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RESEARCH ARTICLE

Formulation Development and Evaluation of a Polyherbal Suspension Containing *Curcuma longa, Ocimum sanctum* and *Azadirachta indica* with Improved Antimicrobial Activity

R. S. Shivarkar[1,2*], S. B. Bhise[3], V. Rama Mohan Gupta[4], N. S. Kulkarni[1] and M. C. Upadhye[1]

[1]Department of Pharmacognosy, PES Modern College of Pharmacy for Ladies Moshi, Pune - 421105, Maharashtra, India; rahulshivarkar19@gmail.com

[2]Jawaharlal Nehru Technological University, Hyderabad - 500085, Telangana, India

[3]A202, Navkar Residency, Near Bibwewadi Police Chowki, Pune - 411037, Maharashtra, India

[4]Pulla Reddy Institute of Pharmacy, Annaram, Gummudidala, Hyderabad - 502313, Telangana, India

Abstract

A lack of global political will to mobilise resource to fight tuberculosis is major challenge in ending tuberculosis. The polyherbal formulations are best alternative, as they are economic, environmentally friendly and easily available than modern drugs. In present study, a polyherbal suspension with extracts of *C. longa, A. indica* and *O. sanctum* was developed and characterized. The developed suspension was found satisfactory with respect to odour, colour, taste, pourability, pH, viscosity, zero microbial count, particle size, percentage ease of disposability, aesthetic characteristic, sedimentation, zeta potential and does not show the crystal growth, polyherbal formulation exhibited significantly inhibited the growth of H37Rv and MIC is also comparable to those of standard agents.

Keywords: Antimicrobial, Polyherbal Formulation, Tuberculosis

1. Introduction

An estimated 10.6 million people became ill with Tuberculosis (TB) in 2021 compared with 10.6 million who died in 2020 from Tuberculosis as per WHO Report 2022. Relative to 2020, the incidence rate of TB increased by 3.6 in 2021 indicating a 2% decrease annualy[1].

Due to the incidence of Multi-Drug Resistant Tuberculosis (MDR-TB), there is an increase in the death rate in the world since 1980[2]. This situation is due to irregularity in TB treatment and current drug therapy failing to treat the disease. For treatment of MDR-TB second-line drugs have been used which showed side effects with only a 50% cure rate. Moreover, the first line and second line of drugs are costly[3]. Only two new drugs introduced such as Delamanid and Bedaquiline which are found unsafe clinically. Since 2015, there are new cases of MDR-TB and continuous addition of Rifampicin-Resistant TB (RR-TB) in patients with Rifampicin-Resistant TB (RR-TB)[4]. In the case of acquired drug resistance, only second-line drugs must be used but are found equally costlier. Therefore, for the control of TB, there is an immediate requirement for modern methods of drug treatment[5]. Folklore medicine especially natural drugs have proven its potency and found the best

*Author for correspondence

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alternative medicines as per WHO[6]. Herbs have long held an important place in the treatment of ailments. Over 350 natural products, many plant species; have been used in traditional medicine and antimicrobial activities[7]. Medicinal plants and their isolated active principles or phytoconstituents created a big opportunity to formulate many dosage forms which can control microbial diseases especially, TB. In the present research work, an attempt was made to isolate the active compounds from medicinally important plant extracts and evaluate them and whole extracts for their pharmacognostic study and antimicrobial/anti-tubercular activity.

2. Materials and Methods

2.1 Plant Extracts

Hydroalcoholic extracts of *Curcuma longa* rhizomes, *Azadirachta indica* leaves, *Ocimum sanctum* leaves will be prepared. The reagents used for the preparation are of analytical grades. Some other chemicals were procured from Sigma-Aldrich, USA. CMC sodium, propyl paraben, methyl paraben and sucrose.

