter C. B., Tilney N. L., Hancock W. W., and Sayegh M. H. (1998). Late blockade of T cell costimulation interrupts progression of experimental chronic allograft rejection. *The Journal of clinical investigation*, 101(11): 2309-2318.

Chauhan A. and Agarwal M. (2009). Assement of contaspective efficency of aqueous extract of *Aegle Marmols corr. L* in male albino rats. *Human fertility*, 12: 107-118.

Chomczynski P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*, 15(3): 532-534.

Clarke C. J., Hales A., Hunt A. and Foxwell B. M. (1998). IL□10□mediated suppression of TNF□α production is independent of its ability to inhibit NFκB activity. *European journal of immunology*, 28(5): 1719-1726.

Coutinho A. E. and Chapman K. E. (2011). The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Molecular and Cellular Endocrinology*. 335(1):2–13.

Creed T. J., Lee R. W., Newcomb P. V., Mambro A. J., Raju M. and Dayan C. M. (2009). The effects of cytokines on suppression of lymphocyte proliferation by dexamethasone. *The Journal of Immunology*, 183(1): 164-171.

Crozier A., Jaganath I. B., and Clifford M. N. (2009). Dietary phenolics: Chemistry, bioavailability and effects on health. *Natural Product Reports,* 26(8):1001–1043.

Das K., Tiwari R.K.S., Shrivastava D.K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: current methods and future trends. *Journal of medicinal plants Research*, 4(2): 104-111.

Devasagayam T.P.A., Sainis K. B. (2002). Immune system and antioxidants especially those derived from Indian medicinal plants. *Indian journal of experimental biology*, 40: 639-655.

Dhama K., Latheef S.K., Mani S., Samad H. A., Karthik K., Tiwari R., Khan R.U., Alagawany M.(2015). Multiple Beneficial Application and Modes of Action of Herbs in Poultry Health and Production: A review. *Internaataional Journal of Pharmacology*, 11 (3): 152-176.

Dickinson D. A. and Forman H. J. (2002). Cellular glutathione and thiols metabolism. *Biochemical pharmacology*, 64(5-6): 1019-1026.

Dinis-Oliveira R. J., Duarte J. A., Remiao F., Sanchez-Navarro A., Bastos M. L. and Carvalho F. (2006). Single high dose dexamethasone treatment decreases the pathological score and increases the survival rate of paraquat-intoxicated rats. *Toxicology*, 227(1-2):73-85.

Djordjevic V. B. (2004). Free radicals in cell biology. *International review of cytology*, 237: 57-91.

Edreva A., Velikova V., Tsonev T., Dagnon S., Gürel A., Aktaş L. and Gesheva, E. (2008). Stress-protective role of secondary metabolites: diversity of functions and mechanisms. *General and Applied Plant Physiology,* 34(2): 67-78.

Ellman G. L. (1959). Tissue sulfhydryl groups. *Archives of biochemistry and biophysics*, 82(1): 70-77.

Embuscado M. E. (2015). Spices and herbs: Natural sources of antioxidants–a mini review. *Journal of Functional Foods*, *18*: 811-819.

Forstermann U. and Sessa W.C. (2012). Nitric oxide synthases: regulation and function. *European Heart Journal*, 33(7): 829–837.

Foti M. C. (2007). Antioxidant properties of phenols. *Journal of Pharmacy and Pharmacology*, *59*(12): 1673-1685.

Freshney R. I. (2005). Cytotoxicity. Culture of animal cells.

Gabay C. (2006). Interleukin-6 and chronic inflammation. *Arthritis Research & Therapy*, 8(2), S3.

Gadani S. P., Cronk J. C., Norris G. T. and Kipnis J. (2012). IL-4 in the brain: a cytokine to remember. *The Journal of Immunology*, 189(9): 4213-4219.

Gaschler M. M. and Stockwell B. R.(2017). Lipid peroxidation in cell death. *Biochemical and biophysical research communications*, 482(3):419-425.

Gessani S., McCandless S., and Baglioni C. (1988). The Glucocorticoid Dexamethasone Inhibits Synthesis of Interferon by Decreasing the Level of Its mRNA. *The Journal of Biological Chemist*, 263(16): 7454-7457.

Ghosh A.K., Banerjee S., Mullick H.I., Banerjee J. (2011). Zingiber officinale: A natural gold. *International Journal of Pharma and Biosciences,* 2(1):283-294.

Gold M. E., Wood K. S., Byrns R. E., Fukuto J., and Ignarro L. J. (1990). NG-methyl-L-arginine causes endothelium-dependent contraction and inhibition of cyclic GMP formation in artery and vein. *Proceedings of the National Academy of Sciences*, *87*(12): 4430-4434.

Greenrod W. and Fenech M.(2003). The principal phenolic and alcoholic components of wine protect human lymphocytes against hydrogen peroxide- and ionizing radiation-induced DNA damage in vitro. *Mutagenesis.* 18(2):119–126.

Gupta A. and Chaphalkar S.R(2016). Immunosuppressive and Cytotoxic Potential of Flavonoids from *Mitragyna parvifolia, Mangifera indica* and *Aegle marmelos*. *Journal of Pharmacology and Toxicological Studies*, 4(1): 1-5.

Gupta A., Shaikh A. C. and Chaphalkar S. R. (2016). Exploration of Active Metabolites from *Aegle Marmelos* and *Emblica Officinalis* for Determining its Immunosuppressive Properties. *Journal of Progressive Research in Biology*. 3(1): 119-126.

Gupta V.K., Sharma S.K. (2006).Plants as Natural Antioxidants. *Natural product radiance*, 5(4): 326-344.

Halliwal B.(1995). Antioxidant characterization methodology and mechnaism. *Biochemical Pharmacology*, 49(10): 1341-1348,

Halliwell B. (2012). Free radicals and antioxidants: updating a personal view. *Nutrition reviews*, 70(5): 257-265.

Halliwell B. and Chirico S.(1993). Lipid peroxidation: its mechanism, measurement, and significance. *The American journal of clinical nutrition*, 57(5):715S-725S.

