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FORMULATION DEVELOPMENT AND EVALUATION OF MICROSPHERE OF *ZEPHYRANTHES CITRINA* EXTRACT FOR ANTIMICROBIAL ACTIVITY

Namita Yadav*, Ashish Jain, Mahadev Kanere, Akhlesh Kumar Singhai
School of Pharmacy, LNCT University, Bhopal (M.P.)

***Correspondence Info:**
Namita Yadav
School of Pharmacy, LNCT University, Bhopal (M.P.)
Email:
namitayadav0504@gmail.com

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ABSTRACT

The study focuses on the formulation development and evaluation of microspheres containing *Zephyranthes citrina* extract for antimicrobial activity. The crude extract yield varied significantly with the solvents used, with methanol extraction yielding 2.32% compared to 0.46% with petroleum ether. Phytochemical analysis revealed the methanolic extract to be rich in alkaloids, glycosides, carbohydrates, and tannins/phenolic compounds, whereas the petroleum ether extract was limited to saponins and triterpenoids/steroids. The solubility study highlighted the extract's high solubility in methanol, making it suitable for microsphere formulation. Particle size analysis of the microspheres ranged from 407.7 nm to 513.9 nm, with MS 2 exhibiting the most uniform size distribution (PI of 0.337) and stable zeta potential (-0.4 mV to -2.2 mV). SEM analysis confirmed the spherical and smooth morphology of the microspheres. Antimicrobial testing against E. coli showed that the microsphere formulations had enhanced activity (zones of inhibition: 9 mm and 14 mm for 0.5 mg/ml and 1 mg/ml, respectively) compared to the crude extract (7 mm). Stability studies indicated that the optimized microsphere formulation MS 2 maintained its integrity and stability over 90 days. These findings suggest that the microsphere formulation of *Zephyranthes citrina* extract offers improved antimicrobial efficacy and stability.

Keywords: *Zephyranthes citrina*, microspheres, antimicrobial activity, methanolic extract, phytochemical analysis, particle size, zeta potential, stability study, *E. coli*.

INTRODUCTION

In recent years, the focus on plant-based therapeutics has gained significant momentum, owing to their potential efficacy, safety, and lower incidence of side effects compared to synthetic drugs. *Zephyranthes citrina*, commonly known as the yellow rain lily, is one such plant that has shown promise in the realm of antimicrobial therapy. *Zephyranthes* species have been traditionally used in various medicinal applications, and recent studies have indicated their potential in combating microbial infections due to their bioactive constituents (Ahmad *et al.,* 2006). *Zephyranthes citrina* is a member of the Amaryllidaceae family and is known for its rich phytochemical profile, including alkaloids, flavonoids, and phenolic compounds. These bioactive compounds are recognized for their antimicrobial, antioxidant, and anti-inflammatory properties (Mahajan & Rawal, 2018). The antimicrobial activity of *Zephyranthes citrina* is particularly noteworthy, as it presents a natural alternative to combat antibiotic-resistant bacterial strains, which pose a significant challenge in clinical settings (Balouiri *et al.,* 2016).

The formulation of microspheres is an advanced drug delivery strategy designed to enhance the efficacy and controlled release of therapeutic agents. Microspheres are spherical, free-flowing particles ranging from 1 to 1000 micrometers in diameter and can encapsulate drugs, protecting them from degradation and ensuring a sustained release profile (Chellampillai & Pawar, 2012). This delivery system is particularly advantageous for phytochemicals, which often suffer from stability and bioavailability issues (Singh *et al.,* 2019).

The encapsulation of *Zephyranthes citrina* extract in microspheres aims to leverage these benefits, ensuring a sustained and targeted release of the antimicrobial constituents. This approach not only enhances the therapeutic potential of the extract but also improves patient compliance by reducing the frequency of administration (Chellampillai & Pawar, 2012).