2.2 Development of Polyherbal Suspension by Using Hydro-alcoholic Extract of Plant Material

The powdered material of test extract was obtained through cold hydro-alcoholic percolation process. In this, dried powder of 10 g was added to 100 ml of petroleum ether and mixed thoroughly for 24 hours using a rotatory shaker at 120 rpm. Then followed by centrifugation of extract for 15 minutes at 5000 rpm and the supernatant was transferred to a cleaned beaker and further it was dried. To the dry powder, 100 ml of 70% ethanol and 30% water were added and then incubated for 24 hours on a rotatory shaker at 120 rpm. The polyherbal suspensions of test extracts were done by the trituration method. The precise contents of polyherbal formulation were determined as the documented doses of the respective herbal agents[8-10]. These documented doses were 1000 mg/kg, 1500 mg/kg and 1.25 in 1 ml of *C. longa, Azadirachta indica* and *O. sanctum* respectively. The polyherbal suspensions were labelled as F1, F2, F3, F4 and F5. The details composition of the suspension was presented in Table 1.

The uniform smooth paste formulation of the extract was prepared by using tween 80 with, methyl and propylparaben and sodium carboxy methyl cellulose. The paste was then rinsed with distilled water to form the suspension. A mechanical stirrer with a speed of 500 rpm is used for homogenous mixing of the dispersion. Then, the final volume of suspension was made to 100 ml.

3. Evaluation of Polyhedral Formulation

All the polyherbal formulations of studied drugs were evaluated for various parameters such as pH, viscosity, sedimentation ability, re-dispersibility, aesthetic characteristics, and microbiological tests.

Table 1. Composition of the polyherbal formulation

Name of the Ingredients	For 3000ml (in gm)				
	F1	F2	F3	F4	F5
Curcuma longa rhizome extract	0.60	1.2	1.8	2.4	3
Azadirachta indica leaves Extract	0.60	1.2	1.8	2.4	3
Ocimum sanctum leaves extract	7.5	15	22.5	30	37.5
Cabaoxy methylcellulose (Thickening, stabilizing agent)	0.5% w/v	0.5% w/v	0.5% w/v	0.5% W/V	0.5% W/V
Propylparaben (as a preservative)	0.1% w/v	0.1% w/v	0.1% w/v	0.1% w/v	0.1% w/v
Methylparaben (as a preservative)	0.2% w/v	0.2% w/v	0.2% w/v	0.2% w/v	0.2% w/v
Sucrose syrup	600	600	600	600	600
Distilled water q. s.	q.s	q.s	q.s	q.s	q.s

3.1 Determination of Color and Odour (Aesthetic Characteristics)

The colour, odour, taste, and pourability were thoroughly and visually inspected after 1 hour of their formulation at 37°C[11].

3.2 Determination of pH

The pH was measured by potentiometrically using a standard buffer solution.

3.3 Determination of Sedimentation Rate

The polyherbal formulations were subjected to the measurement of sedimentation which was performed by the addition of 100 ml quantity in a 100 ml graduated measuring cylinder. The measuring cylinder was further stoppered and inverted a few times in order to get uniform dispersion, subsequently waited to settle for 3 minutes and the resultant volume was estimated as sediment. The study was carried out for 10 days and maintained at ambient temperature.

3.4 Viscosity Determination

The viscosity of the polyherbal suspensions was performed by employing a Brookfield viscometer (Model –LDVVE). Spindle no. S-00 was placed centrally in the test sample adapter. The temperature of the sample was 25 ± 0.1 °C throughout the experiment period. The reading was noted after allowing the equipment to stabilize for 15 mins.

3.5 Determination of Re-dispersibility

The polyhedral suspensions were added to 100 ml graduated measuring cylinder and waited for some time to settle the particles of the formulation[12]. The cylinder was rotated at anangle of 180° and the number of time inversions required to settle the suspension particles were observed.

3.6 Microbiological Studies

The polyherbal formulations were tested for micro bacteria presence such as *Escherichia coli,* and *Salmonella* including the viable aerobic count.

3.7 Measurement of Crystal Growth by Polarized Light Microscopy

Crystals growth of polyherbal formulation was determined under cross-polarized light of crystal. The crystal polymorph elicited various crystalline habits, which results in crystal shape differences and dissolution rates. By dispersing the solid particles onto a glass slide all samples are formed the images were composed at 40x magnification and the 1st-order red compensator was in place.

3.8 Standardization of Selected Suspension (F5)

The F5 suspension was selected and studied for particle size, preservative efficacy, drug content, and accelerated stability studies.

3.8.1 Particle Size Studies

The Eyepiece micrometre was calibrated using the stage micrometre. The mount was prepared by placing one drop of uniformly dispersed suspension on the slide and by placing the covers lip over it. Then, the dimensions of 50 particles were measured after focusing the slide on a compound microscope. The average particle size and range of the particle size were calculated.

