EJPPS
EUROPEAN JOURNAL OF PARENTERAL AND PHARMACEUTICAL SCIENCES

EJPPS – European Journal of Parenteral and Pharmaceutical Sciences Volume 28 Issue 2

Evaluation of the *in-vitro* antifungal activity of *Holoptelea Integrifolia* ethanolic extract loaded in microemulsion.

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Abstract: -

Microemulsions improve the transdermal delivery of several drugs over conventional topical preparations such as emulsions and gels: enhanced drug solubilization, increased skin flux, and decreased diffusion coefficient. Microemulsion-based systems find significant improvement in the topical delivery of antifungals. We believe that drug-loaded microemulsion will show better antifungal activity by better penetration into the skin and fungal cells. Antifungal agents are mostly lipophilic and easily formulated in topical vehicles. Microemulsions were prepared by the phase titration method. Formulations of the same drug and Excipient ratio and different concentrations were optimized with selected parameters like pseudo ternary phase diagram, particle analysis size, zeta potential validation, entrapment efficiency, and drug release studies performed by dialysis bag diffusion techniques at a temperature (37ºC). The study continued for 24 hours. The maximum amount of drug Holoptelea integrifolia release is 90% within 8hr. The study was monitored at 37ºC. Successfully done preparation, characterization, and drug release study of Microemulsion drug loaded.

1. Introduction

Superficial fungal infections like deep skin mycoses are better treated when the drug is administered topically than any other route. It minimizes the deleterious effects of the drug and produces the local action at the site of application more effectively. Antifungal agents are lipophilic and are easily formulated in topical vehicles. It is advantageous to choose microemulsion as a topical vehicle for antifungal agents of ease of preparation due to spontaneous formation, thermodynamic stability, transparent and elegant appearance, increased drug loading, enhanced penetration through the biological membranes, increased bioavailability compared to conventional dosage forms like gel, cream, etc. microemulsion have been regarded as a more effective topical vehicle than its conventional skin applications like cream and gel. Being a transparent and thermodynamically stable system,

microemulsions are formed spontaneously with relative ease of manufacture. Such a system has better scale-up potential demonstrating its industrial feasibility as well. When applied to the skin, these Nano-structured vehicles exhibited better solubilization of drugs and higher skin permeation compared to conventional formulations when applied on the skin. Enhanced drug solubilization, increased flux across the skin, and decreased diffusion coefficient are significant attributes of the microemulsion system owing to the internal phase in Nano size droplet, ultralow interfacial tension with enhanced surface free energy. The effect of formulation components of the microemulsion and trends in selecting new excipients constituting oil phase, surfactant, and cosurfactant has been highlighted herein and in future orientations. Microemulsion-based systems find significant improvement in the topical delivery of antifungals. We believe that drug-loaded microemulsion will show better antifungal activity through better penetration into the skin and fungal cells(Ganie & Yadav, 2014) (Srinivas Reddy et al., 2008), (Rangsimawong et al., 2018).

Besides being antibacterial, H. integrifolia has broad antifungal potential. The alcoholic leaf and stem extracts of H. integrifolia were studied for antifungal activity against five fungal strains: Candida Tropicana, Candida krusei, Candida albicans Aspergillus niger, and Saccharomyces cerevisiae using the agar well diffusion method(El-Aswar, Gaber, Zahran, & Abdelaleem, 2020).

In principle, microemulsions can deliver drugs to patients via several routes, but the topical application of microemulsions has gained increasing interest. The three main factors determining the transdermal permeation of drugs are the mobility of the drug in the vehicle, the release of the drug from the vehicle, and the permeation of the drug into the skin. These factors affect either the thermodynamic activity that drives the drug into the skin or the permeability of the drug in the skin, particularly the stratum corneum. Microemulsions improve the transdermal delivery of several drugs over conventional topical preparations such as emulsions and gels. Isopropyl myristate (IPM) is used as a permeation enhancer in transdermal formulations, but the mechanism of its action is poorly understood. Nonionic surfactants are widely used in topical formulations as solubilizing agents, but some recent results indicate that they may also affect the skin barrier function. It is interesting to explore the effects of these components in the organized microemulsions structures. Microemulsions may affect the permeability of drugs in the skin. In this case, the components of microemulsions serve as permeation enhancers(G. P. Guimarães et al., 2014; Geovani Pereira Guimarães et al., 2014) (Katagi, Kulkarni, Munnolli, Benni, & Akki, 2015), (El-Aswar et al., 2020), (Rizwani, Mahmud, Shareef, Perveen, & Ahmed, 2012), (Maqsood, Masood, Nawaz, Shahzadi, & Arslan, 2019), (Shao, Xi, & Zhang, 2018).