He F. (2011). Bradford Protein Assay. *Bio-protocol*, 1(6): e45.

Hsouna A. B., Trigui M., Mansour R. B., Jarraya R. M., Damak M. and Jaoua S. (2011). Chemical composition, cytotoxicity effect and antimicrobial activity of *Ceratonia siliqua* essential oil with preservative effects against Listeria inoculated in minced beef meat. *International Journal of Food Microbiology*, 148(1): 66-72.

Huang J.J. and Gaudio P.A. (2010). Ocular inflammatory disease and uveitis manual: diagnosis and treatment. Baltimore: Lippincott Williams & Wilkins.

Inoue M., Sato E. F., Nishikawa M., Park A. M., Kira, Y., Imada, I. and Utsumi, K. (2003). Mitochondrial generation of reactive oxygen species and its role in aerobic life. *Current medicinal chemistry*, 10(23):2495-2505.

Ithayaraja C. M. (2011). Mini-Review: Metabolic Functions and Molecular Structure of Glutathione Reductase. *International Journal of Pharmaceutical Sciences Review and Research,* 9(2):104-115.

Jagetia G. C., Venkatesh P. and Baliga, M. S. (2003). Evaluation of the radioprotective effect of *Aegle marmelos* (L.) Correa in cultured human peripheral blood lymphocytes exposed to different doses of γ□radiation: a micronucleus study. *Mutagenesis*, 18(4): 387-393.

Jagetia G.C., Rao S.K., Baliga M.C. and Babu K.S. (2004). The Evaluation of Nitric Oxide Scavenging Activity of Certain Herbal Formulations in vitro: A Preliminary Study. *Phytotherapy Research*. 18(4): 561–565.

Janossy G. and Greaves M. F. (1971). Lymphocyte activation: I. Response of T and B lymphocytes to phytomitogens. *Clinical and Experimental Immunology*, 9(4): 483.

José H. J., Berenice S. G. A. and Cecilia V. R. (1997). Induction of antioxidant enzymes by dexamethasone in the adult rat lung. *Life sciences*, *60*(23):2059-2067.

Kaiser P. (2010). Advances in avian immunology-prospects for disease control: a review. *Avian Pathology*, 39(5): 309-324.

Kamalakkannan N. and Prince P. (2004). Antidiabetic and anti-oxidant activity of Aegle marmelos extract in streptozotocin-induced diabetic rats. *Pharmaceutical biology*, 42(2): 125-130.

Kasinathan N. K., Subramaniya B. R., Pandian, I. and Sivasithamparam N. D.(2014). *Aegle marmelos* fruit extract abates dextran sodium sulfate induced acute colitis in mice: Repression of pro-inflammatory cytokines during colonic inflammation. *Biomedicine & Preventive Nutrition*, 4(2): 307-317.

Kesari A. N., Gupta R. K., Singh S. K., Diwakar S., and Watal G. (2006). Hypoglycemic and antihyperglycemic activity of *Aegle marmelos* seed extract in normal and diabetic rats. *Journal of ethnopharmacology*, 107(3): 374-379.

Khaligh F., Sadeghi G., Karimi A. and Vaziry A. (2011). Evaluation of different medicinal plant blends in diet for broiler chickens. Journal of Medicinal Plants Research, 5(10):1971-1977.

Khan S. MRCP, Sewell W.A. C. (2006).Oral immunosuppressive drugs. *Clinical Medicine*, 6 (4): 352-355.

Kim H. P., Son K. H., Chang H. W. and Kang S. S. (2004). Anti-inflammatory plant flavonoids and cellular action mechanisms. *Journal of pharmacological sciences*, 96(3): 229-245.

Kondratyuk T. P. and Pezzuto J. M.(2004). Natural product polyphenols of relevance to human health. *Pharmaceutical Biology*, *42*(1): 46-63.

Krushna G. S. S., Kareem M. A., Reddy V. D., Padmavathi P., Hussain S. A. and Kodidhela, L. D. (2011). *Aegle marmelos* fruit extract attenuates isoproterenol-induced oxidative stress in rats. *Journal of clinical biochemistry and nutrition*, 50(3): 199-204.

Kumar G., Srivastava A., Sharma S. K. and Gupta Y. K. (2012). Safety evaluation of an Ayurvedic medicine, Arogyavardhini vati on brain, liver and kidney in rats. *Journal of ethnopharmacology*, 140(1): 151-160.

Kumar A. S.(2016). Medicinal plants: Future source of new drugs. *International Journal of Herbal Medicine*, 4(4): 59-64.

Kumar D., Arya V., Kaur R., Bhat Z. A., Gupta V. K. and Kumar V. (2012). A review of immunomodulators in the Indian traditional health care system. *Journal of Microbiology, Immunology and Infection*, 45(3): 165-184.

Kumar S., Bodla R. B. and Bansal H.(2016). Antioxidant activity of leaf extract of *Aegle marmelos* Correa ex Roxb. *Pharmacognosy Journal*, *8*(5): 447-450.

Kumar V., Modi P.K. and Saxena K.K. (2013). Exploration of histoprotective activity of aqueous extract of the *Tinospora cordifolia* : An experimental study. *Asian Journal of Pharmaceutical and Clinical Research*, 6(2): 87-91.

Kumari K. D. K. P., Weerakoon T. C. S., Handunnetti S. M., Samarasinghe K. and Suresh T. S. (2014). Anti-inflammatory activity of dried flower extracts of *Aegle marmelos* in Wistar rats. *Journal of ethnopharmacology*, 151(3):1202-1208.

Lampronti I., Martello D., Bianchi N., Borgatti M., Lambertini E., Piva R., Jabbar S. Choudhuri M.S.K., Khan M. T. H., Gambari, R. (2003). In vitro antiproliferative effects on human tumor cell lines of extracts from the Bangladeshi medicinal plant *Aegle marmelos Correa*. *Phytomedicine*, *10*(4): 300-308.

