

## Formulation and Evaluation of Antimicrobial Potential of Glycerosomes Containing *Allium sativum*, *Zingiber officinale* Extracts

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### Abstract

The aim of the present investigation is to evaluate the antimicrobial potential of *A. sativum* and *Z. officinalis* extracts mediated glycerosomes formulation by thin film of lipid hydration method. The water content present in the *A. sativum* and *Z. officinale* was 42.3 and 27.2 % respectively. The yield of *A. sativum* and *Z. officinale* extract using ethanol as solvent through the Soxhlation process was found to be  $4.7 \pm 1.05$  and  $12.7 \pm 1.108$  % respectively. The crude drug of *A. sativum* and *Z. officinale* showed foreign organic was found to be 0.2 and 0.21 % and volatile oil was found to be 1.8 and 2.4 % only. The ash study revealed that total, acid-insoluble, water-soluble, and sulfated ash values were for the crude drugs of *A. sativum* and *Z. officinale*. The ethanol extracts showed maximum extractive yield. The phytochemicals detected in *A. sativum* bulb ethanol extract are Alkaloids, Carbohydrates, Cardiac glycosides, Phenols, Sterols, Triterpenoids, Saponins, Flavonoids, and Fixed oils and in *Z. officinale* rhizome ethanol extract are Alkaloids, Carbohydrates, Cardiac glycosides, Anthraquinone glycosides, Proteins and Amino acids, Triterpenoids, Saponins, Flavonoid, and Fixed oils. The prepared glycerosome was spherical with a slightly irregular shape. The negative potential of glycerosomes demonstrates the greater stability of the vesicular dispersion. The ASEE glycerosome formulations (F1 and F2) exhibited better antibacterial activity and greater antifungal activity against the gram-negative bacteria in comparison with the ZOEE glycerosome formulations (F3 and F4). The present research work strongly concluded that the *A. sativum* and *Z. officinale* and their glycerosome formulations are good candidate drugs and formulations for the safe and effective treatment of microbial infections.

**Keywords:** *A. sativum*, *Z. officinalis*, thin film of lipid hydration, glycerosomes, Antibacterial activity, Antifungal activity

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## Introduction

Natural products derived from plants and their secondary metabolites are the comprehensive choices of biological activities in humans. Phytonutrients are found to fight against numerous diseases. Worldwide and about 80 to 85 % World's population trusts herbal medicines for the treatment of many diseases [1]. The major phytoconstituents found in the herbal plant (Natural product) are Alkaloids, Glycosides, Carbohydrates, Proteins, Flavonoids, Saponins, Phytosterols, Tannins, etc. Herbal medicines are used for the health promotion of chronic and life-threatening diseases [2]. The chemical compounds derived from the plants play important activities that are antidiabetic, antimicrobial, antifungal, antiaging, anti-inflammatory, anticancer, antioxidant, wound healing, and many more [3].

The novel drug delivery system (NDDS) is a new approach that includes new development, formulations, techniques, and methodologies for delivering natural and pharmaceutical products to the body in a safe and cost-effective manner desiring the patient requirements. Application of NDDS to herbal medicine help in increasing the efficacy of the active constituents and reducing the side effects of herbal compounds. The herbal compound delivery may face some biopharmaceutical problems, which can be solved very effectively by designing the herbal constituents as a novel drug delivery system [4,5].

Glycosome was introduced into the drug delivery system by Manaca, *et al.* The concept of Glycosome was developed from the stability problem of liposomes. Glycosomes are formed by the phospholipid and glycerol in varying concentrations which form bilayer fluidity. Glycosome is a modified form of the liposome. Glycosomes are composed of a phospholipid, water, and glycerol in more amounts in varying concentrations [6]. Liposome exhibit some stability problems as some lattice gaps exist between hydrophilic and hydrophobic bilayers. This could be stabilized by the use of a surfactant. The glycerol is used as a surfactant (Edge activator), thus enhancing the stability of liposomes in the form of Glycosome particles [7]. Glycerol ameliorates the deformability index of liposomal bilayers, thus enhancing skin penetration. Glycosome is predominately used in transdermal and topical delivery of drugs. Both hydrophilic and hydrophobic drugs can be delivered by the Glycosome [8]. The aim of present investigation is to evaluate antimicrobial potential of *A. sativum* and *Z.officinale* extracts mediated glycosomes formulation.

## Materials and Methods

### Materials

The healthy fresh clove of *A. sativum* (About 0.5 to 1 kg) and *Zingiber officinale* (About 1 to 2 kg) was collected from the local market of Hamirpur District of Himachal Pradesh. Dragendorff's reagent, Tollen's reagent, Mayer's reagent and Biuret reagent were procured from Loba Chemie Pvt. Ltd., Mumbai. Wagner reagent, Benedict's reagent, Fehling's solution A & B and Ninhydrin were obtained from Merck Pvt. Ltd., Mumbai. Hager's reagent was obtained from Ozone International Ltd., Mumbai. Molisch's reagent was procured from Stan Bio, Calcutta. Millon's reagent was obtained from Otto Kemi Pvt. Ltd., Mumbai. Erythromycin and Ciprofloxacin were procured as gift sample from Piramal HC Ltd., Goa, India. The gram-positive bacteria like *Staphylococcus aureus* (NCTC 3761) was procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India and the gram-negative bacteria like *Pseudomonas aeruginosa* (NCTC 8201) from National Institute of Immunology (NII), New Delhi, India.

## Methods

### Organoleptic characters of *A. sativum* and *Z. officinale*

Sensory evaluation-visual macroscopy, colour, odour, taste, fracture and surface are the common features helped in identification of the crude drug [9].

### Processing (Drying) of collected *A. sativum* bulbs and *Z. officinale* rhizomes:

The bulbs and tuber were washed thoroughly with fresh water 2 to 3 times. The garlic and ginger was cutted into small pieces by using a hand operated small cutter mill. Now the small pieces of garlic and ginger were air (shade) dried in 12 h day (without exposing to sunlight) and night cycle each for 72 h. After it, the semi dried garlics and ginger were air dried in a clean, cool, dry and dark place at room temperature of  $25 \pm 2$  °C and  $45 \pm 5$  % RH (Relative Humidity) for another 7 days. The dried garlic and ginger were weighed by using electronic digital balance periodically to measure weight loss and accordingly the moisture loss was determined. In due course of drying the garlics and ginger were critically observed no fungal growth should develop in the garlic and ginger. The dried garlic and ginger was stored in an airtight container in a dark place for further study [10,11].

### Size reduction of *A. sativum* bulbs and *Z. officinale* rhizomes:

The dried plant material (Bulbs and Rhizomes) was disintegrated by feeding it into a hammer mill which had in built sieves. Usually, the bulbs were reduced to a very coarse powder particle size between 30 and 40 meshes. The very coarse powder form of garlic bulb was stored in an air tight closed amber coloured glass container in a dark place away from light at low controlled temperature and relative humidity [12,13].

### Extraction of *A. sativum* and *Z. officinale* by Soxhlation:

The very coarse powder form of crude garlic and ginger was extracted by Soxhlation method by using ethanol (Analytical grade) as solvent. A total amount of 150 g very coarse powder was extracted with 300 ml of ethanol solvent [14].

The extraction was done at a temperature of 30 to 35 °C, depending on boiling point of solvent. Extraction was continued unless until the solvent in siphoning tube becomes colourless. For each solvent, around four to five cycles were run to obtain thick slurry. The thick slurry was then concentrated under reduced pressure using rotary evaporator to obtain crude extract. [15]

$$\text{Yield of extract (\%)} = (W_1 / W_2) \times 100 \dots\dots(1).$$

Where,  $W_1$  is the weight of extract in almost towards dried form in g and  $W_2$  is the initial weight of crude drug (g) in very coarse powder form.

