Evaluation of phytochemical characteristics and pharmacological activities of *Allium cepa*

A thesis submitted toward fulfillment of the requirements for the degree of

Master of Engineering in Biomedical Engineering

Submitted by

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CERTIFICATE OF RECOMMENDATION

We hereby recommend that the thesis entitled **"Evaluation of phytochemical characteristics and pharmacological activities of *Allium cepa* "** carried out under our supervision by Nisha Banerjee may be accepted in fulfillment of the requirement for awarding the Degree of Master in Biomedical Engineering of Jadavpur University. The project, in our opinion, is worthy for its acceptance.

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Certificate of Approval

The forgoing thesis is hereby approved as a creditable study of an engineering subject carried out and presented in a manner satisfactory to warrant its acceptance as a prerequisite to the degree for which it has been submitted. It is understood that by this approval the undersigned do not necessarily endorse or approve any statement made, opinion expressed or conclusion drawn therein but approve the thesis only for the purpose for which it is submitted.

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of his **Master of Engineering in Biomedical Engineering** studies during academic session 2016-2017.

All information in this document has been obtained and presented in accordance with academic rules and ethical conduct.

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Abstract

Onion (*Allium cepa*) is a widely cultivated plant all over the world and traditionally this plant was used for treatment of many ailments. It has major groups of phytonutrients include polyphenols, flavonoids, tannin etc., all of which are observed to have potential anti-oxidant, anti-allergic, antimicrobial activity. In this project have to investigate effects of anti allergic activity on mast cell mediated allergic models as well as have to evaluate antioxidant property and antibacterial activity against human pathogenic bacteria.

Recently, Allergic diseases such as asthma, allergic rhinitis, atopic dermatitis, and food allergy afflict up to 20% of the human population in most countries and are believed to be increasing in prevalence. There are so many allopathic medicines for allergy available in market but they have enormous serious side effects such as drowsiness, hallucinations, double vision etc. Whereas through this project from natural resource we can evaluate the anti allergic drug which has less side effects. As well as by this project can be evaluate the antimicrobial property from natural resource which doesn't kill off the another healthy bacteria like the other drugs do and the antioxidant property of plant can be used as potential radical scavenger against deleterious damages caused by the free radicals.

In this project ethanolic extract powder of *Allium cepa* is extracted by maceration using ethanol as a solvent. The extract is subject to qualitative phytochemical tests and had examined the quantum of phytochemical present in the extract. Administered plant extract in some specific concentrations to mice for evaluating the mast cell stabilization and estimation of histamine elevation in the plasma. Antimicrobial activity of the plant extract is evaluated using the agar well diffusion method. By repeating this test the mean diameters of the inhibition zones are recorded. The free radical scavenging activity (RSA) or antioxidant activity is performed using DPPH (1, 1-diphenyl-2-picrylhydrazyl) method.

The study of growth curve against different doses of onion extract showed that onion has different phenomenon for both type bacteria such like gram positive bacteria and gram negative bacteria. Onion demonstrated that they are more effective to inhibit to grow gram negative bacteria whereas the activity against gram positive bacteria is less.

The raised number of intact mast cells intimates that the Onion skins stabilized the mast cell degranulation at dose of 300 mg/kg) and it virtues further work towards the isolation of phytoconstituents from this plant. This finding provides evidence that COR inhibits mast cell-derived immediate-type allergic reactions and mast cell degranulation.

Contents

Chapter 1

Introduction

Natural Product is chemical substance generated by living organisms and easily presented in nature.[1] These chemical substances can be extracted from plants, animals and micro-organisms' cells, tissues.[2] A range of structurally diversity, often novel chemical compounds constitute a crude extract which is extracted from these resources. Pharmacognosy presents the study of identifying, selecting and processing of natural products for medicinal purposes. Generally, the natural product compound has form of biological activities and that type of compound known as Active ingredients, such as a structure develop to become a discovery lead. In this way recently many medicines are acquired directly from natural resources.

In Mesopotamian and Egyptian times, the production of perfumes or pharmaceutically - active oils and waxes was made the history of the extraction of natural products, this was a major business of them. The extraction pots from about 3500 BC were found in 250km south of Baghdad by archeological excavations, which were made from a hard, sandy material presumably air - dried brick earth.[3] The concept of natural product emerged in the early 19^{th} century with foundation of organic chemistry. The principles of organic chemistry had been established in 1789 by the Frenchman Antoine Lavoisier in his

work *Traité Élémentaire de Chimie*. At the end of 18th century he concentrated on the isolation of biological substances from plants, because they had an interesting pharmacological activity. He showed that plants are the main source of such compounds, especially alkaloids and glycosides. In 1815, Eugène Chevreul isolated cholesterol from animal tissue which is a crystalline substance, belongs to the steroids, and in 1820 strychnine, was isolated which is an alkaloid. In middle of 19th century, the concept of natural product turned in new way by the German Justus von Liebig. . The concept of isolation of organic compound from plants was extended to include animal material. In 1884, Hermann Emil Fischer focused on the study of carbohydrates and purines. He was awarded the Nobel Prize in 1902 for his great invention. He also succeeded to produce a variety of synthetic carbohydrates including glucose and mannose. A new direction was opened in front the history of natural product, after the discovery of penicillin by Alexander Fleming in 1928 from fungi and other microorganisms. [4]

The interested product must be isolated and purified from natural products, which are complex mixtures of the components of natural sources. Isolation of a natural product dependent on circumstances, either to the isolation of "adequate quantities" of pure chemicals for chemical structure elucidation, degradation chemistry, biological testing, and other research needs or to the isolation of "analytical quantities" of the interested substance, where the main concentration is on identification and quantification of the substance (e.g. in biological tissue or fluid), and where the isolated part quantities depends on the applied analytical method. The active agent of natural resources can be isolated and purified that dependent on the structure, stability, and quantity of the natural product presented in that natural resource. To achieve these two distinct grades of product, the methods of isolation mainly applied. These two distinct grades are in similar manner distinct, but generally involve extraction, precipitation, adsorptions, chromatography, and sometimes crystallizations. The isolated substance becomes chemically homogeneous in both cases by purification, i.e. specific combined separation and analytical methods such as LC-MS methods are chosen as independently. Structure determination is almost inevitable for early isolation, especially those purified natural product that is associated with even an important pharmacologic activity.

Nowadays, pollution, unhealthy lifestyle, environmental toxins raises the risk of diseases. Overuse and/or misuses of allopathic drugs are also a major concern for their side effects on our health. WHO developed and lunched 'WHO Traditional Medicine Strategy 2014–2023' in 2013, and emphasized to raise universal healthcare through traditional and complementary medicines and to ensure the quality, safety and effectiveness of such medicines. This is the reason that the world is searching for cost effective, easily available, better physiological compatible traditional systems of medicine and holistic approach to avert such problem and provide the basic healthcare to all.[5]

Food allergies have become a serious problem for many people and its prevalence is increasing around the world (Sicherer & Sampson, 2007, 2010).Type I allergies including food allergies, atopic dermatitis, and hay fever (Ring, Kramer, Schafer, & Behrendt, 2001) may be partly due to changes in lifestyle, changes in the environment such as planting many of the same varieties of plants and trees, and air-pollution such as NOx and PM2.5 from automobiles and industries. In the development of antiallergic medicines, suppressing β-hexosaminidase release is a common target, because degranulation of rat basophilic leukemia (RBL-2H3) cells is linked to the release of leukotriene and histamine (Chukaew, Ponglimanont, Karalai, & Tewtrakul, 2008; Shimoda et al., 2006; Yoo, Sok, & Kim, 2013). Besides, it is desirable to treat allergies using drugs that do not have sideeffects, and potent antiallergic substances from food ingredients may contribute to the decrease in the number of allergy patterns andhave a found effect on body.

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Chapter 2

Literature Review

Onion (Allium cepa L.) has been valued as a food and a medicinal plant since ancient times. It is widely cultivated, second only totomato, and is a vegetable bulb crop known to most cultures and consumed worldwide [1]. It is a short duration horticultural crop [2] grown at low latitudes. It is commonly known as “Queen of the kitchen,” due to its highly valued flavor, aroma, and unique taste, and the medicinal properties of its flavor compounds.[3][4] Onion is used throughout the year, for example in curries, in the form of spices, in salads, as a condiment, or cooked with other vegetables, such as boiled or baked. It is also used in different forms of processed food, e.g. pickles, powder, paste, and flakes, and it is known for its medicinal values.[5]

Along with their nutritional values, Onion have been found to show bactericidal or bacteriostatic properties in invitro as well as in invivo model also.[6] It has been reported that onion has antibacterial activity. The presented thiosulfinates in onion the antibacterial and antifungal activities against a variety of Gram-negative and Gram-positive bacteria.[7] It is reported that the antibiotic activity of 1mg of allicin, which is a (+)-S-methyl-l-cysteine sulfoxide, is equal to that of 15 IU of penicillin.[8] Recent investigations have also shown an inhibitory effect by aqueous extract on various pathogenic bacterial and fungal species.[9][10][11] Fistulosin, which can be isolated from

roots of welsh onion, exhibit antifungal activities against various fungal species. Particularly P. roqueforti and A. oryzae demonstrate high sensitivity towards this alkaloid compound.[12] It has been reported that concentrated essential oils extract has their antimicrobial activities against a wide range of a commonly food contaminating bacteria and fungi to maintain their qualities.[13]

Post surgical wounds form scar which can be caused for painful pruritic and raised erythematous.[14] The wound caused by post surgical wound is usually emotionally and cosmetically unsatisfactory to the patients, besides it this scar constantly reminds them the circumstances for which the scar was the result.[15] Recently topical onion extract gel is used as a new armamentarium in scar treatment procedure. By inhibiting the fibroblast function, moreover reducing the formation of ground substance which is necessary for the formation of scar matrix, the Allium cepa extract effected on the scar treatment.[16][17] Various studies have shown that hydration is related to scar improvement. The topical petrolatum-based ointment is able to improve scars in patients by this mechanism.[18][19] It is reported that, topical onion extract gel shows significant result with the improvement of the scar whereas the petrolatum-based ointment cannot do it. [20]

Leishmania spp. is intracellular hemoflagellates that are responsible for leishmaniasis disease. This parasites infects the macrophages of skin and viscera to produce disease, they mainly attack vertebrate hosts. [21] Fever, weight loss and hepato-splenomagaly with biochemical abnormalities hyper-globulinemia and pancytopenia are the common symptoms of this disease. [22] The observation of the experiment suggest that the natural resistance of various strains of Leishmania to the active constitutent(s) of onion does not occur at high frequency.[23] Even in aqueous juice, the active antileishmanial compounds present in very low concentration. [24]

Onion is enriched with sulfur containing active component mainly, S-alkyl cysteine sulfoxides, which can easily disintegrated into variety of thiosulfinates and polysulfides through the action of enzyme allinase in time of extraction. Decomposed products are

reactive, volatile, odor producing and lachrymatory and this is present in the oils of onion.[25] These decomposed products have antidiabetic, antibiotic, hypocholesterolaemic, fibrinolytic and many more biological activities. There are nonvolatile sulfur containing peptides and proteins present which possess various therapeutic agents. [26][27]

