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Characterisation of Lectin from *Pisum sativum* and *Agaricus bisporus* and their Immunomodulatory Effects on Earthworm Coelomocytes *(Immunomodulatory studies)*

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Abstract:- The paper is focused on lectin characterisation from Pisum sativum and Agaricus bisporous by different experimental methods and assays such as antimicrobial effect, Haemagglutination and Sugar specificity. Lectins recognise cellular and molecular moieties and attach them by their sugar specificity. These are highly specific to carbohydrate moieties and the binding is reversible. Invertebrate system sample was taken to check the immunomodulatory effect of lectins. Trypan blue test for cell viability and catalase assay for antioxidant mechanism were assessed in this respect. And presence of lectin restores cell viability and nullifies free radical mediated toxicity. This paper will be valuable source for more studies of lectins and other related area of research.

Keywords:- *Immunomodulatory, Coelomocytes, Lectin, Stress Induced, PBS, HBSS, Trypan Blue Assay, Catalase Test.*

I. INTRODUCTION

The immune system is a defensive system that defends the body from infection. immune system contains a variety of cells and molecules. Any change in the immune response, including activation or suppression of any component or stage of the immune response, is referred to immune system modulation. As a result, immunomodulators are drugs that are used to support the immune system.

One of the most important trials is the regulation of the immune system to cure diseases. Many recent experiments have made significant progress in the study of ethnomedicinal plants as immunomodulatory agents. Immunopharmacology is a field of pharmacology that focuses on immunomodulators. The development of immunomodulatory and anti-tumor drugs based on natural compounds has piqued interest.

Plant extracts are primarily investigated for their potential immunomodulatory properties in various parts of the world. Many new insights into the function of carbohydrate-protein interactions in the immune system have come from advances in glycobiology and immunology.

The current study comprises four main themes:

- Isolatioin and characterisation of specific plant lectins like pea lectin and mushroom lectin
- Immunomodulatory lectins general characteristics
- Activation of the innate immunity cells by these lectins on coelomocytes an invertebrate immunity providing system.

Several plant lectins have immunomodulatory effects that are caused by glycan interactions. Immune cells have moieties on their membranes. Such interactions can cause signal transduction, resulting in the production of specific cytokines and the induction of effective immune responses against tumours or microbial infections. As a result, immunomodulatory lectins may have medicinal uses or aid in the identification of sugar targets for new therapeutic strategies.

Pea lectin and Mushroom lectin has been selected for this study.

PEA LECTIN: Some plant lectins have immunomodulatory properties that are triggered by interactions with glycan moieties on immune cell surfaces. Similarly thus, interactions can cause signal transduction, resulting in the production of cytokines and the induction of effective immune responses against tumours or microbial infections. Immunomodulatory lectins may thus have therapeutic potential or help in the identification of sugar targets for new therapeutic strategies.

MUSHROOM: Immunomodulatory effects of many plant lectins are caused by their association with glycan moieties on immune cell surfaces. Such interactions can cause signal transduction, resulting in the production of specific cytokines and the induction of effective immune responses against tumours or microbial infections. Immunomodulatory lectins may thus have therapeutic potential or help in the identification of sugar targets for new therapeutic strategies. Food lectins may have therapeutic efficacy in the treatment of autoimmune diseases that are characterised by inflammation.

Anatomy of the earthworm immune system and immune effector pathways

Earthworms are protostomian species with a genuine coelom cavity filled with coelomic fluid, which includes a variety of immune cells, coelomocytes, and humoral factors as well as a stable hydrostatic skeleton. Earthworms' main immune cells are coelomocytes, which originate in the cavity's mesenchymal lining. Several essential mechanisms are used by earthworms to protect themselves against pathogens. The phagocytosis of coelomocytes, like that of vertebrates, is regulated by humoral components known as opsonins, which coat the particle and therefore aid phagocytosis. They can also contain nitric oxide and reactive oxygen species. Furthermore, to counteract the harmful effects of ROS, coelomocytes have a host of defence mechanisms in action. Among them are superoxide dismutase, which catalyses the conversion of superoxide to hydrogen peroxide and oxygen, as well as glutathione peroxidases and catalases, which catalyse the degradation of hydrogen peroxide. Therefore, cell viability and catalase activity was estimated in lectin treated earthworms.