## PHARMACOGNOSTIC EVALUATION OF *A. SATIVUM* AND *Z. OFFICINALE* POWDER:

### Determination of Moisture Content (Loss on Drying):

About 1.5 g of crude drugs coarse powder form was weighed into a flat and thin porcelain dish [16]. The drug was dried in an oven at 105 °C up to a constant weight. The drug was cooled and reweighed. The loss in weight is usually recorded as moisture content of crude drug and it was calculated by using the following equation.

$$\text{Moisture loss (\%)} = [(W_1 - W_2) / W_1] \times 100 \dots\dots\dots(2)$$

Where,  $W_1$  is the initial weight of crude drug in g and  $W_2$  is the final weight of crude drug in g after drying.

### **Determination of Foreign Organic Matter:**

Accurately 100 g of sample was weighed, and it was spread on a white tile uniformly and inspected visually using a lens. The foreign organic matter was separated manually [17]. After complete separation, the collected foreign organic matter was weighed and the percentage in w/w present in the sample was calculated by using the following formula.

$$\text{Foreign Organic matter (\%)} = (W_1 / W_2) \times 100 \dots\dots(3)$$

Where,  $W_1$  is the weight of foreign organic matter in g and  $W_2$  is the initial weight of crude drug (100 g).

### **Determination of Foreign Organic Matter:**

The volatile oil content of crude drug was determined by using Clevenger apparatus. About 25 g of very coarse powder drug was taken with 250 ml of water in a distillation flask. The flask was heated through heating mantle and continued till no more oil was collected. On completion of distillation, it was allowed to cool up to room temperature to separate volatile oil and water layers and the volume of volatile oil was recorded [18].

### **Determination of Ash values:**

Accurately 5 g of air-dried powdered drug was accurately weighed in tarred silica crucible. It was incinerated gradually by increasing the heat (Temperature not more than 450 °C) until free from carbon, cooled and weighed accordingly [19]. It was then kept in a desiccator.

$$\text{Total ash value (\%)} = (w/W) \times 100 \dots\dots\dots(4)$$

Where,  $w$  is the weight of obtained ash in g and  $W$  is the weight of crude drug in very coarse powder form taken in g.

### **Determination of acid insoluble ash value:**

The ash from the dish used for total ash was washed with 25 ml of dilute hydrochloric acid using 100 ml beaker and boiled for 5 min. The residue was filtered through an ash-less filter paper. Then the residue was washed twice with hot water [20]. The crucible was ignited, cooled and weighed.

$$\text{Acid insoluble ash value (\%)} = (w_1/w_2) \times 100 \dots\dots\dots(5)$$

Where,  $w_1$  is the weight of obtained acid insoluble ash in g and  $w_2$  is the weight of crude drug in very coarse powder form taken in g.

### **Determination of water insoluble ash value:**

The ash from the dish used for total ash was washed with 25 ml of water and boiled for 5 min. The insoluble matter was collected on an ash-less filter paper. Then the residue was washed twice with hot water [21]. The crucible was ignited to a constant weight at low temperature, cooled and weighed.

$$\text{Water insoluble ash value (\%)} = (W/w) \times 100 \dots\dots\dots(6)$$

Where,  $W$  is the weight of obtained water insoluble ash in g and  $w$  is the weight of crude drug in very coarse powder form taken in g.

### **Determination of sulphated ash value:**

About 5 g of powdered drug was taken in a silica crucible and ignited thoroughly. The residue was cooled and moistened with 1 ml of dilute sulphuric acid [22]. It was heated gently until white fumes were no longer evolved and again ignited until all black particles had disappeared. The crucible was allowed to cool, and the weight of the residue was taken.

$$\text{Sulphated ash value (\%)} = (P1/P2) \times 100 \dots\dots(7)$$

Where, P1 is the weight of obtained sulphated insoluble ash in g and P2 is the weight of crude drug in very coarse powder form taken in g.

**Determination of extractive values:**

About 5 g of powdered drugs of very coarse form was transferred into a dry 250 ml conical flask and macerated with 100 ml of each solvent (Water, Ethanol, n-butanol, Petroleum ether and Chloroform respectively) in a stoppered flask for 24 h. In the first 6 h, the flask was frequently shaken and then allowed to stand for 18 h. Then the mixture was filtered into 50 ml of cylinder [23]. When sufficient filtrate was collected, 25 ml of filtrate was transferred to a weighed, thin porcelain dish. The filtrate was evaporated to dryness by placing the dish on a boiling water bath. The drying was completed in a Hot air oven at 100 °C. The extract was cooled in a desiccator and weighed. The percentage (w/w) of extractive with reference to the air-dried drug was calculated.

**PRELIMINARY PHYTOCHEMICAL SCREENING OF *A. SATIVUM* AND *Z. OFFICINALE* EXTRACTS:**

**Detection of alkaloids:**

About 20 mg of ethanolic crude extract of *A. sativum* bulbs and *Z. officinale* rhizomes was taken and stirred separately with a few drops of dilute hydrochloric acid [24,25]. The solution was filtered through Whatman filter paper No.1. The filtrates were used for the following tests.

**Wagner's test:** The filtrate (1 ml) was treated with a few drops of Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of Alkaloids.

**Mayer's test:** The filtrate (1 ml) was treated with a few drops of Mayer's reagent (Potassium Mercuric Iodide solution). Formation of a yellow-coloured precipitate indicates the presence of Alkaloids.

**Dragendorff's test:** The filtrate (1 ml) was treated with a few drops of Dragendorff's reagent (Solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of Alkaloids.

**Hager's test:** The filtrate was treated with a few drops of Hager's reagent (Saturated solution of picric acid). The presence of alkaloids was confirmed by the formation of yellow coloured precipitate.

**Detection of Carbohydrates:**

The ethanolic crude extract of *A. sativum* bulbs and *Z. officinale* rhizomes dissolved individually in 5 ml distilled water and filtered. The filtrate was used to test for the presence of Carbohydrates [26,27].

**Molisch's test:** The filtrate was treated with 2 drops of 10 % solution of Molisch's reagent (Alcoholic  $\alpha$ -naphthol solution) in a test tube followed by the addition of 1ml of concentrated sulphuric acid. Formation of the violet ring at the junction indicates the presence of Carbohydrates.

**Fehling's test:** The filtrate was hydrolyzed with 0.5 ml of dilute hydrochloric acid. The solution was neutralized with alkali and heated with equal volume of Fehling's A and B solutions. Formation of red precipitate indicates the presence of reducing sugars.



**Benedict's test:** The filtrate was treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

**Barfoed test:** The Barfoed reagent was added to a small amount of each extract and boiled in a water bath for a few minutes. Reddish precipitate indicates the presence of Carbohydrate.

**Detection of cardiac glycosides:** The ethanolic crude extract of *A. sativum* bulbs and *Z. officinale* rhizomes was stirred separately with a few drops of dilute hydrochloric acid [28,29]. The solution was filtered through Whatman filter paper No.1 and then subjected to a test for Glycosides.

**Keller killiani test:** A few mg of the extracts was taken separately boiled with 5 ml of 70 % alcohol for 2 to 3 min and filtered. To the filtrate, 2 ml of water and 1 ml of strong lead acetate solution were added. The chloroform layer was separated and evaporated slowly in a porcelain dish. To the resultant residue, 1 ml of glacial acetic acid containing one drop of Ferric chloride was added and this was carefully transferred to the test tube containing 1 ml of sulphuric acid. A reddish-brown ring at the junction of two layers indicates a positive result for the deoxy sugars.