3.8.2 Zeta Potential Determination

By using the Dynamic Light Scattering (DLS) method with a computerized inspection system –Malvern Zeta sizer Nano–ZS series at 25 ± 0.5, the zeta potential of suspension was studied to understand dispersive system behavior.

3.8.3 Preservative Efficacy Test

In order to ensure the formulation is free from microbial growth or the presence of any contamination the preservative efficacy test was conducted. This test was determined as per the Indian pharmacopeia 2011 against, *C. albicans* (ATCC 10231); *A. niger* (ATCC 16404); *E. coli* (ATCC 8739); *P. aeruginosa* (ATCC 9027); *S. aureus* (ATCC 6538).

3.8.4 Accelerated Stability Studies

The accelerated stability studies were conducted for polyherbal formulation by incubating the samples at various temperature points (25°C, 40°C and 31°C) and at a time intervals of 0, 1, 2 and 3 months. The shelf life of the formulations was revealed from the generated data of stability studies predicted. Further, all the above-studied parameters were determined every 15 days to 90 days.

3.8.5 Determination of the Antitubercular Activity of Polyherbal Formulation by Microplates Almar Blue Assay (MABA)

3.8.5.1 Materials

Clinical isolates of *Mycobacterium tuberculosis* (H37Rv) ATCC 27294 (American Type Culture Collection, Manassas, VA). Kanamycin and Amikacin were procured (Sigma Aldrich, USA). Cultures were incubated in 500 ml nephelometer flasks on a rotary shaker (Equitek, India) at 150 rpm and 37°C until they reached an optical density of 0.4 to 0.5 at 550 nm (EPOCH, BioTek-Agilent, USA). Bacteria were washed and suspended in 20 ml of phosphate-buffered saline and passed through an 8-mm-pore-size filter to eliminate clumps. The filtrates were aliquoted, stored at -20°C, and used within 30 days. A stock solution of kanamycin (1 mg/ml) and amikacin (1 mg/ml) was prepared from chemically pure powder (Sigma Aldrich, USA), filter sterilized, and kept in aliquots at -20°C until use.

3.8.5.2 Experimental Procedure

Antimicrobial potential of polyherbal formulation F5, standard drugs, kanamycin and amikacin were determined against *Mycobacterium tuberculosis* (H37Rv) ATCC 27294 in 96-well Microplates Almar Blue Assay (MABA) (black view plates; Lowbrow, India) as per the method described by Collins and Franzblau (1997) with minor modification. Briefly:

- Initial standard drug dilutions were prepared in either dimethyl sulfoxide or distilled deionized water,and subsequent two-fold dilutions were performed in 0.1 ml of 7H9GC (no Tween 80) in the microplates.
- Addition of 1/10 ml to wells resulted in final bacterial titers of 2 × 105 FU/ml for H37Rv.
- Wells containing kanamycin and amikacin only were used to detect autofluorescence.
- Additional control wells consisted of bacteria only (B) and medium only (M).
- The plates were incubated at 37°C. Starting at day 4 of incubation, 20 µl of Almar blue solution (Thermos Fisher Scientific, Waltham, USA) and 12.5 µl of 20% Tween 80 were added to one B well and one M well, and plates were further incubated at 37°C.
- Then, wells were observed at 12 and 24 h for a color change from blue to pink and for a reading of ≥50,000 Fluorescence Units (FU).
- Fluorescence was determined by employing the multimode microplate reader (BioTek-Agilent, USA) in bottom-reading mode with excitation at 530 nm and emission at 590 nm.
- Further, plates were incubated at 37°C, and results were recorded at 24 h post-reagent addition.
- Minimum inhibitory concentration is defined as the lowest concentration of drug that prevented a color change. For fluorometric MICs, background subtraction was performed on all wells with a mean of triplicate M wells.

3.8.5.3 Statistical Analysis

All analyses were performed with the program SAS (SAS Institute Inc., USA). A general linear analysis of variance of ranked data was conducted to determine significant differences followed by Tukey's posthoc test significance was determined at $P<0.05$.

4. Results

4.1 Physicochemical Investigations

The polyhedral formulation was studied viz. pH, weight per ml, viscosity, and sedimentation are presented in Tables 2 and 3.