This research is supposed to pave the way for further research in this under-researched field, which has the potential to uncover carbohydrate targets for novel prophylactic and therapeutic strategies.

II. METHODOLOGY MATERIALS AND METHODS

- *Isolation of lectins (Pea and Mushroom)*

Materials: Peas, PBS buffer, motor and pestle, ammonium sulphate, dialysis bag.

PBS buffer preparation: Nacl (8g), Kcl (0.2g), Na2HPO4 (1.44g), KH2PO4 (0.245g) for 1000ml.

- *PBS buffer*

Green peas: Peas were soaked overnight before the day of experiment. Ten grams of peas were homogenised with 100ml of PBS buffer and kept in incubator shaker overnight. Homogenised sample was centrifuged 3500 rpm for about 30 mins. Supernatant was collected as crude protein; this was precipitated by salting out process with ammonium sulphate 80% saturation which is 27.22g of ammonium sulphate and supernatant was kept under magnetic stirrer for 30 mins. Centrifuged for 15 mins at 3500rpm.pellet was collected. dialysis was performed with the same buffer (PBS) twice to remove the salt. (Amount of solid ammonium sulphate added to 48mls of 0% saturated buffer to get 80% saturated buffer was 27.22g, final volume was made upto 62.79 mls at 25°c. (Tzi Bun Ng et al. Appl Biochem Biotechnol. 2015 Nov)

- *Mushroom (Button mushroom)*

Matertials: PBS buffer, button mushroom (fruiting bodies), ammonium sulphate, dialysis bag 10 grams of mushroom fruiting bodies were taken and are homogenised with PBS and were kept in incubator shaker overnight. Homogenised sample centriguged at 3500rpm around 30 mins and supernatant was collected as crude protein and ammonium sulphate precipitation was conducted salting in process with 20mls of 0% saturated buffer to get 60% saturated buffer of 7.90g. final volume was made upto 22.67 mls at 25°c. supernatant was collected, 22.67g of ammonium sulphate was added and kept under magnetic stirrer for 30 mins and centrifuged 15 mins at 3500rpm and pellet was collected. salt was removed by dialysis where it was dialysed against the same buffer (PBS) twice to remove salt off. Samples were kept in freezer for further experimental process.

- *Antimicrobial activity of lectins*:

Materials: Lectin samples, 2 test samples given below, nutrient agar, muller hinton agar, autoclaved glasswares, gel punchers,

Two microbial test samples were taken. Bacillus and penicillium to understand both antibacterial and antifungal activities. Test samples were grown on media for bacillus Nutrient agar was taken as media and for Penicillium Muller hintons agar. Media was prepared and poured on autoclaved petri plates and loopful culture was taken and by using spread plate method microbes were inoculated, plates were dried and solidified before spreading test samples so that agar can absorb bacteria more readily (inoculation was done under LAF prior 15 mins UV sterilization to avoid contamination). 3 punch wells were made with gel puncher gentle suction were made to avoid rugged wells. 2 petri plates were used for single lectin and vice versa for comparision. lectins were added in the well and one well was taken as control and kept for incubation in incubator at 37°c for 1 day and were observed for zone of inhibition to understand the antimicrobial activity. (Bayot ML, Bragg BN. Antimicrobial Susceptibility Testing)

- *Haemagglutination:*

Materials: ABO blood group samples, test samples, sterilised slides, ethanol, cotton, lancet, capillary tubes.

10 glass slides were taken. A+, B+, O+, AB+ test samples were drawn from lancet prick and placed on slides. 1 set with 4 slides of samples and 1 control where 1-2 drops of pea lectin were added in 5 slides and were mixed thoroughly with capillary tubes. Vice versa with Mushroom lectins with test samples. And were observed for Results.

- *Sugar specificity test: Estimation of sugars by DNS method.*

Materials: Glucose, Lactose, DNS, Distilled water, water bath, calorimeter, lectin samples.

Glucose preparation: 1g of glucose in 100ml of water
Lactose preparation: 1g of lactose in 100ml of water

DNS reagent preparation: 1g DNS to 20ml 2N NaoH and 50ml water containing 30g of sodium potassium tartarate and make it upto the volume of 100ml.

