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

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Abstract

The aim of the present investigation is to evaluate the Antiarthritic potential of *A. sativum* and *Z. officinalis* extracts mediated glycosomes formulation by a thin film of lipid hydration method. The *in vitro* anti-arthritic activity of *A. sativum* and *Z. officinale* ethanol extract glycerosome was determined by Protein denaturation inhibition and egg albumin denaturation inhibition methods. The protein denaturation inhibition activity of ASEE glycosome formulations F1 and F2 are 60.2±1.02 and 64.8±0.72 %, which are well comparable with the protein denaturation inhibition activity shown by the standard drugs. The egg albumin denaturation inhibition activity of ASEE glycerosome formulations F1 and F2 are 52.5±0.51 and 58.6±0.74 %, which are well comparable with the egg albumin denaturation inhibition activity shown by the standard drugs. The protein denaturation inhibition activity of ZOEE glycosome formulations F3 and F4 are 68.9±0.97 and 70.9±0.81 %, which are well comparable with the protein denaturation inhibition activity shown by the standard drugs. The egg albumin denaturation inhibition activity of ZOEE glycosome formulations F3 and F4 are 61.4±0.64 and 65.7±0.88 %, which are well comparable with the protein denaturation inhibition activity shown by the standard drugs. The ZOEE glycosome formulations (F3 and F4) showed greater antiarthritic activity than the ASEE glycosome formulations (F1 and F2). Even ZOEE glycosome formulations (F3 and F4) showed greater antiarthritic activity than the standard drugs Celecoxib and Cupferron marketed tablets. The present research work strongly concluded that the *A. sativum* and *Z. official and* their glycosome formulations are good candidate drugs and formulations for the safe and effective treatment of arthritis.

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

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].

Glycerosome was introduced into the drug delivery system by Manaca, *et al*. The concept of Glycerosome was developed from the stability problem of liposomes. Glycerosomes are formed by the phospholipid and glycerol in varying concentrations which form bilayer fluidity. Glycerosome is a modified form of the liposome. Glycerosomes 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 Glycerosome particles [7]. Glycerol ameliorates the deformability index of liposomal bilayers, thus enhancing skin penetration**.** Glycerosome is predominately used in transdermal and topical delivery of drugs. Both hydrophilic and hydrophobic drugs can be delivered by the Glycerosome [8,9].

The aim of present investigation is to evaluate Antiarthritic potential of *A. sativum* and *Z.officinalis* extracts mediated glycerosomes 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. The bovine serum albumin was purchased from Merck, India. The distilled water was prepared in own research laboratory. The concentrated hydrochloric acid and sodium hydroxide were purchased from LobaChemie, Mumbai, Inida. The Celecoxib and Cupferron tablets were purchased from the local market of Hamirpur District of Himachal Pradesh. The albumin from the chicken egg white was purchased from Sigma-Aldrich, USA. The disodium hydrogen phosphate, potassium dihydrogen phosphate, and sodium chloride were purchased from Hi-Media, New Delhi. All the other chemicals used were of analytical grade and procured from authorized dealer.

Methods

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 [10,11].

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 [12]. The glycerol concentration was taken in variable concentrations demonstrating the different *A. sativum* and *Z. officinale* glycerosome 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* Glycerosomes:

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±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 Glycerosomes were separated by filtration using Dialysis technique and kept in cold temperature for further study [13,14].

Table 1. Formulation design of various glycerosome 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 GLYCEROSOME FORMULATIONS:

Glycerosome appearance, size, and shape:

The appearance of the glycerosomes was examined using a transmission electron microscope. Samples were prepared for negative staining as follows that are glycerosomes 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 [15].

Glycerosome surface charge (Zeta Potential):

The Zeta sizer was used for the determination of surface charges of all the Glycerosomal formulations. The Zeta potential (mV) of all the formulations was measured by laser doppler electrophoresis at the same concentration as used for particle analysis [16].

***IN VITRO* ANTI-ARTHRITIC ACIVITY:**

Preparation of saline phosphate buffer pH 6.4:

The saline phosphate buffer of pH 6.4 was prepared by using 1.79 g of disodium hydrogen phosphate, 1.36 g of potassium dihydrogen phosphate and 7.02 g of sodium chloride. The three chemicals were weighed and dissolved in double distilled water. Finally, the volume of the solution was adjusted to 1000 ml. The pH of the final solution was determined by using digital pH meter. The pH of the solution was adjusted to 6.4 by using dilute solution of HCl and NaOH.

***In vitro* anti-arthritic activity of *A. sativum* glycerosome by Protein denaturation inhibition method:**

Test drugs:

The normal saline water was used as normal (Negative) control (Test group I). The marketed conventional tablet of Celecoxib and Cupferron are used as standard (Positive) control (Test groups II and III). The glyecerosome formulations F1 (30 % Glycerol) and F2 (30 % Glycerol) containing 100 mg of *A. sativum* ethanolic extract, are treated as test controls (Test groups IV and V).