Previous research has demonstrated the antimicrobial efficacy of various plant extracts when formulated into microspheres. For instance, studies on microsphere formulations of plant extracts have shown improved stability, sustained release, and enhanced bioavailability, leading to better clinical outcomes (Saeed *et al.,* 2012). Given the promising results from these studies, the development of *Zephyranthes citrina* extract-loaded microspheres represents a logical progression in the quest for effective and natural antimicrobial therapies.

The primary objective of this study is to formulate and evaluate microspheres containing *Zephyranthes citrina* extract for their antimicrobial activity. This involves optimizing the formulation parameters, characterizing the microspheres, and assessing their antimicrobial efficacy against selected microbial strains. The study aims to contribute to the growing body of knowledge on plant-based antimicrobials and their advanced delivery systems, potentially paving the way for new, effective treatments in the fight against microbial infections.

MATERIALS AND METHODS

Material

Petroleum ether from Ranken and methanol from Molychem were used as solvents for extraction. Sodium hydroxide, concentrated sulphuric acid, copper sulphate, lead acetate, mercuric chloride, ammonium sulphate, ferric chloride, hydrochloric acid, and Follin-Ciocalteu's reagent were all sourced from Merck for various chemical reactions and assays. Sodium nitrite from Sunchem, ninhydrin and potassium sodium tartrate from Himedia, and sodium citrate and sodium carbonate from s.d.fine-CHEM Ltd were essential for analytical procedures and buffer preparation.

Plant collection

The medicinal plant *Zephyranthes citrina* (300 gm) was collected. After cleaning, plant parts (whole plant) were dried under shade at room temperature for 3 days and then in oven dried at 45°C till complete dryness. Dried plant parts were stored in air tight glass containers in dry and cool place to avoid contamination and deterioration. Authentication of selected traditional plant - Medicinal plant *Zephyranthes citrina* was authenticated by a plant taxonomist in order to confirm its identity and purity.

Extraction

In the current investigation, plant material was extracted utilizing the Soxhlet apparatus and a continuous hot percolation method. *Zephyranthes citrina* powder was added to a soxhlet apparatus thimble. At 60°C, soxhlation was carried out with petroleum ether acting as a non-polar solvent. After being dried, the exhausted plant material (marc) was extracted again using an ethanol solvent. For every solvent, the extraction process was carried out until no visual color shift was seen in the siphon tube, and the absence of any residual solvent upon evaporation confirmed that the extraction was complete.

The obtained extracts were evaporated at 40°C in a Buchi-type rotating vacuum evaporator. Weighing the dried extract allowed us to calculate the % yield for each extract using a formula:

$$\% \text{ Yield} = \frac{\text{Weight of extract}}{\text{Weight of Plant Material used}} \times 100$$

Prepared extracts was observed for organoleptic characters (percentage yield, colour and odour) and was packed in air tight container and labelled till further use (Baidya *et al.,* 2002).

Phytochemical investigation

To find out if specific phytoconstituents were present or absent, an extensive qualitative phytochemical analysis was used in an experiment. The precipitate formation or color intensity was utilized to gauge how the body would react to various tests. Standard operating procedures were employed (Purohit *et al.,* 2014).

Test for Carbohydrates

Molisch's Test: A few drops of the Molish reagent (naphthol) were combined with the 1 ml aqueous solution of *Zephyranthes citrina* extract and conc. Sulfuric acid, or H2SO4, was added dropwise along the test tube wall. At the junction, a purple color ring forms when two liquids combine. It indicates the presence of carbohydrates.

Fehling's Test: 2 ml of *Zephyranthes citrina* extract aqueous solution were added after equal amounts of Fehling A and Fehling B solution (1 ml each) were combined. Boil it for 5-10 minutes on water bath. Formation of reddish brown coloured precipitate due to cuprous oxide formation shows the presence of reducing sugar.

Benedict's test: *Zephyranthes citrina* extract and Benedict's reagent were combined in equal amounts in a test tube and heated in a water bath for five to ten minutes. Depending on the amount of reducing sugar present in the test solution, appears green, yellow or red which shows the presences of reducing sugar.