**Legal test:** The extracts were treated with 1 ml sodium nitroprusside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of Cardiac glycosides.

**Kedde's test:** A small quantity of residue of the extracts was taken in alcohol, decolorized with lead acetate and filtered. The filtrate was evaporated to dryness and residue was dissolved in a few drops of methanol. Two to three drops of Kedde's reagent (1 % 3.5 ml dinitro-benzoic acid in methanol) followed by two drops of 2N methanolic NaOH were added. Blue or violet colour indicates the presence of Cardiac glycosides.

**Guignard's test:** About 0.2 g of extracts was taken in a test tube, followed by the addition of a few drops of chloroform to enhance the enzymatic activity. The strip of filter paper was moistened with the Sodium picrate solution. The strip was removed, dried and inserted between the split cork stoppers that were fitted into the neck of the test tube containing reaction mixture. The test tube and contents were then warmed at 30 to 35 °C for 0.5 h. The appearance of a red colour on the paper indicates the presence of HCN evolution and hence indicates the presence of Cyanogenetic glycosides.

#### **Detection of anthraquinone glycosides:**

**Borntrager's test:** About 0.1 g of extracts was boiled with 5 ml of 10 % sulphuric acid for 2 min. It was filtered while hot, cooled and the filtrate was shaken with equal volume of benzene. The benzene layer was allowed to separate completely from the lower layer, which was pipetted out and transferred out to a clean test tube [30]. Then half of its volume of aqueous ammonia (10 %) was added, shaken gently and the layers were allowed to separate. The lower ammonia layer will show red, pink colour due to presence of free anthraquinone.

**Modified Borntrager's test:** The extracts were treated with 1 ml of Ferric Chloride solution and immersed in boiling water for about 5 min. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with a few drops of ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of Anthracene glycosides [31].

### Detection of Gums and Mucilages:

**Ruthenium red test:** The extracts were added to a solution containing 0.08 g of ruthenium red in 10 ml of 10% solution of lead acetate; it stains the mucilage to red colour [32].

**Molisch's test:** The aqueous or alcoholic solution (2 ml) of the extracts was shaken with 10 % alcoholic solution of  $\alpha$ -naphthol and concentrated sulphuric acid was added along the side of the test tube. A violet ring at the junction of two liquids confirms the presence of carbohydrates, gums, and Mucilage [33].

**Test with 95 % Alcohol:** When 95 % alcohol was added to the extracts, gums precipitated out. The precipitate was insoluble in alcohol [34].

### Detection of proteins and amino acids [35,36]

**Biuret test:** About 2 ml of the extracts, 2 ml of 10 % NaOH solution and 2 to 3 drops of 1 %  $\text{CuSO}_4$  solution were mixed. The appearance of violet or purple colour confirms the presence of Proteins.

**Ninhydrin test:** To the extracts, 0.25 % w/v ninhydrin reagent was added and boiled for a few minutes. Formation of blue colour indicates the presence of Amino acids.

**Xanthoproteic test:** About 2 ml of the extracts was boiled with 1 ml of conc.  $\text{HNO}_3$ , cooled and subsequently 40 % NaOH solution was added drop by drop to it. Appearance of coloured solution indicates the presence of Proteins.

**Millon's test:** About 2 ml of the extracts and 2 ml of Millon's reagent were boiled, subsequently cooled, and then few drops of  $\text{NaNO}_2$  were added to it. Appearance of red precipitate and red coloured solution indicates the presence of Proteins.

### Detection of tannins and phenolic compounds [37]

**Lead acetate test:** The lead acetate was added to the extract. Tannins get precipitated.

**Ferric chloride test:** The extracts were treated with 3 to 4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of Phenols.

**Gelatin test:** The extracts were added with 1 % gelatin solution containing sodium chloride. Formation of white precipitate indicates the presence of Tannins.

### Detection of steroids and sterols [38]

**Salkowski's test:** The extracts were treated with 1 ml of chloroform and filtered. The filtrates were treated with few drops of conc. sulphuric acid, shaken and allowed to stand. The appearance of golden yellow colour indicates the presence of Sterols.

**Liebermann Burchard test:** The extracts were treated with chloroform and filtered. The filtrate was treated with a few drops of acetic anhydride, boiled and cooled. The cooled solution was mixed with a few drops of concentrated sulphuric acid. Formation of the brown ring at the junction indicates the presence of Phytosterols.

### Detection of triterpenoids [39]

**Tin and thionyl chloride test:** For the detection of triterpenoids, the extracts were dissolved in chloroform. A piece of metallic tin and 1 drop of thionyl chloride were added to it. The presence of pink colour confirms the result.

**Salkowski test:** Few drops of concentrated sulphuric acid are added to the ethanol extracts and shaken. The standing lower layer turns to golden yellow colour indicating positive results.

**Liebermann Burchard test:** To the chloroform solution of the extracts few drops of acetic anhydride are added and mixed well. One ml of concentrated sulphuric acid is added from the side of the test tube. A reddish-brown ring indicates Triterpenes.

#### **Detection of saponins [40,41]**

**Foam test:** A few mg of residue of the extracts was taken separately in a test tube with a small amount of sodium bicarbonate along with water and shaken vigorously. Formation of stable froth indicates the presence of Saponins.

**Haemolytic test:** About 2 ml of 1.8 % NaCl was taken in two test tubes. To one test tube, 2 ml distilled water was added, whereas to another test tube, 2 ml of 1 % extracts was added. About 5 drops of blood was added to each tube and gently mixed the contents. The appearance of haemolysis under the microscope in the tube containing the extract indicates the presence of Saponins.

#### **Detection of flavonoids [42,43]**

**Magnesium turning method:** About 20 mg of crude extracts was mixed with 10 ml of methanol and filtered; 2 ml of filtrate solution was taken and mixed with concentrated HCl. Magnesium ribbon was added until a pink- tomato red colour was gauged, indicating the presence of Flavonoids.

**Ferric chloride test:** Few drops of neutral ferric chloride solution were added to little quantities of alcoholic extracts. A blackish green colour production indicates the phenolic nucleus.

**Lead acetate test:** Few drops of lead acetate solution (10 %) were added to alcoholic solution of test extracts. Appearance of yellow precipitate indicates a positive test.

**Zinc hydrochloric acid reduction test:** The alcoholic solution of the extracts was treated with a pinch of zinc dust and few drops of concentrated HCl; magenta colour is produced after a few min.

**Sodium hydroxide test:** For the detection of flavonoids, the extracts were first dissolved in water. It was filtered and the filtrate was treated with sodium hydroxide. A yellow colour confirms the presence of Flavonoids.

**Sulphuric acid test:** The extracts were first dissolved in water. It was filtered and the filtrate was treated with sodium hydroxide. A yellow colour confirms the presence of flavonoids. A drop of H<sub>2</sub>SO<sub>4</sub> when added, the yellow colour disappears.

#### **PREPARATION OF A. SATIVUM AND Z. OFFICINALE GLYCEROSOMES**

The basic principle of Lipid Thin Film Hydration method for preparation of Glycerosomes includes formation of thin dried Lipid film by slowly evaporating the organic solvents followed by hydration as well as sonicating the hydrated lipid which ultimately converts into formation of Glycerosomes [44,45].

##### **Preparation of Organic Phase:**

A clean and dry round bottom flask of capacity 250 ml was taken. To the flask, the lipid components, Lecithin (30 mg/ml), Stearylamine (10 % of lecithin) and Cholesterol (2 mg/ml) were taken. To the flask, 10 ml of Methanol and Chloroform in mixture form (3: 1 v/v) were added. The flask was shaken for complete dissolve of lecithin. The Chloroform was used as co-solvents. The organic solvent mixture was kept for further processing.



