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Hygrophilla Auriculata Extract Loaded Microemulsion gel for treatment of skin cancer

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Abstract

Globally, skin cancer is the most common type of cancer and is on the rise. It is broadly divided into Melanoma and Non-melanoma skin cancer, or Keratinocyte carcinoma, which are malignancies produced from melanocytes and epidermal cells, respectively. One of the most deadly malignant illnesses, cancer can spread to other tissues and organs and exhibit unchecked and aberrant cell division. Therefore, achieving a safe formulation for the treatment of skin cancer is important. The aim of this research is to formulate the Hygrophila Auriculata Flower extract Loaded Microemulsion Gel with small droplet size, high drug concentration, and high stability for skin application. A magnetic stirrer was used to create the Hygrophila Auriculata Flower extract Loaded Microemulsion, which was examined for morphology, clarity, dilution, zeta potential, particle size, stability, and chromatography. The cytotoxicity study of the formulation was performed in A431 cells and compared with a marketed formulation. The smallest particle size of the emulsion droplets improves the bioavailability of the drug, playing a vital role in skin penetration and effectiveness against skin cancer. Hence, the study predicts that the Hygrophila Auriculata Flower extract Loaded Microemulsion Gel could be effective for the treatment of skin cancer in the future.

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INTRODUCTION

In addition to being extremely aware of their health, people these days are also highly considerate of their external appearance. One of the most deadly malignant illnesses, cancer, can spread to other tissues and organs and exhibit unchecked and aberrant cell division. When normal, healthy cells or tissues are destroyed by the unchecked proliferation of malignant cells, the result is a mass known as a tumor. Among all solid tumors, skin cancer is the most common type.

Skin cancer is listed as a deadly disorder, and its incidence is on the rise. It is broadly divided into melanoma and non-melanoma skin cancer, which are malignancies produced from melanocytes and epidermal cells, respectively. These groups primarily account for 95% of skin cancer cases, with other skin malignancies making up a relatively small portion. Treatment for skin cancer places a significant financial burden on the US healthcare system, costing $8.2 billion on average annually. It was established that at least 543,000 Americans are currently coping with an aggressive melanoma diagnosis. They represent a threat category that could benefit from improved melanoma screening. There were more than

331,722 new cases of skin cancer in 2022, with approximately 2 to 3 million non-melanoma cancer cases and 132,000 melanoma cancer cases.

Some individuals with a variety of lifestyle factors never develop cancer, whereas others with none do. The following elements may raise the risk of causing skin cancer: ultraviolet B (UVB) radiation from the sun is more closely associated with melanoma, and research indicates that ultraviolet A (UVA) radiation may also cause melanoma.

The need for achieving a safe formulation for the treatment of skin cancer is crucial. In this research, we formulated the Hygrophila Auriculata flower extract loaded Microemulsion Gel. Hygrophila Auriculata (K. schum), also known as Asteracantha longifolia, Auriculata schum, or Barleria longifolia, Kokilaksha, belongs to the family Acanthaceae and is a wild herb typically found in damp areas along riverbanks, ditches, and rice fields in India. In this research, we used only the flower part of this plant because it is rich in flavonoid content. According to studies [1], flavonoids prove to be useful drugs in the fight against skin cancer. Flavonoids are categorized as medications with low water solubility and high lipophilicity under the Biopharmaceutical Classification System (BCS)-II [2].

Flower dried powder of Hygrophila Auriculata is not directly used for skin cancer treatment because it consists of pollen grains, which can lead to itchiness and rashes on the skin and have less bioavailability. Therefore, we decided to extract the flower dried constituents. The extract is not directly used for treatment and cannot easily pass through the skin, so a suitable formulation is required. Microemulsions have been used in recent years to improve the stability and bioavailability of lipophilic constituents. Our extract belongs to BCS class-II, making it suitable for the formulation of a microemulsion. However, microemulsions are difficult to apply to the skin, so our aim in this research is to formulate the Hygrophila Auriculata flower extract loaded Microemulsion Gel for the treatment of skin cancer and to perform their characterization. The optimized formulation was evaluated using the A431 cell line and compared with marketed formulations used in the treatment of skin cancer [3].

MATERIALS AND METHODS

Materials:

The plant species Hygrophila Auriculata was discovered in the Mankarnika River of Palkhed, Tal-Niphad, Dist. Nashik 422209, India. The plant was identified and authenticated based on morphology and microscopic/macroscopic examination by the Department of Botany, Maratha Vidya Prasarak Samaj K.R.T. Arts, B.H. Commerce, and A.M. Science (K.T.H.M.) College of Nashik - 422002, India. Other materials used include castor oil (Research Lab), surfactant Tween 80 (Research Lab), and Carbopol (Research Lab).