This test is done to understand the lectins which they specific to. Glucose and lactose were used as standard solutions. 8 test tubes were taken where for pea lectin 3 tubes of samples and 1 control and with mushroom lectin 3 tubes of

samples and 1 control were taken. 0.25ml of glucose and 0.75 of water was added in the first tube followed by 0.5ml of glucose and 0.5ml of water.0.75ml of glucose and 0.25ml of water. And control with glucose of 1ml. 0.1ml of lectin was added in all the tubes. 2 sets followed by pea and mushroom lectins. These were kept for room temperature for about 30 mins and 3ml of DNS was added to each test tube and kept for water bath for about 10 mins at 100°c. 6ml of water was added to all the test tubes and absorbance was taken at 540nm.color was observed to understand the specificity of the lectins. (MEPUR H. RAVINDRANATH, SAKUNTHALA MUTHUGOUNDER, in Autoantibodies (2007)

➢ *Immunomodulatory effect of lectins on coelomocyte: (Mushroom and pea lectin) Coelomocyte cells extraction:*

Materials: PBS buffer, HBSS, Earthworm, ice cubes, sterilised glasswares. HBSS preparation:

Components: Prepare 800mL of distilled water in a suitable container. Add 8g of NaCl to the solution. Add 400mg of KCl to the solution. Add 100mg of MgSO4-7H2O to the solution. Add 100mg of MgCl2- 6H2O to the solution. Add 60mg of Na2HPO4-2H2O to the solution. Add 60mg of KH2PO4 to the solution. Add 1g of D-Glucose (Dextrose) to the solution. Add 350mg of NaHCO3 to the solution. Add Phenol red. Add 140mg of CaCl2 to 200mL of distilled water. Autoclave the solutions separately, bring them to room temperature and mix them together in an LAF.

Method: Earthworms were washed under normal tap water and were cleaned thoroughly in Distilled water and transferred to sterilised petri plates and were kept in PBS of 3ml and were treated with ice cubes which gives cold shock where coelomocytes were released in media. Petri plates are then kept in co2 incubator for incubation. Next day 1 drop from plates were taken in glass slide and observed under microscope. Direct observation can be done in phase contrast microscope. same procedure is done with HBSS instead of PBS. (Isolation and culture of coelomocytes "Nandhitha Madhusudhan; Preetha Nair; Kale, R. D. Dynamic Soil, Dynamic Plant 2009)

➢ *Cold shock method to isolate coelomocyte*

- *Catalase test*

Materials: Working solution- (cobalt chloride, sodium phosphate, sodium Bicarbonate), phosphate buffer- (potassium Dihydrogen phosphate, Sodium hydrogen phosphate), Hydrogen peroxide, coelomic fluid from earthworm.

- *Reagent's preparation:*

Cobalt chloride – 2.03gm in 100ml of Distilled water Sodium phosphate – 1g in 100ml of Distilled water Sodium Bicarbonate – 9g I 100ml distilled water
Working solution: 10ml of cobalt, 10ml Na_3PO_4 and 90ml of $NaHCO_3$ were mixed well. Phosphate buffer: pH 7
KH2PO4 – 0.68g in 100ml of DW Na2HPO4 – 0.890g in 100ml of DW
Mix phosphate buffer while setting experiment in ratio (1:1.5) which is (10:15) Hydrogen peroxide – 0.1134ml of 30% in 100ml of DW

Working solution: Hydrogen peroxide mixed in 100ml phosphate buffer.

Incubation was done in water bath or in room temperature for 10min at 37°C. working solution was added 6000 µl in all the tubes. And this was kept under room temperature for 10min in dark and was then absorbance was taken at 440nm. (Hadwan, M.H. Simple spectrophotometric assay for measuring catalase activity in biological tissues. BMC Biochem 19, 7 (2018)

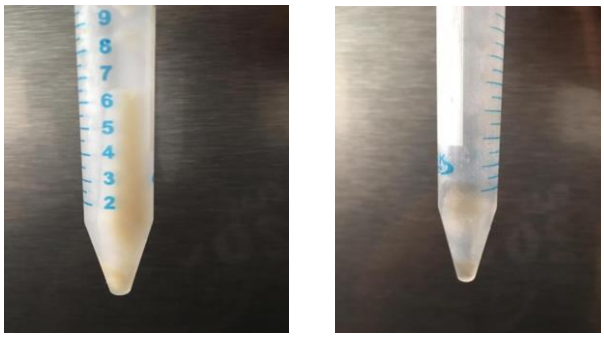
➢ *Trypan blue assay*

Materials: Trypan blue dye, test samples, glass slides, cover slip, ethanol, microscope.