### Preparation of Aqueous phase of *A. sativum* ethanol extracts (ASEE) and *Z. officinale* ethanol extract (ZOEE)

The required quantity of ASEE and ZOEE was taken in the beaker. To the beaker, a few ml of ethanol was added and mixed. To the above mixture, variable concentrations (30 and 40 %) of glycerol in the Phosphate buffer of pH 7.4 were added. All the components were uniformly mixed and a homogeneous mixture of aqueous phases containing ASEE was prepared. The glycerol concentration was taken in variable concentrations demonstrating the different *A. sativum* and *Z. officinale* glycrosome formulations. The glycerol concentration was fixed to 30 and 40 % based on the scientific work literature and their successful formulation result.

### Preparation of *A. sativum* and *Z. officinale* Glycrosomes:

The round bottom flask containing organic phase was fitted to the Rotary evaporator. The organic solvents were slowly evaporated by simultaneous rotation of flask at speed of 100 RPM over controlled heated water bath at temperature of  $37 \pm 2$  °C and application of vacuum (under reduced pressure of 250 mm of Hg). The drying process continued for 2 h. On completion of the drying process, a thin film of lipid was formed in the inner wall of the round bottom flask. To the dried lipid film, about 5 ml of aqueous phase containing ASEE and ZOEE were added. The lipid film was hydrated using a glycerol solution in a phosphate buffer of pH 7.4 with variable concentration in the proportion of 3:1 % v/v as per the formulation design. On hydration, an emulsion containing multilamellar lipid vesicles was formed containing ASEE and ZOEE, which was kept at rest for 1 h. The vesicular mixture was transferred to the beaker. Then the vesicular mixture was sonicated using an ultrasonicator at a speed of 1000 RPM for 5 min under ice bath. After sonication, the prepared ASEE and ZOEE Glycrosomes were separated by filtration using Dialysis technique and kept in cold temperature for further study [46,47].

**Table 1. Formulation design of various glycrosome formulations (F1 to F4) using ethanol extract of *A. Sativum* and *Z. Officinale***

Ingredients(mg/10 ml)	F1	F2	F3	F4
ASEE	100	100	--	--
ZOEE	--	--	100	100
Lecithin	300	300	300	300
Sterylamine	30	30	30	30
Cholesterol	10	10	10	10
Glycerol	30	40	30	40

ASEE - *A. Sativum* ethanol extract and ZOEE - *Z. Officinale* ethanol extract

### CHARACTERIZATION OF ASEE AND ZOEE GLYCROSOME FORMULATIONS

#### Glycrosome appearance, size, and shape:

The appearance of the glycrosomes was examined using a transmission electron microscope. Samples were prepared for negative staining as follows that are glycrosomes were diluted with a 10 % (v/v) glycerol–water solution and gently dropped on a film-coated copper grid. After 20 min of drying, a drop of phosphotungstic acid (2 %, v/v) was added to the film.

After air-drying for 3 h at room temperature, the film was observed under a transmission electron microscope [48].

#### **Glycosome surface charge (Zeta Potential):**

The Zeta sizer was used for the determination of surface charges of all the Glycosomal formulations. The Zeta potential (mV) of all the formulations was measured by laser doppler electrophoresis at the same concentration as used for particle analysis [49].

#### **ANTIBACTERIAL ACTIVITY:**

##### **Antibacterial activity of ASEE Glycosome:**

##### ***Determination of Minimum Inhibitory Concentration (MIC):***

The minimum inhibitory concentration (MIC) of ASEE and ZOEE glycosome formulations was determined with the selected test organisms by the paper disk diffusion technique. The ASEE and ZOEE glycosome formulations were diluted with DMSO to give concentrations of 5, 10, 15, and 20 mg/ml of ASEE and ZOEE containing glycosome formulations. The lowest concentration of the test drug that could inhibit the bacterial growth was considered as MIC [50].

##### ***Antibacterial activity:***

The antibacterial activity of the ASEE and ZOEE glycosome formulations was investigated against 2 bacterial strains by the paper disk diffusion technique. Each ASEE and ZOEE glycosome formulations was re-dissolved in ethanol to make a 1000 µg/ml solution and then filtered. From this solution, 80 µL aliquots were transferred onto blank paper disks (6 mm diameter) and dried. The dried disks were placed onto Mueller Hinton agar medium (Merck) previously inoculated with a bacterial suspension (*ca.* 10<sup>8</sup> CFU/ml) and incubated at 37±1 °C for 24 h. Plates were then examined for the presence of growth inhibition zones, and their diameters were measured. The Erythromycin (Against gram-positive bacteria) and Ciprofloxacin (Against gram-negative bacteria) at dose of 125 µg/ml were used as standard drugs (Positive control). A disk loaded with 80 µL ethanol served as the negative control. The experiments were carried out three times and the results are presented as mean, standard deviation and standard error of mean [52].

#### **ANTIFUNGAL ACTIVITY:**

The antifungal activity of the ASEE and ZOEE glycosome formulations was determined by disc diffusion method using the test samples, reference, and blank discs. The reference standard (Clotrimazole) and the ASEE and ZOEE glycosome formulations (10 mg/ml) were dissolved in DMSO (Di-methyl sulfoxide). Clotrimazole (25 µg/ml) was used as a reference (Positive control) standard drug. Solvent control (only DMSO) was also maintained throughout the experiment. The selected micro-organism is *Candida albicans* [53].

Each the ASEE and ZOEE glycosome formulations was re-dissolved in DMSO to make a 1000 µg/ml solution and then filtered. From this solution, 80 µL aliquots were transferred onto blank paper disks (6 mm diameter) and dried. The dried disks were placed onto Mueller Hinton agar medium (Merck) previously inoculated with a fungal suspension and incubated at 28±1 °C for 48 h. Plates were then examined for the presence of growth inhibition zones, and their diameters were measured. Clotrimazole (25 µg/ml) was used as positive control. A disk loaded with 80 µL DMSO served as the negative control [54].

## Results and Discussions

### Organoleptic characters of *A. sativum* and *Z. officinale*:

The bulbs of *A. sativum* and rhizomes of *Z. officinale* possess characteristic odour and pungent taste. This might be due to the presence of characteristic chemicals like volatile oils or phenolic compounds.

**Table 2. Organoleptic characteristics of *A. sativum* bulbs and *Z. officinale* rhizomes.**

S. No.	Parameters	<i>A. sativum</i> bulbs Characters	<i>Z. officinale</i> rhizomes Characters
1	Size	2.5 to 4 cm	2.5 to 7 cm
2	Shape	Ovoid	Tuber
3	Colour	Whitish to purple	Brownish
4	Odour	Characteristics	Characteristics
5	Taste	Pungent	Pungent
6	Fracture	Spongy	Splintery
7	Surface	Smooth	Scaring

### Processing (Drying) of collected *A. sativum* and *Z. officinale*:

From the drying experimental study, it was assessed that tely water content present in the freshly collected bulbs of *A. sativum* was 42.3 % rhizomes of *Z. officinale* was 27.2 %.

### Extraction and Yield of *A. sativum* and *Z. officinale*:

The Soxhlation method was found to be efficient method for the successful extraction of the dried *A. sativum*. The yield of *A. sativum* bulb and *Z. officinale* tuber extract using ethanol as solvent was found to be  $4.7 \pm 1.05$  % and  $12.7 \pm 1.108$  % (Data calculated in the form of mean  $\pm$  standard deviation, n = 3).

### Pharmacognostic evaluation of dried *A. sativum* bulbs:

The moisture content of very coarse powder form of *A. sativum* bulbs and *Z. officinale* rhizomes was 4.5 % and 4.31 %. This result explains that this crude drug is less prone to microbial decomposition and enzymatic deactivation, suggesting more stable for longer periods. The crude drug of *A. sativum* and *Z. officinale* rhizomes showed foreign organic matter of only 0.28 % and 0.21 %. This study assures that this crude drug is purer and more qualitative.