Experimental method:

The *in vitro* anti-arthritic activity of ASEE glycerosomes was performed by protein denaturation method as per the standard procedure mentioned in the literature [17,18]. The Bovine serum albumin (BSA) solution was prepared in the strength of 5 % w/v by using saline phosphate buffer solution as solvent. The pH of the BSA solution was adjusted to 6.4 by using HCl. About 5 ml of solution consisting of 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of double distilled water was used as Normal (Negative) control solution. About 5 ml of solution containing 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of Celecoxib solution using the Celecoxib marketed tablet (Equivalent to Celecoxib strength of 100 µg/ml) was treated as primary standard (Positive) control solution. Separately, another 5 ml of solution containing 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of Cupferron solution using the Cupferron marketed tablet (Equivalent to Cupferron strength of 100 µg/ml) was treated as secondary standard (Positive) control solution.

About 5 ml of solution of ASEE glycerosomes consisting of 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of ASEE glycerosome formulation F1 and F2 (Equivalent to

ASEE strength of 100 µg/ml calculated with respect to yield of ASEE glycerosome) separately were used as test control solutions [19].

All the above mentioned five sample solutions were incubated separately by using the laboratory grade incubator (Royal Scientific Incubators, RSW 107, Tamil Nadu, India) at 37 °C for 15 min. After incubation, the sample solution mixture was heated separately at 60 °C for 5 min. This heating activity will induce the protein denaturation process. Now the samples were cooled to room temperature.

The absorbance of all five processed sample solutions was determined by using the UV-Visible spectrophotometer at λ_{max} of 660 nm. From the observed absorbance, the percentage inhibition of protein (BSA) was calculated by using the following equation as mentioned below [20].

$$\mathbf{PDI\ (\%) = [(AC_{660} - AT_{660})/\ AC_{660}] \times 100\ \ldots.(1)}$$

Where PDI is protein denaturation inhibition, AC_{660} and AT_{660} are absorbance of control and test solutions.

In vitro anti-arthritic activity of *A. sativum* glycerosome by egg albumin denaturation inhibition method:

The *in vitro* anti-arthritic activity of ASEE glycerosomes was performed by egg albumin denaturation method as per the standard procedure mentioned in the literature. The egg albumin solution was prepared in the strength of 5 % w/v by using saline phosphate buffer solution as solvent. The pH of the egg albumin solution was adjusted to 6.4 by using HCl. About 5 ml of solution consisting of 4.5 ml of egg albumin (5 % w/v) and 0.5 ml of double distilled water was used as Normal (Negative) control solution. About 5 ml of solution containing 4.5 ml of egg albumin (5 % w/v) and 0.5 ml of Celecoxib solution using the Celecoxib marketed tablet (Equivalent to Celecoxib strength of 100 µg/ml) was treated as primary standard (Positive) control solution. Separately, another 5 ml of solution containing 4.5 ml of egg albumin (5 % w/v) and 0.5 ml of Cupferron solution using the Cupferron marketed tablet (Equivalent to Cupferron strength of 100 µg/ml) was treated as secondary standard (Positive) control solution. About 5 ml of solution of ASEE glycerosomes consisting of 4.5 ml of egg albumin (5 % w/v) and 0.5 ml of ASEE glycerosome formulation F1 and F2 (Equivalent to ASEE strength of 100 µg/ml calculated with respect to yield of ASEE glycerosome) separately were used as test control solutions [21,22].

All the above mentioned five sample solutions were incubated separately by using the laboratory grade incubator (Royal Scientific Incubators, RSW 107, Tamil Nadu, India) at 37 °C for 15 min. After incubation, the sample solution mixture was heated separately at 60 °C

for 5 min. This heating activity will induce the protein denaturation process. Now the samples were cooled to room temperature. The absorbance of all five processed sample solutions was determined by using the UV-Visible spectrophotometer (Shimadzu 1900i, Japan) at λ_{max} of 660 nm. From the observed absorbance, the percentage inhibition of protein (BSA) was calculated by using the following equation as mentioned below.

$$\mathbf{EADI\ (\%) = [(AC_{660} - AT_{660})/\ AC_{660}] \times 100 \ldots. (2)}$$

Where EADI is egg albumin denaturation inhibition, AC_{660} and AT_{660} are absorbance of control and test solutions.

***In vitro* anti-arthritic activity of *Z. officinale* glycerosome by protein denaturation inhibition method:**

Test drugs:

The normal saline water was used as normal (Negative) control (Test group I). The marketed conventional tablet of Celecoxib and Cupferron are used as standard (Positive) control (Test groups II and III).

The glyecerosome formulations F3 (30 % Glycerol) and F4 (30 % Glycerol) containing 100 mg of *Z. officinale*ethanolic extract, are treated as test controls (Test groups IV and V).

Experimental method:

The *in vitro* anti-arthritic activity of ZOEE glycerosomes was performed by protein denaturation method as per the standard procedure mentioned in the literature [23,24]. The Bovine serum albumin (BSA) solution was prepared in the strength of 5 % w/v by using saline phosphate buffer solution as solvent. The pH of the BSA solution was adjusted to 6.4 by using HCl. About 5 ml of solution consisting of 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of double distilled water was used as Normal (Negative) control solution. About 5 ml of solution containing 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of Celecoxib solution using the Celecoxib marketed tablet (Equivalent to Celecoxib strength of 100 µg/ml) was treated as primary standard (Positive) control solution. Separately, another 5 ml of solution containing 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of Cupferron solution using the Cupferron marketed tablet (Equivalent to Cupferron strength of 100 µg/ml) was treated as secondary standard (Positive) control solution. About 5 ml of solution of ZOEE glycerosomes consisting of 4.5 ml of Bovine serum albumin (BSA – 5 % w/v) and 0.5 ml of ZOEE glycerosome formulation F1 and F2 (Equivalent to ZOEE strength of 100 µg/ml calculated with respect to yield of ZOEE glycerosome) separately were used as test control solutions.