Barfoed's Test: In the aqueous solution of *Zephyranthes citrina* extract, 1 ml of Benedict solution was added and heated for boiling. In the presence of monosaccharides red colour indication was seen due to formation of cupric oxide.

Tests for Alkaloids

Dragendorff's Test: 1 ml of *Zephyranthes citrina* extract was taken. Little drops of acetic acid and Dragendroff's reagent were combined with alcohol and thoroughly shaken. The presence of alkaloids indicates by the presence of an orange red precipitate.

Wagner's Test: In acetic acid 1ml of *Zephyranthes citrina* extract was dissolved. Few drops of Wagner's reagent were added. The presence of alkaloids indicated the reddish-brown precipitate.

Mayer's Test: A few drops of Mayer's reagent were added to 1 ml of acetic acid that had been dissolved with *Zephyranthes citrina* extract. The presence of alkaloids was indicated by the formation of a dull white precipitate.

Hager's Test: 1-2 ml of *Zephyranthes citrina* extract was dissolved in acetic acid. To it 3 mL of Hager's reagent was added; the formation of yellow precipitate indicated by the presence of alkaloids.

Test for Saponins

Froth Test: Add 1 milliliter of Zephyranthes citrina extract to distilled water and give it a good shake. Stable froth formation showed the presence of saponin.

Test for Triterpenoids and Steroids

Libermann-Burchard Test: In chloroform, the *Zephyranthes citrina* extract was dissolved. Following a water bath heating and cooling process, 1 mL of acetic acid and acetic anhydride were added. Next, a few drops of strong sulfuric acid were applied along the test tube's sides. The emergence of blue green color indicates the presence of steroids.

Salkowski Test: The extract of *Zephyranthes citrina* was dissolved in chloroform, and then an equivalent volume of sulfuric acid was added. The development of bluish red to cherry red color in the chloroform layer and green fluorescence in the acid layer were signs that steroids were present.

Test for Tannin and Phenolic Compounds

Ferric Chloride Test: A certain amount of distilled water was used to dissolve *Zephyranthes citrina* extract. A few drops of a diluted ferric chloride solution should be added to it. The presence of tannins was revealed by the formation of a dark blue color.

Gelatin Test: In the distilled water, a certain amount of *Zephyranthes citrina* extract was dissolved.10% sodium chloride and 2 milliliters of 1% gelatin solution were added. The formation of a white precipitate indicates the presence of phenolic chemicals.

Lead Acetate Test: A small amount of distilled water and *Zephyranthes citrina* extract were dissolved in a test tube, and then a few drops of lead acetate solution were added. The presence of phenolic compounds is shown by the formation of white precipitate.

Test for Flavonoids

Shinoda's Test: A few magnesium turnings and little drops of concentrated hydrochloric acid to 1 ml of *Zephyranthes citrina* extract in alcohol were added. It was heated on a water bath. When the formation of red to pink colour occurred, indicated the presence of flavonoids.

Test for Glycosides

Borntragers Test: Sulfuric and sulfuric acid solutions were diluted and added to 3 milliliters of the test solution. After five minutes of boiling, the filtrate was extracted. An equal amount of either chloroform or benzene was added to the cool filtrate, and it was thoroughly shaken. After the organic solvent layer had been separated, ammonia was added. The production of pink to red

color in the ammonical layer was indicated by the presence of anthraquinone glycosides.

Keller Killiani Test: 3 ml of glacial acetic acid, 1 drop of 5% ferric chloride, and 2 ml of test solution were put to a test tube. Carefully add 0.5 milliliter of sulfuric acid concentrate. The development of a blue color in the acetic acid layer suggested the presence of cardiac glycosides.

Test for fats and oils

Solubility test

- To 2-3 ml of alcoholic solution of *Zephyranthes citrina* extract, add few ml. of chloroform and solubility was observed.
- To 2-3 ml of alcoholic solution of *Zephyranthes citrina* extract. Add few ml. of 90% ethanol and solubility was observed.