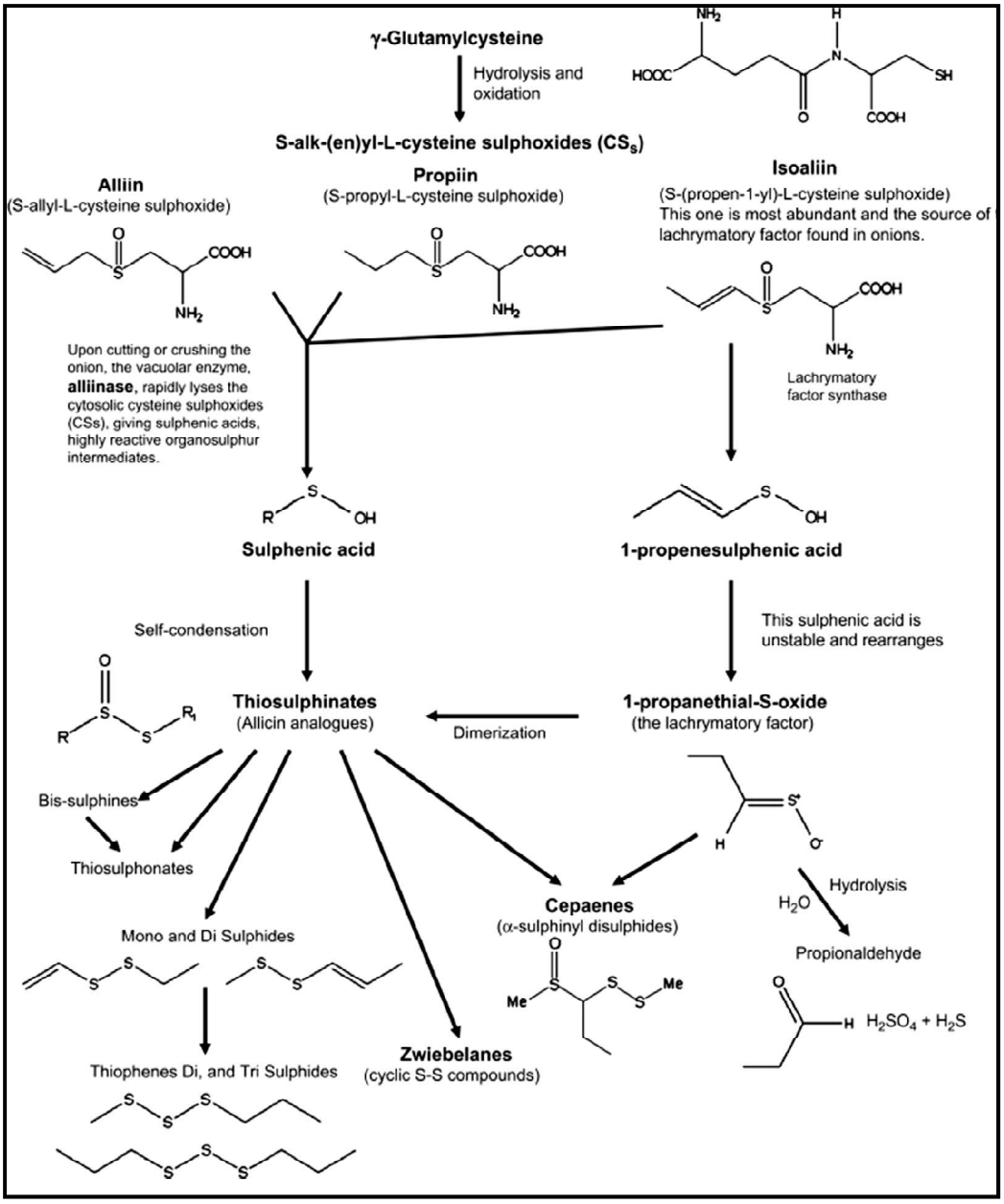

Fig. 2.1 During metabolic pathways organo sulfur compounds form in processed onion. [28]

Evidence from several investigation ssuggests that the biological and medical functions of alliums are mainly due to their high organo sulfur compounds content [29]. The primary sulfur-containing constituents in whole onions and garlic are the *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs), such as alliin, andγ-glutamylcysteines, which, besides serving as important storage peptides, are biosynthetic intermediates for corresponding ACSOs; from these, and by different metabolic pathways in each vegetable, volatiles such as allicin, and lipid-soluble sulfur compounds such as di allyl sulfide(DAS) and di allyl di sulfide(DADS), are originated [30]. These compounds provide onions with their characteristic odor and flavor, as well as most of their biological properties.

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Chapter 3

Materials and Methodology

3.1 Collection, Extraction and Preparation of Plant Sample

The skins of Onions (*Allium cepa*) were collected from local market of Jadavpur and Ultadanga localities in Kolkata. The collected skins were washed thoroughly by fresh tap water and distilled water in several times to remove dirt (Fig.3.1). Maceration method had chosen as extraction procedure to extract bioactive compounds from plant sample. 30% (v/v) Ethanol was taken as the solvent (menstruum) of this maceration process and macerated for 5-6 days with shaking occasionally. After maceration the produced extract filtered by Whatman filter paper under vacuum pressure (Fig. 3.2 A). Filtered extract solution concentrated by Rotary Evaporator at 45ºC and lyophilized by Freeze dryer machine at -80ºC for 24 hours (Fig. 3.2 B). The lyophilized Onion skin powder stored at -20ºC in freezer for further use in future.

Fig. 3.1 Onion skins with Onion bulb

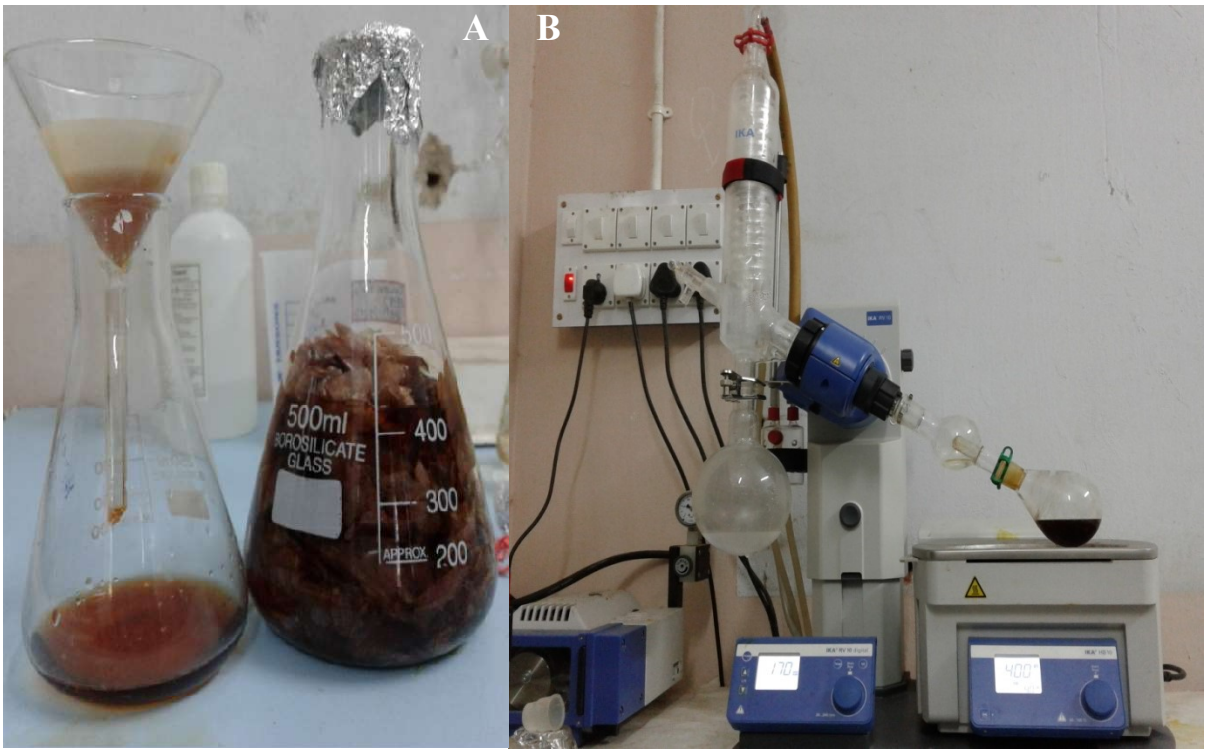

Fig.3.2: A. Macerated Ethanol extract of skins of Onion, B. Sample loaded on Rotary Evaporator

3.2 Screening Tests for phytochemicals of Extract

Phytochemical Screening tests were done to evaluate the qualitative properties of sample plant extract. The standard protocols for these qualitative tests are following:

3.2.1 Test for Phenols:

Lead Acetate test: 10% lead acetate solution was added into 3ml extract in glass test tube. The presence of phenols was indicated by white precipitate formation.

3.2.2 Test for Flavonoids:

2ml of filtrate aqueous plant extract was added with around 3ml of dilute ammonia. Succeeded by inclusion of 1ml concentrated H_2SO_4 (Sulphuric Acid). The presence of flavonoids was indicated by yellow coloration.

3.2.3 Test for Alkaloids:

Dragendorff's test: Little amount of plant extract was stirred with 6ml of 1% HCl (Hydrochloric Acid) in water bath for 5 mins and filtered. Dragendorff reagent (Potassium bismuth iodide solution) was added with that filtered extract. Orange red precipitate indicated the presence of alkaloids in extract.

3.2.4 Test for Saponins:

0.5g of plant extract shook with 10ml distilled water in test tube. The presence of saponin was shown by formed stable and persistent froth which was persisting in water bath for 4-5 mins.

3.2.5 Test for Tannins:

Approximate 0.1g of powder of plant extract was boiled in 4ml distilled water in glass test tube. Then few drops of 0.1% ferric chloride were added in filtered plant extract and blue-black or brownish green coloration indicated the presence of tannin in plant extract.

3.2.6 Test for Phlobatannins:

Aqueous extract of sample was boiled with 1% aqueous HCl (Hydrochloric Acid) and the presence of phlobatannins was observed by deposition of red precipitate at the bottom of the test tube.

3.2.7 Test for Terpenoids:

Salkowski test: The crude plant extract was mixed with 1ml of Chloroform and 1ml of concentrated H_2SO_4 respectively and intense reddish brown coloration indicated the presence of terpenoids in the sample.

3.2.8 Test for Reducing Sugars:

Fehling's test: The ethanolic plant extract which was made by about 0.2g of crude powder in 1ml ethanol, was added to 3ml of distilled water and mixed very well. In a test

tube 1mlof Fehling's A solution and Fehling's B solution was taken and boiled and then poured in the aqueous ethanolic plant extract. The presence of reducing sugar was detected by changing in colour reaction.

3.3 Determination of Total Phenolic Content (TPC)

To evaluate the quantitative amount of Total Phenolic Content in the plant extract was followed by Folin-Ciocalteu reagent (Singleton and Rossi, 1965). 1ml of plant extract was mixed up with 0.5ml Folin-Ciocalteu (FC) reagent which was 1:1 diluted with distilled water and kept in incubator for 5mins at 22°C. The mixture followed by addition of 4ml of Na_2CO_3 (sodium carbonate) solution (75g/L) and incubated at 22°C for 90 mins. The absorbance of this mixture was measured at 765nm using spectrophotometer. Gallic acid was taken as standard to construct the calibration curve. The formula was used for determining the total content of phenolic in plant extract is,

$$C = C^1 \times V/m$$

Where, C= total phenolic content in Gallic Acid Equivalent (GAE), in mg/g,

C^1= Concentration of Gallic acid estimated from the Standard Curve in mg/ml,

V= Volume of plant extract in ml,

m= Weight of plant extract in g. [1]

3.4 Determine of Total Flavonoid Content

To determine the quantity of Total Flavonoid Content in plant extract, Aluminium chloride method was applied. 0.5ml distilled water and 0.3ml of 5% (w/v) $NaNO_2$ (sodium nitrite) solution were added in 0.5ml of plant extract. The mixture was kept in the incubator for 5mins at 25°C. After that immediately 0.3ml of 10% of $AlCl_3$ (Aluminium Chloride) was added. This was followed by addition of 2ml of 1M NaOH (sodium hydroxide) in the reaction mixture after 5 mins. The absorbance of the mixture was measured at 510nm using spectrophotometer. The standard curve was made by

different concentration of quercetin. To evaluate the total flavonoid content in the plant extract a linear equation was construct from the standard curve. [2]

3.5 Quantitation of Antioxidant activity by using DPPH

DPPH method was used to determine the ability of scavenging of the stable free radicals. 2.5ml of 0.04% 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in methanol was mixed with 0.5ml of different concentrations of plant extract individually. The absorbance was taken at 517nm after 30mins incubation at room temperature in dark. The Methanol solution was taken as control and standard antioxidant curve was made by using Citric acid. The formula for calculation of the percentage inhibition of free radical is,

$$\text{RSA (\%)} = \frac{\text{OD of Control} - \text{ODof Sample}}{\text{OD of Control}} \times 100$$

3.6 Determination of Antimicrobial Activity

The antimicrobial activity of onion skin extract was observed on *Escherichia coli* (gram negative bacteria) and *Staphylococcus aureus* (gram positive bacteria) respectively which are common human pathogenic bacteria.