Method: All the test samples were taken in a sterilised glass slides separately.1 drop of trypan blue was added to each slide. Ratio of test sample and trypan blue was taken was 1:1 ratio. And kept for incubation for 10 mins at room temperature and covered with cover slip. Later observed under microscope at 10x ,40x and 100x magnification and cell viability was counted. But in this experiment we are just focusing on the sustainability of the cells. cell counting is not mandatory. (Warren Strober1 1National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD).

III. RESULTS

Isolation and ammonium sulphate precipitation of pea and Mushroom lectin. Supernatant was collected as crude protein. After ammonium sulphate precipitation, Dialysis carried out to remove excess salt and impurities. Pellet was collected and preserved in freezer for further experimental process.



(a) (b)

Fig 1. Pea and mushroom lectin preserved in freezer in a centriguged tube after dialysis. (a) pea lectin (b) Mushroom lectin.

➢ *Antimicrobial activity of lectins:*

Zone of inhibition test is also known as Kirby-Bauer test which is used to measure and test antimicrobial activity of different samples. Where Bacillus and penicillum bacteria was grown and was incubated with the lectin samples. strain is

susceptible to the agent so; zone of inhibition was formed around the test sample which showed Antimicrobial activity of the lectins. size of the inhibition gives the level of antimicrobial activity present in the sample. Medium zone was observed which means antimicrobial is potent. Zone of inhibition around mushroom lectin sample and pea lectin sample in bacillus culture plate was seen.

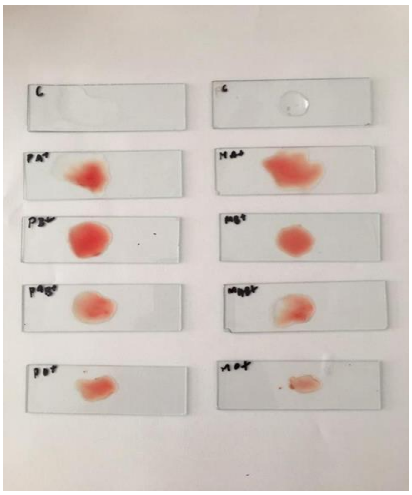
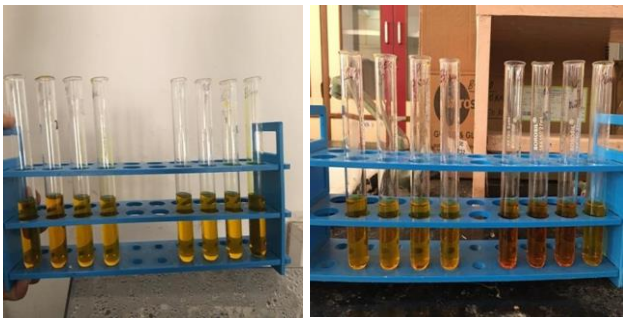


Fig 2. Showing agglutination of blood group when lectins were added A and B blood group showing agglutination, because of presence of glucose moiety. Haemagglutination: A, B and AB both positive and negative showed agglutination.

Sugar specificity: Reducing sugars has the property of reducing a number of reagents, one of the most important of which is 3,5- DNS. When this is reduced to 3 amino 5 nitro salicylic acid in an alkaline solution, it becomes 3 amino 5 nitro salicylic acid. The object of this test is to see if there is a carbonyl group present. In a lactose assay, mushroom and pea lectin produced no results, but in a glucose assay, pea lectin demonstrated specificity by producing a red colour.