All the above mentioned five sample solutions were incubated separately by using the laboratory grade incubator (Royal Scientific Incubators, RSW 107, Tamil Nadu, India) at 37 °C for 15 min. After incubation, the sample solution mixture was heated separately at 60 °C for 5 min. This heating activity will induce the protein denaturation process. Now the samples were cooled to room temperature. The absorbance of all five processed sample solutions was determined by using the UV-Visible spectrophotometer (Shimadzu 1900i, Japan) at λ_{max} of 660 nm. From the observed absorbance, the percentage inhibition of protein (BSA) was calculated by using the equation 2 as mentioned above [25].

***In vitro*anti-arthritic activity of *Z. officinale* glycerosome by egg albumin denaturation inhibition method:**

The *in vitro* anti-arthritic activity of ZOEE glycerosomes was performed by egg albumin denaturation method as per the standard procedure mentioned in the literature. The egg albumin solution was prepared in the strength of 5 % w/v by using saline phosphate buffer solution as solvent. The pH of the egg albumin solution was adjusted to 6.4 by using HCl. About 5 ml of solution consisting of 4.5 ml of egg albumin (5 % w/v) and 0.5 ml of double distilled water was used as Normal (Negative) control solution. About 5 ml of solution containing 4.5 ml of egg albumin (5 % w/v) and 0.5 ml of Celecoxib solution using the Celecoxib marketed tablet (Equivalent to Celecoxib strength of 100 µg/ml) was treated as primary standard (Positive) control solution. Separately, another 5 ml of solution containing 4.5 ml of egg albumin (5 % w/v) and 0.5 ml of Cupferron solution using the Cupferron marketed tablet (Equivalent to Cupferronstrength of 100 µg/ml) was treated as secondary standard (Positive) control solution. About 5 ml of solution of ZOEE glycerosomes consisting of 4.5 ml of egg albumin (5 % w/v) and 0.5 ml of ZOEE glycerosome formulation F3 and F4(Equivalent to ZOEE strength of 100 µg/ml calculated with respect to yield of ZOEE glycerosome) separately were used as test control solutions [26].

All the above mentioned five sample solutions were incubated separately by using the laboratory grade incubator (Royal Scientific Incubators, RSW 107, Tamil Nadu, India) at 37 °C for 15 min. After incubation, the sample solution mixture was heated separately at 60 °C for 5 min. This heating activity will induce the protein denaturation process. Now the samples were cooled to room temperature. The absorbance of all five processed sample solutions was determined by using the UV-Visible spectrophotometer (Shimadzu 1900i, Japan) at λ_{max} of 660 nm. From the observed absorbance, the percentage inhibition of protein (BSA) was calculated by using the equation 2 as mentioned above.

Results and Discussion

Characterization of ASEE and ZOEE Glycerosome formulations:

Vesicle size and shape:

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

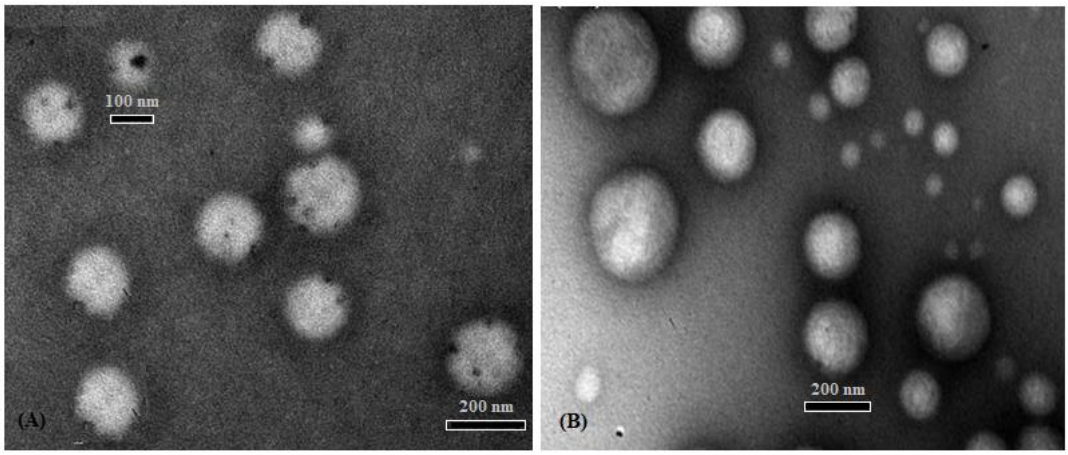

Fig 1. TEM Photographs of (A) ASEE Glycerosome formulation and (B) ZOEE Glycerosome formulation.

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

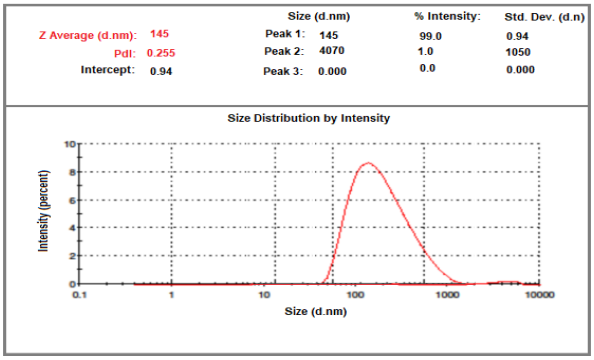

Fig 2. Vesicle size distribution of ASEE Glycerosome formulation F1.