3.6.1 Evaluation of activity using bacterial suspension culture

To evaluate the activity of ethanol extract of Onion skins on the 'log phase' stages of bacterial cultures, the following steps were followed,

1. Two separated 80ml of Nutrient Broth (NB) medium (Himedia) were prepared in two different 250ml conical flasks for medium of suspension cultures of E.Coli and S.aureus respectively. The Nutrient Broth was taken as 13g in 1 L distilled water.
2. Primary inoculums were inoculated using bacteriological loop from E.coli and S.aureus streaked culture plates into NB medium contained conical flasks.

3. The conical flasks were incubated for 24 hours in shakers at 37ºC which enabled the growth of bacteria actively in the 'log phase'.
4. In the next day, the two separated 80ml conical flasks were poured equally in 50ml falcon tubes and labeled as control and treated for both E.Coli and S.aureus respectively.
5. 1ml distilled water and 1ml of 100mg/ml of Allium cepa extract were added in control and treated tubes respectively.
6. The absorbances of these cultures were measured at 600nm for 5 hours. The data was recorded for 10 times at 30 minutes intervals.
7. The drug action was monitored on the culture in 'log phase' both graphically and microscopically.
8.

3.6.2 Counting Total Bacterial Cells

Total Bacterial cells were counted by using hemocytometer for the given time period of 5 hours. This experiment was done for determining the total number of cells present after treatment with the plant extracts. The calculation for counting the total number of bacterial cells presented was,

$$\text{Total number of bacterial cells} = n \times d \times 10^4 \text{ cells/ml}$$

Where, n= total number of cells in one square corner,

d= dilution factor,

10^4 used to extrapolate the total number of cells present in 1 ml.

100µl of the suspension cultures were plated on nutrient agar plates and incubated at 37ºC overnight to observe the growth of colonies.

3.6.3 Determination of Minimum Inhibition Zone of ethanol extract of Onion skins

The antimicrobial assay was performed using the agar well diffusion method by measuring zone of inhibition against E.Coli and S.aureus. The succeeding steps were followed to do this experiment,

1. Primary bacterial cultures were inoculated using bacteriological loop from E.coli and S.aureus streaked culture plates into two 5ml contained NB mediums.
2. After 24 hours incubation, 1ml of primary bacterial cultures of both bacteria E.Coli and S.aureus were poured in both 50ml lukewarm sterile molten NB-agar solutions, which were made by 13g of NB in 1 L distilled water and 2.5% (w/v) agar-agar (Merck, CAS No. 9002-18-0) respectively.
3. Thereafter, these lukewarm bacteria inoculated molten NB-agar solutions poured into different petri plates and allowed the agar to completely gel without disturbing it.
4. After solidifying the petri plates were punched by using well puncher and 150µl of the different doses of plant extract and streptomycin as standard drug were loaded into the wells of both bacterial plates.
5. The zones of inhibition were measured after overnight incubation in 37ºC and hence the minimum inhibitory concentration (MIC) of plant extract was determined.

3.6.4 Analysis of growth curves of E.Coli and S.aureus

The analysis of growth curves of gram negative bacteria, E.Coli and gram positive bacteria, S.aureus was performed in following fashioned,

1. Six separated 50ml of Nutrient Broth (NB) medium (Himedia) were prepared in six different 100ml conical flasks for medium of E.Coli and S.aureus respectively. The Nutrient Broth was taken as 13g in 1 L distilled water.
2. 1ml of 100mg/ml Allium cepa extract and 1ml of E.Coli and S.aureus bacteria at the logarithmic phage respectively, were added into the 50ml culture solutions. The same amount of sterile distilled water was taken as positive control and same amount of 150µg/ml Streptomycin was taken as negative control.
3. The conical flasks were incubated for 24 hours in shaker at 37ºC condition, during incubation period the absorbance of these cultures were measured at 600nm at 2 hours intervals.

4. The bacterial growth curve was made by graphically to analysis the effect of Allium cepa L. on E.Coli and S.aureus growth.[3]

3.7 Activity of Onion Skins on Primary Cell Line

This experiment was done to study the activity of ethanol extract of Onion skins on normal cell line. Here human peripheral blood mononuclear cells (PBMCs) was taken as primary cell line and was determined the cytotoxic level of plant extract.

PBMCs were isolated from peripheral blood and identified by its round nuclei for the presence of monocytes and lymphocytes (T cells, B cells and NK cells) cells. The cell fraction corresponding to red blood cells and granulocytes (neutrophils, basophils and eosinophils) was removed from whole blood by density gradient centrifugation. A gradient medium with a density of 1.077 g/ml separates whole blood into two fractions. PBMCs made up the population of cells that remain in the low density fraction (upper fraction), whilst red blood cells and polymorphonuclear leukocytes have a higher density and were found in the lower fraction.

3.7.1 Isolation and Culture of PBMCs

The following stages are followed to separate the human peripheral mononuclear cells from the human blood sample,

1. 5ml fresh human blood was collected in EDTA coated vacutainer and kept as that can be used within maximum 2 hours at room temperature.
2. To prevent of any blood clots, 1:3 dilution of the whole blood was made by 0.9% autoclaved chilled saline (0.9g of NaCl in 100ml Millipore water).
3. In a 15ml falcon conical centrifuge tube 2.5ml HiSep LSM 1077 (Himedia) was taken and 7.5ml of diluted blood was layered over this. There was a sharp inter phase was observed between these two layers, so, extra precaution was taken to avoid the mixing of these two layers.
4. The blood sample was centrifuged at room temperature for 15 minutes.

5. After centrifugation, the blood sample separated into 4 layers. Among those layers the topmost faded yellow layer of plasma thrombocyte layer was discarded and the following white ring layer of lymphocytes was drawn out very precautiously in 15ml another falcon conical centrifuge tube. This white ring layer was together with half of HiSep layer. The lowermost layer which contained with red pellet of RBC's was also discarded.
6. The collected lymphocytes and HiSep mixture was added with an equal volume of sterile filtrated serum free RPMI-1640 (Himedia) media. After mixing the sample was centrifuged at 800 rpm for 10 minutes.
7. The same washing process was done for removing the excess platelets, HiSep and plasma cells.
8. The obtained PBMCs was suspended in complete RPMI 1640 medium with 10% Fetal Bovine Serum and 2% Penicillin-Streptomycin and maintained at 37^0C in a humidified incubator with 5% CO_2.

The PBMCs were cultured in RPMI 1640 with 10% FBS and 2% Penicillin- Streptomycin solution. Passaging was done for two days at 24 hr. intervals.

3.7.2 Cytotoxic activity on PBMCs

The cytotoxic level of ethanol extract of Onion skins was performed by using tetrazolium assay or MTT assay on Peripheral Blood Mononuclear cell line. This assay is based on the reduction of tetrazolium dye MTT 3-(4, 5- dimethylthiazol- 2- yl)-2,5 diphenyltetrazolium bromide (Merck) by mitochondrial dehydrogenase to form insoluble, dark blue formazan product. Only viable cells with dehydrogenase activity are able to reduce significant amounts of the MTT dye to formazan. Finally, the optical density for each well at 590 nm was measured using a spectrophotometer.

The isolated cells were seeded in 96 well plates (0.1×10^6 cells per well) and cultured at 37ºC for 24 hours in a humidified incubator with 5% CO_2. When cells reached 60% confluence after 24 hours, cells were treated with various concentrations of ethanol extract of Onion skins test samples as 25µg/ml, 50µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml and 1000µg/ml and the cells were incubated 37ºC for 24 hours in a humidified incubator with

5% CO_2. MTT (3 mg/ml in PBS) was added to the cells of each well and incubated for 3-4 hours. After that, the MTT was removed and DMSO (100 µl/well) was added to dissolve the formazan crystal. Absorbance was measured at 590 nm and the percentage of cell viability was calculated as,

$$\text{Perentage of viable cell} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

3.8 Determination of Anti allergic activity on In Vivo model

3.8.1 Experimental Animals

This experiment was carried out with specific-pathogen-free female BALB/c mice of weighing 16-24g and approximately same ages. Those mice were kept in the Animal House of Bengal School of Technology. All mice were housed in polypropylene cages at specific pathogen-free conditions as five mice per cage in manner. They were allowed to acclimatize to the laboratory conditions for 7 days prior to this experiment. They were housed under temperature controlled room where temperature maintained at 23±2ºC and relative humidity of 50-55% throughout the study. Mice had free access to their normal diet and water ad libitum and exposed them into a proper day and night cycle (12 hours light-dark cycle). Each mouse was used only for once as they were euthanized at the end of the experiment. The animals were procured as per the animal ethical guidelines of government of India.

3.8.2 Determination of Acute toxicity level and Dose selection

Acute toxicity test was done to get preliminary information of the toxic nature of ethanol extract of *Allium cepa*. The result of this test was used to select the dose levels for the mice. The ethanol extract of skins of Onions was administered by orally in the dose range of 50,100, 200, 300 and 2000 mg/kg to different groups of mice of single dose. The mice

were examined for 30mins intervals up to 4 hours and checked the mortality test for 24 hours. Meanwhile the signs of toxicity were also observed, such like excitation, tremors, twitches, motor co-ordination, righting reflex and respiratory changes. The mice exhibited no mortality and stayed safe at decided highest dose, 2000 mg/kg of body weight. So, this observation and result established that the dose of this anti allergic activity was selected at three dose levels 50 mg/kg, 100 mg/kg, 200 mg/kg and 300 mg/kg of body weight.[4][5]

Fig. 3.3 The Dose of different concentrations of the ethanol extract of Onion Skins which were administered to the animals.

3.8.3 Animal Treatment

After seven days acclimatization, the mice were randomly divided into groups and subgroups to achieve the objective of this experiment. Two major model groups were created, which were known as Mast cell stabilization group and Systemic anaphylaxis group. Each group was divided into seven individual groups and each group was consisting of 5 mice. Group I denoted as control group and served normal diet to them. Group II denoted as challenged group or negative control group, they were also served normal diet. Group III denoted as standard drug group which was positive control group and served Di- Sodium Cromoglycate (DSCG) or Cromolyn sodium salt (≥95%) (Sigma-Aldrich, CAS Number: 15826-37-6) by inject i.p. (intraperitoneal injection) through

tuberculin syringe due to its poor absorption through oral route (Fig 3.4 B). Group from IV-VII were treated with doses of ethanol extract of skins of onions at 50 mg/kg, 100 mg/kg, 200 mg/kg and 300 mg/kg respectively doses by oral gavage (Fig. 3.4 A). All the plant extracts were suspended in distilled water and were given to rats once daily by oral gavage. Same grouping did for both major groups, Mast cell stabilization group and System anaphylaxis group and daily delivered their drugs as mentioned ways. [6]