The volatile oil content of the crude drug was found to be very less i.e., 1.8 % and 2.4 % only. The ash study revealed that total, acid insoluble, water soluble and sulphated ash values were 3.8, 0.51, 8.55, and 0.78 % for *A. sativum* bulbs; 5.3, 1.6, 10.8, and 0.33 % for *Z. officinale* rhizomes respectively. The colour of *A. sativum* bulbs and *Z. officinale* rhizomes was slightly brownish. All the extracts showed a characteristic odour. The appearance of *A. sativum* bulbs and *Z. officinale* rhizomes extracts were very slightly sticky. The extractive values were 18.6, 5.7, 3.3, 1.1, and 0.25 % for *A. sativum* bulbs; 12.8, 11.2, 8.6, 2.14, and 0.85 % of *Z. officinale* rhizomes for water, ethanol, n-butanol, Petroleum ether and chloroform respectively. The ethanol extracts showed maximum extractive yield, whereas chloroform exhibited minimum extractive yield.

**Table 3. Physical evaluation of very coarse powder form of *A. Sativum* and *Z. officinale***

S. No.	Parameters	% w/w	% w/w
01	Moisture content	$4.5 \pm 0.08$	$4.31 \pm 0.12$

02	Foreign organic matter	0.28 ± 0.02	0.21 ± 0.08
03	Volatile oil content	1.8 ± 0.17	2.4 ± 0.11
04	Total ash	3.8 ± 0.22	5.3 ± 0.18
05	Acid insoluble ash	0.51 ± 0.11	1.6 ± 0.22
06	Water soluble ash	8.55 ± 0.16	10.8 ± 0.20
07	Sulphated ash	0.78 ± 0.12	0.33 ± 0.24

All values are expressed as mean ± standard deviation (n = 3).

**Table 4. Physical and extractive values of bulb extract of *A. sativum* and rhizome extract of *Z. officinale*.**

Sl. No.	Specifications	% w/w	% w/w
01	Colour	Creamy whitish	Slightly brownish
02	Odour	Characteristics	Pungent
03	Appearance	Slightly sticky	Very slightly sticky
04	Water soluble extractives	18.6 ± 0.55	12.8 ± 0.71
05	Ethanol soluble extractives	5.7 ± 0.38	11.2 ± 0.44
06	n-butanol soluble extractives	3.3 ± 0.29	8.6 ± 0.35
07	Petroleum ether	1.1 ± 0.29	2.14 ± 0.54
08	Chloroform	0.25 ± 0.31	0.85 ± 0.28

All values are expressed as mean ± standard deviation (n = 3).

#### Phytochemical screening of *A. sativum* extracts:

Table 5 shows the phytochemicals detected in *A. sativum* bulb and *Z. officinale* rhizome ethanol extract. The tests for Alkaloids, Carbohydrates, Cardiac glycosides, Phenols, Sterols, Triterpenoids, Saponins, Flavonoids, and Fixed oils were positive. The phytochemicals that are Gums and Mucilage, Tannins, Phenols and steroids are absent in ethanol extract of *A. sativum* bulb and *Z. officinale* rhizom. Thus *A. sativum* bulb and *Z. officinale* rhizomethanolic extract contains Triterpenoids and Flavonoids in abundant manner, Saponins in moderate form and Alkaloid, Carbohydrates, cardiac glycosides, Phenol, Sterols, and Fixed oils in fair manner.

**Table 5. Phytochemical constituents detected in bulb and rhizome ethanol extracts of *A. sativum* and *Z. officinale*.**

Phytochemicals	Test	<i>A. sativum</i>	<i>Z. officinale</i>	<i>A. sativum</i>	<i>Z. officinale</i>
		Result		Remark	
Alkaloids	Wagner's test	+	+	+	+++
	Mayer's test	+	+		
	Dragendorff's test	+	+		
	Hager's test	-	+		
Carbohydrates	Molisch's test	+	+	+	+
	Fehling's test	+	+		
	Benedict's test	-	+		
	Barfoed test	+	-		
Cardiac	Keller killiani test	+	+		

glycosides	Legal test	+	+	+	++
	Kedde's test	+	-		
	Guignard's test	-	+		
Anthraquinone glycosides	Borntrager's test	-	+	-	+
	Modified Borntrager's test	-	-		
Gums and mucilages	Ruthenium red test	-	-	-	-
	Molisch's test	-	-		
	Test with 95% Alcohol	-	-		
Proteins and amino acids	Biuret test	-	+	-	+
	Ninhydrin test	-	+		
	Xanthoproteic test	-	+		
	Millon's test	-	+		
Tannins	Lead acetate test	-	-	-	-
	Ferric chloride test	-	-		
	Gelatin test	-	-		
Phenols	Ferric chloride test	+	-	+	-
	Lead acetate test	+	-		
	Gelatin test	-	-		
Steroids and sterols	Salkowski's test	+	-	+	-
	LiebermannBurchard test	+	-		
Triterpenoids	Tin and thionyl chloride test	+	+	+++	+
	Salkowski test	+	+		
	Liebermann Burchard test	+	+		
Saponins	Foam test	+	+	++	+++
	Haemolytic test	+	+		
Flavonoids	Magnesium turning method	+	+	+++	++
	Ferric chloride test	+	+		
	Lead acetate test	+	+		
	Zinc hydrochloric acid reduction test	+	+		
	Sodium hydroxide test	+	+		
	Sulphuric acid test	+	+		
Fixed oil and fats	Fat test	+	+	+	+

(+) sign indicates present and (-) sign indicates absent.

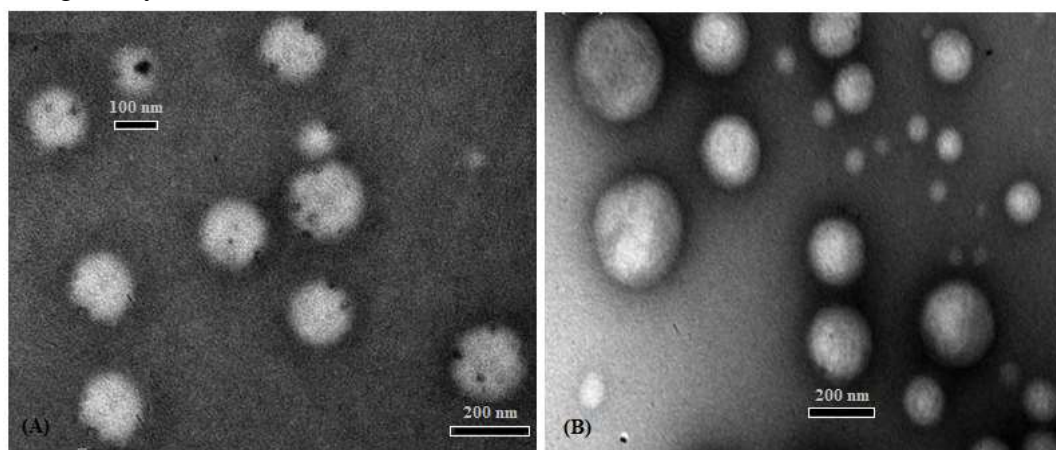
+++ (Abundantly present), ++ (Moderately present and + (Fairly present).