Latheef S.K., Dhama K., Wani M.Y., Samad H. A., Barathidasan R.(2013). Ameliorate effect of four herbs (*Tinospora cordifolia, Azadirachta indica, Withana somnifera* and E care SE herbal) on the pathogenesis of infectious chicken anaemia virus. *International Journal of Current Research,* 5(8): 2327-2331.

Lavelle G. C., Sturman L., and Hadlow W. J. (1972). Isolation from mouse spleen of cell populations with high specific infectivity for scrapie virus. *Infection and immunity*, *5*(3): 319-323.

Lee K. W., Bode A. M. and Dong Z. (2011). Molecular targets of phytochemicals for cancer prevention. *Nature Reviews Cancer*, *11*(3): 211.

Lee K.W., Everts H., Kappert H.J., Freshner M., Losa R., Beynen A.C.(2003). Effects of dietary essential oils componenst on growth performance, digestive enzymes and lipid metabolism in female broiler chichkens. *British Poultry Sciences*, 44(3): 450-457.

Lee S.I., Kim B.S., Kim K.S., Lee S., Shin K.S., Lim J.S. (2008). Immunosuppressive activity of punicalagin via inhibition of NFAT activation. *Biochemical and Biophysical Research Communications,* 371(4): 799-803.

Levic J., Carbarkapa I., Todorovic G., Pavkov S., Sredanovic S., Cohil-galonja T. and Kostadinovic L.(2011). *In virto* antimicrobial activity of essential oil from plant family Lamiaceae. *Romanian biotechnological letters*, 16: 6034-6041.

Liang Y.C., Huang Y.T., Tsai S.H., Lin-Shiau S.Y., Chen C.F., Lin J.K. (1999). Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. *Carcinogenesis,* 20(10):1945–1952.

Madesh, M., Benard, O. and Balasubramanian, K. A. (1998). Glutathione modulates lipid composition of human colon derived HT-29 cells. *The International Journal of Biochemistry & Cell Biology*, 30(12): 1345-1352.

Mahady G.B., Gyllenhall C., Fong H.H, Farnsworth N.R. (2000). Ginsengs: a review of safety and efficacy. *Nutrition in Clinical Care*, 3:90-101.

Mahima A. R., Deb R. and Latheef S. K. HA Samad er al. (2012). Immunomodulatory and therapeutic potentials of herbal, traditional/indigenous and ethnoveterinary medicines. *Pakistan Journal of Biological Sciences*, 15: 754-774.

Maity P., Hansda D., Bandyopadhyay U., and Mishra D. K. (2009). Biological activities of crude extracts and chemical constituents of Bael, *Aegle marmelos* (L.) Corr. *Indian Journal of Experimental Biology*, 47(11):849-61.

Majeedi S. F., Roqaiya M., Jahan D., Khan A. A.(2015). Immunomodulatory herbs of Unani medicine: A review. *International Journal of Herbal Medicine*, 3(4): 19-21.

Makare N., Bodhankar S. and Rangari V. (2001). Immunomodulatory activity of alcoholic extract of *Mangifera indica L*. in mice. *Journal of Ethnopharmacology*, 78(3): 133-137.

Manandhar B., Paudel K. R., Sharma B, Karki R. (2017). Phytochemical profile and pharmacological activity of *Aegle marmelos Linn*. *Journal of Integrative Medicine*, 16(3): 153-163.

Mansour R. B., Gargouri B., Bouaziz M., Elloumi N., Jilani I. B., Ghrabi Z. and Lassoued, S. (2011). Antioxidant activity of ethanolic extract of inflorescence of Ormenis Africana in vitro and in cell cultures. *Lipids in health and disease*, 10(1): 78.

Marcocci L., Packer L., Droy-Lefaix M. T., Sekaki A. and Gardès-Albert M.(1994). Antioxidant action of Ginkgo biloba extract EGb 761. *Methods in enzymology,* 234: 462-475.

Martens S., Preuss A., and Matern U. (2010). Multifunctional flavonoid dioxygenases: flavonols and anthocyanin biosynthesis in *Arabidopsis thaliana* L. Phytochemistry 71: 1040–1049.

Mehrotra S., Mishra K.P., Maurya R., Srimal R.C., Yadav V.S., Pandey R., Singh V.K (2003). Anticellular and immunosuppressive properties of ethanolic extract of *Acorus calamus* rhizome. *International Immunopharmacology*, 3(1): 53-61.

Mehta M. and Shike M. (2014). Diet and physical activity in the prevention of colorectal cancer. *Journal of the National Comprehensive Cancer Network*, *12*(12): 1721-1726.

Mer M. and Richards G. A. (1998). Corticosteroids in life-threatening varicella pneumonia. *Chest*, 114(2): 426-431.

Mills S. and Bone K. (2000). Principle and practice of Phytotherapy modern herbal medicine. *Complementary medicine*, 34-37.

Mirshafiey A, Khorramizadeh MR, Saadat F, Rehm BH (2004). Chemopreventive effect of M2000, a new antiinflammatory agent. *Medical Science Monitor*, 10(10):105-109.

Moniruzzaman M., Rokeya B., Ahmed S., Bhowmik A., Khalil M. I. and Gan S. H. (2012). In vitro antioxidant effects of *Aloe barbadensis* Miller extracts and the potential role of these extracts as antidiabetic and antilipidemic agents on streptozotocin-induced type 2 diabetic model rats. *Molecules*, 17(11): 12851-12867.

Mounika N., Nazneen A., Shashikanth P., Prusty K. B. and Rao J. V.(2017). Evaluation of Hepatoprotective and Antioxidant activity of Root extracts of *Aegle Marmelos* in carbon tetrachloride induced Hepatotoxicity wistar albino rats. *International Journal of Pharmaceutical Sciences and Research*, 4113-4128.

Mujeeb F., Bajpai P. and Pathak N.(2014). Phytochemical evaluation, antimicrobial activity and determination of bioactive components from leaves of *Aegle marmelos*. *BioMed research international*, 1-11.