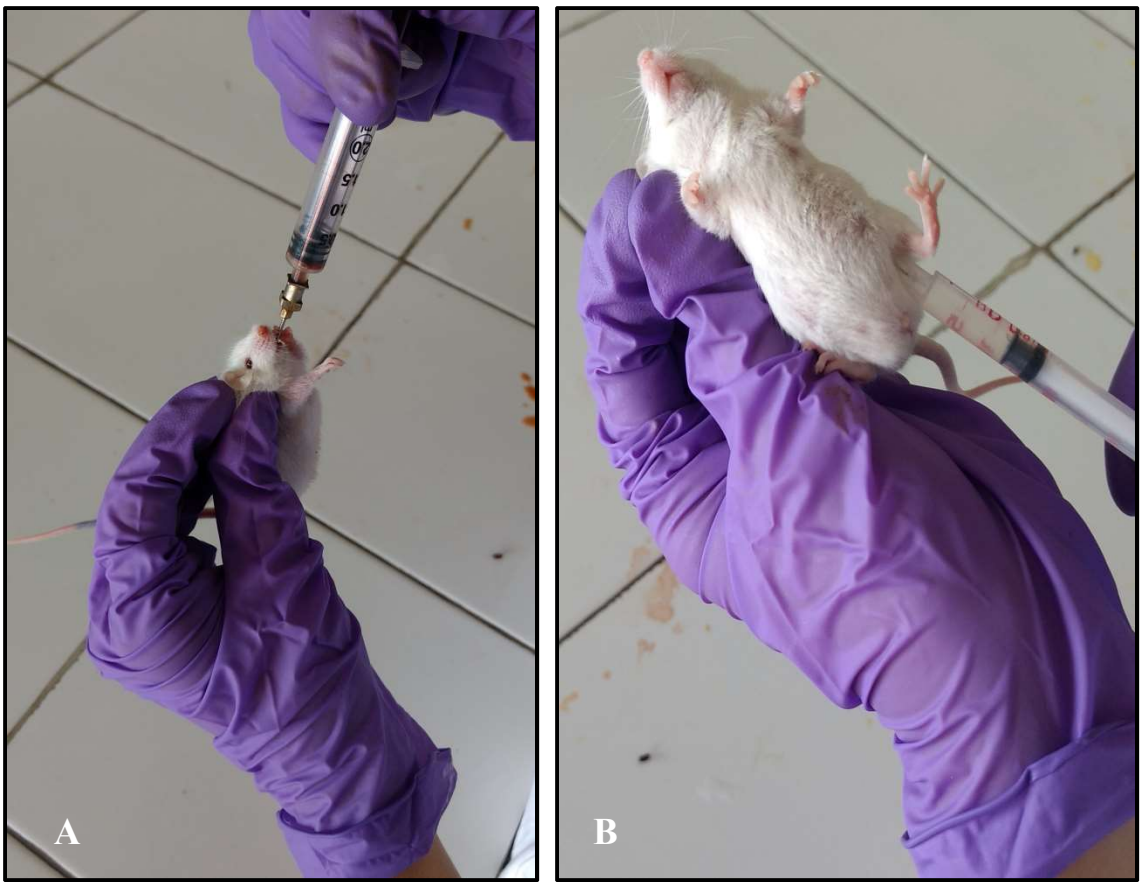

Fig. 3.4 Different administration processes of the drugs at different doses of tests and standard, A. Onion Skins Extract was administered by orally and B. Standard drug, DSCG was administered by intraperitoneal injection

3.8.4 Preparation of Peritoneal Mast Cell (PMC)

Earlier than the isolation process of peritoneal mast cell of mice, the following described steps were successively done:

1. Every surgical procedure was performed in clean condition and surgical forceps and scissors were sterilized and the Aluminum Dissecting Tray with paraffin wax cleaned to avoid contamination.
2. Each mouse of the group of mast cell stabilization was sacrificed by keeping them in the anesthetic ether filled small vacuum desiccators and inhaled them over dose of anesthetic ether. The ethanol extract of *Allium cepa* L. at doses of 50 mg/ kg, 100mg/kg, 200mg/kg and 300mg/kg and the DSCG at doses of 10mg/kg were given to mice daily for 5-6 days prior to the collection of peritoneal fluid which contained mast cells.[7]
3. The sacrificed mouse was placed on the dissecting tray and the abdominal skin sterilized by 70% ethanol solution (as a standard antiseptic) filled gauze swab.
4. After that, the abdominal skin was lifted up with forcep and an incision into the abdominal skin was done using surgical scissor. The cutis layer of skin was cut open crosswise at approximately 2cm length by surgical scissor to expose the abdominal wall. (Fig. 3.5)

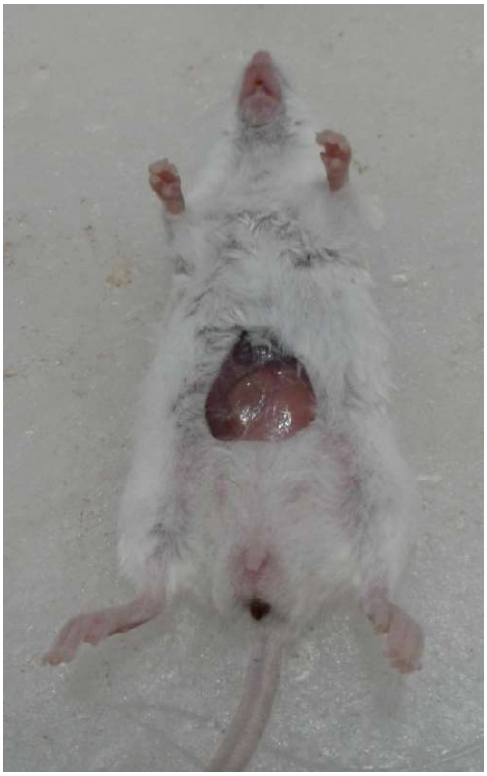

Fig. 3.5 Sacrificed mouse with exposed abdominal wall

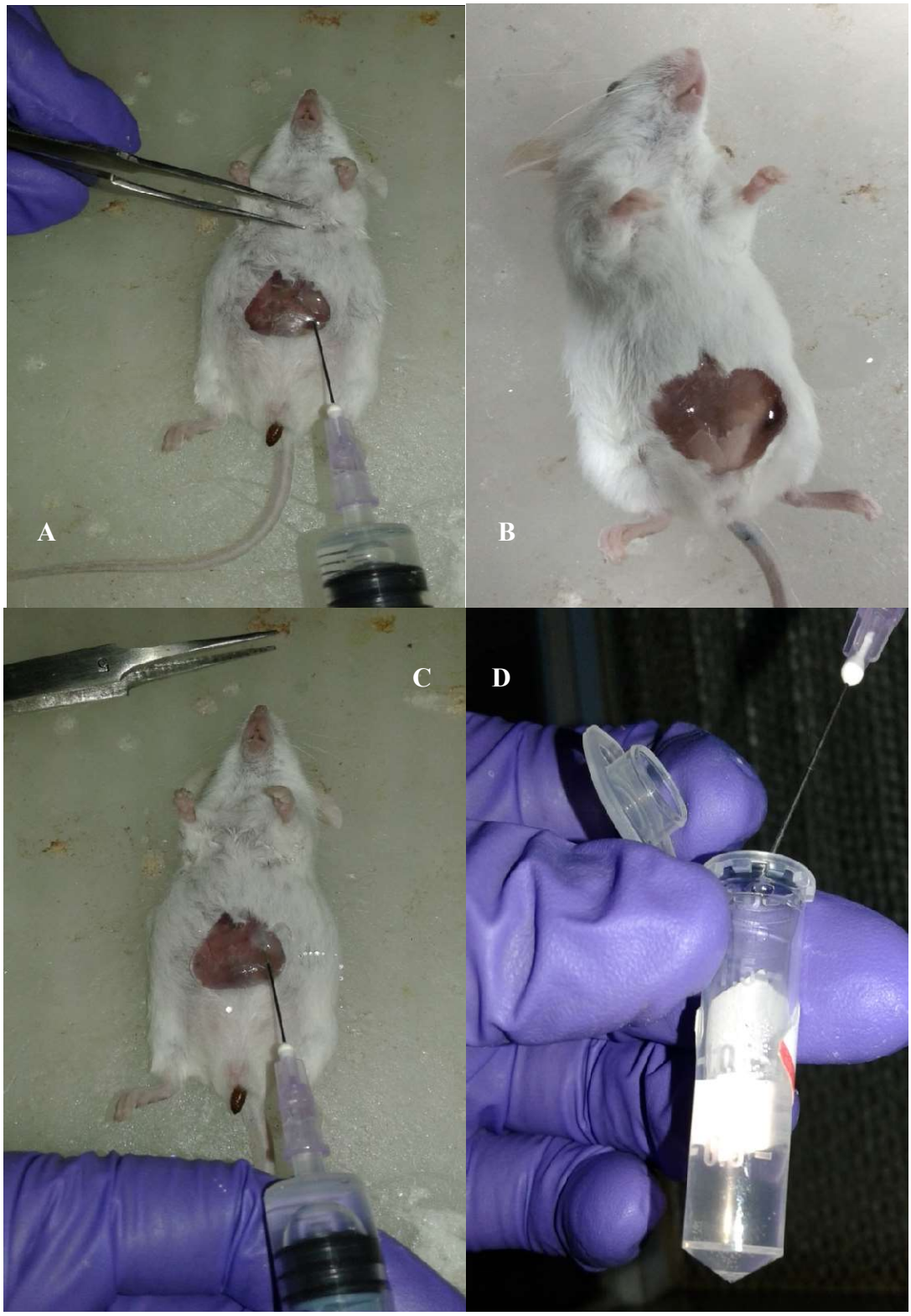

Fig. 3.6. A. 5ml PBS solution was injecting in intraperitoneal cavity, B. PBS filled intraperitoneal cavity, C. After 10s intraperitoneal fluid was collecting,D.collected intraperitoneal fluid.

The isolation procedure of peritoneal mast cells were followed the following steps,

1. A 5ml syringe was filled with 3ml of chilled Phosphate Buffer Saline (PBS) (Himedia) and 2ml of air.
2. This air and PBS filled syringe was slowly injected into the peritoneal cavity at the linea alba to prevent disintegration of many tiny and thin lymphatic and blood vessels and leaking of blood into the peritoneal cavity.
3. A bloated cavity was shown after the previous step where the subcutaneous tissue was separated from the internal organs and the peritoneal cavity was filled with PBS.
4. The mouse was placed on the surface of wax filled dissection tray and gentle abdominal massaged for 10s.[8] This procedure will allow the mast cells to take apart from tissue and passing over in the PBS solution.
5. Afterwards, the injected air was removed by syringe. The cell suspension was drawn out from the peritoneal cavity by another 5ml syringe. The suction process of collecting fluid should be very carefully to prevent attachment of surfaces of highly vascularised organ to the tip of syringe and disintegration of thin vessels. Thus, peritoneal cells which contained with mast cells as well also flushed out from the mouse's peritoneal cavity.
6. The peritoneal cells containing fluid was collected into siliconized micro centrifuged tubes which containing 1ml of RPMI-1640 (pH 7.2-7.4) (Himedia) and stored on ice bucket until later use (Fig 3.6. A., B., C., D.). [8][9]

3.8.5 Mast cell stabilization activity

To study the mast cell stabilization activity of ethanol extract of skins of onions, the collected peritoneal fluids which contained mast cells were used. The following stages were followed to perform this experiment,

1. Cell viability was checked using trypan blue dye(Himedia) (0.4%) by trypan blue exclusion test. 50µl of the mast cell suspension were placed in cryovial tube and

to obtain 1 dilution added equal amount of 0.4% trypan blue dye to cell suspension and mixed by pipetting up and down. Thereafter, mixture was incubated for less than three minutes at room temperature. If cells are counted after approximately five minutes, viability will be inaccurate due to cell death, so within five minutes the cell countered using hemocytometer by 10µl of cell suspension.Determined cell viability using the formula below,

$$\text{Viability (\%)} = \frac{\text{Live cell count}}{\text{Total cell count}}$$

2. The collected and stored peritoneal fluid, contained of mast cells were washed for thrice with RPMI-1640 media at lower speed centrifugation method in 500-600 rpm for 10 minutes. The supernatant was discarded and the pellet of mast cells was resuspended in the RPMI-1640 media.[10]
3. Mast cells from control and treated groups were seeded in 96 well plate ($10x10^4$ cells per well) and incubated with compound 48/80 (Sigma-Aldrich, C2313) at 1µg/ml concentration for 10 minutes at 37 °C in a humidified incubator with 5% CO_2.
4. After 10 minutes, the cells were stained with 0.1% toludine blue (Sigma-Aldrich, CAS Number 92-31-9) and observed under high power microscope at 400x magnification and counted the numbers of degranulated mast cells and intact mast cells from total number of 100 cells by using hemocytometer.[11]
5. Calculated the percentage of the intact mast cells as,

$$\text{Percentageofintactmastcells} = \frac{\text{Total number of mast cells} - \text{Totalnumber of degranulated cells}}{\text{Total number of mast cells}} \times 100$$