2. Material & Methods

The plant Holoptelea integrifolia was collected from the local area of Satpura (22.4667_N_78.4333_E_type: forest region: in – Madhya Pradesh), CTAB, TWEEN80, and IPM were procured from Titan biotech ltd.(India), Sigma-Aldrich(India), and Pallav Chemical and SolventPvt. Ltd.(India)

2.1 Method of Preparation

The compositions' microemulsion was devised using four-component IPM oil as the oil phase, C-TAB surfactant, tween 80 as a Co-surfactant, and distilled water as the aqueous phase. Batches were designed for different surfactant–co-surfactant ratios (S/Cos ratio). In the present study, three S/Cos ratios, 1:1, 2:1, and 3:1, were tried.

The microemulsion was prepared by the phase titration method constructing the phase diagram. Pseudo-ternary phase diagrams were constructed to investigate the effect of surfactant on the co-surfactant ratio on the area of microemulsion existence region.

The extract was dissolved into the oil phase, and an S/C mixture was added. To achieve homogeneity, the mixture was stirred magnetically (Magnetic stirrer, Remi, India). Water was added dropwise to obtain a clear, transparent microemulsion. The various batches were formulated(Salimi & Shirani, 2021).

2.2 Particle size and dispersion

Droplet size distributions in pharmaceutical and cosmetic emulsions are essential for stability and biopharmaceutical considerations. Dynamic light scattering, also called photon correlation spectroscopy (PCS), is used to analyze the fluctuations in the intensity of scattering by droplets due to Brownian motion. Dynamic light scattering has been widely applied in the study of emulsions and microemulsions to determine emulsion and microemulsion droplet size and shape. In these experiments, the intensity of scattered light is measured at various angles for different concentrations of the emulsion or microemulsion droplets(Lu et al., 2018).

2.3 Zeta potential

Dispersion stability was assured after determining zeta potential. The zeta potential measurement was performed at 25ºC with the help of the HORIBA scientific nanoparticle analyzer. Software provided by the manufacturer was used to calculate z average and zeta potential. Data on particle size(Gupta, Bansal, Ali, Gabrani, & Dang, 2014).

2.4 In Vitro Drug Release Study

The batches with a lower polydispersity index and higher zeta potential were selected to evaluate

their drug release (A1 and A3). In vitro drug release study of these batches was performed in a diffusion cell using a dialysis membrane– 150 (Code No. LA150); HiMedia Laboratories Pvt. Ltd. Mumbai - soaked in distilled water for 12h. this Dialysis membrane is A regenerated seamless cellulose tubing wherein the membrane is partially permeable, having a molecular weight cut off between 12,000 to 14,000. The main similarity between the dialysis bag and the cell membrane is that they both serve to protect the cell. Due to its semi-permeable nature, the dialysis bag mimics the cell membrane's diffusion property. Its structure is the main difference between the dialysis bag and the cell membrane. The selection of the membrane was based on previous references.

Gao Y, Zuo J, Bou-Chacra N, Pinto Tde J, Clas SD, Walker RB, Löbenberg R. In vitro release kinetics of antituberculosis drugs from nanoparticles assessed using a modified dissolution apparatus. Biomed Res Int. 2013;2013:136590. doi: 10.1155/2013/136590. Epub 2013 Jul 10. PMID: 23936771; PMCID: PMC3723057.

The receiver chamber was filled with phosphate buffer pH 7.4, and the temperature of the medium was thermostatically controlled at 37±1ºC. Accurately measured, 1 ml of microemulsion was applied to the donor compartment. 1ml sample was withdrawn throughout 24 hours and analyzed using a spectrophotometer at 242nm, using phosphate buffer of pH 7.4 as blank.

2.5 In Vitro Antifungal Assay

The antifungal activity was assessed by using the cup plate method. The activity was observed against Candida Albicans. The single model organism was based on the results of Kabir MA, Hussain MA, and Ahmad Z. Candida albicans: A Model Organism for Studying Fungal Pathogens. ISRN Microbiol. 2012 Sep 29;2012:538694. doi: 10.5402/2012/538694. PMID: 23762753; PMCID: PMC3671685.