Müllberg J., Geib T., Jostock T., Hoischen S. H., Vollmer P., Voltz N, Heinz D., Galle P.R., Klouche M. and Rose-John S. (2000). IL-6 receptor independent stimulation of human gp130 by viral IL-6. *The Journal of Immunology*, 164(9): 4672-4677.

Murphy M. P. (1999). Nitric oxide and cell death. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1411(2-3), 401-414.

Nagarathna P.K.M, Reena K, Sriram Reddy, Johnson Wesley (2013). Review on Immunomodulation and Immunomodulatory Activity of Some Herbal Plants. *International Journal of Pharmaceutical Sciences Review and Research*, 22(1): 223-230.

Nahak G. and Sahu R. K.(2010). In vitro antioxidative acitivity of *Azadirachta indica* and *Melia azedarach* leaves by DPPH scavenging assay. *Nature and Science*, 8(4): 22-28.

Nathan C. (1992). Nitric oxide as a secretory product of mammalian cells. *The FASEB journal*, *6*(12):3051-3064.

Ncube N.S., Afolayan A.J., Okoh A.I. (2008). Assessment techniques of antimicrobial properties of natural compounds of plants origin: current methods and future trends. *African Journal of Biotechnology*, 7(12): 1797-1806.

Nimse S.B., Pal D.K. (2015).Free radicals, natural antioxidants and their reaction mechanisms. RSC Advances, 5: 27986 –2800.

Noori S., Naderi G.A., Hassan Z.M., Habibi Z., Bathaie S.Z., Hashemi S.M.(2004). Immunosuppressive activity of a molecule isolated from *Artemisia annua* on DTH responses compared with cyclosporin A. *International Immunopharmacology*, 4(10): 1301-1306.

Ohkawa H., Ohishi N. and Yagi K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, 95(2): 351-358.

Oppenheim J. J. (2001). Cytokines: past, present, and future. *International journal of hematology*, *74*(1): 3-8.

Orwa C., Mutua A., Kindt R., Jamnadass R. and Simons A. (2009). Agroforestree database: a tree species reference and selection guide version 4.0. *World Agroforestry Centre ICRAF, Nairobi, KE*.

Ozaki T., Habara K., Matsui K., Kaibori M., Kwon A. H., Ito S., Nishizawa M. and Okumura T. (2010). Dexamethasone inhibits the induction of iNOS gene expression through destabilization of its mRNA in proinflammatory cytokine-stimulated hepatocytes. *Shock*, *33*(1):64-69.

Padmanabhan P., Jangle S. N.(2012). Evaluation of DPPH Radical Scavenging Activity and Reducing Power of Four Selected Medicinal Plants and Their Combinations. *International Journal of Pharmaceutical sciences and Drug Research*, 4(2): 143-146.

Pant M., Ambwani T. and Umapathi V. (2012). Antiviral activity of Ashwagandha extract on infectious bursal disease virus replication. *Indian Journal of Science and Technology*, 5(5): 2750-2751.

Patil R. H., Kumar M. N., Kumar K. K., Nagesh R., Kavya K., Babu R. L., Ramesh G. T. and Sharma S. C. (2017). Dexamethasone inhibits inflammatory response via down regulation of AP-1 transcription factor in human lung epithelial cells. *Gene*, 645: 85-94.

Rahman A, Imran H, Iqbal L, STaqvi SI, Fatima N, Yaqeen Z.(2016). Dry and ripe fruit of *Aegle marmelos L*.: a potent source of antioxidant, lipoxygenase inhibitors and free radical scavenger. *Pakistan Journal of Pharmaceutical Sciences*, 29(4):1127–1131.

Rahman M.M., Uyangaa E., Han W. Y., Kim S.B., Kim J. H., Choi J. Y., Eo S.K.(2012). Ehancement of Th1- biased protective immunity against avian influneza H9N2 virus vial oral co-adminstration of attenuated salmonella enterica serovar typhimurium expressing chicken interferon α and interleukin-18 along with an inactivated vaccine. *BMC vaterinary research*, 8:105.

Rahman S. and Parvin, R. (2014). Therapeutic potential of *Aegle marmelos* (L.)-An overview. *Asian Pacific journal of tropical disease*, *4*(1): 71-77.

Raja W. W. and Khan S. H. (2017). Estimation of some phytoconstituents and evaluation of antioxidant activity in *Aegle marmelos* leaves extract. *Journal of Pharmacognosy and Phytochemistry*, *6*(1): 37-40.

Rajaram A., Vanaja G. R., Vyakaranam P., Rachamallu A., Reddy G. V., Anilkumar K., Arunasree K.M., Dhyani A., Prasad N.K., Sharma S. and Joshi M. C. (2017). Anti-inflammatory profile of *Aegle marmelos* (L) Correa (Bilva) with special reference to young roots grown in different parts of India. *Journal of Ayurveda and integrative medicine*, 9: 90-98.

Ramakrishna Y.G., Savithri K., Kist M. and Devaraj S.N. (2015). *Aegle marmelos* fruit extract attenuates Helicobacter pylori lipopolysaccharide induced oxidative stress in Sprague Dawley rats. *BMC Complementary and Alternative Medicine,* 15:375.

Rathee D., Kamboj A., Sachdev R. K. and Sidhu S. (2017). Hepatoprotective effect of *Aegle marmelos* augmented with piperine co-administration in paracetamol model. *Revista Brasileira de Farmacognosia*, 28(1): 65-72.

Reddy V. P. and Urooj A. (2013). Antioxidant properties and stability of *Aegle marmelos* leaves extracts. *Journal of food science and technology*, *50*(1):135-140.

Ricciotti E. and FitzGerald G. A. (2011). Prostaglandins and inflammation. *Arteriosclerosis, thrombosis, and vascular biology*, 31(5):986-1000.

Rincon M. (2012). Interleukin-6 from an inflammatory marker to target for inflammatory diseases. *Trends in immunology*, 33(11): 571-577.