### Characterization of ASEE and ZOEE Glycosome formulations:

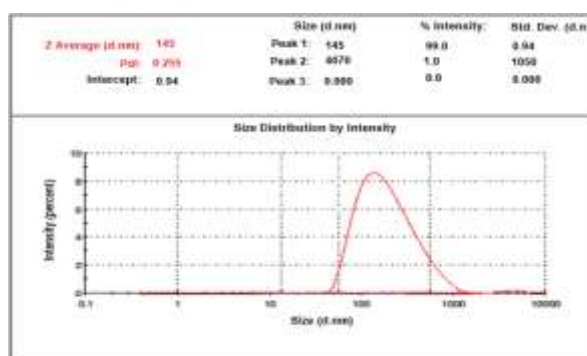
#### *Vesicle size and shape:*

The size and shape of the glycosome are determined by Transmission Electron Microscope (TEM). The shape of the prepared Glycosome vesicles was spherical and smooth with slight irregularity.

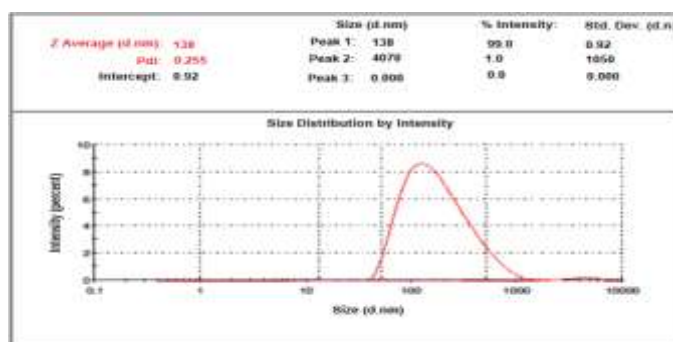


**Fig 1. TEM Photographs of (A) ASEE Glycosome formulation and (B) ZOEE Glycosome formulation.**

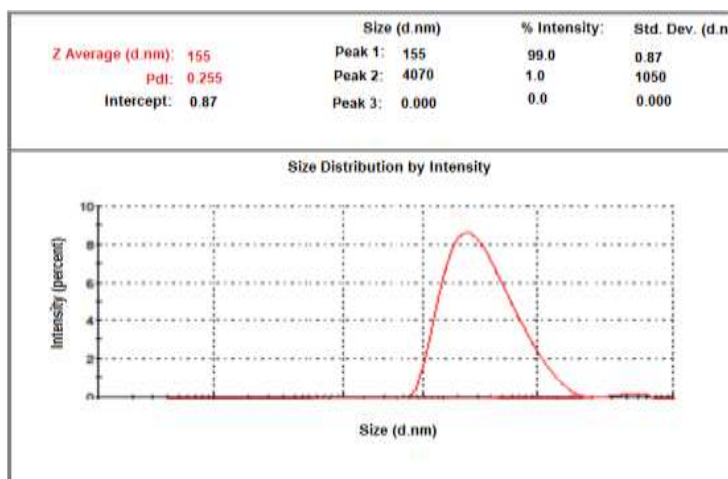
The vesicle size of ASEE and ZOEE glycosomes are found to be small on a nanometer scale that is  $145 \pm 0.94$ ,  $138 \pm 0.92$  for the ASEE Glycosome formulations F1 and F2 and  $155 \pm 0.87$ , and  $131 \pm 0.84$  nm of ZOEE Glycosome formulations F3 and F4.



**Fig 2. Vesicle size distribution of ASEE Glycosome formulation F1.**



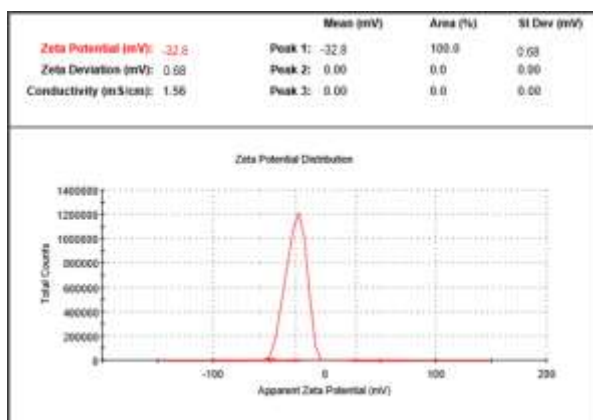
**Fig 3. Vesicle size distribution of ASEE Glycosome formulation F2.**



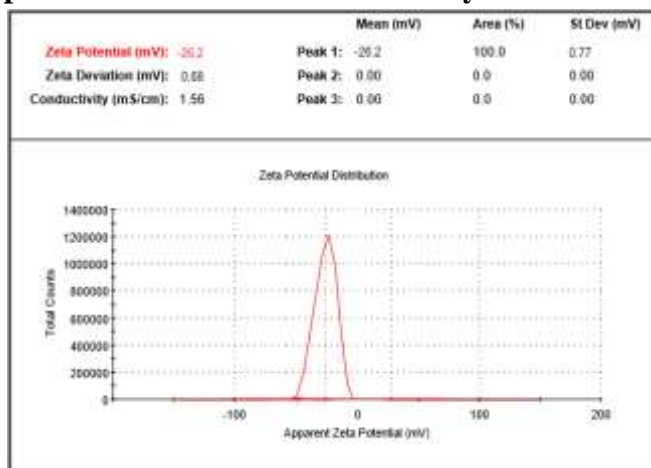
**Fig 4. The vesicle size distribution of ZOEE Glycrosome formulation F3.**

### ***Zeta potential:***

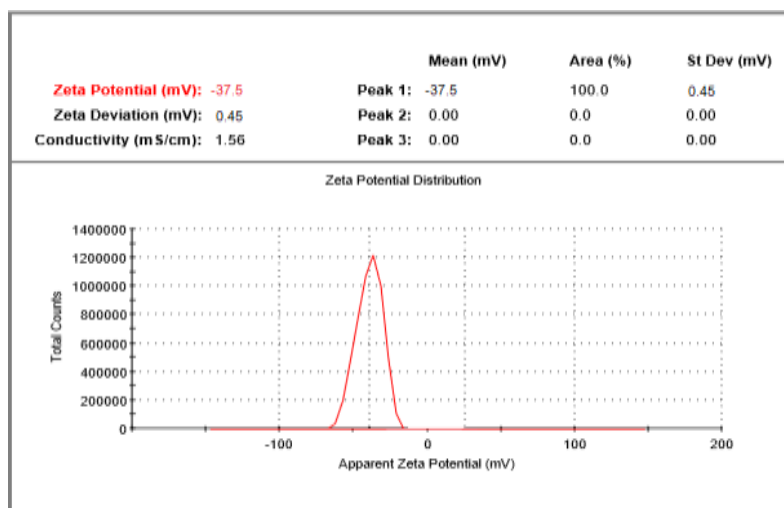
The surface charge (Potential) of ASEE and ZOEE Glycrosome formulations F2 and F4 was found to be more than the ASEE and ZOEE Glycrosome formulations F1 and F3. This might be due to the presence of more proportion of Glycerol in the formulations. The negative potential of glycrosomes demonstrates the greater stability of the vesicular dispersion.