Sabouraud dextrose agar (SDA) media was used for in vitro antifungal activity. Accurately weighed, 4.7 g of media was added to distilled water (100 mL) and heated to boiling point to dissolve the SDA media completely. It was sterilized by autoclave at 121°C for 15 min (15 psi). After autoclaving, it was cooled to 40- 50°C. Microbial suspension of the test organism was prepared. The resultant microbial suspension was added to the media and mixed well. The media was then transferred to the Petri plate. The topical microemulsion was applied to previously prepared cavities in solidified media under laminar airflow in an aseptic area (Class 100). The plate was then incubated for 18 h, and the zone of inhibition was measured (Khumpirapang, Klayraung, Tima, & Okonogi, 2021; Yin et al., 2021).

2.6 Stability evaluation

The physical stability of herbal drug-loaded microemulsion was investigated via clarity, particle size

analysis, and phase separation, observed at 2-8˚C and room temperature for three months. Centrifugation was performed at 12000rpm 3g for 30 min at 25˚C to assess the physical stability of the microemulsion. Phase separation and concentration of herbal drugs were investigated monthly to judge the optimal storage temperature. Chemical stability was evaluated on drug-loaded formulations stored at 2-8˚C and room temperature, with the PG content determined by UV spectrophotometry at 274nm(Pumival, Tadtong, Athikomkulchai, & Chittasupho, 2020)

3. RESULTS AND DISCUSSION

Extraction

Table 1.: Extractive value of *Holoptelea integrifolia* in ethanol solvent.[45].

Plant	Ethanol
Holoptelea Integrifolia	**11%w/w**

4. **Table 2. : % yield of an extract of leaves *Holoptelea integrifolia*.**

S.No.	Solvent	Extraction time (In hrs.)	Color of extract	Yield (gm)	% Yield
1.	Ethanol	**48**	**Blackish green**	**4.5**	**11.11%**

5. **Note: 40gm of air-dried, coarsely powdered Holoptelea integrifolia was taken for extraction**

3.1 Microscopy

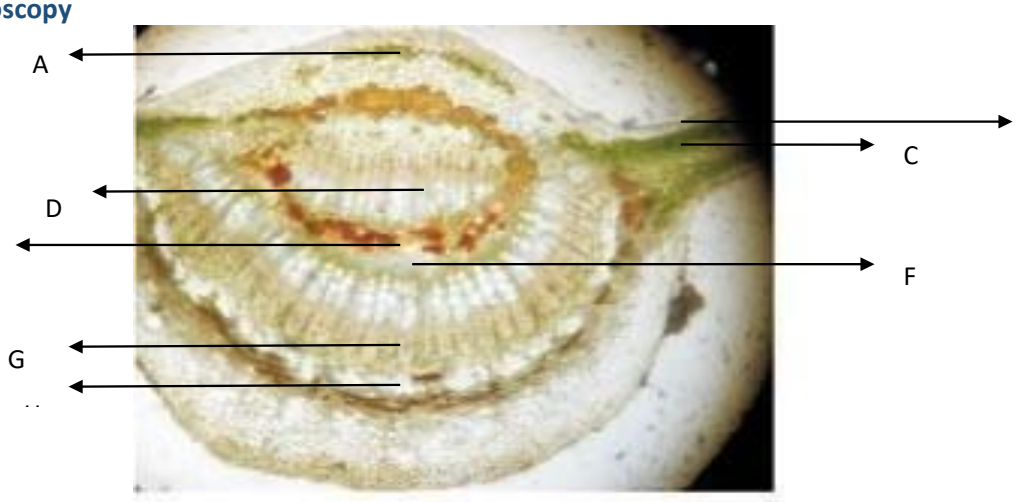


Fig.1. Transverse section of H. integrifolia leaf. A: Upper epidermis; B: Spongy parenchyma; C: Palisade cells; D: Oil glands; E: Phloem; F: Xylem; G: Collenchyma; H: Lowerepidermis

3.2 Result and Discussion

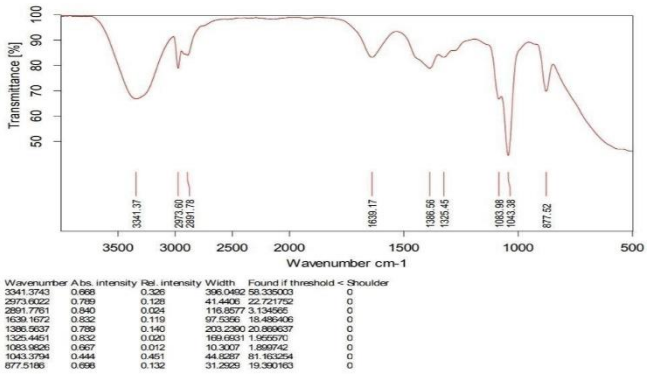
The leaves from Holoptelea integrifolia were authenticated and collected from a local area off Satpura (MP). The dried leaves were coarsely powdered and successively extracted in solvent ethanol. *Holoptelea integrifolia* extracts yielded 4.3 gm in ethanol with a blackish-green color.