Robbers J.E. and Tylor V.E. (2002). Herbs of choice: the therapeutic use of phytomedicines. *International Journal of Pharma Sciences*, 3(2): 199-203.

Roopashree T.S., Dang R., Rani RH. S. and Narendra C. (2008). Antibacterial activity of antipsoriatic herb: *Cassia tora*, *Momordica charantia* and *Calendula officinalis*. *International Journal of Applied Research in Natural Products*, 1(3): 20-28.

Rose N. R. and Friedman H. (1976). Manual of clinical immunology. *Manual of Clinical Immunology.*

Sabu M. C. and Kuttan R. (2004). Antidiabetic activity of Aegle marmelos and its relationship with its antioxidant properties. *Indian Journal of physiology and pharmacology*, 48(1): 81-88.

Schmidt J., Fleissner S., Heimann-Weitschat I., Lindstaedt R., Pomberg B., Werner U. and Szelenyi I. (1994). Effect of corticosteroids, cyclosporin A and methotrexate on cytokine release from monocytes and T cell subsets. *Immunopharmacology,* 27: 173-179.

Schroder K., Hertzog, P. J., Ravasi, T. and Hume, D. A. (2004). Interferon-γ: an overview of signals, mechanisms and functions. *Journal of leukocyte biology*, *75*(2): 163-189.

Shahidi F. and Zhong Y. (2010). Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews*, *39*(11): 4067-4079.

Shaikh A. C., Gupta A. and Chaphalkar S. R. (2016). Exploration of active metabolites from *Aegle Marmelos* and *Emblica Officinalis* for determining its immunosuppressive properties. *Journal of Progressive Research in Biology,* 3(1): 119-126.

Sharma G. N. and Dubey S. K. (2005). Medicinal value of Bael: A review. *International Journal of Current Pharmaceutical Research*, 1(3): 20-31.

Sharma G. N., Dubey S. K., Sati N. and Sanadya J.(2011). Phytochemical screening and estimation of total phenolic content in Aegle marmelos seeds. *International Journal of pharmaceutical and clinical research*, *3*(2): 27-29.

Sharma P., Kumar P., Sharma R., Gupta G., Chaudhary A.(2017). Immunomodulators : Role of medicinal plants in immune system. *National Journal of Physiology, Pharmacy and Pharmacology*, 7(6): 552-556.

Shivhare Y., Singour P. K., Patil U. K. and Pawar R. S. (2010). Wound healing potential of methanolic extract of Trichosanthes dioica Roxb (fruits) in rats. *Journal of Ethnopharmacology*, 127(3): 614-619.

Shroff F. M. (2017). What is ayurvedic health care and how is it applicable to the Modern Day?. *Journal of Nutrition and Human Health*, 1(2):17-29.

Siddique N. A., Mujeeb M., Najmi A. K. and Akram M.(2010). Evaluation of antioxidant activity, quantitative estimation of phenols and flavonoids in different parts of *Aegle marmelos. African Journal of Plant Science.* 4 (1): 001-005.

Sies H., Koch O. R., Martino E., and Boveris A. (1979). Increased biliary glutathione disulfide release in chronically ethanol-treated rats. *FEBS letters*, *103*(2), 287-290.

Singanan V., Singanan M. And Begum H. (2007). The Hepatoprotective Effect of Bael Leaves (*Aegle Marmelos*) in Alcohol Induced Liver Injury in Albino Rats. *International Journal of Science & Technology*, 2(2): 83-92.

Singh N., Tailang M. and Mehta, S. C. (2016). A review on herbal plants as immunomodulators. *International Journal of Pharmaceutical Sciences and Research*, 7(9): 3602-3610.

Singh R. P., Banerjee S. and Rao A. R. (2000). Effect of *Aegle marmelos* on biotransformation enzyme systems and protection against free□radical□mediated damage in mice. *Journal of pharmacy and pharmacology*, *52*(8): 991-1000.

Smolinski A. T. and Pestka J. J. (2003). Modulation of lipopolysaccharide-induced proinflammatory cytokine production in vitro and in vivo by the herbal constituents apigenin (*chamomile*), ginsenoside Rb1 (*ginseng*) and parthenolide (feverfew). *Food and Chemical Toxicology*, 41(10): 1381-1390.

Spelman K., Burns J. J., Nichols D., Winters N., Ottersberg S., and Tenborg M. (2006). Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. *Alternative medicine review*, 11(2): 128-150.

Stef D. S., Gergen I., Trasca T. I., Monica Harmanescu S. L., Ramona B. and Heghedus M. (2009). Total antioxidant and radical scavenging capacities for different medicinal herbs. *Romanian Biotechnological Letters*, 14(5): 4705-4710.

Stirling R.G. and Chung K.F. (2000). Future treatments of allergic diseases and asthma. *British Medical Bulletin*, 56(4):1037-1053.

Stuehr D. J. and Nathan C. F. (1989). Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *Journal of Experimental Medicine*, 169(5): 1543-1555.

Stuehr D. J., and Nathan C. F. (1989). Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *Journal of Experimental Medicine*, *169*(5): 543-1555.

Subramaniam D., Giridharan P., Murmu N., Shankaranarayanan N. P., May R., Houchen C. W., Ramanujam R.P., Balakrishnan A., Vishwakarma R.A. and Anant S. (2008). Activation of apoptosis by 1-hydroxy-5, 7-dimethoxy-2-naphthalene-carboxaldehyde, a novel compound from *Aegle marmelos*. *Cancer research*, *68*(20), 8573-8581.

Sudarshan S., Fairoze N., Ruban S. W., Badhe S.R. and Raghunath B.V.(2010). Effect of aqueous extract and essential oil of ginger and garlic s decontaminant in chicken meat. *The Journal of Poultry Science* 3: 58-61.

Surai P. F. (2016). Antioxidant systems in poultry biology: superoxide dismutase. *Journal of Animal Research and Nutrition*, *1*(1): 1-17.