4.2 Effects of Polyherbal Formulations on Sedimentation Volumes

The F value of the Formulations is near to 1, which indicates the volume of sediment is equal to the original volume of the suspension. The formed formulation is a flocculated suspension.

4.3 Microbiological Investigations

The presence of microbiological burden, microbial counts, or bacteria in the test polyherbal formulations were investigated by *E. coli*, and *Salmonella* and its findings are presented in Table 4.

Table 2. Physico-chemical properties of polyherbal formulations

Formulations	PH	Weight per ml	Viscosity
F1	4.63	0.0206	160cps
F2	4.59	0.0205	280cps
F3	4.14	0.0208	200cps
F4	4.71	0.0209	800cps
F5	4.50	0.0207	110cps

Table 3. Effects of polyherbal formulations on sedimentation volumes

Time in days	Initial Volume (Vo) ml	Ultimate Volume (Vu) ml					Sedimentation volume F (Vu/Vo)				
		F1	F2	F3	F3	F5	F1	F2	F3	F4	F5
0	100	100	100	100	100	100	1	1	1	1	1
1		100	98.2	99.2	95.2	100	1	0.982	0.992	0.952	1
2		100	94.4	96.2	93.2	100	1	0.944	0.982	0.934	1
3		100	94.4	95.0	90.2	100	1	0.944	0.962	0.902	1
4		100	94.4	95.0	88.2	100	1	0.944	0.950	0.882	1
5		100	94.4	95.0	86.4	100	1	0.944	0.950	0.864	1
6		100	94.4	95.0	84.5	100	1	0.944	0.950	0.845	1
7		100	94.4	95.0	84.5	100	1	0.944	0.950	0.845	1
8		100	94.4	95.0	84.5	100	1	0.944	0.950	0.845	1
9		100	94.4	95.0	84.5	100	1	0.944	0.950	0.845	1
10		100	94.4	95.0	84.5	100	1	0.944	0.950	0.840	1

Table 4. Microbial counts and presence of bacteria in polyherbal formulations

Formulation	Total Microbial count C.F. U		Test for E. coli	Test for Salmonella
	Bacteria	Fungi		
F1	-ve	-ve	-ve	-ve
F2	-ve	-ve	-ve	-ve
F3	-ve	-ve	-ve	-ve
F4	-ve	-ve	-ve	-ve
F5	-ve	-ve	-ve	-ve

4.4 Crystal Growth

The experimental findings revealed that crystal growth was not observed in either formulation F1-F5 when detected under the microscope at 40°C ± 2°C and 75% relative humidity for the duration of 3 months, suggesting the stability of the formulations.

4.5 Evaluation of Selected Oral Suspension (F5)

Polyherbal formulation of F5 was investigated for particle size, microbial load, efficacy and stability studies.

4.5.1 Analysis of Particle Size

The formulation was further subjected to determined particle size and presented in Table 5. It revealed the highest particles at the 20-60 µm size range.

Table 5. Analysis of particle size of polyherbal suspension

Particle size (µm.)	Mean (µm.)	Particles present in sample (µm.)
10-20	15	-
20-30	25	30
30-40	35	35
40-50	45	25
50-60	55	10
60-70	65	-
70-80	75	-
80-90	85	-
90-100	95	-
100-110	105	-

4.5.2 Preservative Efficacy Test

The preservative usefulness was determined against, *Escherichia coli*, *Pseudomonas mirabilis* and *Staphylococcus aureus*. It was found that polyherbal formulation was found to be effective against the above bacteria (Table 6).

4.5.3 Accelerated Stability

The polyherbal formulation was subjected to an accelerated stability profile for shelf life. Other paradigms mainly weight per ml, viscosity, pH, sedimentation, particle size, aesthetic characteristics and microbial assays.