3.3 Qualitative analysis.

Preliminary phytochemical tests were performed on the ethanolic extract of Holoptelea integrifolia leaves to identify various chemical constituents such as carbohydrates, proteins, steroids, fat, and oil. Ethanoic extract possessed alkaloids, glycosides, flavonoids, protein, and steroids. The result has been Alkaloids identification test, Mayer, Wagner test, dragendroff, hagers test & Steroids & Protein test Libermann-Burchard test Liebermann test & Biuret, Ninhydrin test, Glycosides Killer killani, Borntrager test, Flavonoids identification Test of Lead acetate, Shinoda test in show present (+) in the extract of Holoptelea integrifolia leaves Carbohydrates Molisch‟s, Fehling‟s, Benedict‟s test give absent (-) results with Ethanol Extract, give positive results with Ethanol Extract.

3.4 Drug excipients compatibility

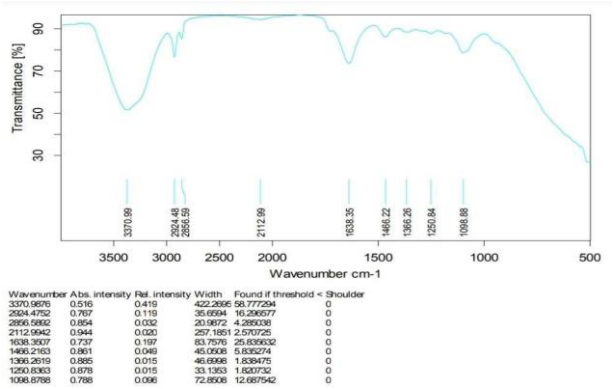
Drug excipients interaction by IR spectrum



Wavenumber	Abs. intensity	Rel. intensity	Width	Found if threshold < Shoulder	
3341.3743	0.668	0.326	396.0492	58.335003	0
2973.6022	0.789	0.128	41.4406	22.721752	0
2891.7761	0.840	0.024	116.8577	3.134565	0
1639.1672	0.832	0.119	97.5356	18.486406	0
1386.5637	0.789	0.140	203.2360	20.869637	0
1325.4451	0.832	0.020	169.6631	1.955570	0
1083.9826	0.667	0.012	10.3007	1.899742	0
1043.3794	0.444	0.451	44.8287	81.163254	0
877.5186	0.698	0.132	31.2929	19.390163	0

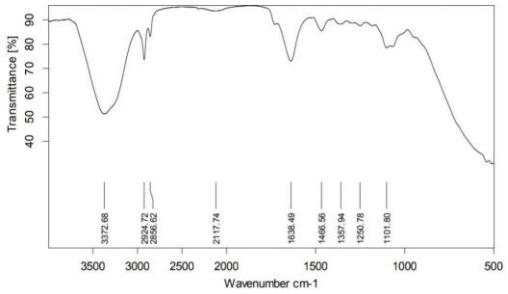
Fig no. 2: FTIR of the drug-loaded microemulsion.

FTIR studies shown in fig no.5 revealed that there was no appearance of any new peak and disappearance of existing peaks, which indicated that there was no chemical interaction between the drug and excipients used. However, slight shifts are seen, which are not that prominent.



Wavenumber	Abs. intensity	Rel. intensity	Width	Found if threshold < Shoulder
3370.9876	0.516	0.419	422.2695	58.777294 0
2924.4752	0.767	0.119	35.6994	16.296577 0
2856.5892	0.854	0.032	20.9872	4.285038 0
2112.9942	0.944	0.020	257.1851	2.570725 0
1638.3507	0.737	0.197	83.7576	25.835632 0
1466.2163	0.861	0.049	45.0508	5.835274 0
1366.2619	0.885	0.015	46.6998	1.838475 0
1250.8363	0.878	0.015	33.1353	1.820732 0
1098.8788	0.788	0.096	72.8508	12.687542 0

Fig no. 3: FTIR of optimized microemulsion



Wavenumber	Abs. intensity	Rel. intensity	Width	Found if threshold < Shoulder
3372.6759	0.513	0.402	419.7578	59.672883 0
2924.7159	0.737	0.126	34.8070	18.352827 0
2856.6166	0.832	0.035	20.7307	5.043393 0
2117.7440	0.937	0.021	311.4609	2.630437 0
1638.4877	0.730	0.197	80.2379	27.654940 0
1466.5559	0.855	0.053	44.8229	7.710544 0
1357.9348	0.882	0.017	46.5641	2.032306 0
1250.7820	0.876	0.016	32.7636	2.105797 0
1101.7963	0.785	0.096	87.4834	11.482986 0

Fig no. 4: FTIR of *Holoptelea integrifolia* ethanolic extract.