Suriyamoorthy P., Mary M. R. F., Subrhamanian H. and Kanagasapabathy D. (2014). Anti hyperlipidemic effect of aqueous extract of Aegle marmelos and Camellia sinensis in oil fed dyslipidemic rats. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6*(2): 338-341.

Tiwari A., Jatawa S., Paul R.(2011). Indian Medicinal Plants : A Rich Source of Natural Immuno-Modulator. *Internatioal journal of pharmacology*, 7(2): 198-205.

Tiwari P., Kumar B., Kaur M., Kaur G., Kaur H. (2011). Phytochemical screening and Extraction: A Review. *Internationale Pharmaceutica Sciencia*, 1(1): 98-106.

Trease G.E. and Evans W.C. (2002). Pharmacognosy. 15th Ed. London: Saunders Publishers, 42–44: 221–229.

Upadhya S., Shanbhag K.K., Suneetha G, Balachandra Naidu M. and Upadhya S. (2004). A Study of hypoglycemic and antioxidant activity of *Aegle marmelos* in alloxan induced diabetic Rats. *Indian Journal of Physiology and Pharmacology*. 48 (4): 476-480.

Uttara B., Singh A. V., Zamboni P., Mahajan R.T.(2009). Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. Current Neuropharmacology, 7: 65-74.

Vardhini S. P., Sivaraj C., Arumugam P., Ranjan H.K.T. and Baskar M. (2018). Antioxidant, anticancer, antibacterial activities and GC-MS analysis of aqueous extract of pulps of *Aegle marmelos* (L.) Correa. *The Journal of Phytopharmacology*, 7(1): 72-78.

Verma S., Bahorun T., Singh R. K., Aruoma O. I. and Kumar A.(2013). Effect of *Aegle marmelos* leaf extract on N-methyl N-nitrosourea-induced hepatocarcinogensis in Balb/c mice. *Pharmaceutical biology*, 51(10): 1272-1281.

Wershil B.K., Furuta G.T., Lavigne J.A., Choudhury A.R., Wang Z-S. and Gall, S.J. (1995). Dexamethasone or cyclosporin A suppress mast cell-leukocyte cytokine cascades. Multiple mechanisms of inhibition of IgE and mast cell-dependent cutaneous inflammation in the mouse. *Journal of Immunology*, 154 (3): 1391- 1398.

Xuana B., Dua X., Lia X. and Shen Z. (2015). A new potent immunosuppressive isoflavanonol from *Campylotropis hirtella*. *Natural Product Research*, 30(12): 1423-1430.

Zhang X.F., Cui Y., Huang J.J., Zhang Y.Z., Nie Z., Wang L.F., Yan B.Z., Tang Y.L. and Liu Y. (2007). Immunostimulating properties of diosgenyl saponins isolated from *Paris polyphylla*. *Bioorganic & medicinal chemistry letters*, 17(9): 2408-2413.

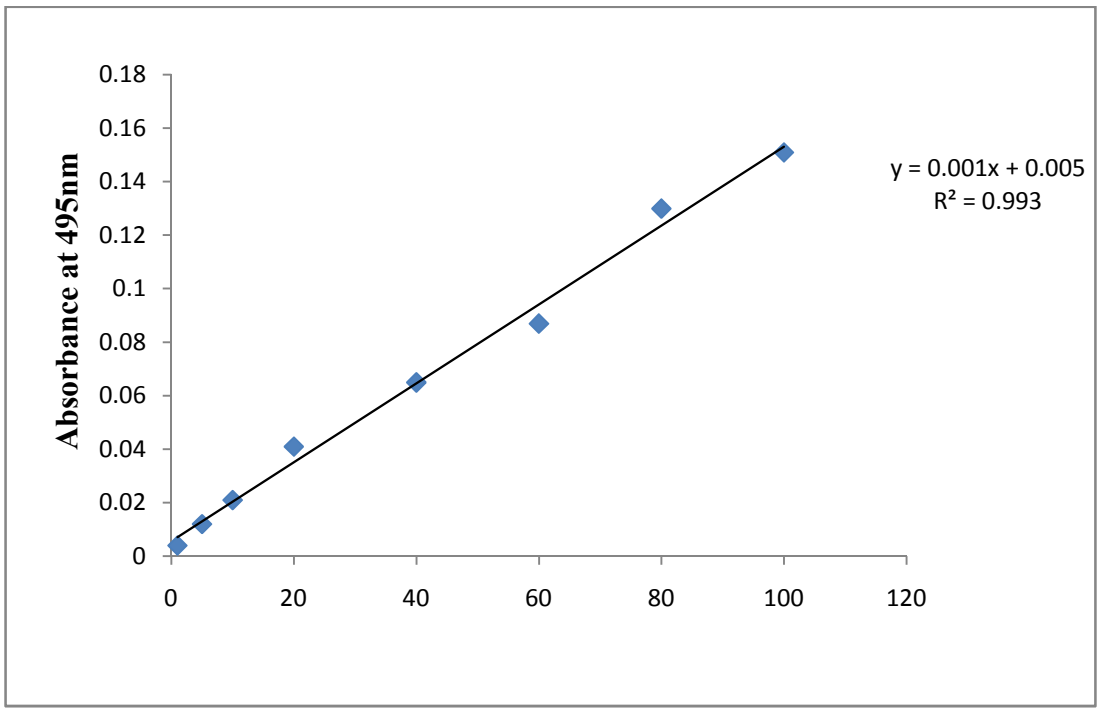
Zheng M., Storz G. (2000). Redox sensing by prokaryotic transcription factors. *Biochemical Pharmacology*, 59: 1-6.

Zhu Y.N., Zhao W.M., Yang Y.F., Liu Q.F., Zhou Y., Tian J., Ni J., Fu Y.F., Zhong X.G., Tang W. and Zhou R.(2006). Periplocoside E, an effective compound from *Periploca sepium Bge*, inhibited T cell activation in vitro and in vivo. *Journal of Pharmacology and Experimental Therapeutics*, 316(2):662-669.