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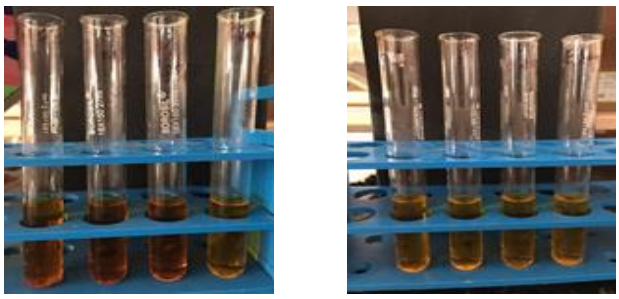

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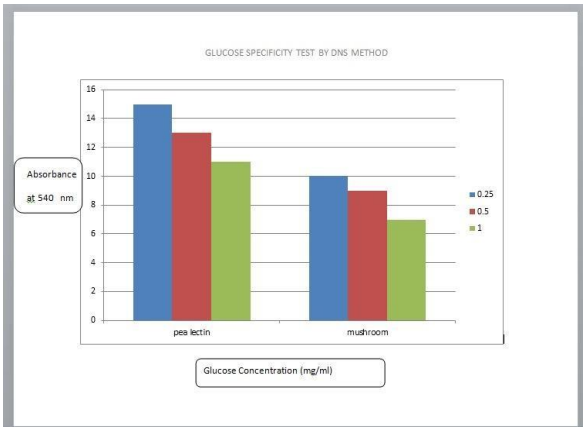

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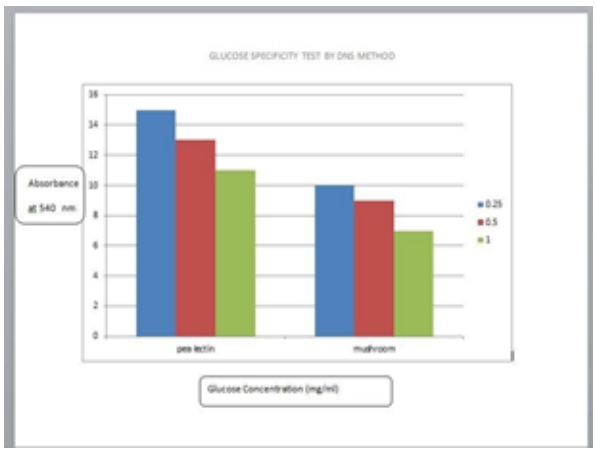
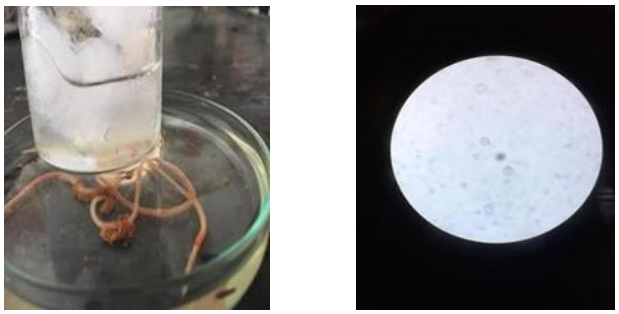


(f)

Fig 3. Showing sugar specificity of pea and mushroom lectins by glucose and lactose assay by dinitro-salicylic colorimetric method (a) Lectins showing no specificity to the lactose (b) Pea lectin showing specificity to glucose (c) Pea lectin sample showing change in colour in glucose assay (d) No reaction in lactose assay when test sample mushrrom was estimated (e) Graph showing glucose sugar speicificity test by DNS method. (f) Graph showing lactose sugar speicificity test by DNS method.

Coelomocyte: Coelomic fluid is expelled through dorsal pores when stress is induced. Cold shock method was used, after 10 mins of cold shock the petri plates were taken and incubated which showed coelomic cells next day observed in microscope



(a) (b)

Fig 4. Showing cold shock method on earthworms and microscopic view of cells. (a) Cold shock method, where earthworms were soaked in PBS (b) Microscopic view of coelomocytes in 40x magnification.