3.8.6 Anti Anaphylaxis Activity

To observe the anti anaphylaxis activity of ethanol extract of onion skins, few steps were taken,

1. The DSCG drug administered at intraperitoneally area of controlled group and different doses of onion skins extract as 50 mg/ kg, 100mg/kg, 200mg/kg, 300mg/kg were administered respective groups for 5-6 days as daily basis.
2. 4 days later, mast cell degranulator, Compound 48/80 injected intraperitoneally injection through tuberculin injection to mouse of each groups as 0.8mg/kg body weight of compound 48/80.
3. Mortality was monitored and recorded for 1 hour after administration of compound 48/80. [4],[11]
4. The percentage of mortality by inducing anaphylactic shock was calculated as,

$$\text{Mortality (\%)} = \frac{\text{Total number of dead mice}}{\text{Total number of experimental mice}} \times 100$$

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Chapter 4

Result and Discussion

4.1 Qualitative analysis of phytochemicals in crude extract

Through performing the phytochemicals screening test of crude extract of Onion skins, the presence of different phytochemicals such like Phenols, Flavonoids, Alkaloids, and Tannins etc. were confirmed. These results suggest that there are various kind of pharmacological activities presented in this extract. (Table 4.1)

Sl. No.	Test Performed	Results
1	Phenols	+
2	Flavonoids	+
3	Alkaloids	±
4	Saponins	+
5	Tannins	+
6	Phlobatannins	+
7	Terpenoids	+
8	Reducing Sugars	+

Table 4.1. Phytochemical Screening Result of crude Onion Skins extract

4.2 Results of Total Phenolic Content

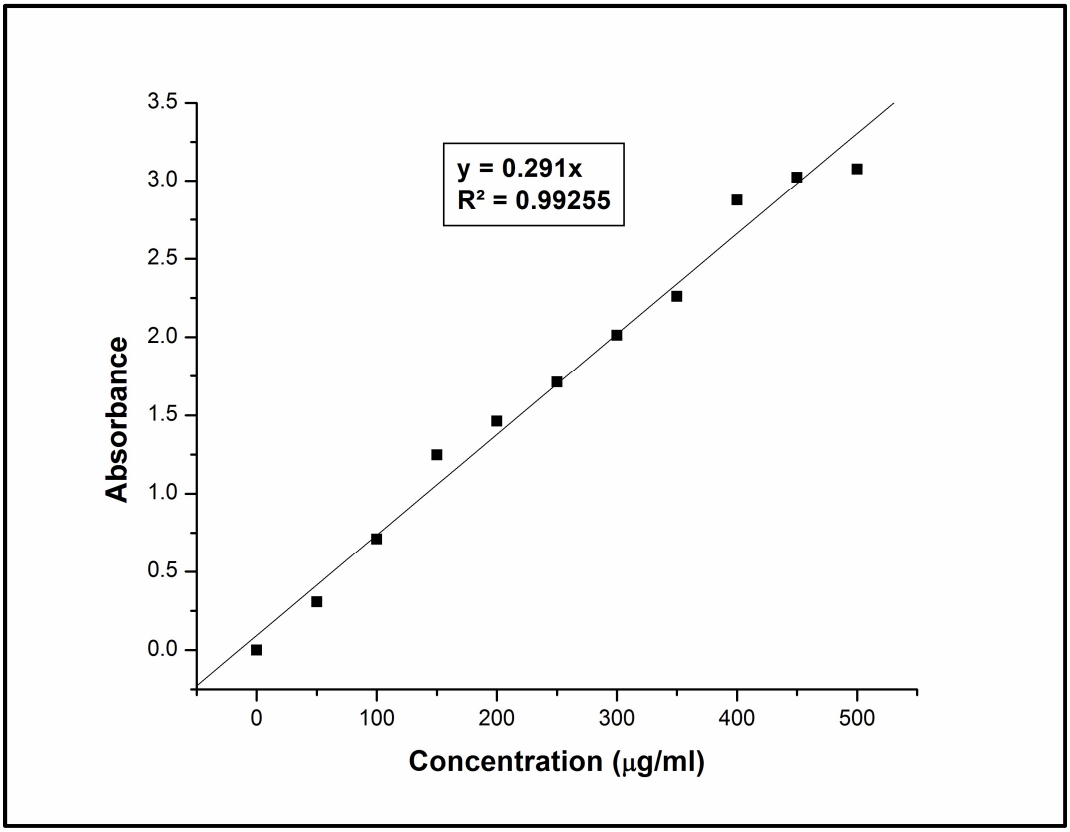

Fig. 4.1. Standard curve of Gallic Acid

The result of Total Phenolic Content showed that 240.55mg/g Gallic Acid Equivalent (GAE) was presented in the ethanol extract of Onion skins. The Standard Curve of gallic acid was required to estimate the Total Phenolic Content of plant extract (Fig. 4.1). The result indicated that onion skins are enriched with polyphenolic compounds. In onion skins gallic acid and ferulic acid present as major polyphenolic compounds, this has anti diabetic, anti ageing, anti cancer properties.[1] Phenolic acids are effective modulators, which has has significant synergistic, enhanced, and additive effects on NF-κB activity.[2] Mainly phenolic compound contained plants are used as spices, such like onion is widely used as spice in our daily recipes.[3] The phenolic compound has antimicrobial activity on pathogenic bacteria which may retard microbial invasion. For this reason now a days they are used in preservation of foods at high concentration.[4]

4.3 Results of Total Flavonoid Content

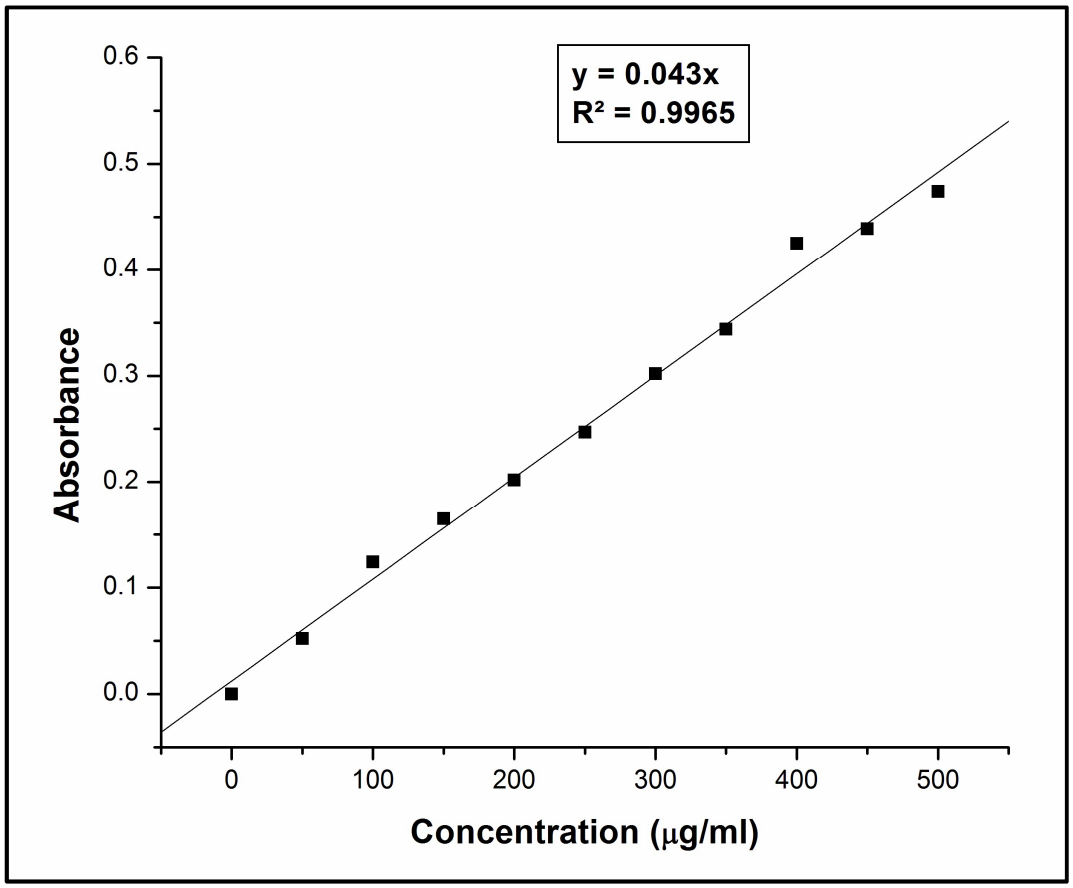

Fig. 4.2 Standard Curve of Quercetin

The result of Total Favonoid Content showed that 295μg/ml of flavonoid content was presented in the ethanol extract of Onion skins. Quercetin was used to construct the Standard Curve for estimating the Total Phenolic Content of plant extract (Fig. 4.2). The result demonstrated that the content of flavonoid is more present in Onion skins rather than phenol contents. Mainly quercetin 3,4′-diglucoside, quercetin 4′-glucoside, and isorhamnetin 4′-glucoside are present in the skins of Onions. Among them quercetin 4′ -glucoside, that is highly bioavailable, exhibits more effect on suppression of the type I allergy. [5]

4.4 Result of Free Radical Scavenging Activity

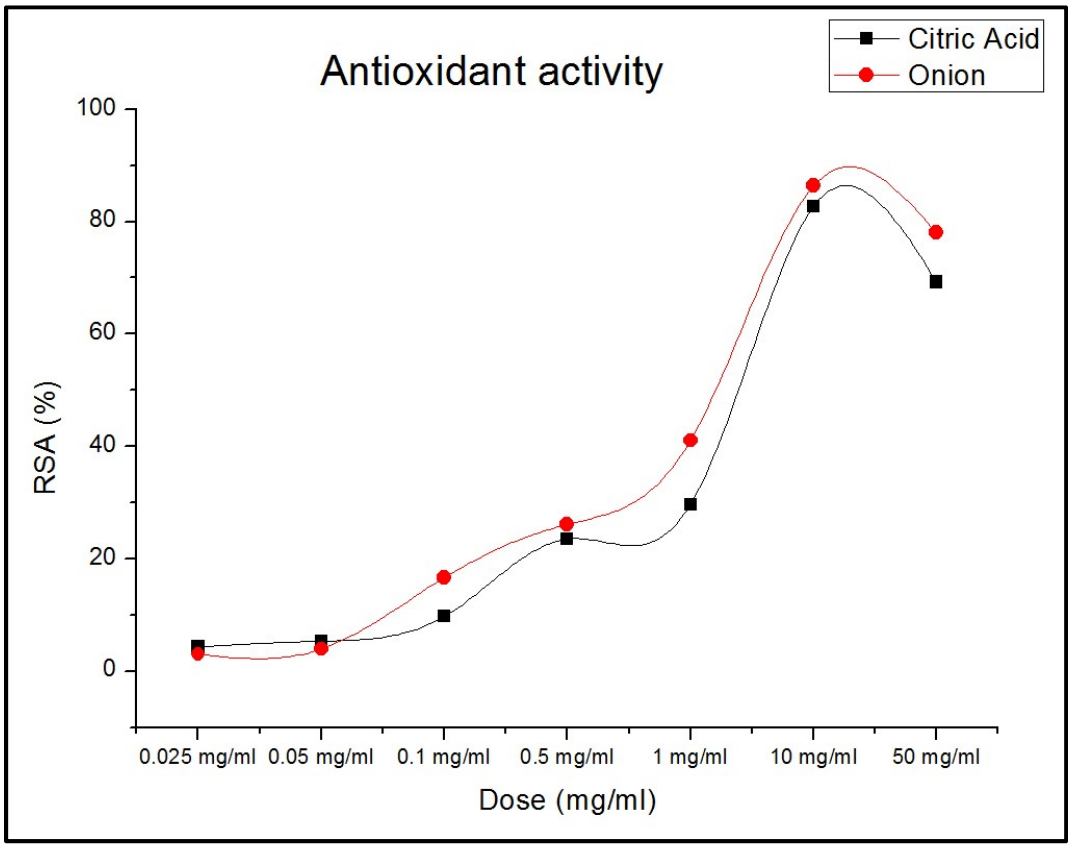

Fig. 4.3 Dose dependent free radical scavenging activity by using DPPH method

By using 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution as a free radical, the free radical scavenging activity (RSA) of Onion skins were performed. Citric acid at same concentration was taken as standard anti oxidant. The result showed that 2.99987% to 78.04339% DPPH free radical scavenging activity were presented in 0.025mg/ml to 50mg/ml concentrations of skins of Onions (Fig. 4.3). The percentage of RSA was decreased after the dose of 10mg/ml of Onion Skins sample. Onion is enriched with polyphenol compounds, mainly flavonoids, which is the main reason of mediation of the activity of free radical scavenging for onion.[1] Antioxidant has potential to perform dual role from the view point of food safety, as prevention of autoxidation of food and as antimicrobial.[6] Antioxidant mainly exists in front line of defense line against reactive species and by scavenging initiating radicals, binding metal ions or removing damaged

molecules, they acts at different levels of the oxidation process. Some specific antioxidants from diet or vitamin supplements improve the condition of lung function and control the asthma problem in asthmatic children and adults.[7]