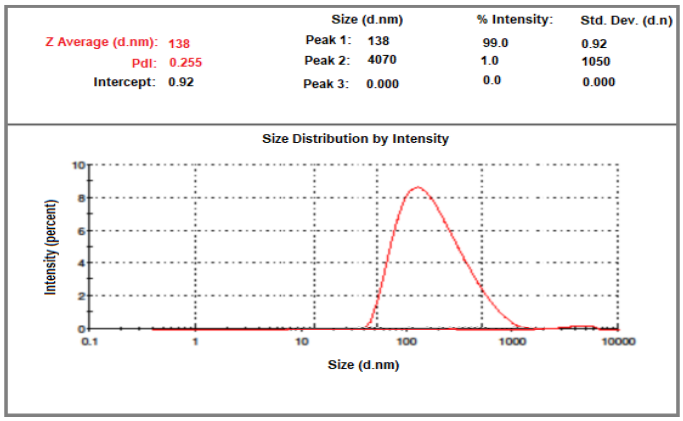

Fig 3. Vesicle size distribution of ASEE Glycerosome formulation F2.

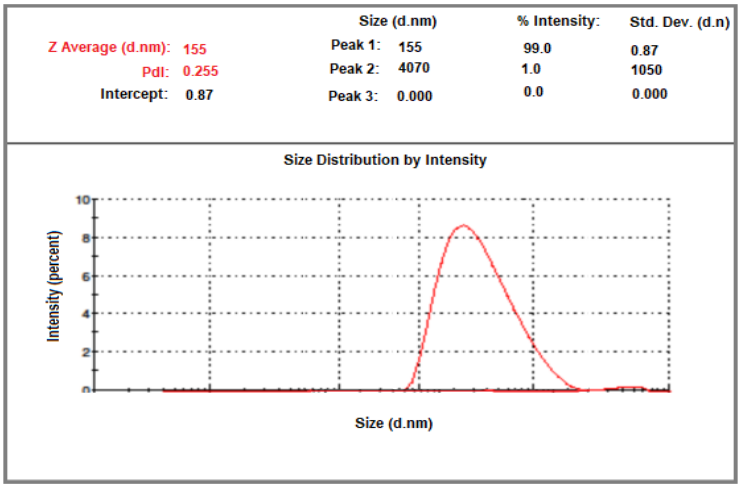

Fig 4. The vesicle size distribution of ZOEE Glycerosome formulation F3.

Fig 5. Vesicle size distribution of ZOEE Glycerosome formulation F4.