4.5.4 Antitubercular Activity against Mycobacterium tuberculosis (H37Rv) ATCC 27294 by Microplates Almar Blue Assay (MABA)

For H37Rv, the reduction of Alamar Blue dye with test formulation, as determined by fluorometric measurement, paralleled the increase in FU during incubation in 7H9GC for 5 days, after which the fluorescence appeared to increase more rapidly (Figure 1). The standard drugs kanamycin and amikacin significantly inhibited the H37Rv growth. The MIC were much lower for these agents. Polyherbal

Table 6. Effects of polyherbal formulation on preservative efficacy

Microorganism	Preservative efficacy				
	Day 0	7 days	14 days	21 Days	28 days
E. coli,					
10^5	10	6	NIL	NIL	NIL
10^6	5	10	1	NIL	NIL
P. mirabilis					
10^5	17	10	5	1	NIL
10^6	8	5	3	1	NIL
S. aureus					
10^5	10	4	7	1	NIL
10^6	15	3	4	1	NIL

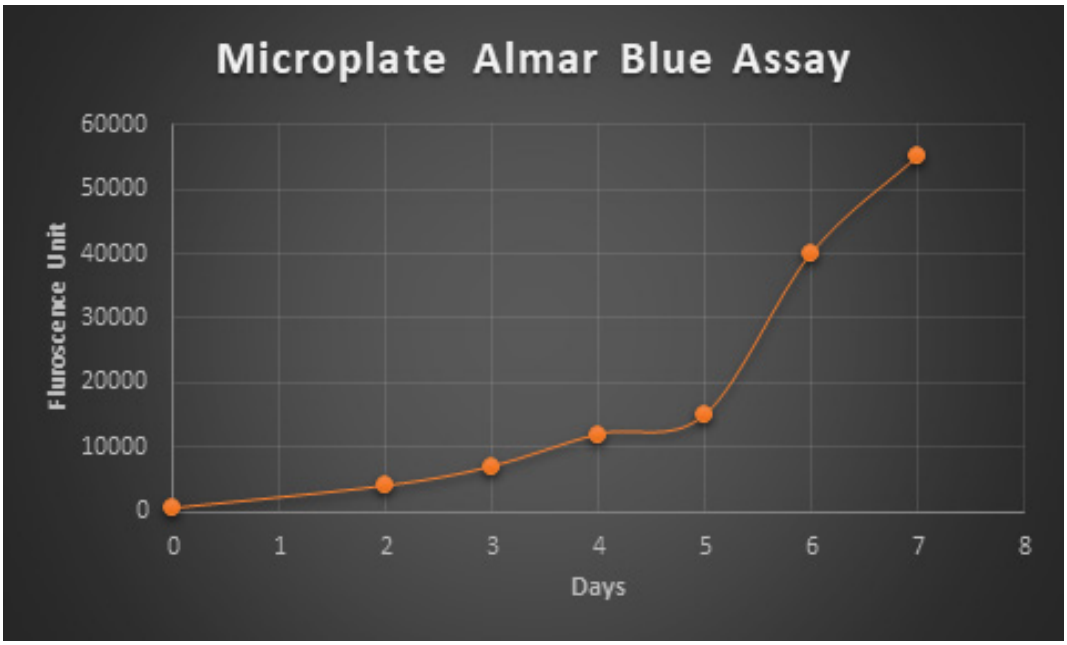

Figure 1. Alamar blue reduction FU during growth of *M. tuberculosis* H37Rv.

formulation also exhibited significantly inhibited the growth of H37Rv and MIC is also comparable to those of standard agents.

MICs of the polyherbal formulation F5 and a standard antibiotic against H37Rv are presented in Table 7.

MICs of polyherbal formulation F5 greater found to be comparable to that of standard drugs.

4.5.5 pH Studies

The pH of the polyherbal formulation F5 was investigated at various time intervals with different temperatures. The findings are presented in Table 8.

4.5.6 Weight per ml

The property of weight in ml was determined at various time intervals, the findings suggested no significant alternations were observed Table 9.

4.5.7 Viscosity

The viscosity was determined early and later for 30 days and found to exhibit non-significant alteration (Table 10).

4.5.8 Sedimentation Volume

The sedimentation volumes were investigated at 25°C, 31°C and 40°C for 3 months and found to have no changes (Table 11).

4.5.9 Determination of Particle Size

Polyherbal formulation F5 was subjected for particle analysis at various temperatures and results are presented in Table 12.