4.5 Results of activity using bacterial suspension culture

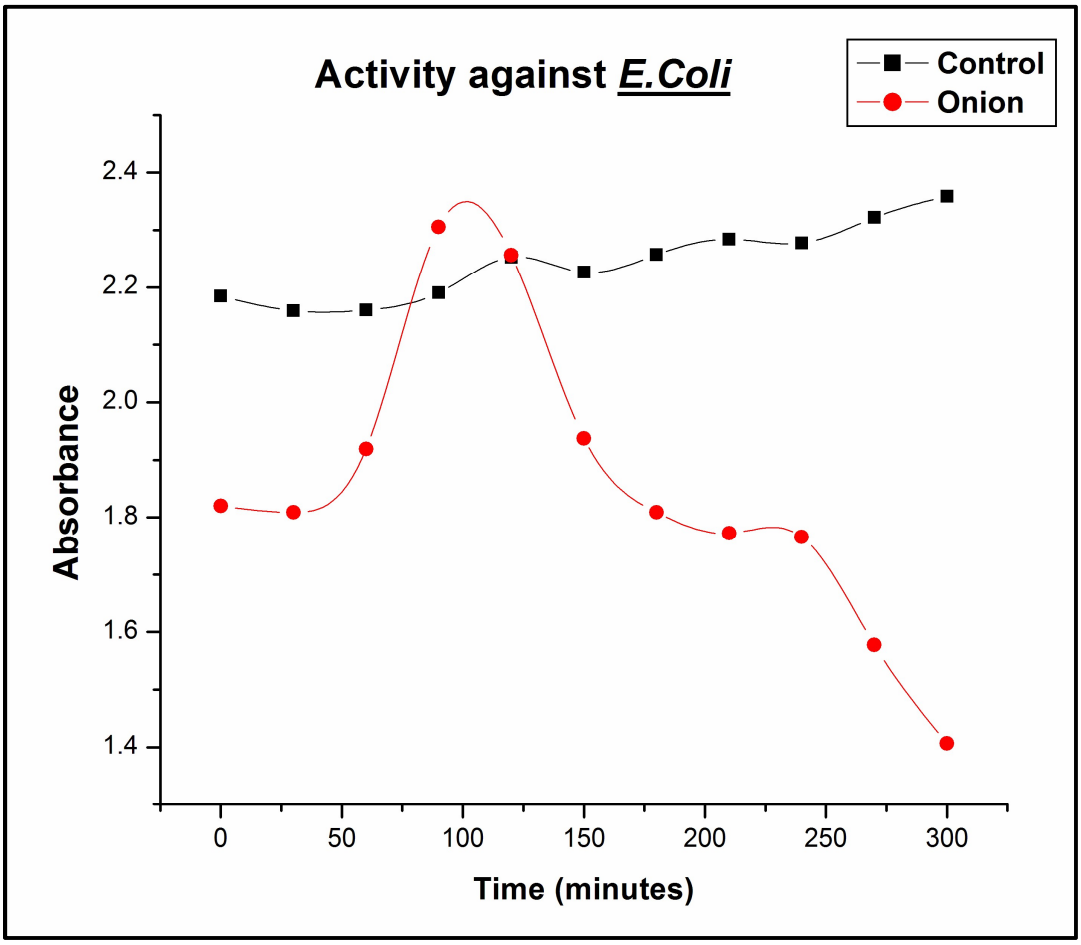

Fig. 4.4The antimicrobial activity of the onion extract against the growth of *E. coli* in suspension culture, demonstrated by absorbance vs. time graph

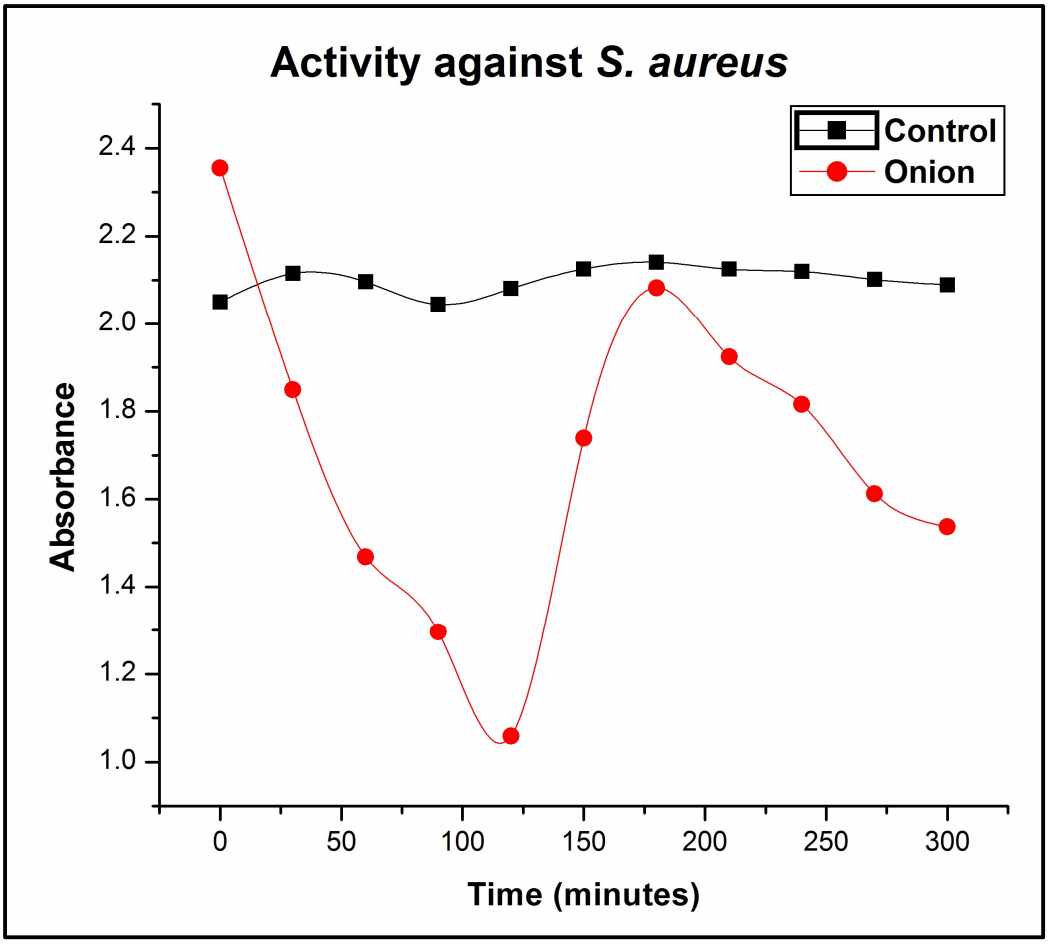

Fig. 4.5 The antimicrobial activity of the onion extract against the growth of *S. aureus* in suspension culture, demonstrated by absorbance vs. time graph

The graphically results of anti microbial activity of the onion extract against the growth of *E. coli* and *S.aureus* in suspension culture indicated that the sample extract inhibited the growth of both bacteria (Fig.4.4 and Fig.4.5). Microscopic images demonstrated that plasmolysis of the both bacterial cells in treatment with onion extracts (Fig. 4.6). The cells treated with onion exhibited effects only at much milder degrees. Through this experiment it was demonstrated that onion extract is more bactericidal for gram negative bacteria than gram positive bacteria.

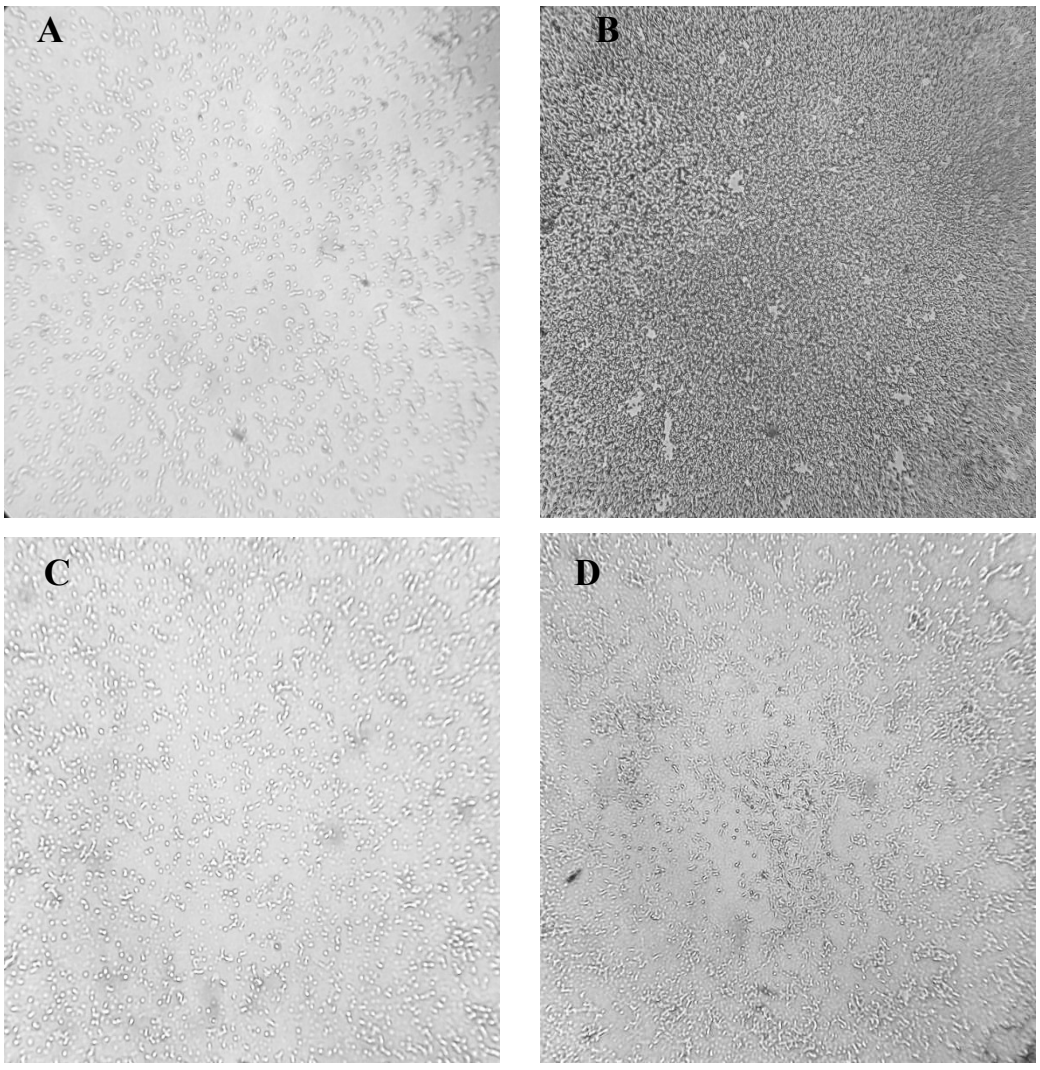

Fig. 4.6 Microscopic evaluation of the bacterial cells after treatment with the extract, after 6 hour through 40X microscope. A: *E.coli* cells as Control, B. Treated with onion extract against *E.coli* , C. *S.aureus* cells as standard, D. Treat with onion extract against *S.aureus.*