Solubility study

Qualitative solubility of *Zephyranthes citrina* in different solvents was determined according to USP NF, 2007. Approximately 1 mg of *Zephyranthes citrina* was weighed and transferred into a 10 ml test tube and dissolved in the respective solvents (1 ml each of methanol, DMOS ethanol, and water) (Jain *et al.,* 2020)**.**

Formulation of microspheres by Solvent Evaporation method

Microspheres with extract *(Zephyranthes citrina)* as the core material were created by means of the solvent evaporation procedure. Extract (*Zephyranthes citrina*), HPMC, and EC were dissolved in a 1:1 ethanol and dichloromethane solution at room temperature. This was added to 250 milliliters of water with 0.01% Tween-80 and kept between 30 and 40 degrees Celsius. The mixture was then agitated at 300 revolutions per minute for 45 minutes in order to allow the volatile solvent to evaporate. The resulting microspheres were filtered, given a water wash, and then baked at 37°C to dry them (Fartyal *et al.,* 2011).

Evaluation parameter of extract loaded microsphere

Particle size

When it comes to characterization of microspheres, one of the most important parameters is their particle size. The Malvern Zeta sizer (Malvern Instruments) was used to measure the size of microspheres. The sample was placed in a disposable sized cuvette after the dispersions were diluted with Millipore filtered water to the proper scattering intensity at 25°C (Singh *et al.,* 2008).

Zeta potential

The particle charge and movement velocity in an electric field were determined by measuring the zeta potential. In this experiment, Zetasizer Malvern equipment was used to evaluate the microspheres after they were diluted 10 times with distilled water. All samples were sonicated for 5-15 minutes before zeta potential measurements (Volic *et al.,* 2022).

Scanning Electron Microscopic (SEM)

The extract-loaded microspheres' morphological characteristics were obtained using the electron beam from a scanning electron microscope. A vacuum-sputter coater was used to coat the microspheres with a thin coating (2–20 nm) of gold, palladium, or platinum.

Following the preparation, the specimen was exposed to an electron beam, which caused secondary electrons known as auger electrons to develop. From this interaction between the electron beam and the specimen's atoms, only the electrons scattered at 90° were selected and further processed based on Rutherford and Kramer's Law for acquiring the images of surface topography (Ahmed *et al.,* 2020).

Antibacterial activity of Microsphere by Well diffusion assay

Preparation of Nutrient Agar Media

28 g of Nutrient Media was dissolved in 1 litre of distilled water. pH of media was checked before sterilization. Media was sterilized in autoclave at 121^{o}C at 15 lbs pressure for 15 minutes. Nutrient media was poured into plates and placed in the laminar air flow until the agar was get solidified.

Well Diffusion Assay

The bacterial suspension of *E. coli* was standardized to 10^8 CFU/ml of bacteria and kept into the shaker. Then, 100µl of the inoculums from the broth (containing 10^8 CFU/ml) was taken with a micropipette and then transferred to fresh and sterile solidified Agar Media Plate (Mohammadi-Sichani *et al.,* 2012). The agar plate was inoculated by spreading the inoculums with a sterile spreader, over the entire sterile agar surface. Three wells of 6 mm were bored in the inoculated media with the help of sterile cork-borer. The microsphere (0.5 and 1 mg/ml) and extract (1 mg/ml) solution were then added to the wells for inoculation. The sample was loaded in 100 µl. It was allowed to diffuse for about 30 minutes at room temperature and incubated for 18-24 hours at 37^{o}C. Following incubation, plates were checked to see if a clear zone formed around the well, indicating that the chemicals under test had antimicrobial activity. A measurement of the zone of inhibition (ZOI) in millimeters was made.

Zones were measured with a ruler, held on the back of the inverted Petri plate, to the closest millimeter. A few inches above a black, non-reflective background was where the Petri plate was held. The diameters of the zone of complete inhibition (as judge by unaided eye) were measured, including the diameter of the well (Manandhar *et al.,* 2019).