Method:

First, the flowers of Hygrophila Auriculata were collected. The best time for collection is during the rainy and winter seasons (September to December). After collection, the flowers were placed on a tray, covered with a clean cloth, and shade-dried for 7 days. The dried flower material was then ground into a powder and passed through a screen (No. 40). The powdered material was kept in an airtight container for further examination.

Extraction and Isolation:

Flavonoids were isolated from the flowers of H. Auriculata. The air-dried flower parts containing flavonoids were ground, and a measured amount of the ground flower was placed into a Soxhlet thimble showed in Figure 1. The extraction was performed in two batches: 20 g of powder in 200 ml of solvent and 25 g of powder in 250 ml of solvent for 7 hours. The flask was placed on water, and the solvent was heated slowly. The condenser was placed on top of the Soxhlet holder, and cooling water was turned on. The condenser condensed the vapor entering the chamber, allowing it to condense back as liquid. This operation continued until a sufficient amount of extract was separated by siphoning. After the process was completed, the flask containing ethanol was weighed. An increase in weight indicated that some extract had been extracted by the solvent. The distillation process was then introduced by reassembling the condenser, where ethanol was evaporated and recovered in a conical flask, leaving the extracted material in the round-bottom flask showed in Figure 2.

Figure 1 Soxhlet

Figure 2 Distillation

Analysis and Partial Purification of the Plant Extract by TLC:

Thin layer chromatography (TLC) was performed for the ethanolic extract of Hygrophila Auriculata. The TLC profile of this plant (flower part) extract indicated the presence of various phytochemicals. Different solvent systems were used to identify the active pharmaceutical ingredient (API) in the flower part. TLC investigation provided important information about the polarity of chemical constituents. Choosing a good solvent system for the mobile phase is crucial for targeting the class of flavonoids. Qualitative analysis of Hygrophila Auriculata flower extract was performed using TLC.

TLC was performed using two solvent systems:

According to the paper published by Teena Magline Immaculate V: Solvent used for first qualitative analysis: Toluene: Ethyl acetate: Formic acid (3:2:0.15)[4]

According to the Ayurvedic Pharmacopoeia of India: Solvent used for second qualitative analysis: Toluene: Ethyl acetate (1.75:0.25)[5]

Three spots were plotted:

- Extract + methanol
- Methanol + ethanolic extract
- Ethanolic extract[6]

Screening of Oils & Preparation of Extract Loaded Microemulsion:

Microemulsion was prepared using a magnetic stirrer by taking a constant ratio (3:4) of oil/surfactant. Castor oil and Tween 80 were mixed properly in a mortar and pestle. After 15 minutes, the flower extract was added with continuous trituration for 15 minutes. The mixture was then stabilized for 15 minutes. In another beaker, water was taken, and the above mixture was added dropwise with continuous stirring under a magnetic stirrer [7].

Characterization of Extract Loaded Microemulsion:

1. **Clarity Test:** Performed using a clarity apparatus.
2. **Dilution Test:** The microemulsion was subjected to a dilution test to determine its visual quality (clear, less clear, or milky). The basis for the dilution test is the emulsion's miscibility with the liquid that constitutes its phase. After 24 hours of storage, the microemulsion produced by dilution with the continuous phase should not exhibit any separation.
3. **Droplet Size and Zeta Potentials:** Determined by Nanoplus. The microemulsion system was diluted with distilled water and measured with Nanoplus [8].
4. **Stability Study:** The microemulsion was stored in an aseptic area at 25-35°C for 2 months.

Preparation of Microemulsion Gel:

Water was added to a beaker, followed by a measurable amount of Carbopol with continuous stirring. After stabilizing for 30 minutes, the microemulsion was added with continuous stirring.

Cell Culture:

The cell line was obtained from the National Center for Cell Science (NCCS), Pune, India (A431 cell line

- Human epidermoid squamous carcinoma cell line). A431 cells were cultured in DMEM High Glucose with 10% Fetal Bovine Serum (FBS). The cells were incubated in a CO2 incubator with 5% CO2 for further studies [9].

