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Design and Development of Self-preserving and Preservative-free Herbal Liquid Oral Formulation

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ABSTRACT

Microbial contamination is one of the major inevitable concerns associated with herbal liquid formulations, which may originate from herbal raw materials. Inclusion of preservatives in herbal liquid formulations has been of considerable value for many years. Anti-microbial preservatives are normally added to prevent microbial proliferation while the product on shelf and during in use conditions. The properties of these preservatives are due to certain functional groups, which are usually harmful to living cells and might therefore be associated with certain risks when used in humans and they are the leading causes of adverse reactions and have negative and potentially life threatening side effects, because they not only act on microorganism but may also interfere with human cells. In this study, we have made an effort to develop a preservative-free and self-preserving liquid oral formulation by understanding and applying alternative principles of preservation (approaches other than using preservatives) by taking Ashoka herb extract as a prototype. Our series of formulation trials using different vehicle systems, which reduce the water activity by controlling the pH and osmotic conditions successfully yielded a vehicle system that could be used for the manufacturing of stable preservative-free/self-preserving herbal liquid oral formulations. Ashoka formulations were found to be physically, chemically and microbiologically stable during the six months of accelerated stability studies.

INTRODUCTION

The renewed interest on herbal medicines worldwide has significantly escalated their demand. Manufacturing and commercialization of these medicines to meet these increase demand makes it imperative to evolve a systemic approach for development of herbal formulation. In order to meet the large scale demand and manufacture of herbal product, a robust techniques, scientifically relevant, systematic and well-designed methodologies are warranted to meet the stringent regulatory requirements and quality of the product. But, one of the major challenges in standardizing herbal raw materials or formulations is microbial contamination. Most herbal raw materials for pharmaceutical products support some forms of microbial growth, depending on their nutritive properties and moisture contents (Stevic *et al.*, 2012) and these microbial contaminants invariably get transferred to the final product. Formulation of herbal dosage forms is unique and poses greater challenges compared to non-herbal dosage forms. This is mainly due to the complexity of input herbal materials and also due to the lack of complete characterization, batch to batch variation and the absence of ample data. One of the most important challenges in formulating herbal dosage forms is to control the microbial burden or bioburden in almost all types of dosage forms such as tablets, capsules and also in liquid orals/liquid. Herbal extracts can be difficult to formulate, especially in liquid oral dosage forms, which are prone to be contaminated with microorganisms that survive in the final formulation. Thus, microbial bioburden is always a risk (Kamil and Lupuliasa, 2011). Many pharmacopoeias (European Pharmacopoeia, Indian Pharmacopoeia, United States Pharmacopeia) and regulatory agencies have included the limits for bioburden, which are not uniform across the globe; however, they are unequivocal in setting up a limit for bio-burden. Inclusion of preservatives in several types of drug formulations has been of considerable value for many years.

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However, preservatives are often connected to the leading causes of adverse reactions. For example, parabens interact with mitochondrial cells and induce male infertility; sodium benzoate causes hyperactivity in children; potassium sorbate cause mutagenic effects in lymphocytes (Mamur *et al.*, 2010; McCann *et al.*, 2007; Tavares *et al.*, 2009). An alternative approach to develop herbal liquid formulations without preservatives is to use water activity-lowering agents like sorbitol, propylene glycol, glycerol, xylitol, etc.(Galmarini *et al.*, 2008; Lilia *et al.*, 2010; Stella *et al.*, 1994; Martin and Linwood, 1957), which are known to reduce the free water available in finished product. Amount of free or unbound water in the products can play a significant role in maintaining the microbial quality of the product. As free water enables the metabolism of microorganisms and therefore in certain cases resulting in the production of toxins or other harmful substances which affects microbiological stability, chemical stability, organoleptic properties and also nutritional values (Leistner, Undated; Leistner and Gould, 2002; Ramirez-Jimenez *et al.*, 2003). This study describes the formulation and development of optimized Ashoka (*Saraca indica*) liquid formulation without sucrose and preservative using water activity-lowering agents to keep the microbial count well within limits throughout the shelf-life of the product.

MATERIALS ANDMETHODS

Standardized Ashoka bark water extract

The extract was supplied by Phytochemistry Division of R&D Center, The Himalaya Drug Company, Makali, Bangalore, India. The extract was spary dried powder without any preservatives. The extract underwent detailed analytical studies and was standardized with respect to markers such as polyphenols and catechins.

Chemicals

Sucrose, citric acid monohydrate, sodium citrate, sorbitol, xylitol, propylene glycol, glycerin and purified water. All chemicals were of European Pharmacopoeial grades.

Dose selection of Ashoka extract for liquid oral formulation

The daily human dose of Ashoka extract/active is 900 mg. The liquid preparations were prepared in such a way that each ml contains 30mg of active, so that dose of the formulation is equal to 15ml twice daily (~900 mg of herbal extract).

Scheme of trial formulations prepared

- Ashoka dry extract liquid oral formulation with sucrose and without preservatives
- Ashoka dry extract liquid oral formulation without sucrose and without preservatives

Formulation of Ashoka liquid with and without sucrose

Ashoka liquid was prepared using sucrose with concentration in the range of 60% to 75%w/w. The other ingredient was glycerin (20-25%), citric acid and sodium citrate were added to maintain a suitable pH. The sugar/sucrose-free Ashoka liquid was prepared using varying concentrations of glycerin, sorbitol and xylitol. The composition details are glycerin (20-77%), sorbitol (0-70%), xylitol (5-10%) and buffer to maintain the pH. The liquid orals were prepared as per the standard and well accepted methodology reported by Aulton and Taylor, 2013.