Stability studies

After the formulation of the extract-loaded microspheres was packed, it was put in the stability test chamber and stabilized for three months at accelerated testing temperatures of 25^0C±2^0C and 60 ±5% RH and 40^0C±2^0C and 70 ±5% RH.

The formulation was checked for evaluation parameter particle size and Zeta potential studies at the interval of 30, 45, 60, 90 days (3 month) months. The formulation was tested for stability under accelerated storage condition for 3 months in accordance to International Conference on Harmonization (ICH) guidelines. Formulation was analyzed for the change in evaluation parameter particle size and zeta potential studies.

RESULTS AND DISCUSSION

The percentage yield of crude extracts from *Zephyranthes citrina* varied significantly between the solvents used. Petroleum ether extraction resulted in a yield of 0.46%, whereas methanol extraction yielded a much higher 2.32%. This difference is indicative of

the higher solubility and efficiency of methanol in extracting the bioactive compounds from the plant material.

Phytochemical testing revealed that the methanolic extract of *Zephyranthes citrina* was rich in a variety of bioactive compounds, including alkaloids, glycosides, carbohydrates, and tannins/phenolic compounds. In contrast, the petroleum ether extract showed limited phytochemical presence, with notable absence of alkaloids, glycosides, and carbohydrates but presence of saponins and triterpenoids/steroids. This disparity underscores the solvent-specific extraction of different classes of phytochemicals, which could influence the antimicrobial efficacy of the extracts.

The solubility study indicated that the *Zephyranthes citrina* extract was freely soluble in methanol and ethanol, and soluble in DMSO and water. This high solubility in organic solvents makes methanol an ideal candidate for extracting and further formulating the bioactive compounds into microspheres, ensuring efficient encapsulation and release. The particle size analysis of the microsphere formulations showed a range from 407.7 nm to 513.9 nm. The polydispersity index (PI) values varied, with MS 2 showing the most favorable value of 0.337, indicating a relatively uniform particle size distribution. Zeta potential measurements revealed that all formulations had negative surface charges, with values ranging from -0.4 mV to -2.2 mV.

The slightly higher zeta potential for MS 5 suggests greater stability against aggregation in suspension.

SEM analysis of the optimized formulation MS 2 confirmed the spherical morphology and smooth surface of the microspheres. This structural integrity is crucial for controlled release applications, as it influences the drug release kinetics and stability of the formulation.

The antimicrobial activity of the *Zephyranthes citrina* plant extract and its microsphere formulations was evaluated against *E. coli*. The plant extract showed a moderate zone of inhibition of 7 mm, whereas the microsphere formulations exhibited enhanced antimicrobial activity with zones of inhibition of 9 mm and 14 mm for concentrations of 0.5 mg/ml and 1 mg/ml, respectively. This significant increase in antimicrobial activity upon encapsulation can be attributed to the sustained release and better penetration of the active compounds from the microspheres.

The stability study of the optimized microsphere formulation MS 2 over a period of 90 days under different temperature and humidity conditions demonstrated excellent physical and chemical stability. The particle size and zeta potential showed minimal changes, indicating that the microspheres maintained their integrity and dispersion stability. This stability is essential for the practical application and long-term storage of the formulation.

Table 1: Composition of microsphere formulation

S. No	Formulations (Code)	Polymer HPMC (mg)	Polymer Ethyl cellulose (mg)	Tween-80 (%)	Extract (mg)	Temperature °C	Solvent ratio(1:1) ethanol/DCM
1	MS 1	300	50	0.01%	100	30-40°C	5ml:5ml
2	MS 2	250	100	0.01%	100	30-40°C	5ml:5ml
3	MS 3	200	150	0.01%	100	30-40°C	5ml:5ml
4	MS 4	150	200	0.01%	100	30-40°C	5ml:5ml
5	MS 5	100	250	0.01%	100	30-40°C	5ml:5ml