Cytotoxicity Studies

Cytotoxicity studies were performed on the A431 cell line using the MTT assay (3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium). The MTT assay is a colorimetric assay used for determining cell proliferation and cytotoxicity based on the reduction of the yellow-colored water-soluble tetrazolium dye MTT to formazan crystals. Cells were seeded in 200 µl cell suspension in a 96-well plate containing DMEM medium with 10% FBS at a required cell density (5*10^3 cells per well) without the test agent. Appropriate concentrations of the test agent were added, and the plate was incubated at 37°C for 48 hours in a 5% CO2 atmosphere. After incubation, the plates were removed from the incubator, the spent media was discarded, and MTT reagent was added to a final concentration of 0.5 mg/ml of the total volume. The plate was wrapped with aluminum foil to avoid exposure to light and returned to the incubator for 3 hours at 37°C (Note: Incubation time may vary for different cell lines; within one experiment, incubation time should be kept constant for comparisons). The MTT reagent was then removed, and 100 µl of solubilization solution (DMSO) was added. Gentle stirring in a gyratory shaker enhanced dissolution, and occasionally pipetting up and down was required to completely dissolve the MTT formazan crystals, especially in dense cultures. Absorbance was checked using a spectrophotometer or an ELISA reader at 570 nm, with 630 nm used as a reference wavelength [3][10].

RESULTS AND DISCUSSIONS

Qualitative Analysis:

Qualitative analysis of Hygrophila Auriculata flower extract was performed using TLC with two solvent systems.

First System: According to the paper published by Teena Magline Immaculate V. (Reference No-4), the solvent used for the first qualitative analysis was Toluene: Ethyl acetate: Formic acid (3:2:0.15). An orange spot was observed under UV light with Rf values of 0.3, 0.2, and 0.4, confirmed by the Ayurvedic Pharmacopoeia of India.

Second System: According to the Ayurvedic Pharmacopoeia of India, the solvent used for the second qualitative analysis was Toluene: Ethyl acetate (1.75:0.25). The ethanolic extract spot showed an Rf value of 0.77, matching the standard, indicating the presence of flavonoids showed in Figure 3.

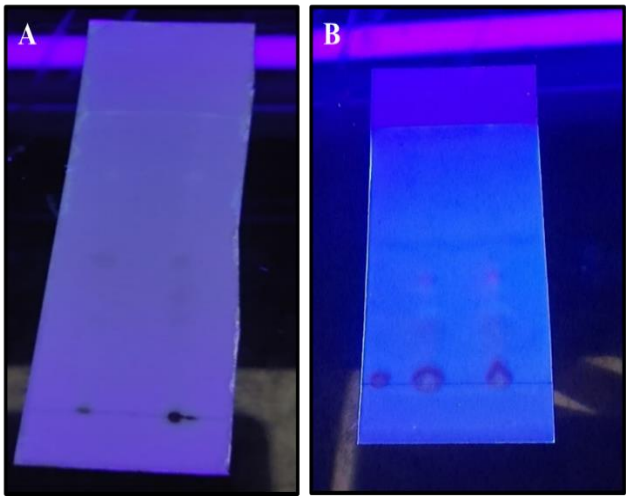

Figure 3 TLC

Screening of Oil (Surfactant)

Hygrophila Auriculata flower extract contains flavonoids, which belong to BCS class-II. After performing a solubility study in different solvents, it was found that Hygrophila Auriculata flower extract exhibited maximum solubility in castor oil and Tween 80.

In a microemulsion system, improved thermodynamic stability of the microemulsion formulation results from the reduction in the free energy needed for the formation of the emulsion. The formation of the microemulsion is influenced by the choice of surfactant and oil ratio. A 1:1 ratio is not stable because phase separation is seen.

Figure 4 A) 1:1; B) 1:3; C) 3:4

Figure 5 Dilution Test

In a 1:4 ratio, precipitation of the microemulsion was observed after some time. The 3:4 ratio was found to be the most stable. Hence, 3:4 is selected for further preparation.

Characterisation of Microemulsion

Dilution Test: Water was added to the microemulsion, and it was easily miscible with the system, indicating that the prepared microemulsion is O/W type as shown in Figure 5 [11].

Stability of Microemulsion: The microemulsion is a clear, thermodynamically stable, isotropic liquid containing a mixture of oil, water, and surfactant. Microemulsions are thermodynamically stable O/W emulsions with a mean droplet size of approximately 100-400 nm without phase separation, creaming, or cracking. The selected microemulsion was placed in aseptic conditions for 2 months, and no phase separation occurred after this period.

Droplet Size and Zeta Potential: Droplet size of microemulsion globules is an important parameter for evaluating the bioavailability of the drug. Particle size is evaluated to ensure that the microemulsion is an efficient dosage form with good stability. The particle size of the selected formulation was found to be 154 nm, and the PDI was 0.259.