Table 7. MICs of polyherbal formulation and standard kanamycin and amikacin *Mycobacterium tuberculosis* H37Rv

Treatment and groups	MIC µg/mL (Mean±SEM)
Control	>320±0.2
Polyherbal formulation F5	7.42±0.1
Kanamycin	2.19±0.1
Amikacin	0.88±0.2

Table 8. The effects of polyherbal formulation F5 on the pH at various temperatures

Formulation No. 5	Time	Temp		
		25 °C	**35** °C	**40** °C
1st Month	Initial	4.48	4.48	4.49
	After 15 days	4.50	4.50	4.49
2nd Month	Initial	4.50	4.50	4.52
	After 15 days	4.52	4.52	4.53
3rd Month	Initial	4.54	4.54	4.56
	After 15 ml	4.55	4.55	4.57

4.5.10 Ease of Re-dispersibility

The ease of re-dispersibility was evaluated on 15 days and 30 days with stability batches and data suggested 100% re-dispersibility of polyherbal formulation F5 (Table 13).

4.5.11 Zeta Size

The zeta-potential was determined for the polyherbal formulation and found to be −19.3 ± mV and -17.7 ± mV at the 0th and 180th days respectively (Figure 2). The

Table 9. Effects of polyherbal formulation F5 on weight per ml

Temperature	Before	1st Month	2nd Month	3rd Month
25°C	0.0207	0.0207	0.0207	0.0207
31 °C	0.0207	0.0207	0.0207	0.0207
40 °C	0.0207	0.0207	0.0207	0.0207

Table 10. Effects of polyherbal formulation F5 on viscosity

Formulation	Temperature (°C)	Viscosity			
		Before	One month	Two months	Three months
No. 5	25	110cps	115cps	120cps	120cps
	31 °C	110cps	115cps	120cps	120cps
	40 °C	110cps	115cps	120cps	120cps

Table 11. Effects of polyherbal formulation F5 on sedimentation volume at different temperatures

Time in days	Initial Volume	At 25 °C		At 31 °C		At 40 °C	
		Ultimate Volume (Vu)	Sed. Volume (Vu/V0)	Ultimate Volume (Vu)	Sed. volume (Vu/V0)	Ultimate volume (Vu)	Sed. volume (Vu/V0)
0	100	100	1	100	1	100	1
1		100	1	100	1	100	1
2		100	1	100	1	100	1
3		100	1	100	1	100	1
4		100	1	100	1	100	1
5		100	1	100	1	100	1
6		100	1	100	1	100	1
7		100	1	100	1	100	1
15		100	1	100	1	100	1
25		100	1	100	1	100	1
35		100	1	100	1	100	1
45		100	1	100	1	100	1
60		99	0.99	99	0.99	99	0.99
75		99	0.99	99	0.99	99	0.99
90		99	0.99	99	0.99	99	0.99

Table 12. Particle size at 25°C, 31°C, and 40°C

Range (µm)	Particles size range (µm)			
	Day 1	37°C Day 90	25°C Day 90	40°C Day 90
10-20	-	-	-	-
20-30	30	32	30	30
30-40	35	30	35	35
40-50	24	28	20	25
50-60	11	10	15	10
60-70	-	-	-	-
70-80	-	-	-	-
80-90	-	-	-	-
90-100	-	-	-	-
100-110	-	-	-	-

findings revealed that a negative charge indicates larger half-lives with greater high biodistribution, thus more stability.

The polyherbal formulation F5 was investigated on various paradigms and found to elicit non-significant alternations pH, weight per ml, microbial count, viscosity, sedimentation volume, ease of re-dispersibility and particle size analysis. Thus, it is concluded that the polyhedral formulation no. 5(F5) was found to be stable, and uniform along with better readily dispersible oral suspension.

Table 13. Ability of polyherbal formulation F5 on re-dispersibility

Polyherbal formulation F5	Time intervals	Re-dispersibility at various temperatures (%)		
		25°C	31°C	40°C
1st Month	After 15 Days	100	100	100
	After 30days			
2nd Month	After 15 Days			
	After 30days			
3rd Month	After 15 Days			
	After 30days			