Excess extract eluent was dissolved in 1ml of ethanol, appropriately diluted with ethanol, and absorbance was determined spectrophotometrically at 272nm of separation chemical constituents.

Table:3. Results for separation of Chemical Constituents of leaves extract

S. No	Eluent	Compound	Reference
1.	Ethanol	Red Mixture	Steroidal triterpenes, glycosides, and tannins

Composition of Microemulsion Batch

Visual inspection

The samples were identified as microemulsions that appear transparent/translucent and easily flowable liquid. The samples were identified as emulsions when they appeared milky or translucent. Various batches of microemulsion are formulated and presented in Fig 5.

Fig. 5: microemulsion batches s/co. Mix. (1:1), (2:1) & (3:1), conc

Construction of Pseudo-ternary phase DiagramResult and discussion

1:1, 2:1, and 3:1 ratios resulted in an equal area of existing micro-emulsion; hence, the one that solubilized more extract was selected for the optimized batch. The translucent microemulsion region is presented in phase diagrams. Based on visual observation, the rest of the region represents the turbid and conventional emulsion. The area of the microemulsion region changed slightly in size with the increasing ratio of surfactant to co-surfactant. At the optimum S/C value, the cosurfactant was inserted into the cavities between the surfactant molecules precisely, and the formed microemulsion will have the maximum so-labialization capacity. The liquid crystal or bi-continuous structure area was more significant in ratio 2:1 because tween 80 is polar & has the tendency to incorporate into the water highly, and the relatively lower Tween 80 content in the microemulsion systems decreased the hydrophilicity of the surfactant mix.

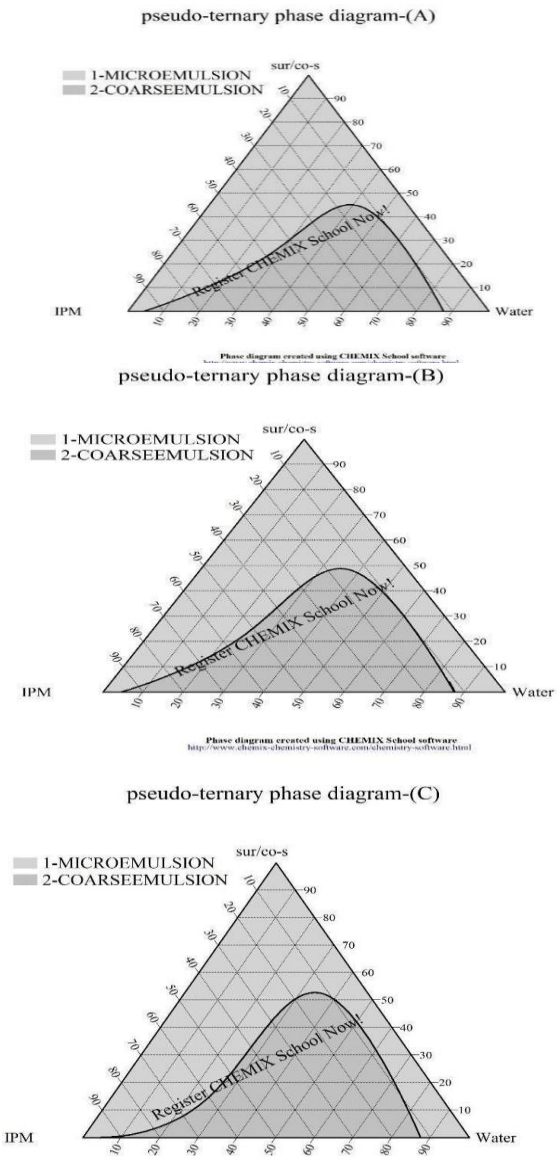


Fig no. 6: pseudo-ternary-phase diagram. (A)- sur/co-s mix ratio 1:1 conc. (B)- sur/co-smix ratio 2:1 conc. (C)- sur/co-s mix ratio 3:1 conc.

3.5 Determination of Particle size & poly disparity index

In the present study, the average particle size of all formulations was 41.43 to 1354.63 nm. The average particle size and polydispersity index for all batches are listed in the table. A higher concentration of surfactant resulted in more acceptable droplet size. The mean particle size and width of batch A-2 are shown in Fig. 7. The polydispersity index of all batches was 0.5033 to 1; thus, the microemulsion formulation showed a narrow distribution width and considerably small particle size.