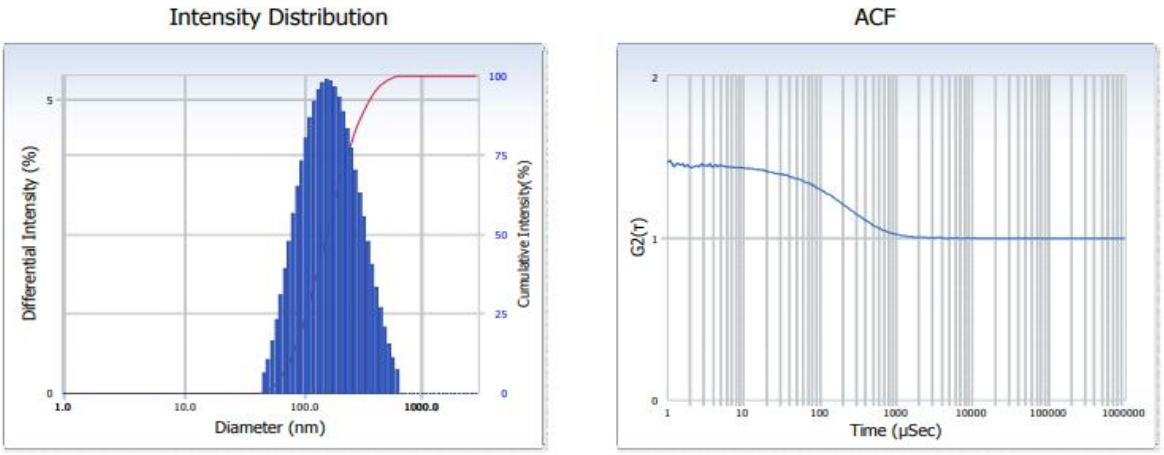

Figure 6 Particle Size

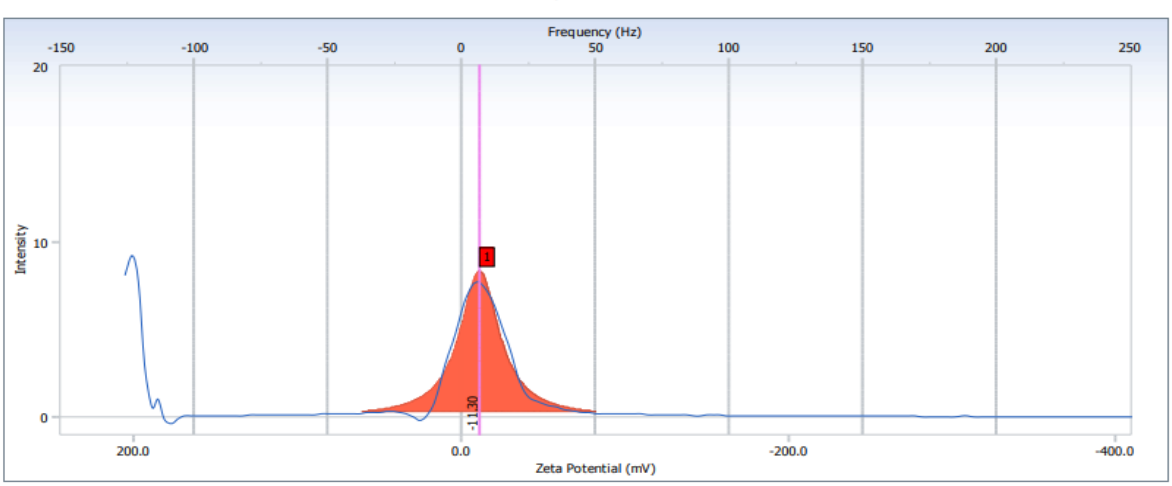

Figure 7 Zeta Potential

Table 1 Details of Samples

Sr.No.	Name of Sample/code	Concentration	Cell line
1	Test formulation (gel)	5(31,25,62,125,250 & 500µg/ml)	A431
2	5-FU Formulation (gel)	250 µg/ml	A431

Table 2 Detail of test compound concentration

Sr. No	Test Compounds	Cell line	Concentrations
1	Untreated	A431	No treatment
2	Standard (5-FU gel)	A431	250µg/ml
3	Blank	A431	media without cell
4	Test formulation	A431	(10,50,100,250,500µg/ml)