***Zeta potential*:**

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

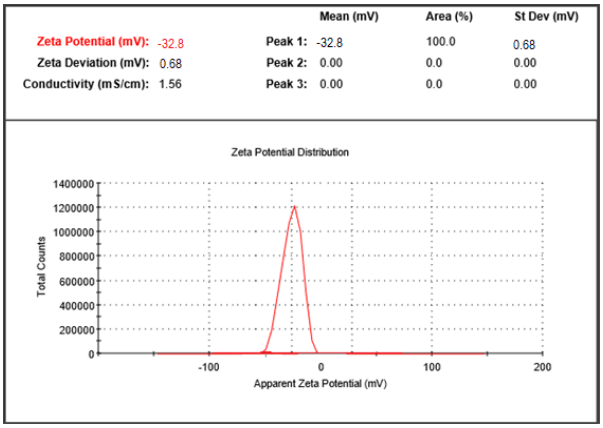

Fig 6. Zeta potential distribution of ASEE Glycerosome formulation F1

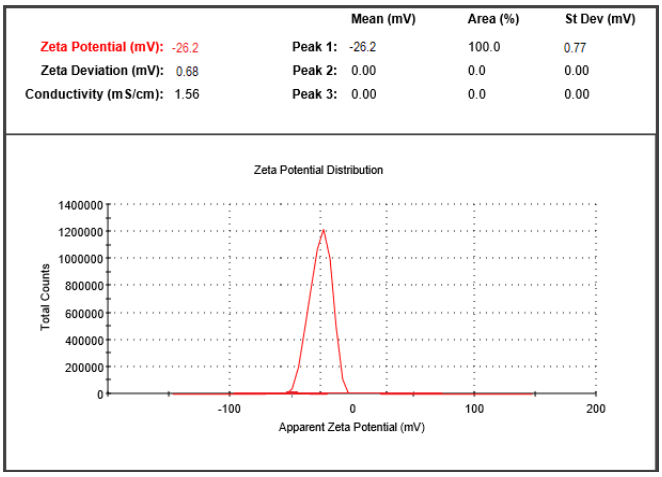

Fig 7. Zeta potential distribution of ASEE Glycerosome formulation F2

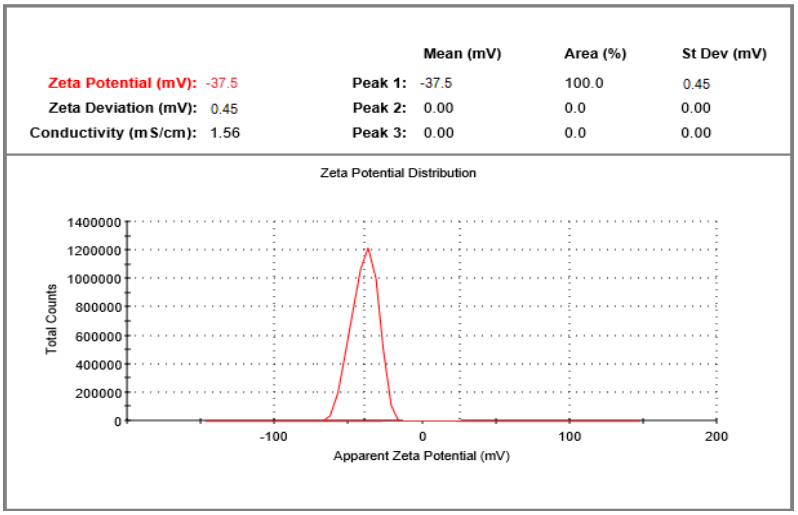

Fig 8. Zeta potential distribution of ZOEE Glycerosome formulation F3.

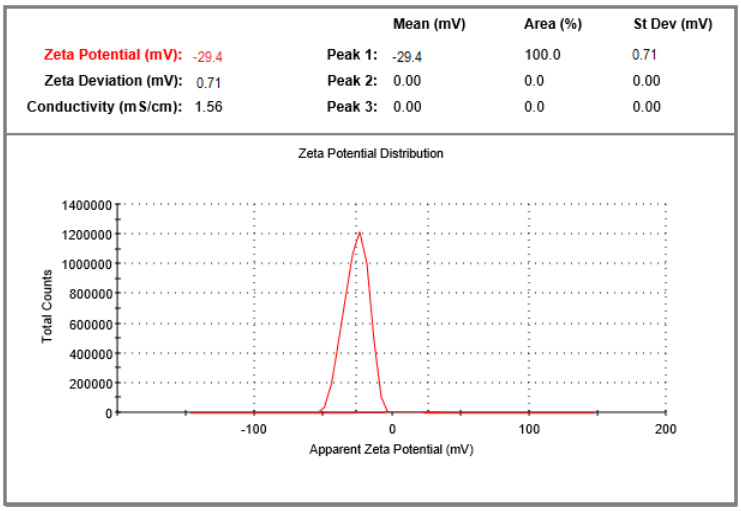

Fig 9. Zeta potential distribution of ZOEE Glycerosome formulation F4

Table 2. Zeta potential of ASEE and ZOEE glycerosome formulations

S. 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.

Anti-arthritic activity:

***In vitro anti-arthritic activity of A. sativum glycerosome by Protein denaturation inhibition method*:**

The protein (BSA) denaturation inhibition activities of standard drugs Celecoxib and Cupferron marketed tablets are 76.8±0.88 and 71.4±0.69 %. In comparison to the protein denaturation activity exhibited by Normal saline water, standard drugs exhibited statistically significant activity with a 99 % confidence level (**p<0.01). The protein denaturation inhibition activity of ASEE glycerosome formulations F1 and F2 are 60.2±1.02 and 64.8±0.72 % (With the statistical significance of 95 % confidence level that is $p < 0.05$), which are well comparable with the protein denaturation inhibition activity shown by the standard drugs. The protein denaturation inhibition activity shown by glycerosome formulation F2 is more than the glycerosome formulation F1. It might be due to more availability of drugs that is phytoconstituents of *A. sativum*, as because of more stability of Glycerosome formulation F2 due to involvement of more Glycerol content.

Table 3. Protein denaturation inhibition activity of ASEE glycerosome formulations.

Sl. No.	Test group	Control	Treatments	Protein denaturation Inhibition (%) (X±S.D.)
1	I	Negative	Normal saline water	--
2	II	Standard 1	Celecoxib tablet	76.8±0.88**
3	III	Standard 2	Cupferron tablet	71.4±0.69**
4	IV	Test 1	ASEE Glycerosome (F1)	60.2±1.02*
5	V	Test 2	ASEE Glycerosome (F2)	64.8±0.72*

All data are expressed as mean ± standard deviation (N = 3). Standard error of mean (SEM) <0.589. Statistical significance: (*p<0.05) and (**p<0.01) One-way ANOVA followed by Dunnett test.

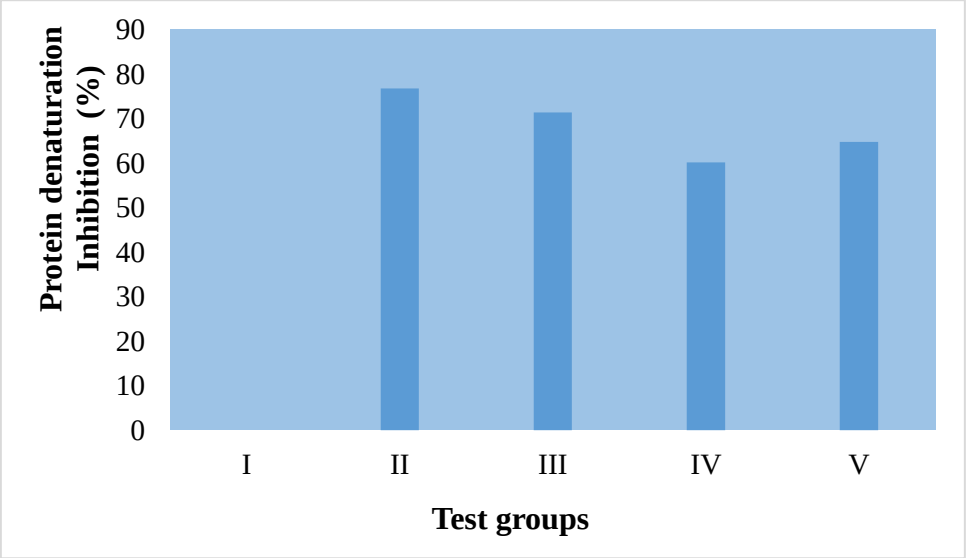

Fig 10. Protein (BSA) denaturation inhibition activity of ASEE glycerosome formulations