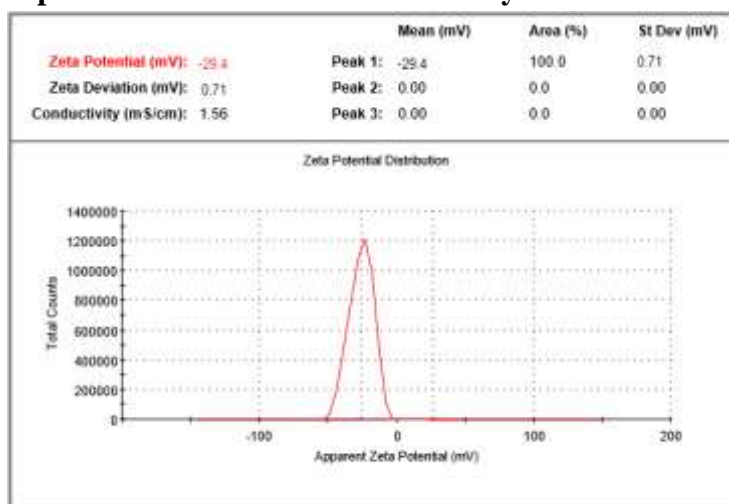
**Fig 6. Zeta potential distribution of ASEE Glycrosome formulation F1**



**Fig 7. Zeta potential distribution of ASEE Glycrosome formulation F2**



**Fig 8. Zeta potential distribution of ZOEE Glycosome formulation F3.**



**Fig 9. Zeta potential distribution of ZOEE Glycosome formulation F4**

**Table 6. Zeta potential of ASEE and ZOEE glycosome formulations.**

Sl. No.	Formulations	Zeta potential (mV)
1	F1	-32.8±0.68
2	F2	-26.2±0.77
3	F3	-37.5±0.45
4	F4	-29.4±0.71

Data are presented as Mean ± Standard deviation (n=3). Standard error of mean < 0.542.

### **Antimicrobial activity of Glycosome formulations:**

#### ***Antibacterial activity of ASEE glycosome formulations:***

The antibacterial activity of ASEE glycosome formulations was carried out by disk diffusion method. It has been observed that the Glycosome formulation F2 exhibited greater antibacterial activity than the Glycosome formulation F1. The antibacterial activity

shown by the ASEE glycosome might be due to the presence of phytoconstituents as drugs that are Alkaloids, Saponins, Flavonoids, and steroids inside the glycosome. The antibacterial activity shown by the ASEE Glycosome formulations was found to be statistically significant.



(a) Inhibitory activity of ASEE Glycosome formulation F1 against *S. aureus*.



(b) Inhibitory activity of ASEE Glycosome formulation F1 against *P. aeruginosa*.



(c) Inhibitory activity of ASEE Glycosome formulation F2 against *S. aureus*.



(d) Inhibitory activity of ASEE Glycosome formulation F2 against *P. aeruginosa*.

**Fig 10. Antibacterial activity of ASEE Glycosome formulations**

**Table 7. Antibacterial activity of ASEE glycosome formulations.**

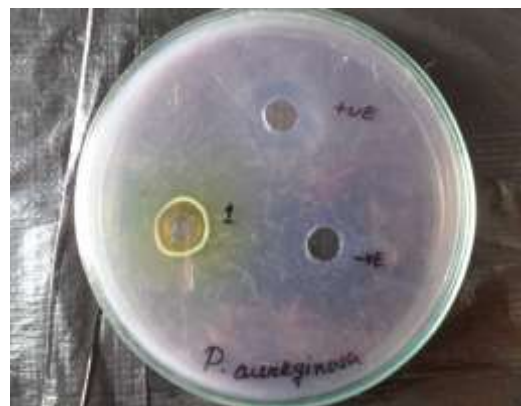
Test Groups	Drugs	Dose	Inhibition zone diameter (mm)	
			<i>Gm +ve</i>	<i>Gm -ve</i>
			<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>
I	Normal Control (Ethanol)	80 $\mu$ L	6.37 $\pm$ 0.71	6.68 $\pm$ 0.88
II	Erythromycin (Standard)	125 $\mu$ g/ml	26.74 $\pm$ 0.97	--
III	Ciprofloxacin (Standard)	125 $\mu$ g/ml	--	10.35 $\pm$ 0.90
IV	ASEE Glycosome (F1)	10 mg/ml	21.67 $\pm$ 1.05	27.6 $\pm$ 1.02
V	ASEE Glycosome (F2)	10 mg/ml	24.22 $\pm$ 0.93	33.68 $\pm$ 0.99

Each data is expressed as zone of inhibition (mm) for 24 h of study. Each value is represented as mean  $\pm$  standard deviation (n = 3). Standard error of mean < 0.606.

**Antibacterial activity of ZOEE glycosome formulations:**



(a) Inhibitory activity of ZOEE Glycosome formulation F3 against *S. aureus*.



(b) Inhibitory activity of ZOEE Glycosome formulation F3 against *P. aeruginosa*.



(c) Inhibitory activity of ZOEE Glycosome formulation F4 against *S. aureus*.



(d) Inhibitory activity of ZOEE Glycosome formulation F4 against *P. aeruginosa*.

**Fig 11. Antibacterial activity of ZOEE Glycosome formulations**

It has been observed that the Glycosome formulation F4 exhibited greater antibacterial activity than the Glycosome formulation F3 against the gram-negative bacteria *Pseudomonas aeruginosa*. This might be due to the more drug content (Phytoconstituents) present in the ZOEE glycosome. This could be due to more stability of glycosome, as because of the presence of more glycerol in the formulation. The antibacterial activity shown by the ZOEE Glycosome formulations was found to be statistically significant.

The possible mechanism of phytoconstituents that are present inside the ZOEE glycosome formulations are inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function, inhibition of energy metabolism, inhibition of the attachment and biofilm formation, inhibition of the porin on the cell membrane, alteration of the membrane permeability, and attenuation of the pathogenicity.



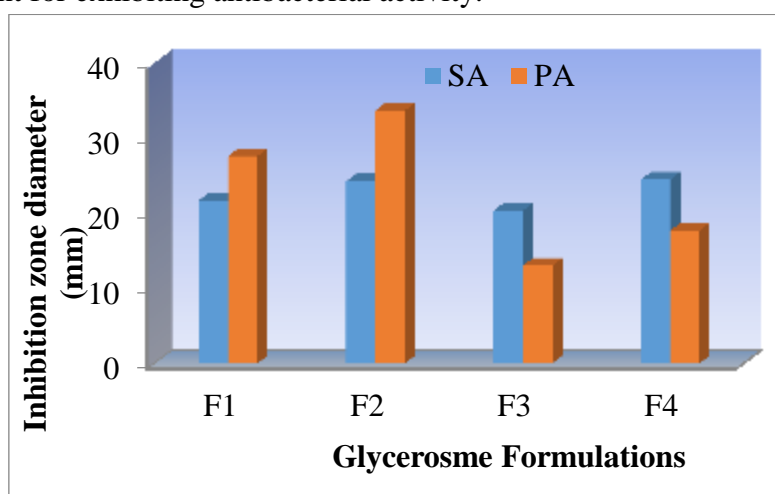
**Table 8. Antibacterial activity of ZOEE glycosome formulations.**

Test Groups	Drugs	Dose	Inhibition zone diameter (mm)	
			<i>Gm +ve</i>	<i>Gm -ve</i>
			<i>Staphylococcus aureus</i> (X±S.D.)	<i>Pseudomonas aeruginosa</i> (X±S.D.)
I	Normal Control (Ethanol)	80 µL	6.37±0.71	6.68±0.88
II	Erythromycin (Standard)	125µg/ml	26.74±0.97	--
III	Ciprofloxacin (Standard)	125µg/ml	--	10.35±0.90
IV	ZOEE Glycosome (F3)	10 mg/ml	20.33±1.01	13.0±1.11
V	ZOEE Glycosome (F4)	10 mg/ml	24.46±0.87	17.67±0.75

Each data is expressed as zone of inhibition (mm) for 24 h of study. Each value is represented as mean ± standard deviation (N = 3). Standard error of mean < 0.641.