Table 3 Corrected absorbance after MTT assay

Treatment	Conc.(µg/ml)	Abs (n1)	Abs (n2)	Abs (n3)	Abs (n4)
Test formulation	500	0.036	0.035	0.03	0.047
Test formulation	250	0.067	0.047	0.041	0.048
Test formulation	100	0.077	0.046	0.046	0.061
Tests formulation	50	0.088	0.051	0.053	0.068
Test formulation	10	0.093	0.065	0.056	0.073
5-FU formulation	250	0.029	0.017	0	0.012
DMEM+ Cell	-	0.103	0.07	0.073	0.08
DMEM	-	0	0	0	0

Table 4 Percentage cell viability by the treatment

Treatment	Conc.(µg/m)	% cell viability (n1)	% cell viability (n2)	% cell viability (n3)	% cell viability (n4)
Test formulation	500	34.95	50.00	41.10	58.75
Test formulation	250	65.05	67.14	56.16	60.00
Test formulation	100	74.76	65.71	63.01	76.25
Test formulation	50	85.44	72.86	72.60	85.00
Test formulation	10	90.29	92.86	76.71	91.25
5-FU formulation	250	28.16	24.29	0.00	15.00
DMEM +Cell	-	100	100	100	100
DMEM	-	-	-	-	-

Table 5 Percentage cell viability (mean ± S.D.)

Treatment	Conc. (µg/ml)	% cell viability (mean)	S.D.
Test formulation	500	46.20	10.40
Test formulation	250	62.09	4.96
Test formulation	100	69.93	6.55
Test formulation	50	78.97	7.21
Test formulation	10	87.78	7.45
5-FU formulation	250	16.86	12.52
DMEM +Cell	-	100	-

The small size of the microemulsion globules confirms the high stability of the formulation and shows the potential for enhanced permeation through the skin membrane. The zeta potential of the selected formulation was found to be 24.23 mV, as shown in Figure 6.

Cytotoxicity Study

The effect on cell proliferation of the test formulation was evaluated after 48 hours of incubation, which induced a concentration-dependent inhibition of cell proliferation as

evaluated by the MTT assay [12]. Test cells were treated with 5 concentrations (10, 50, 100, 250, 500 µg/ml) of the test compound, with Fluorouracil used as an internal positive control (250 µg/ml), DMEM used as a negative control, and wells without cells considered as blanks. After 48 hours, the amount of formazan crystals formed was determined by a microplate reader. The results of the cytotoxicity study (MTT assay) indicate that the test compounds showed cytotoxicity against the A431 cell line after 48 hours of incubation [13]. Results are shown in the figure.

The results of the cytotoxicity study (MTT assay) suggested that the synthetic marketed formulation 5-Fluorouracil shows 20% cell viability at 250 µg/ml, while our herbal formulation at 250 µg/ml

shows 60% cell viability. Increasing the dose of the herbal formulation also increases the percentage of cell viability. At 500 µg/ml, the herbal formulation shows 40% cell viability.

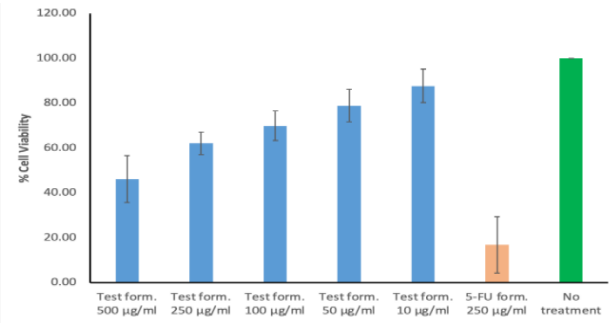

Figure 8 % cell viability after 48hour incubation by various treatment against A431 cell

CONCLUSION

In this research work, the Hygrophila Auriculata flower extract loaded microemulsion gel was formulated using the magnetic stirrer method and evaluated against skin cancer using the A431 cell line. The oil-in-water microemulsion formulated with the flower extract of Hygrophila Auriculata shows good bioavailability due to better solubility. It is solubilized in a high amount of castor oil, which is used as the oil phase, and Tween 80 as the surfactant. The Hygrophila Auriculata flower extract loaded microemulsion has a particle size range of 100-500 nm, showing high physical stability with no phase separation or precipitation. The formulation has a particle size of 154 nm. This formulation shows high penetrability and high cytotoxicity against the A431 cell line.

Ethical Approval

No ethical approval was necessary for this study.

Author Contribution

All authors made substantial contributions to the conception, design, acquisition, analysis, or interpretation of data for the work. They were involved in drafting the manuscript or revising it critically for important intellectual content. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work, ensuring its accuracy and integrity.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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