Groups I, II, III, IV, and V are negative (Normal saline water), Standard 1 (Celecoxib tablet), Standard 2 (Cupferron tablet), ASEE Glycerosome (F1), and ASEE Glycerosome (F2) respectively.

Low content of glycerol in ASEE glycerosome may lead to the low stability of the vesicle which might result in the leakage of *A. sativum* phytoconstituents, thus the availability of phytoconstituents in ASEE glycerosome formulation F1 is less. This results in comparatively less protein denaturation inhibition activity.

***In vitro anti-arthritic activity of A. sativum glycerosome by egg albumin denaturation inhibition method*:**

The egg albumin denaturation inhibition activities of standard drugs Celecoxib and Cupferron marketed tablet are 71.6±0.87and 68.2±0.91%. In comparison to the egg albumin denaturation activity exhibited by Normal saline water, standard drugs exhibited statistically significant

activity with a 99 % confidence level (**p<0.01). The egg albumin denaturation inhibition activity of ASEE glycerosome formulations F1 and F2 are 52.5±0.51 and 58.6±0.74 % (With a statistical significance of 95 % confidence level that is $p < 0.05$), which are well comparable with the egg albumin denaturation inhibition activity shown by the standard drugs. the egg albumin denaturation inhibition activity shown by glycerosome formulation F2 is more than the glycerosome formulation F1. It might be due to more availability of drugs that is phytoconstituents of *A. sativum*, as because of more stability of Glycerosome formulation F2 due to involvement of more Glycerol content.

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Table 4. Egg albumin denaturation inhibition activity of ASEE glycerosome formulations.

S. No.	Test group	Control	Treatments	Egg albumin denaturation Inhibition (%) (X±S.D.)
1	I	Negative	Normal saline water	--
2	II	Standard 1	Celecoxib tablet	71.6±0.87**
3	III	Standard 2	Cupferron tablet	68.2±0.91**
4	IV	Test 1	ASEE Glycerosome (F1)	52.5±0.51*
5	V	Test 2	ASEE Glycerosome (F2)	58.6±0.74*

All data are expressed as mean ± standard deviation (N = 3). Standard error of mean (SEM) < 0.525. Statistical significance: (*p<0.05) and (**p<0.01) One-way ANOVA followed by Dunnett test.

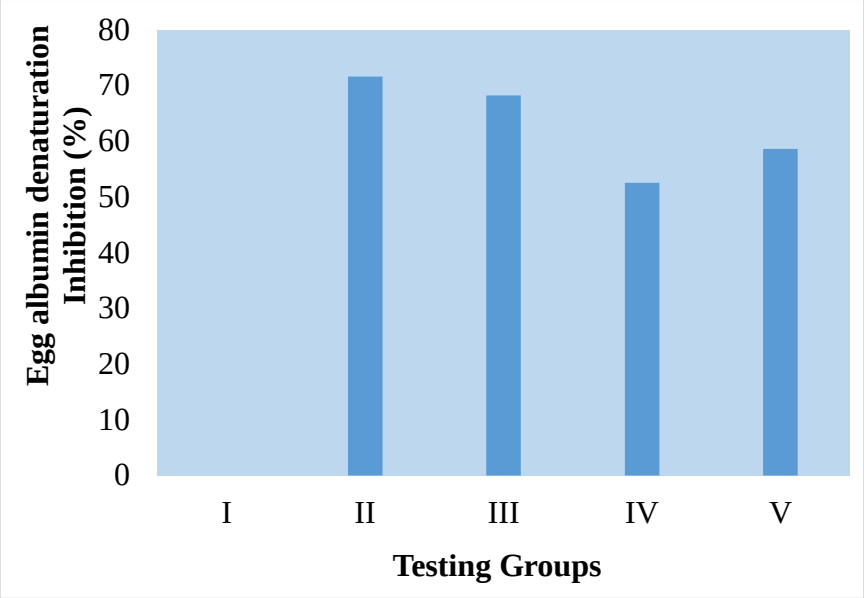

Fig 11. Egg albumin denaturation inhibition activity of ASEE glycerosome formulations

Groups I, II, III, IV, and V are negative (Normal saline water), Standard 1 (Celecoxib tablet), Standard 2 (Cupferron tablet), ASEE Glycerosome (F1), and ASEE Glycerosome (F2) respectively.

***In vitroanti-arthritic activity of Z. officinale glycerosome by protein denaturation inhibition method*:**

The protein (BSA) denaturation inhibition activities of standard drugs Celecoxib and Cupferron marketed tablets are 76.8±0.88 and 71.4±0.69 %.