**Comparative antibacterial activity between ASEE and ZOEE glycosome formulations:**

It has been seen that the ASEE glycosome formulations (F1 and F2) exhibited almost the same antibacterial activity against the gram-positive bacteria in comparison with the ZOEE glycosome formulations (F3 and F4), whereas ASEE glycosome formulations (F1 and F2) exhibited better antibacterial activity against the gram-negative bacteria in comparison with the ZOEE glycosome formulations (F3 and F4). This might be due to the presence of more flavonoid content in ASEE. Also, the ASEE glycosome contains sterols, which are a major constituent for exhibiting antibacterial activity.



**Fig 12. Comparative antibacterial activities of ASEE and ZOEE Glycosome formulations against gram-positive and gram-negative bacteria**

SA - *Staphylococcus aureus* (Gram-positive bacteria) and PA - *Pseudomonas aeruginosa* (Gram-negative bacteria).

**Antifungal activity of ASEE glycosome formulations:**

**Table 9. Antifungal activity of ASEE glycosome formulations.**

Test Groups	Drugs	Dose	Inhibition zone diameter (mm)
			<i>Candida albicans</i> (X±S.D.)
I	Normal Control (Ethanol)	80 µL	3.45±0.92
II	Clotrimazole (Standard)	25µg/ml	22.42±1.02
III	ASEE Glycosome (F1)	10 mg/ml	17.68±0.95
IV	ASEE Glycosome (F2)	10 mg/ml	18.28±1.03

Each data is expressed as zone of inhibition (mm) for 24 h of study. Each value is represented as mean ± standard deviation (N = 3). Standard error of mean < 0.595.

The antifungal activity exhibited by the ASEE glycosome formulations was found to be less than the standard drug Clotrimazole. The ASEE glycosome formulation F2 showed greater antifungal activity than the ASEE glycosome formulation F1.

This might be due to more phytoconstituents content as ASEE glycosome formulation F2 is more stable than the ASEE glycosome formulation F1. The ASEE glycosome formulation produced significant antifungal activity when it was compared with antifungal activity exhibited by the Standard drug Clotrimazole.

**Antifungal activity of ZOEE glycosome formulations:**

The antifungal activity exhibited by the ZOEE glycosome formulations was found to be very less than the standard drug. The ZOEE glycosome formulation F2 showed greater antifungal activity than the ZOEE glycosome formulation F1. This might be due to more phytoconstituents content as ZOEE glycosome formulation F2 is more stable than the ASEE glycosome formulation F1. The ASEE glycosome formulation does not produce significant antifungal activity when it was compared with the antifungal activity exhibited by the Standard drug Clotrimazole. But in comparison with the antifungal activity exhibited by normal control, the ASEE glycosome formulation produces significant antifungal activity.

**Table 10. Antifungal activity of ZOEE glycosome formulations.**

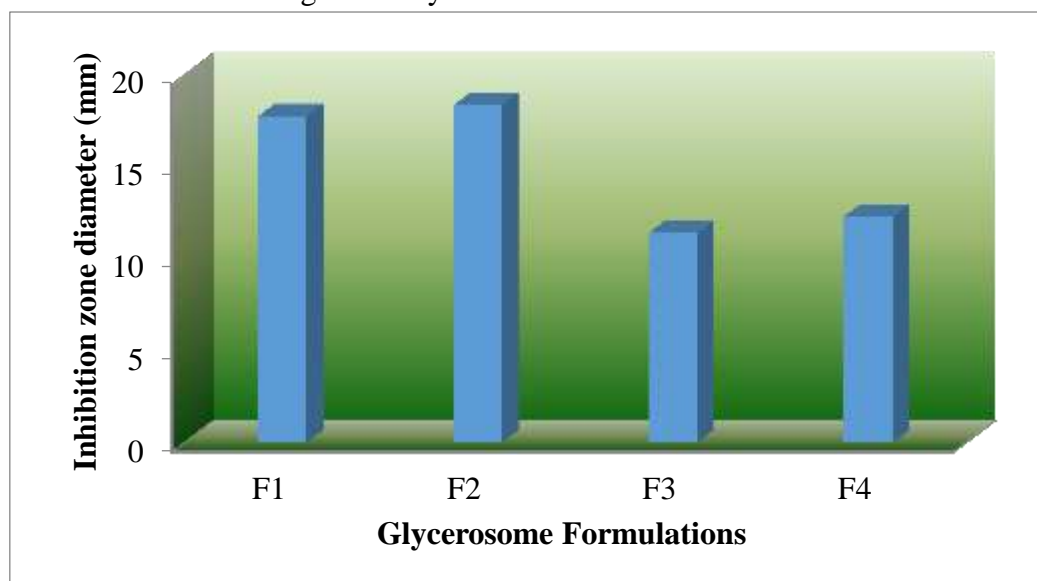
Test Groups	Drugs	Dose	Inhibition zone diameter (mm)
			<i>Candida albicans</i> (X±S.D.)
I	Normal Control (Ethanol)	80 µL	3.45±0.92
II	Clotrimazole (Standard)	25µg/ml	22.42±1.02
III	ZOEE Glycosome (F3)	10 mg/ml	11.35±0.89

IV	ZOEE Glycrosome (F4)	10 mg/ml	12.26±1.07
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Each data is expressed as zone of inhibition (mm) for 24 h of study. Each value is represented as mean  $\pm$  standard deviation (N = 3). Standard error of mean < 0.618.

**Comparative antifungal activity between ASEE and ZOEE glycrosome formulations:**

It has been seen that the ASEE glycrosome formulations (F1 and F2) exhibited greater antifungal activity in comparison with the ZOEE glycrosome formulations (F3 and F4). This property of ASEE glycrosome formulations may be attributed due to the presence of greater flavonoid content in ASEE. Also, the ASEE glycrosome contains sterols, which might be responsible for better antifungal activity.



**Fig 13.** Comparative antifungal activities of ASEE and ZOEE Glycrosome formulations against fungal stain *Candida albicans*.

**Conclusion**

Natural products (Mostly secondary metabolites) derived from plants are showing the biological activities in humans, resulting in fighting against numerous diseases. The aim of the present investigation is to evaluate the antimicrobial potential of *A. sativum* and *Z. officinalis* extracts mediated glycrosomes formulation. The Soxhlation method was found to be an efficient method for the successful extraction of the dried *A. sativum* and *Z. officinale* using ethanol as solvent. The crude drug of *A. sativum* and *Z. officinale* showed foreign organic was found to be 0.2 and 0.21 % and volatile oil was found to be 1.8 and 2.4 % only. The phytochemicals detected in *A. sativum* bulb ethanol extract are Alkaloids, Carbohydrates, Cardiac glycosides, Phenols, Sterols, Triterpenoids, Saponins, Flavonoids, and Fixed oils. The phytochemicals detected in *Z. officinale* rhizome ethanol extract are Alkaloids, Carbohydrates, Cardiac glycosides, Anthraquinone glycosides, Proteins and Amino acids, Triterpenoids, Saponins, Flavonoid, and Fixed oils. The thin film of lipid hydration was an efficient method for successfully preparing ASEE and ZOEE Glycrosomes. The prepared glycrosome was spherical with a slightly irregular shape. The glycrosome preparation was turbid in nature. The shape of the prepared Glycrosome vesicles was spherical and smooth

with slight irregularity. The negative potential of glycosomes demonstrates the greater stability of the vesicular dispersion. The ASEE glycosome formulations (F1 and F2) exhibited better antibacterial activity and greater antifungal activity against the gram-negative bacteria in comparison with the ZOEE glycosome formulations (F3 and F4). The present research work strongly concluded that the *A. sativum* and *Z. officinale* and their glycosome formulations are good candidate drugs and formulations for the safe and effective treatment of microbial infections. The present research work strongly concluded that the *A. sativum* and *Z. officinale* and their glycosome formulations are good candidate drugs and formulations for the safe and effective treatment of microbial infections.

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