Table 2: Percentage Yield of crude extracts of Zephyranthes citrina extract

S. No	Plant name	Solvent	Theoretical weight	Yield (gm)	% Yield
1.	*Zephyranthes citrina*	Petroleum ether	299	1.40	0.46%
2.		Methanol	284	6.60	2.32%

Table 3: Phytochemical testing of extract

S. No.	Experiment	Presence or absence of phytochemical test	
		Pet. Ether extract	Methanolic extract
1.	**Alkaloids**		
1.1	Dragendroff's test	Absent	Present
1.2	Mayer's reagent test	Absent	Present
1.3	Wagner's reagent test	Absent	Present
1.4	Hager's reagent test	Absent	Present
2.	**Glycoside**		
2.1	Borntrager test	Absent	Present
2.2	Legal's test	Absent	Present
2.3	Killer-Killiani test	Absent	Present
3.	**Carbohydrates**		
3.1	Molish's test	Absent	Present
3.2	Fehling's test	Absent	Present
3.3	Benedict's test	Absent	Present

3.4	Barfoed's test	Absent	Present
4.	**Proteins and Amino Acids**		
4.1	Biuret test	Absent	Absent
5.	**Flavonoids**		
5.1	Alkaline reagent test	Absent	Absent
5.2	Lead Acetate test	Absent	Absent
6.	**Tannin and Phenolic Compounds**		
6.1	Ferric Chloride test	Absent	Present
7.	**Saponin**		
7.1	Foam test	Present	Absent
8.	**Test for Triterpenoids and Steroids**		
8.1	Salkowski's test	Present	Absent
8.2	Libbermann-Burchard's test	Present	Absent

Table 4: Solubility study of Extract

Extract	Solvents	Observation/Inference
Zephyranthes citrina	Methanol	Freely Soluble
	Ethanol	Freely Soluble
	DMSO	Soluble
	Water	Soluble

Table 5: Result of Particle size of all formulations

S. No.	Formulations	Particle size (nm)	PI Value
1.	MS 1	419.8 nm	1.098
2.	**MS 2**	**407.7 nm**	**0.337**
3.	MS 3	420.6 nm	1.759
4.	MS 4	513.9 nm	0.374
5.	MS 5	493.9nm	0.374

Table 6: Result of Zeta potential of all formulations

S. No	Formulation	Zeta potential
1	Microsphere (MS 1)	-0.9 mV
2	**Microsphere (MS 2)**	**-0.4mV**
3	Microsphere (MS 3)	-1.2mV
4	Microsphere (MS 4)	-1.5 mV
5	Microsphere (MS 5)	-2.2mV