Table: 4 The measurement of average particle size and polydispersity index

Batch no.	Avg. particle size (nm)	Polydispersity index
	Mean ±S.D.	Mean±S.D.
A-1	45.033 ± 0.467	0.809 ± 0.292
A-2	**41.43± 0.467**	**0.502± 0.0252**
A-3	44.33 ± 0.877	0.641 ± 0.0716
A-4	52.767 ± 0.695	0.167 ± 0.368
A-5	94.63 ± 0.057	0.368 ± 0.613
B-1	46.32 ± 0.047	0.199 ± 0.136
B-2	66.26 ± 0.392	0.167 ± 0.204
B-3	109.47 ± 1.55	0.106 ± 0.0878
B-4	55.11 ± 0.0312	0.0353 ± 0.034
B-5	76.30 ± 0.035	0.340 ± 0.0774
C-1	140.267 ± 0.27	0.138 ± 0.0295
C-2	25.40 ± 0.070	0.0342 ± 0.0266
C-3	98.60 ± 0.060	0.0524 ± 0.056
C-4	99.60 ± 0.020	0.0256 ± 0.6250
C-5	103.67 ± 0.433	0.029 ± 0.1115

The value expressed as mean±S.D., n = 3

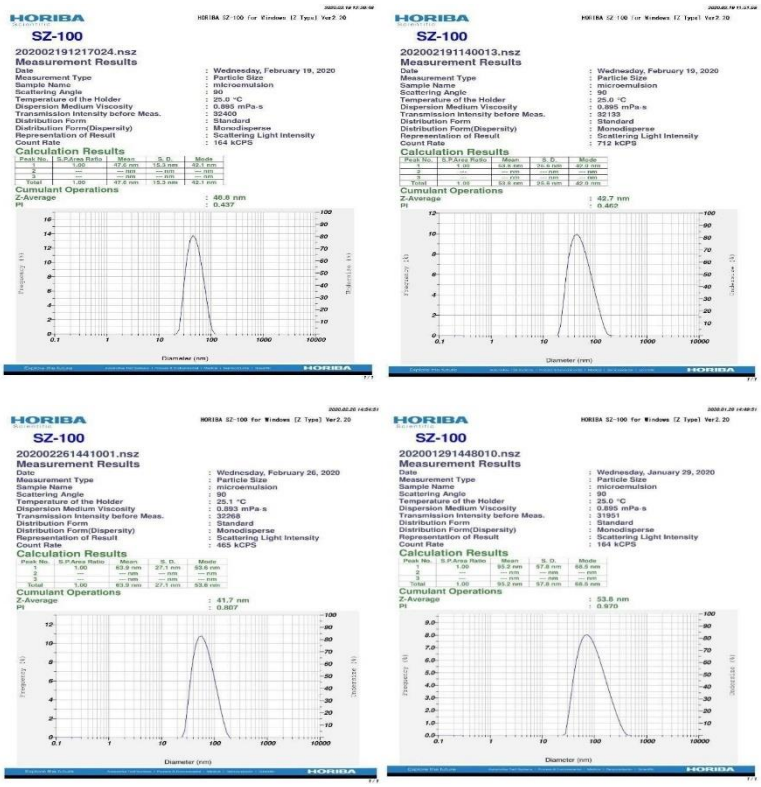


Fig. 7: The Measurement of optimized batches' average particle size distribution.

3.6 Determination of Zeta Potential

The zeta potential analysis is a valuable tool for estimating the stability of dispersed systems. The experimental measurement is indicated in table no. 13, which shows that all batches value in the expectable limit. Hence, particle aggregation would not likely occur due to electrostatic repulsion between the particles. Moreover, positive zeta potential is due to the presence of CTAB. Fig. no.17 indicates the zeta potential measurements of the optimized batch.

Table: 5 Zeta potential measurements of various formulations.