4.6 Results of Total Bacterial Cell Counts

The results of total bacterial cell counting for both bacteria showed that the total number of bacterial cells presented at hemocytometer were approximate 131×10^4 cells/ml and 125×10^4 cells/ml(Table. 4.2). This result indicated that onion extract has more anti microbial activity against *E.coli* than *S.aureus*. The main phytochemicals present in onion are isquercetin and allicin (thio-2-propene-1-sulfinic acid-5-allyl-esters),quercetin where

they can binds with the bacterial DNA gyrase as well as allicin inhibits certain thiol containing enzymes in the microorganisms by the rapid reaction of thiosulfinates.[8]

Escherichia coli	
Test Sample	**$\times 10^4$ cells/ml**
Control	197
Onion	131

Staphylococcus aureus	
Test Sample	**$\times 10^4$ cells/ml**
Control	178
Onion	125

Table 4.2 Total bacterial cell counts for E. coli and S. aureus after treatment with onion extract at 100 mg/ml concentration

4.7 Result of Minimum Inhibition Zone of ethanol extract of Onion skins

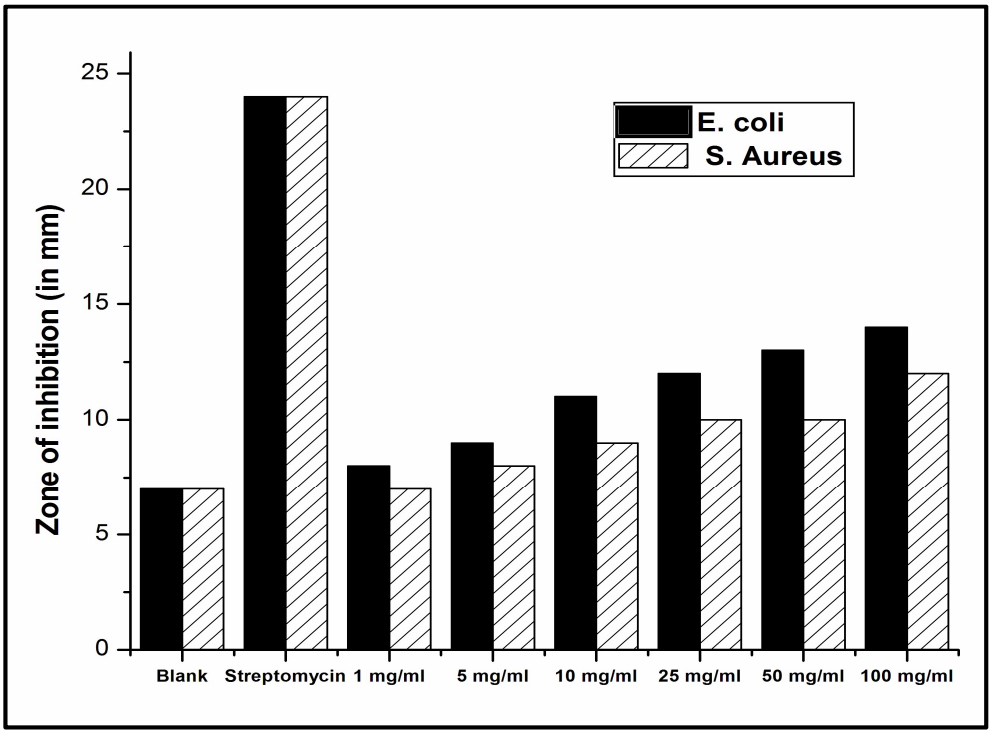

Fig. 4.7 The graph of Zone of inhibition (in mm) of onion skins extract against *E.Coli* and *S.aureus* at different doses

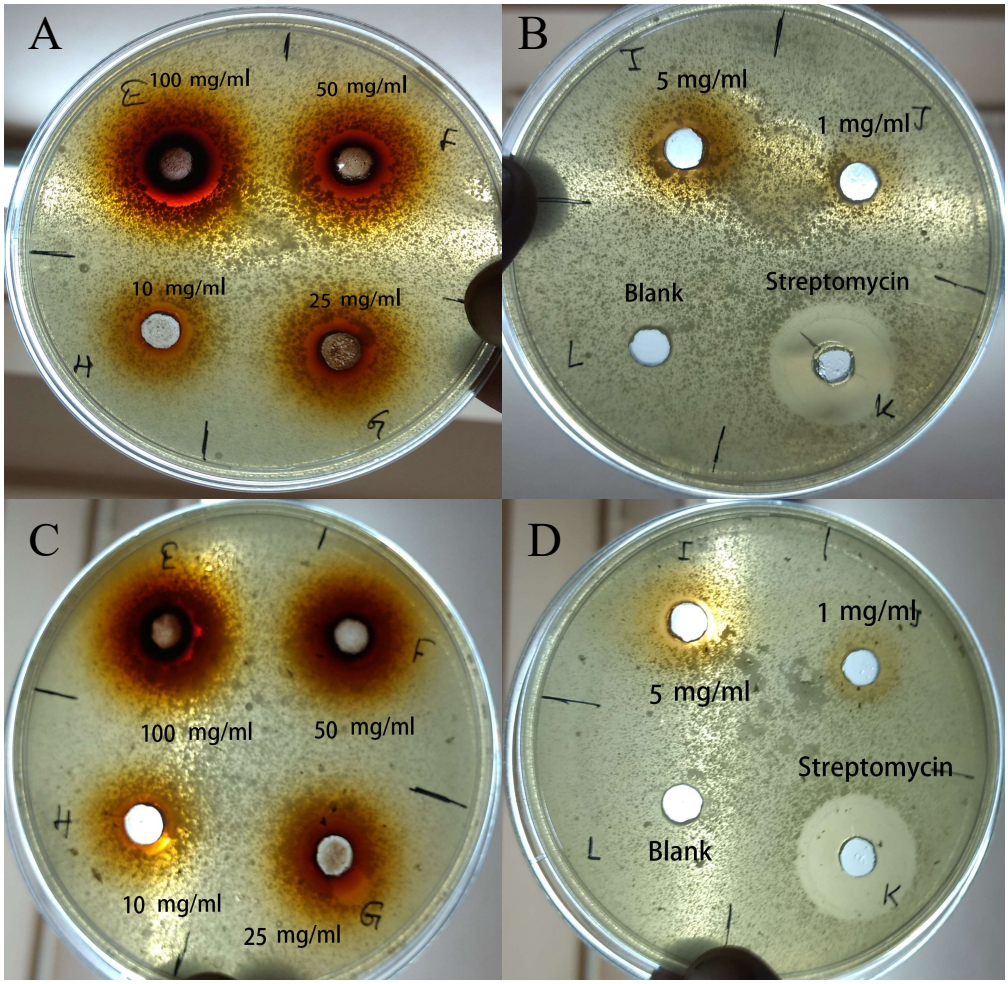

Fig. 4.8 Zone of inhibition (in mm) of onion skins extract against *E.Coli* and *S.aureus* at different doses by the agar well diffusion method

4.8 Results of growth curves of E.Coli and S.aureus

The results of anti microbial activity of the onion extract against the growth of *E. coli* and *S.aureus* indicated that the sample extract inhibited the growth of both bacteria (Fig.4.9 and Fig.4.10). The experiment was performed for 24 hours of treatment with the Onion extract. The result was monitored in 2 hours intervals. The result showed that the growth curve for E.coli was better that the growth curve of the S.aureus, with respect to antibiotic Streptomycin.

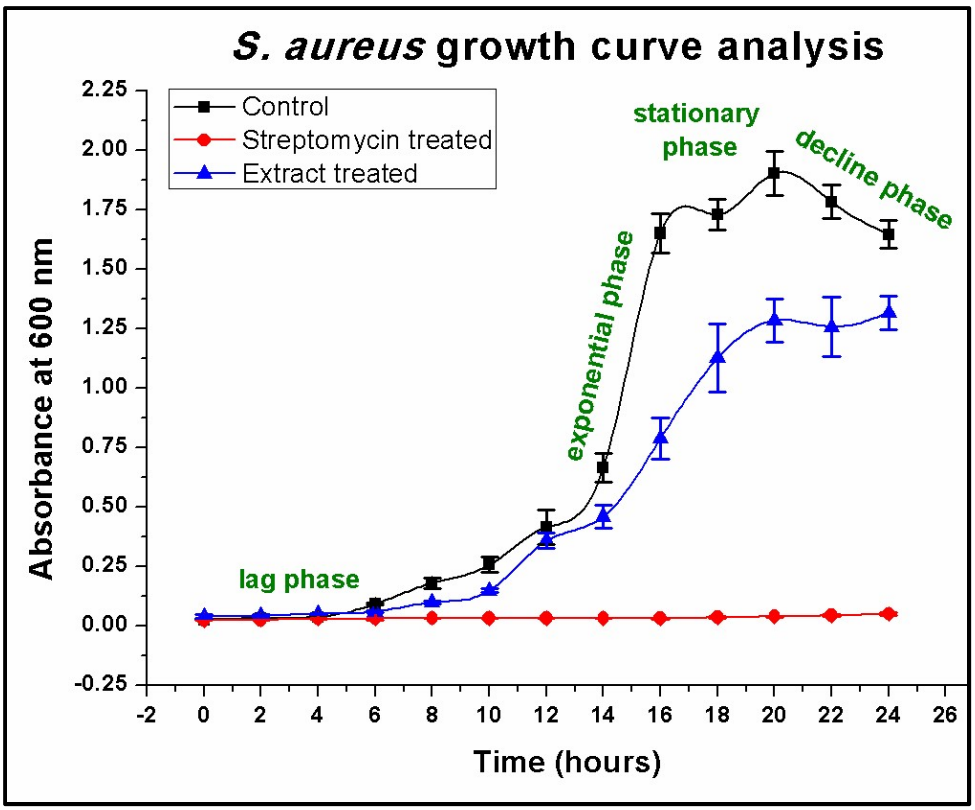

Fig . 4.9 The growth curve of *S.aureus*

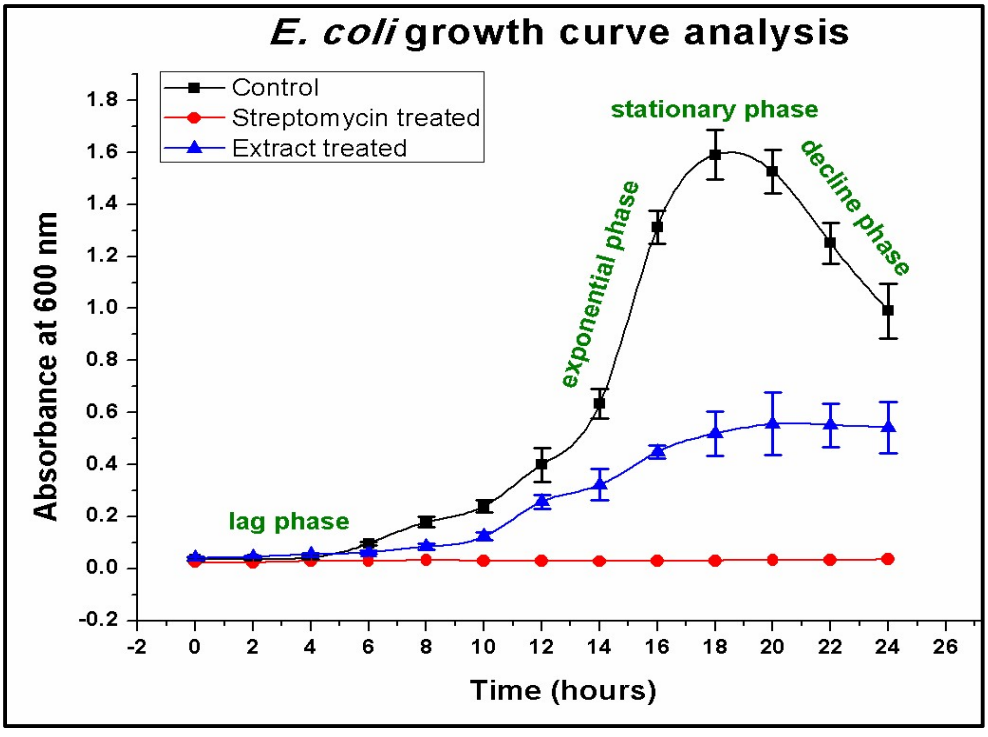

Fig. 4.10 The growth curve of *E.Coli*

4.9 Results of Dose-dependent cytotoxicity study of the Onion skins extracts

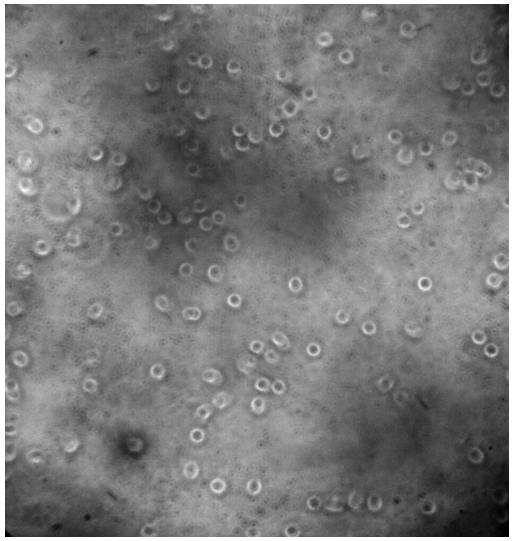

Fig. 4.11 Microscopic (x40) image of Peripheral blood mononuclear lymphocyte cell