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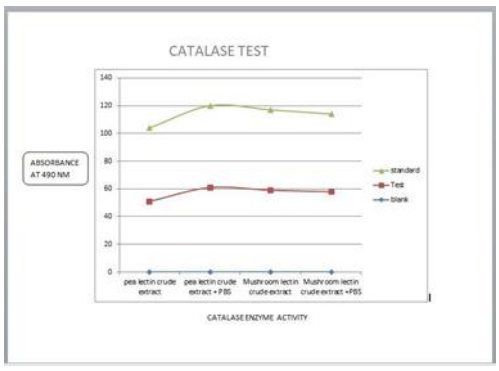
Catalase test: Precise, sensitive spectrophotometric method of measuring the activity of catalase, rapid formation of colored carbonato-cobaltate (III) complex. The activity was found to be directly propotional to the rate of dissociation of hydrogen peroxide.440nm peak was utilised for assessing catalase activity. Ability of cell to produce catalase enzymes was noted. Catalase activity was found to be active in both pea and mushroom incubated earthworm cells. Rapid liberation of oxygen bubble was observed and the tubes changed to green color from purple showed the activity of catalase enzyme.


(a)

(b)


(c)


(d)

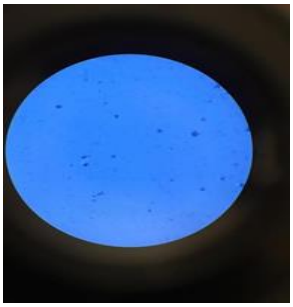


(d)

Fig 5. Showing catalse activity when it is reacted with hydrogen peroxide. (a) Change of color from purple to green showing activity of enzyme. (b) Working solution containing cobalt chloride, sodium phosphate and sodium Bicarbonate. (c) Bubble formation (d) Blank and test samples after reaction. (e) Graph showing catalyse activity of lectins at 490nm.

➢ *Trypan Blue Assay:*

Test was done to identify live and dead cells after treatment test samples Mushroom and pea lectins. The Earthworm cells observed under the microscope after soaking in crude lectin extract was found to be alive. And the cells which were soaked with lectin and PBS was found to be dead. PBS was found to be toxic to Earthworm even in diluted condition as they were incubated with 7 days and all of them died. 1hr maximum exposure showed effectivity to the experiment with different composition showed different results. Lack of food and environmental condition Earthworm was found to be dead and toxicological properties have not been thoroughly investigated yet it is difficult to conclude the death of the earthworms. Crude extract sample showed maximum live cells as they were naturally extracted. Sustainability of the cells were not as only few cells were seen and differentiation is done on the basis of the lectins added and their effect on cells.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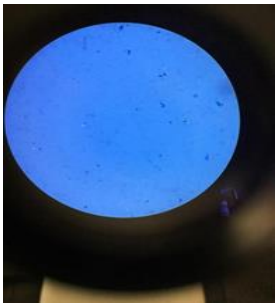
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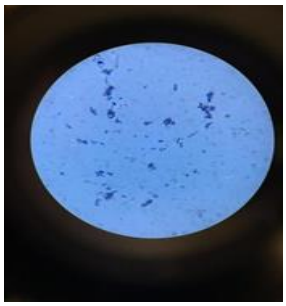
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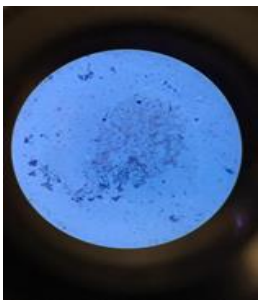
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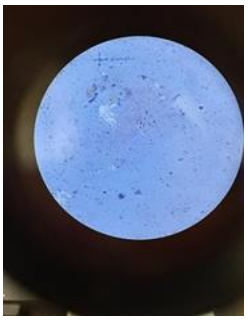
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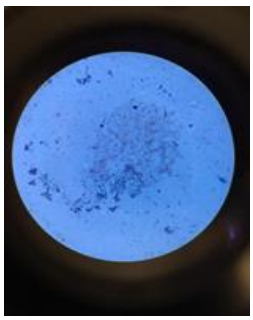
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Fig 6. Showing alive and dead cells soaked in crude extract and crude extract with PBS of both pea and Mushroom lectins. (a) Crude mushroom extract-soaked Earthworm cells (b) Pea crude extract-soaked Earthworm cells (c) Pea lectin with PBS showing maximum dead cells (d) Mushroom lectin with PBS showing maximum dead cells (e) Oil emersed 100x magnification of both alive and dead cells. (f) PBS-soaked cells almost dead.