Batch no.	Zeta potential
	Mean ± S. D
A1	64.2 ± 4.2
A2	64.0 ± 4.2
A3	63.8 ± 4.5
A4	61.6 ± 3.8
A5	38.8 ± 2.25
B1	68.1 ± 4.13
B2	67.9 ± 5.29
B3	65.3 ± 5.25
B4	64.9 ± 4.1
B5	64.3 ± 4.12
C1	90.7 ± 7.0
C2	87.8 ± 6.7
C3	83.9 ± 6.1
C4	71.4 ± 5.14
C5	68.7 ± 4.12

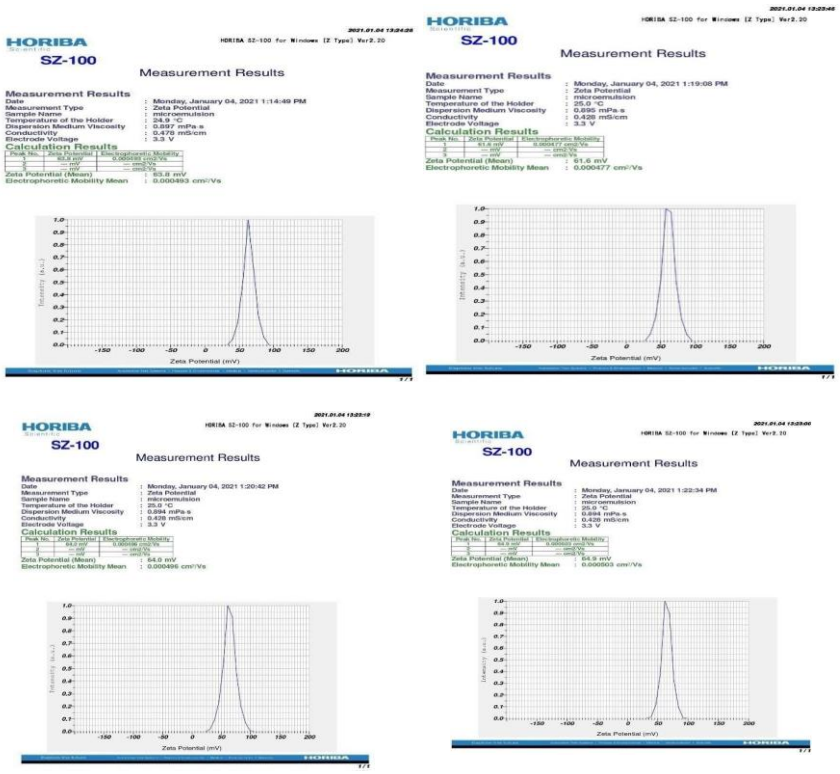


Fig. 8: The Zeta Potential Measurements of Optimized Batch

3.7 In vitro drug release studyResult and Discussion

The collective percentage release from the two batches, A-1 and B-1, was investigated for 24h. The release was slower from batch B-1, i.e., 65.0%, while 82.31% for A1. This release may be related to the surfactant concentration. B-1 had a smaller particle size due to higher surfactant concentration and higher viscosity and exhibited a slower release. Approximately 90% release was found in 8hrs, representing the optimum time for fungicidal effect.

Table 6 Drug release data of optimized microemulsion

Time (h)	Absorbance (nm)	Dilution factor	Conc. (µg/ml)	% Drug release
0	0	0	0	0
1	0.423	20	67.88	12.36±0.157
2	0.727	20	125.24	29.21±0.099
4	1.043	20	185.6	52.46±0.232
6	1.143	30	203.73	82.56±0.146
8	1.304	30	234.11	91.80±0.236
10	1.304	30	234.11	91.82±0.172
12	1.304	30	234.12	91.81±0.236
24	1.305	30	234.11	91.85±0.202