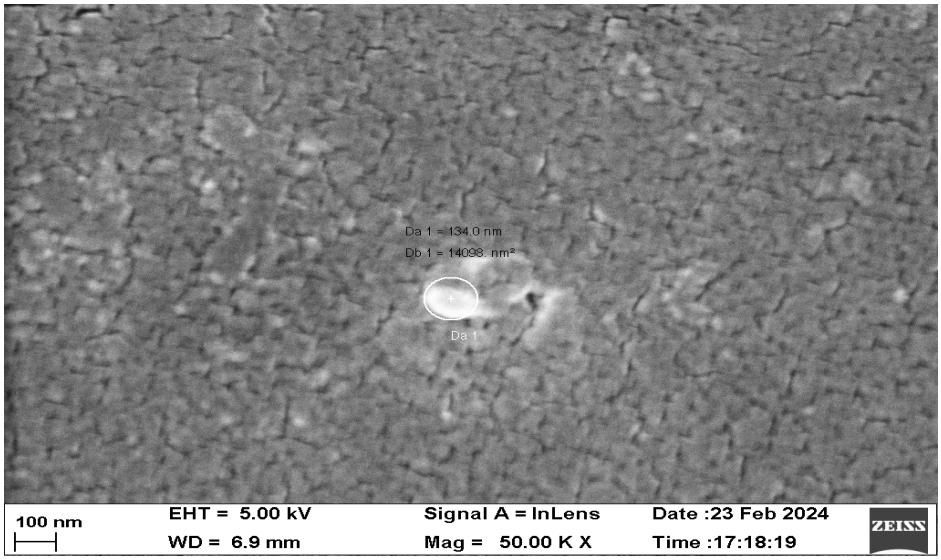

Figure 1: SEM analysis of optimized formulation MS2

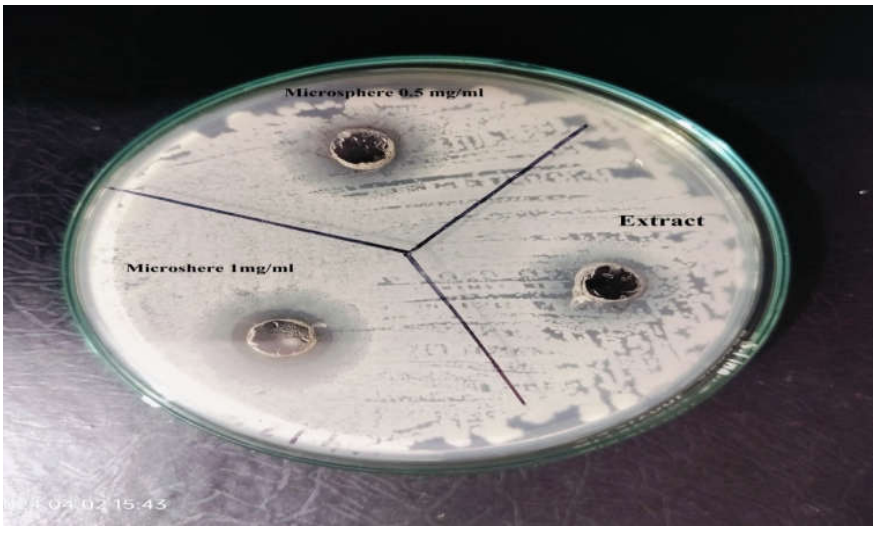

Figure 2: Antimicrobial activity

Table 7: Antimicrobial activity of Formulation and plant extract against *E. coli*

S. No.	Sample Name	Zone of Inhibition (mm)
1.	Plant extract	7 mm
2.	Microsphere formulation (0.5mg/ml)	9 mm
3.	Microsphere formulation (1mg/ml)	14mm

Table 8: Stability Study of Microsphere (MS2) formulation

S. No	Time (Days)	25^0C±2 ^{0}C and 60 ± 5% RH			40^0C±2 ^{0}C and 70 ±5% RH		
		Appearance	Particle size nm	Zeta potential mV	Appearance	Particle size nm	Zeta potential mV
1.	0	Solid Powder	407.7 nm	-0.4 mV	Solid Powder	407.7 nm	-0.4 mV
2.	30	Solid Powder	407.9 nm	-0.6 mV	Solid Powder	407.8 nm	-0.8 mV
3.	45	Solid Powder	408.2 nm	-0.9 mV	Solid Powder	408.6 nm	-1.3 mV
3.	60	Solid Powder	408.4 nm	-1.1 mV	Solid Powder	408.9 nm	-1.6 mV
4.	90	Solid Powder	408.8 nm	-1.4 mV	Solid Powder	409.5 nm	-1.8 mV

CONCLUSION

The results of this study highlight the potential of methanol as an effective solvent for extracting bioactive compounds from *Zephyranthes citrina*. The development of microsphere formulations significantly enhanced the antimicrobial efficacy of the extracts, providing a promising approach for natural antimicrobial therapies. The stable physical and chemical properties of the optimized microspheres further support their potential for clinical applications. Future studies should focus on in vivo evaluations and exploring the mechanisms underlying the enhanced antimicrobial activity of the microsphere formulations.

DECLARATION OF INTEREST

The authors declare no conflicts of interests. The authors alone are responsible for the content and writing of this article.

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