Appendices

A.1. Reagents and Buffers

S. No.	Buffer/ Solution	Composition/ Preparation of Stock solutions
1	Normal saline solution	8.5 g of sodium chloride was dissolved in one liter of distilled water.
2	10% trichloroacetic Acid (TCA) solution	10 g TCA was dissolved in distilled water and the volume was made up to 100 ml with distilled water.
3	0.67% thiobarbituric acid (TBA)	Prepared by taking 0.67 g of TBA in 100 ml of distilled water and warmed up for dissolving TBA.
4	PBS (PH 7.4)	8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 0.94 g Na_2HPO_4 in 1000 ml of distilled water.
5	0.5 M EDTA	18.61 g EDTA was added to 80 ml of distilled water. pH was adjusted with NaOH pellets to 8.0 Volume was maintained to 100 ml with distilled water and autoclaved before use.
6	DTNB (0.01 M)	99 mg of DTNB reagent was dissolved in 25 ml of methanol.
7	1M-Tris	121 g of tris base was dissolved in 950 ml of distilled water. pH was adjusted to 8.9 and the final volume was made up to 1000 ml using distilled water.
8	TCA (50%)	50 g of TCA was dissolved in 100 ml of distilled water.
9	1.25 mM MTT	2.58 mg MTT was dissolved in 5ml of distilled water
10	Dragendroff reagent	0.17 g of bismuth nitrate was added in 2 ml of acetic acid and volume was made up to 8 ml (Solution A). 4 g of potassium iodide was added in 10 ml of acetic acid and volume was made up to 20 ml (Solution B). Solution A and B mixed together and finally volume was made up to 100 ml. Solution was stored at room temperature.
11	Lysis buffer (50 mM HEPES buffer, pH 7.0; 150 mMNaCl)	30 ml of NaCl (500 mM) was mixed with 25 ml of HEPES (100 mM). 0.6 ml of triton X was added to it and finally volume made up to 50 ml.



Standard curves for estimation of protein by Bradford dye method using BSA as a standard

VITA

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ABSTRACT

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Major	:	Molecular Biology and Biotechnology	**Department**	:	Molecular Biology and Genetic Engineering
Thesis title	:	**"Determination of Antioxidative Potential and Cytokines Mediated Immunomodulation due to *in vitro* exposure of *Aegle marmelos* (L.) Corr. in Chicken Lymphocytes Culture System"**			
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Traditional medicine system has always been a part of different cultures and civilizations around the world. India has one of the richest plant based medicine system. Poultry system is vulnerable to new evolving bacterial, viral strains and different environmental stresses which result in huge economic loss. *Aegle marmelos* (L.) Corr. belongs to *Rutaceae* family, commonly known as "bael" in Hindi. Various extracts of *Aegle marmelos* are reported to have antiproliferative activity in splenocytes and peripheral blood mononuclear cells. On the basis of above mention lines, the present study was planned to explore the immunomodulatory and antioxidative potential of aqueous extract of leaves of *Aegle marmelos* (AME) in chicken lymphocytes. To check the presence of various phytoconstituents in AME, various qualitative and quantitative phytochemical analyses were carried out. Further, two cytokines (IL-6, IL-10) and iNOS expression analysis in AME exposed chicken lymphocytes was carried out through quantitative real time PCR. The immunomodulatory potential was explored through lymphocyte proliferation assay, while DPPH assay, NO radical scavenging assay and various cell based assays *viz.* LPO, SOD, Catalase, Reduced GSH and Nitric oxide estimation were conducted to explore antioxidative potential of AME.

The extraction yield of aqueous extract of *Aegle marmelos* (AME) was found to be 14.1%. In biochemical analysis, the extract showed presence of various phytoconstituents. Total phenolic and flavanoids contents in AME was estimated to be 480 GAE mg/g and 371.2 RE mg/g respectively. The maximum non cytotoxic dose (MNCD) of extract was determined to be 75µg/ml in chicken lymphocyte culture system. AME showed immunosuppressive potential by decreasing the proliferation of T and B lymphocytes in mitogen stimulated cells. The percent decrease of 6.20% and 15.5% % in T cell proliferation was observed in case of Con A and PHA stimulated cells respectively. Significant decrease of 9.23% in B cell proliferation was observed due to AME exposure in case of LPS stimulated cells as compared to control.

AME showed significant antioxidative activity in DPPH and Nitric oxide radical scavenging assays with IC_{50} value for DPPH and NO scavenging assays of 233.3 µg/ml and 113.80µg/ml, respectively. After the exposure of the extract in lymphocytes, the level of membrane lipid peroxidation and nitric oxide content was decreased. The level of reduced GSH, SOD and catalase was increased in chicken lymphocytes after treatment with AME. In quantitative real time PCR, the expression level of proinflammatory cytokine IL-6 and inducible nitric oxide synthase was significantly decreased in comparison to control but the expression of anti-inflammatory cytokine IL-10 was significantly increased after the exposure of AME at MNCD. Thus, it could be concluded that AME displayed significant antioxidative and immunosuppressive potential in chicken lymphocytes culture system which could be correlated with the alteration in IL-6, IL-10 and iNOS expression levels. However, there is need for further suitable *in vitro* and *in vivo* analyses to exploit this plant resources based therapeutic preparation for certain disease conditions.