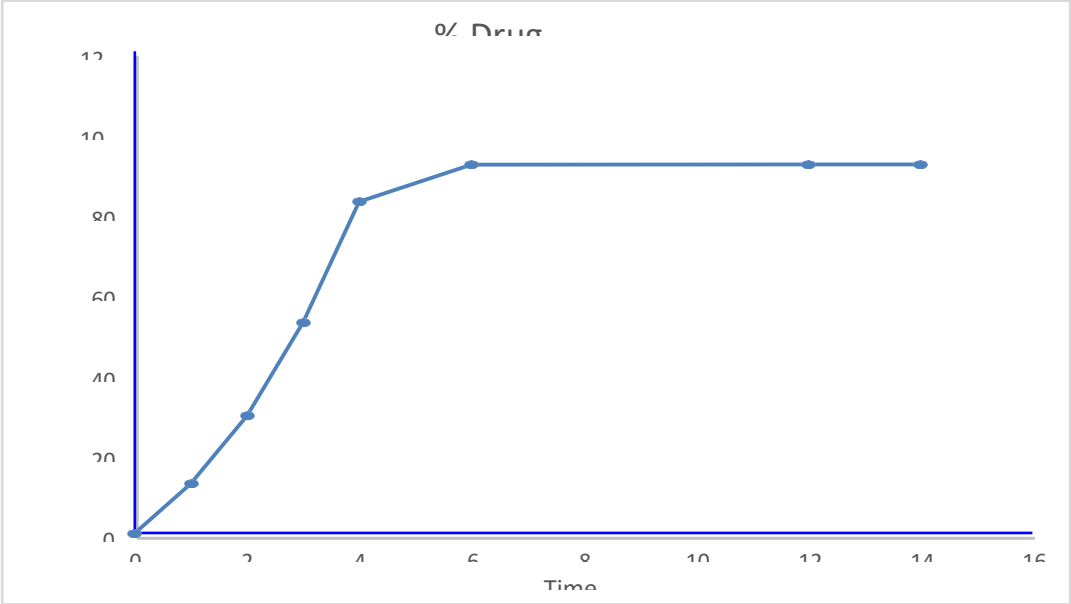


Fig. 9: In-vitro drug release

3.8 In Vitro Antifungal Assay

The in vitro assay for the zone of inhibition was performed on the fungal strain Albicans. The maximum inhibition zone was 1.09±0.82 mm for optimized microemulsion loaded withHoloptelea integrifolia leaves extract. Then 0.66±0.17 mm for the standard marketed antifungal drug itraconazole. This result was followed by 0.59±0.20mm for Holoptelea integrifolia leave extract. Placebo microemulsions had a zone of inhibition of 0.29±0.11mm due to the presence of CTAB. Fig. 20. These results suggest that the herbal drug Holoptelea integrifolia extract-loaded microemulsion could be more efficient than the market drug itraconazole.

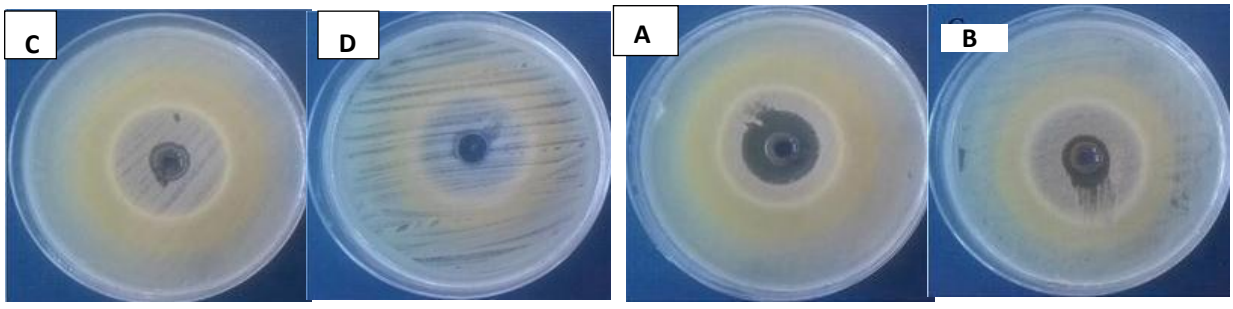


Fig. 10: plate showing zone of inhibition of (A) pure drug (Holoptelea integrifolia) (B) optimized microemulsion loaded with Holoptelea integrifolia leaves extract. (C) markets drug itraconazole. (D) Surfactant C-Tab

3.9 Stability study

Stability studies were performed to detect changes in pH, droplet size, and drug content. The optimized microemulsion was physically stable (16,29), retaining homogeneity and demonstrating no phase separation after three months. No significant changes were observed in the droplet size and degradation within three months. The centrifuge tests demonstrated that optimized microemulsion is physically stable. Three months later, no difference was noticed in the pH, droplet size, drug content, and viscosity between microemulsion stored at 2- 8˚C and room temperature.

Table: 7 Stability analysis result

Characteristics of microemulsion			
Formulation	Size(nm)	PDI	pH
Before storage	46.34±0.07	0.184±0.03	6.51±0.02
After storage	45.23±0.05	0.197±0.01	6.12±0.02

6.CONCLUSION:-

Holoptelea integrifolia leaves are the most investigated part of the plant. Leaf has been a promising agent for treating skin diseases like leukoderma, scabies, ringworm (fungal infection), and eczema. Microemulsion formulation drugs show better antifungal activity by enhancing penetration into the skin and fungal cells. A microemulsion is an emerging trait to develop novel drug delivery systems because of their intelligent working property. It is a novel and exciting strategy using water, oil surfactant, and co-surfactant to prepare Microemulsion. Antifungal agents are generally lipophilic and formulated in topical vehicles. It is superior tochoose microemulsion as a topical vehicle for antifungal agents since ease of preparation due to spontaneous formation, thermodynamic stability, transparent and elegant appearance, increased drug loading, enhanced penetration through the biological membranes, increased bioavailability as compared to conventional dosage forms like gel, cream, etc

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