In comparison to the protein denaturation activity exhibited by Normal saline water, standard drugs exhibited statistically significant activity with a 99 % confidence level (**p<0.01). The protein denaturation inhibition activity of ZOEE glycerosome formulations F1 and F2 are 68.9±0.97 and 70.9±0.81% (With a statistical significance of 99 % confidence level that is $p < 0.01$), which are well comparable with the protein denaturation inhibition activity shown by the standard drugs. From the result, it was revealed that the ZOEE glycerosome formulations exhibited more or less protein denaturation inhibitory activity equal to that of the standard drugs. The protein denaturation inhibition activity shown by glycerosome formulation F2 is more than the glycerosome formulation F1. It might be due to more availability of drugs that is phytoconstituents of *Z. officinale*, as because of more stability of Glycerosome formulation F2 due to involvement of more Glycerol content.

Low content of glycerol in ZOEE glycerosome may lead to the low stability of the vesicle which might result in the leakage of *Z. officinale* phytoconstituents, thus the availability of phytoconstituents in ZOEE glycerosome formulation F1 is less. This results in comparatively less protein denaturation inhibition activity.

Table 5. Protein denaturation inhibition activity of ZOEE glycerosome formulations.

Sl. No.	Test group	Control	Treatments	Protein denaturation Inhibition (%) (X±S.D.)
1	I	Negative	Normal saline water	--
2	II	Standard 1	Celecoxib tablet	76.8±0.88**
3	III	Standard 2	Cupferron tablet	71.4±0.69**
4	IV	Test 1	ASEE Glycerosome (F3)	68.9±0.97**
5	V	Test 2	ASEE Glycerosome (F4)	70.9±0.81**

All data are expressed as mean ± standard deviation (N = 3). Standard error of mean (SEM) < 0.56. Statistical significance: (*p<0.05) and (**p<0.01) One-way ANOVA followed by Dunnett test.

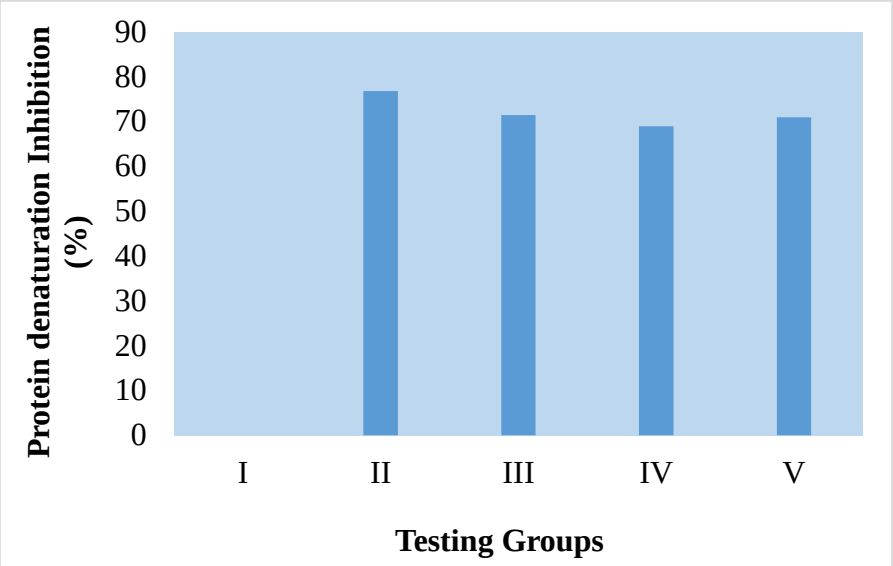

Fig 12. Protein (BSA) denaturation inhibition activity of ZOEE glycerosome formulations

Groups I, II, III, IV, and V are negative (Normal saline water), Standard 1 (Celecoxib tablet), Standard 2 (Cupferron tablet), ZOEE Glycerosome (F1), and ZOEE Glycerosome (F2) respectively.

***In vitroanti-arthritic activity of Z. officinale glycerosome by egg albumin denaturation inhibition method*:**

The egg albumin denaturation inhibition activities of standard drugs Celecoxib and Cupferron marketed tablet are 71.6±0.87and 68.2±0.91%. In comparison to the egg albumin denaturation activity exhibited by Normal saline water, standard drugs exhibited statistically significant activity with a 99 % confidence level (**p<0.01). The egg albumin denaturation inhibition activity of ZOEE glycerosome formulations F1 and F2 are 61.4±0.64 and 65.7±0.88 % (With a statistical significance of 95 % confidence level that is p < 0.05), which are well comparable

with the protein denaturation inhibition activity shown by the standard drugs. From the result, it was revealed that the ZOEE glycerosome formulations exhibited more or less protein denaturation inhibitory activity equal to that of the standard drugs. The egg albumin denaturation inhibition activity shown by glycerosome formulation F2 is more than the glycerosome formulation F1. It might be due to more availability of drugs that is phytoconstituents of *Z. officinale*, as because of more stability of Glycerosome formulation F2 due to involvement of more Glycerol content.

Low content of glycerol in ZOEE glycerosome may lead to the low stability of the vesicle which might result in the leakage of *Z. officinale* phytoconstituents, thus the availability of phytoconstituents in ZOEE glycerosome formulation F1 is less. This results in comparatively less protein denaturation inhibition activity.