(Sonu Ambwani)
Advisor

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सारांश

नाम :	अमनदीप कौर	**परिचयांक** :	51168
षट्मास एवं प्रवेष वर्ष :	प्रथम, 2016–2017	**उपाधि** :	स्नातकोत्तर (कृषि)
प्रमुख :	आण्विक जीव विज्ञान और जैव प्रौद्योगिकी	**विभाग** :	आण्विक जीव विज्ञान और आनुवांशिक अभियांत्रिकी
शोध का शीर्षक :	**''लिफोंसाइटस संर्वधन तंत्र में एगल मार्मेलस (एल) कोर के इन विट्रो एक्सपोजर की वजह से साइटोकाइन द्वारा दोजित प्रतिरक्षा मोड्यूलेशन और अपचायक क्षमता का मूल्यांकन''**		
सलाहकार :	**डॉ0 सोनू अम्बवानी**		

पारंपरिक दवा प्रणाली हमेशा दुनिया भर में विभिन्न संस्कृतियों और सम्यताओं का हिस्सा रही हैं। पादप आधारित औषधि प्रणाली में भारत बहुत सम्पन्न है। नये विकसित जीवाणु, विषाणु उपभेदों एवं विभिन्न पर्यावरणीय तनावों के लिये मुर्गी पालन कमजोर है जिसके परिणामस्वरुप अत्यधिक आर्थिक नुकसान होता है। एगल मार्मेलोस (एल) कोर रुटाऐसी परिवार की सदस्य है, जिसे हिन्दी में 'बेल' नाम से जाना जाता है। एगल मार्मेलोस के विभिन्न सत्वों द्वारा स्पलीनोसाईट्स एवं परिधीय रक्त मोनोन्यूक्लियर कोशिकाओं में एंटीप्रोलिफ्रेटिव सक्रियता पायी जाती है। ऊपर आधारित पंक्तियों के आधार पर, वर्तमान अध्ययन एगल मार्मेलोस की पत्तियों के जलीय सत्वों की प्रतिरक्षात्मक एवं एंटीऑक्सीडेटिव क्षमता को मुर्गी केलिम्फोसाइट्स में पता लगाने पर आधारित किया गया। ए0एम0ई0 में उपस्थित विभिन्न फाइटोकॉस्टिटुऐंटस की जांच करने के लिये, विभिन्न गुणात्मक एंव मात्रात्मक पादप रसायनिक विश्लेषण किये गये। इसके अलावा, ए0एम0ई0 से उपचारित मुर्गी के लिम्फोसाइट्स में दो साइटोकाइनस (आई0एल0 06, आई0एल0 10) एवं आई0 नॉस का मात्रात्मिक रियल टाईम पी0सी0आर0 द्वारा अभिव्यक्ति विश्लेषण किया गया। इम्यूनोमोड्यूलेटरी क्षमता लिम्फोसाइट प्रसार के द्वारा जाँच की गयी, जबकि डीपीपीएच जाँच, नाइट्रिक ऑक्साइड तत्वरुप स्कैवन्जिंगएवं विभिन्न कोशिकाओं पर आधारित जाँच जैसे एलपीओ, एसओडी0, केटालेस, रिडयूसड जीएसएच एवं नाइट्रिक ऑक्साइड अनुमानों द्वारा एएमई की ऑक्सेडेटिव क्षमताओं का पता लगाया गया।

एगल मॉर्मोलोस (एएमई) के जलीय सत्त की निष्कर्षण द्वारा सत्त में विभिन्न पादपघटकों की उपस्थिति देखी गयी। एएमई में कुल फिर्नोलिक एवं फ्लेवनॉइड सामग्री क्रमशः 480 जीएई मिलीग्राम/ग्राम और 371.2 आरई मिलीग्राम/ग्राम आकंलन किया गया। मुर्गी के लिम्फोसाइट्स के संवर्धन प्रणाली में सत की अधिकतम गैर कोशिकाविषी मान 75 माइक्रोग्रा0/मिलीलीटर निर्धारित की गयी। एएमई ने माइटोजन उतेजित कोशिकाओं में टी और बी लिम्फोसाइट्स के प्रसार को कम करके प्रतिरक्षा क्षमता दिखाई। टी कोशिकाओं प्रसार में कॉनए और पीएचए उत्तेजित कोशिकाओं में क्रमशः 6.20 एवं 15.5 प्रतिशत कमी पायी गयी। नियंत्रण की तुलना में एलपीएस उत्तेजित कोशिकाओं में एएमई एक्सपोजर के कारण बी कोशिकाओं के प्रसार में 9.23 प्रतिशत की कमी देखी गयी।

एएमई ने डीपीपीएच और नाइट्रिक ऑक्साइड तत्वरुप स्कैवन्जिंग में आईसी 50 की मात्रा 233.3 माइक्रोग्राम/मिलीलीटर और 113.80 माइक्रोग्राम/मिलीलीटर क्रमशः डीपीपीएच और नाइट्रिक ऑक्साइड तत्वरुप जांचों के साथ महत्वपूर्ण एंटीऑक्सिडेटिव गतिविधि दिखायी। सत्व के एक्सपोजर के बाद फिम्फोसाइट्स झिल्ली में लिपिड परॉआक्सिडेशन एवं नाइट्रिक ऑक्साइड की मात्रा में कमी पायी गयी। एएमई के उपचार के उपरान्त, फिम्फोसाइट्स में रिडयूसड जीएसएच, एसओडी एवं केटालेस का स्तर बढ़ गया था। मात्रात्मक रियल टाइम पीसीआर में, नियंत्रण की तुलना में प्रोइनफ्लेमेटरी साइटोकाइन आई. ए.एल.6 एवं उत्तेजित, नाइट्रिक ऑक्साइड सिन्थेस का अभिव्यक्ति स्तर बहुत कम पाया गया लेकिनएएमई के एमएनसीडी एक्सपोजर के बाद एंटीइनफ्लामेटरी साइटोकाइन आइएल–10 का अभिव्यक्ति से काफी वृद्धि हुयी थी। इससे यह निष्कर्ष निकाला जा सकता है कि एएमई मुर्गी लिम्फोसाइट्स संवर्धन प्रणाली में एंटीऑक्सिडेटिव, इम्यूनोस्पप्रेसिव क्षमताओं के लिये महत्वपूर्ण भूमिका निभाता है जिसे आईएल–6, आईएल–10 और आईएनओएस के अभिव्यक्ति के स्तर में बदलाव से संबधित हो सकता है। तथापि कुछ रोगों की स्थिति में इस पौधे के आधारित चिकित्सकीय हर्बल रचना का लाभ उठाने हेतु इस पर इनविटरो एण्ड इनविवो विश्लेषण आवश्यक है।

(सोनू अम्बवानी)
सलाहकार

(अमनदीप कौर)
लेखिका