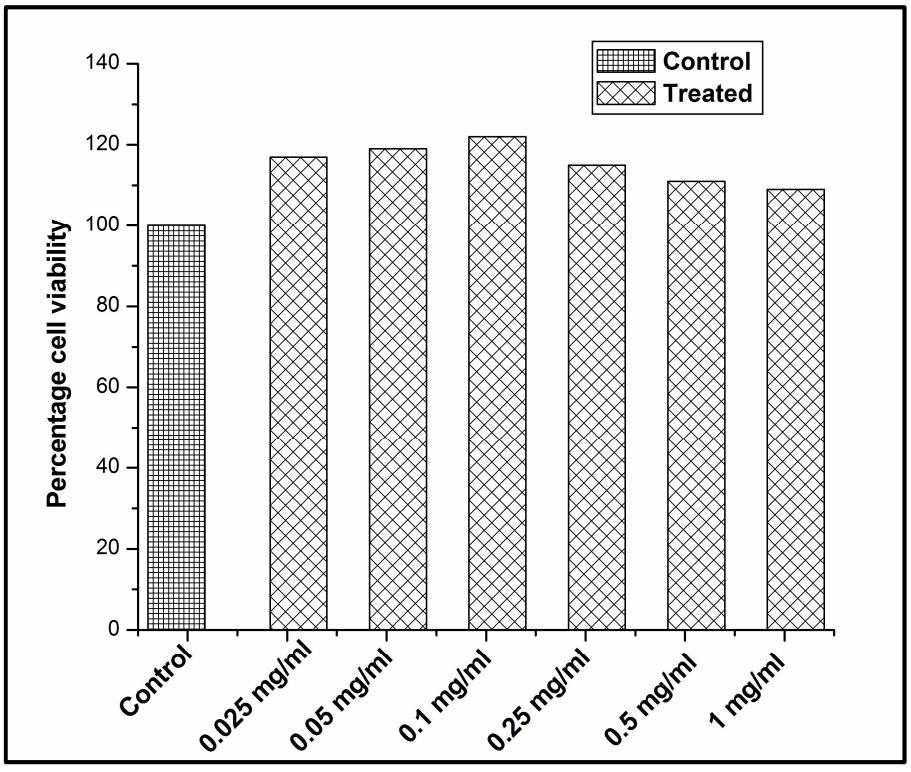

Fig. 4.12 Cytotoxic effect of different concentrations of Onion skins extract on PBMC

Dose-dependent cytotoxicity study of Onion skins extract was determined on peripheral blood mononuclear lymphocyte cell line (Fig.4.11) by performing MTT assay. The MTT assay was executed on PBMC line after 24 hours of treatment with the different concentration of the ethanolic Onion skins extract. Distilled water was kept as control to perform this experiment.

The result of this cytotoxic study was revealed that doses of different concentrations of ethanol extract of Onion skins had low toxicity at 0.1 mg/ml, where the percentage of cell viability was high than other doses (Fig. 4.12). This study demonstrated that onion skins extract raised the growth level of PBMC and almost low cytotoxicity effect observed.

4.10 Effect of extract of Allium cepa L. on percentage protection of mast cell degranulation

In the standard protocol we were able to find out the activity of Allium cepa L. to manage compound 48/80–induced plasma histamine release. Allium cepa L. was administered to the set of mice at concentrations ranging from 50 to 300 mg/kg, p.o. prior to collection of mast cells for 5 to 6 days. The results of the above experiment showed that Allium cepa L. had dose dependent mast cell stabilization activity in terms of percentage protection. In the dose groups of 50, 100, 200 and 300 mg/kg Allium cepa L. protected the degranulation (26 ± 0.94, 52 ± 0.11, $p<0.001$ and 60 ± 1.38, $p<0.05$, respectively) which closely resembles the protection observed in the standard DSCG (73 ± 1.32) which is expressed in the Table 4.3.(Fig. 4. 13)

Drug administered to the animals group	Dose	% Protection
Control group	Treated only saline	95±1.25
Challenged group	Treated with 48/80 at 0.8 mg/Kg	3±0.12$^{###}$
Allium cepa L. treated	Treated with 50 mg/ kg b.w	20±0.35
	Treated with100 mg/ kg b.w	26±0.94**
	Treated with 200 mg/ kg b.w	52±0.11***
	Treated with 300 mg/ kg b.w	60±1.38**
DSCG treated	Treated with 10 mg/ kg b.w	73±1.32***

Table 4.3. Percentage protection of mast cell degranulation upon the treatment with Onion skins ethanol extracts at different doses

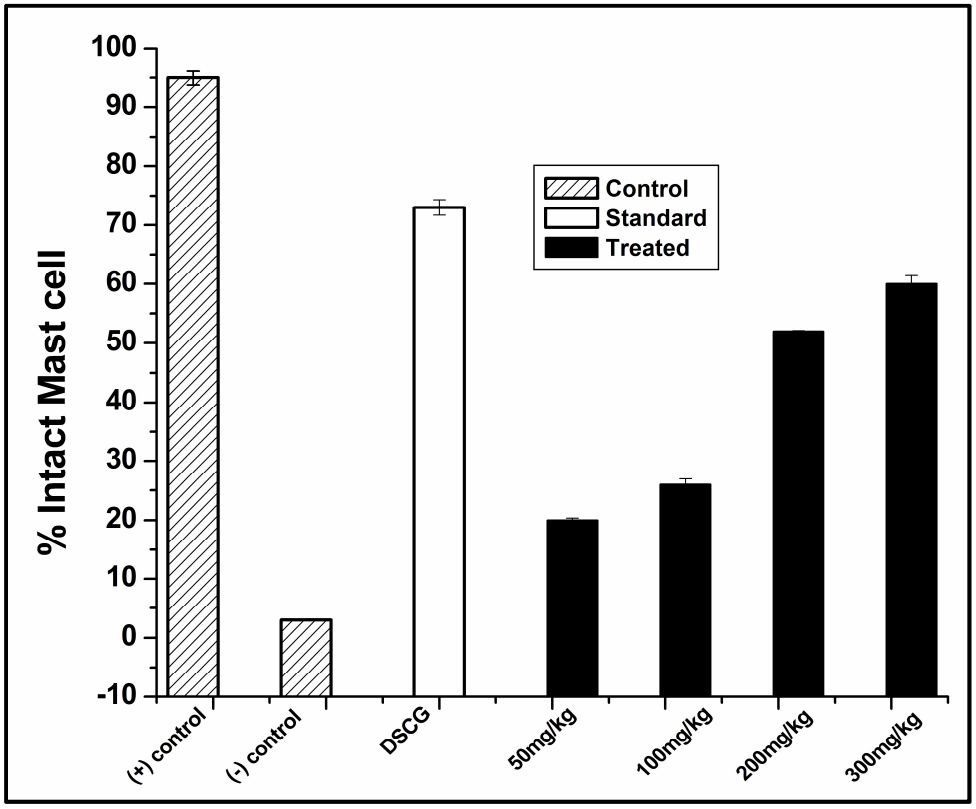

Fig. 4.13. Evaluation of the ethanolic extracts of Onion Skins on mast cell stabilization

4.11 Effect of Allium cepa L. extracts on compound 48/80 induced systemic anaphylaxis

There was significant protection from degranulation (by compound 48/80 induced) of mast cells in Allium cepa L.-treated animals. The lower rate of morality of mice from the anaphylactic shock was 40% at both 200 and 300 mg/kg were expressed in table 2.

The lower rate of morality of mice from the anaphylactic shock was 40% at both 200 and 300 mg/kg expressed in Table 4.4 It is showed by the experiment that the effect of inhibition provided by Allium cepa L. against compound 48/80- induced mast cell degranulation and anaphylaxis were almost similar to that of DSCG. Our experimental results of the present study expressed that the Allium cepa L.extract exert a marked mast cell stabilization and antihistaminic activities in the Bulb/c mice.

Drug	Extract Dose	Challenging agent (Compound 48/80)	% Mortality
Control group of animals	**-**		**0**
Challenged group of animals	**-**	**0.8 mg/Kg**	**100**
Allium cepa L. treated animals	**Treated with 50 mg/ kg b.w**	**0.8 mg/Kg**	**90**
	Treated with100 mg/ kg b.w	**0.8 mg/Kg**	**80**
	Treated with 200 mg/ kg b.w	**0.8 mg/Kg**	**40**
	Treated with 300 mg/ kg b.w	**0.8 mg/Kg**	**40**
DSCG treated animals (10 mg/ kg b.w)	**-**	**0.8 mg/Kg**	**20**

Table 4.4 Effect of Onion skins ethanol extracts at different doses on anaphylactic shock induced by compound 48/80 in mice.

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Chapter 5

Conclusion

From this study it can be concluded that the Skins of Onion has a significant amount of antioxidant, anti microbial, anti allergic properties. These pharmacological activies of onion skins extract exhibited for the presence of enormous secondary metabolites like phenolic, flavonoids compounds.

The study on the microorganisms reveled that this plant extract has anti microbial properties against the common human pathogenic bacteria. The study of growth curve against different doses of onion extract showed that onion has different phenomenon for both type bacteria such like gram positive bacteria and gram negative bacteria. Onion demonstrated that they are more effective to inhibit to grow gram negative bacteria whereas the activity against gram positive bacteria is less.

The evaluation of cytotoxic study revealed that ethanol extract of onion skins has the ability to promote normal Peripheral Blood Mononuclear Cell (PBMC). It suggests that this cytotoxic property may be due to the antioxidant property of onion extract.

The study of the anti allergic activity on animal model showed that the Anti allergic property of ethanol extract of onion skins inhibited the mast cell degranulation which is happened due to the chemical allergen compound 48/80. The result of this study demonstrated that this onion skin has the property which are closed to the DSCG activity. The report suggested that the phytochemicals, mainly flavonoids of this plant extract exhibit this biological activity. As a conclusion it can be say that it is worth to isolate the phytochemical form the onion skins, mainly abandoned from our daily life as a waste product, for benefit of being potential anti allergic drug.

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Chapter 6

Future Aspects of Work

As a future plan of this work it can mention that there are lots of future aspects from this project.

- Through the histamine release study it can be evaluated the signal-transduction pathway which can stimulate the mast cell with compound 48/80 which can be initiated the activation of which leads to histamine release.
- The enzyme linked immune sorbent assay can be used to detect the amount of IgE quantitatively.
- Different phytochemical assay can be examined .
- Testing of resistant bacteria .