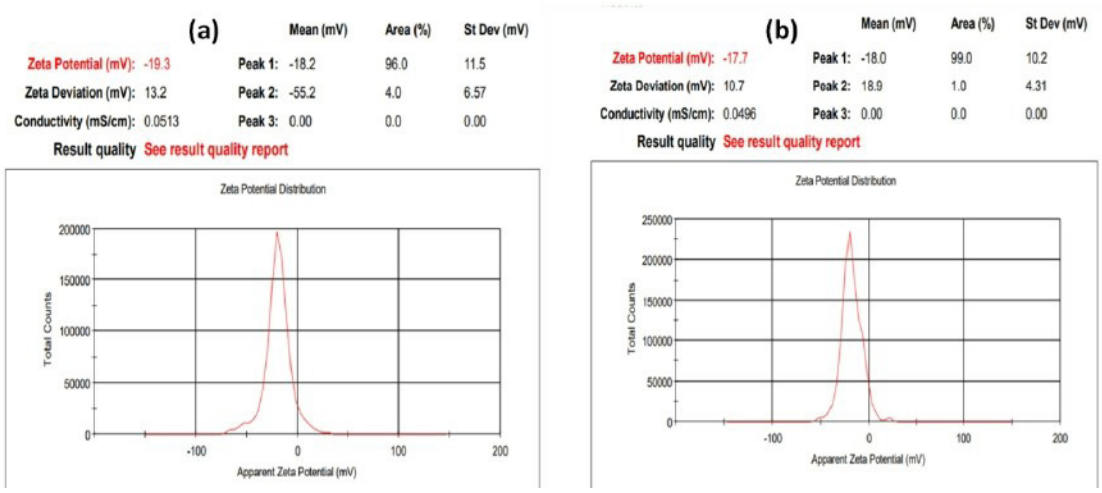

Figure 2. Zeta potential of polyherbal formulation F-5 on 0th day (a) 180th (b) days.

5. Discussion

The polyherbal suspension with extracts of *C. longa*, *Azadirachta indica* and *O. sanctum* was developed using sodium CMC as a thickening agent and characterized. The developed suspension was found satisfactory with respect to odour, colour, taste, and pourability indicating the ideal dosage form. The viscosity of the formulations ranged from 110 to 800 cps while pH was found between 4.14 to 4.71. The viscosity was found excellent with which the formulation can be easily pourable. The sedimentation volume study revealed no formation of any sediment even after a 10-day period time. The viscosity of the formulation also plays a very important role in the avoidance of the sedimentation of the ingredients utilized in suspension[11]. Sodium CMC is a widely used thickening agent, and viscosity modifier in pharmaceutical dosage forms including suspensions, emulsions and gels[13]. All the developed suspensions didn't show the growth of microorganisms like *E. coli* and *Salmonella*. The formulations passed the test for the total microbial count. This suggests the outstanding stability of the formulations with no microbial growth. Even the formulations didn't show the crystal growth at the initial time point and at 3M at 40°C ± 2°C and relative humidity of 75% revealed better stability of the formulations. The particle size of the optimized suspension was in the range of 20-60 microns which is a sufficiently smaller and a good indicator to avoid the grittiness as well as the roughness of the suspension[14]. Preservative efficacy testing of the optimized formulation was determined to check the potential of preservatives (methyl and propyl paraben) used in the formulation against *Candida albicans*, *Aspergillus niger*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Straph. Aureus* and found to have no microbial count after 4 weeks suggesting an effective preservative system. Other paradigms mainly pH, weight per ml, microbial assay, viscosity, sedimentation volume, particle size, percentage ease of disposability and aesthetic characters were found within acceptable limits during stability studies. The stability

study performed on the optimized formulation showed. The developed polyherbal suspension formulation was found to be stable and robust.

Thus, it can be suggested that polyherbal suspension containing extracts of *C. longa*, *Azadirachta indica* and *O. sanctum* exhibited significant and potent antimicrobial and anti-TB activity and supports the adjunct therapy for combating TB.

6. Conclusion

The polyherbal suspensions of extract of *C. longa*, *Azadirachta indica* and *O. sanctum* developed using sodium CMC as a thickening agent was found to be stable, and robust. The developed suspension was found satisfactory with respect to odour, colour, taste, and pourability. The formulations passed the test for the total microbial count as well as preservative efficacy testing. This indicates the excellent stability of the formulations which doesn't support the microbial growth. Thus, present experimental findings suggested the potential approach for the treatment of TB in the form of polyherbal suspension containing extracts of *C. longa*, *Azadirachta indica* and *O. sanctum* having significant antimicrobial and anti-TB activity.

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