Table 6. The Egg albumin denaturation inhibition activity of ZOEE glycerosome formulations

Sl. No.	Test group	Control	Treatments	Egg albumin denaturation inhibition (%) (X±S.D.)
1	I	Negative	Normal saline water	--
2	II	Standard 1	Celecoxib tablet	71.6±0.87**
3	III	Standard 2	Cupferron tablet	68.2±0.91**
4	IV	Test 1	ASEE Glycerosome (F3)	61.4±0.64**
5	V	Test 2	ASEE Glycerosome (F4)	65.7±0.88**

All data are expressed as mean ± standard deviation (N = 3). Standard error of mean (SEM) <0.525. Statistical significance: (*p<0.05) and (**p<0.01) One-way ANOVA followed by Dunnett test.

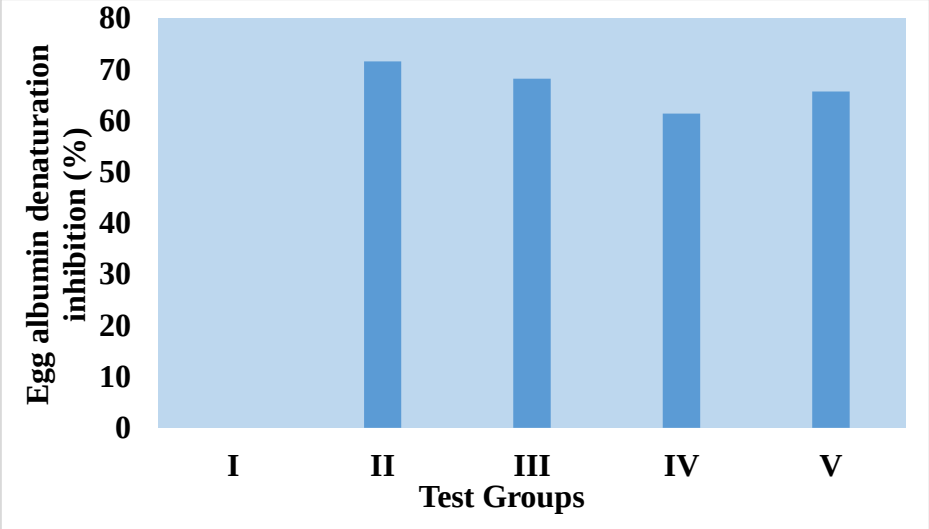

Fig 13. Egg albumin denaturation inhibition activity of ZOEE glycerosome formulations

Groups I, II, III, IV, and V are negative (Normal saline water), Standard 1 (Celecoxib tablet), Standard 2 (Cupferron tablet), ZOEE Glycerosome (F1), and ZOEE Glycerosome (F2) respectively.

Conclusion

The aim of the present investigation is to evaluate the antiarthritic potential of *A. sativum* and *Z. officinalis* extracts mediated glycerosomes formulation. With the objective to design, optimize and develop a glycerosome that might have an efficient anti-arthritis property with no or lesser adverse effects than the existing drug of choice.

The *in vitro* anti-arthritic activity of *A. sativum* and *Z. officinale* ethanol extract glycerosome was determined by Protein denaturation inhibition and egg albumin denaturation inhibition methods.

The protein denaturation inhibition activity of ASEE glycerosome formulations F1 and F2 are 60.2±1.02 and 64.8±0.72 %, which are well comparable with the protein denaturation inhibition activity shown by the standard drugs. The protein denaturation inhibition activity shown by glycerosome formulation F2 is more than the glycerosome formulation F1.

The egg albumin denaturation inhibition activity of ASEE glycerosome formulations F1 and F2 are 52.5±0.51 and 58.6±0.74 %, which are well comparable with the egg albumin denaturation inhibition activity shown by the standard drugs. The egg albumin denaturation inhibition activity shown by glycerosome formulation F2 is more than the glycerosome formulation F1.

The protein denaturation inhibition activity of ZOEE glycerosome formulations F3 and F4 are 68.9±0.97 and 70.9±0.81 %, which are well comparable with the protein denaturation inhibition activity shown by the standard drugs. The protein denaturation inhibition activity shown by glycerosome formulation F4 is more than the glycerosome formulation F3.

The egg albumin denaturation inhibition activity of ZOEE glycerosome formulations F3 and F4 are 61.4±0.64 and 65.7±0.88 %, which are well comparable with the protein denaturation inhibition activity shown by the standard drugs.

The ZOEE glycerosome formulations (F3 and F4) showed greater antiarthritic activity than the ASEE glycerosome formulations (F1 and F2). Even ZOEE glycerosome formulations (F3 and F4) showed greater antiarthritic activity than the standard drugs Celecoxib and Cupferron marketed tablets.

The result of the experimental study revealed that the ASEE and ZOEE glycerosome formulations are significantly showing antimicrobial and antiarthritic activities. The ASEE

glycerosome formulations showed more potent antimicrobial activity than the ZOEE glycerosome formulations. The ZOEE glycerosome formulations showed more potent antiarthritic activity than the ASEE glycerosome formulations.

However extensive research is to be carried out to find out the chemical moiety in quantitative form is responsible for exhibiting antimicrobial and antiarthritic activities through sophisticated instrumental studies. Extensive parameters need to be studied to evaluate the glycerosome formulations and to ascertain the *in vitro* and *in vivo* stability of glycerosome formulations in both formulation and biological aspects.

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 and arthritis.

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