IV. DISCUSSION AND CONCLUSION

Characterisation of pea and mushroom lectin were studied. Imunomodulatory charecteristics were understood. Glycon interaction of plant lectins excerts immunomdoulatory activites, where these glycon moieties are found on immune cells' surfaces. Anti-oxidant system activation stimulates modification towards mildly oxidizing environment which favours immunity providing cell maturation. Zone of inhibition test is easy to substitute one disk for another, contamination and low level of resistance can be identified easiliy. simple to perform and reproducible reagents are not much expensive, no requirement of special equipments. Standardised inoculum can be replaced by interested specimen for example swab of pus or blood culture and results can be obtained within 24 hours. Although it can only be used with rapidly growing organisms. MBC cannot be done using agar diffusion techniques. density of the inoculum cannot be controlled as primary test should be verified by isolates. Haemagglutination shows the hemolysis reaction where hemolysis shows the negative result and no hemolysis shows positive result.it is simple interpretative test and different antigen can be used. Highly specific and used as standard whereas reagents and equipment demands are high, components to be fresh for ex. RBC, these are less sensitive when compared to ELISA. Its time consuming and cannot distinguish IgG from IgM.

DNS method is one of the crucial methods for reducing sugars as the problem may rise with standard and test samples give lower OD values than expected it is because procedure was not carried out properly and absorbance was not measured at correct wavelength. It can be corrected by following the procedure carefully through the experiment and measure absorbance at correct wavelength. Catalyst test is to understand catalyst enzyme produced in the cells of the aerobic organisms as catalyst enzyme protect the cells from destruction by hydrogen peroxide produced in aerobic metabolism and release oxygen. Trypan blue assay has been carried out years to check the cell viability trypan blue is negatively charged and it doesn't bind to cell unless the membrane is compromised it is time sensitive viable exclude trypan blue. Another method for cell viability dye-exclusion, in this if the cells take up the impermeant dye it is considered dead. Cells that become reproductively non-viable i.e., ionising rad: impermeant dye is useless, the viable cells will be small, round and refractive where as non-viable cells will be dark blue, swollen and larger, appropriate precaution should be take while doing the experiment. This is very quick and inexpensive only a small fraction of total cell from a population is required.

- *Current Studies and Future Perspective:*

Lectins are nonimmune proteins or glycoproteins that attach to cell surface carbohydrates and trigger cell agglutination. These are thought to be essential in both the host's defence mechanism and the spread of cancer. Lectins have been found in a host of new sites in recent years. There have been some new lectins discovered, each with its own set of specificities and exploitable properties. Mushrooms have been introduced to a number of culinary and medical authorities. Several species have long been used in traditional Chinese medicine and functional foods in Japan and other Asian countries. Other bioactive constituents isolated from mushrooms include polysaccharides, polysaccharopeptides, polysaccharide-protein complexes, proteases, ribonucleases, ribosome inactivating proteins, antifungal proteins, immunomodulatory proteins, enzymes, lectins, and other bioactive constituents. Mushroom lectins have mitogenic, antiproliferative, antitumor, antiviral, and immune-stimulating properties. The aim of this study is to learn more about mushroom lectins, such as their blood group and sugar specificities, biomedical capacity, and future prospects.

Lectin receptor-like kinases (LecRLKs) are RLK family members with lectin-like extracellular recognition domains, transmembrane domains, and cytoplasmic kinase domains. Signal transduction is believed to be aided by plasma membrane proteins including LecRLKs. The majority of members of the protein family have yet to be functionally characterised in animals. Pisum sativum LecRLK (PsLecRLK) is localised in plasma membrane systems and/or other regions of the cell under salinity stress, and its transcript is upregulated. Overexpression of PsLecRLK in transgenic tobacco plants confers salinity stress tolerance, which alleviates both the ionic and osmotic component of salinity stress. Even when exposed to salinity, the transgenic plants have better Na+ tissue compartmentalization and higher ROS scavenging behaviour, resulting in less membrane destruction, increased efficiency, and yield maintenance. Overexpression of PsLecRLK also causes the expression of certain genes involved in cellular homeostasis to be disrupted. Relief of the osmotic and ionic components of salinity stress, as well as reduced oxidative damage and upregulation of stress-responsive genes in transgenic plants, can be a possible mechanism for increased stress tolerance under salinity stress conditions. PsLecRLK has been identified as a possible candidate for crop enhancement in this study, as well as a new route for more research into its signalling pathway.

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