General procedure for formulation of preservative free Ashoka syrup with sucrose

An accurately weighed quantity of sucrose was dissolved in water to form a clear syrup. Weighed quantity of Ashoka dry extract was dispersed in water and heated at 70^{o}C in a clean stainless steel (SS) vessel and added to the sucrose syrup. A measured quantity of glycerin taken in a clean SS vessel was added under stirring for a few minutes.

The citric acid and sodium citrate were dissolved in water and added as per the Table 1 to the liquid mixture. The formulated liquid was filtered and made up to the final volume with purified water.

General procedure for formulation of preservative-free Ashoka syrup without sucrose

Accurately weighed quantity of Ashoka dry extract was dispersed in water and heated up to 70^{o}C in a clean SS vessel and added to a weighed quantity of glycerin heated to 80 to 85^{o}C. Xylitol was added under stirring and then the mixture was cooled to 50 to 55^{o}C; sorbitol and propylene glycol were added and stirred; and weighed quantity of citric acid and sodium citrate were solubilized in water and were added as per the Table 3. The mixture was filtered through Whatman no. 1 filter paper and made up to the final volume with purified water.

Measurement of water activity

Water activity (Aw) of Ashoka liquid syrup was measured using water activity analyser (Rotronic Ltd.).

Microbiological analysis

Microbiological characteristic of Ashoka oral formulation was studied by measuring parameters, such as, total aerobic microbial count (TAMC), total yeast and mould count (TYMC) and microbiological challenge test (MCT) using Pharmacopeial test.

Marker analysis for polyphenols and catechins

Estimation of total polyphenols

Spectrophotometric method

Samples of trial liquid formulations were subjected to estimation of total pholyphenols. The test solution was dissolved by sonication (for liquid oral). Extracted or dissolved sample was transferred to 250 ml volumetric flask and made up to 250 ml with water and filtered through Whatman No. 1 filter paper. Pyrogallol

from Himedia at 0.025 mg/ml concentration in water was used as standard.

Method

The analysis was performed by taking 2 ml of samples (test and standard) in a 25 ml volumetric flask and adding 1 ml of Folin & Ciocateeu's reagent (Loba chemie) (prepared by 1:1 dilution with water) followed by addition of 10 ml of water and diluted to 25 ml with 29% sodium carbonate. The absorbance was measured after 30 minutes at 760 nm using purified water as blank. The percentage of total polyphenols were then calculated.

Esmitmation of total catechins

High performance liquid chromatography (HPLC)

The sample of trial liquid formulations were subjected to HPLC to estimate total catechins (catechin and epicatechin). One gram sample was dissolved with methanol by sonication and made up to 100 ml with same solvent and filtered through 0.45 µ syringe filter. Catechin and Epicatechin from Chromadex at 0.1 mg/ml concentration with methanol were used as standards.

Gradient elution program	
Time (min)	**Percentage of solution B**
0.01	15
12	25
22	25
30	15

Solvent system

Solution A: 0.1% phosphoric acid in water, Solution B: acetonitrile with gradient elution program. HPLC(Shimadzu, Model: Prominence, LC-20 AD Pump, SIL-20 AC HT auto sampler, SPD-20 A UV-Vis detector, Column-C-18/Phenomenex Luna 5µ (Size 250x4.6 mm), Flow rate-1.0 ml/min and detection at UV 210 nm wavelength. The content of total catechins by sum of percentage catechin and percentage epicatechin.

Evaluation of herbal liquid oral formulation

Different parameters of the oral formulations were assessed, such as, pH, physical appearance (taste and odour), active marker estimations (polyphenol, catechins), specific gravity by refractometer reading (RMR) and water activity (Table 5). Stability studies of the Ashoka liquid formulation with water activity-lowering agents like AS-06, and AS-07 were performed as per International Conference on Harmonization (ICH) guidelines Q1A (R2) (Leistner, Undated). Accelerated stability studies at various time points were performed and results are tabulated.

Statistical analysis

All the values of stability studies were expressed as mean ± SD. The results were analyzed statistically using One-way ANOVA followed by post-hoc tests.

RESULTS

Preformulation studies

Ashoka soft extract was found to be compatible with all excipients used as per the drug–excipient compatibility study.

Formulation and optimization of Ashoka liquid formulation

Liquid oral formulation with various concentrations of excipients were prepared. An optimized formula with Aw-lowering excipient was chosen to evaluate the palatability and effectiveness in reducing the bioburden (evaluated through microbiological challenge test).

Comparative evaluation of Ashoka liquid formulation with sucrose and water activity-lowering agents

The detailed formulation composition of Ashoka oral liquid with sucrose is given in Table 1. The Ashoka trial liquid formulations with sucrose were evaluated for various physicochemical properties, such as, taste, specific gravity, pH, refractometer reading, Aw and microbiological attributes. Micro-biological attributes were evaluated using microbiological challenge test (MCT), total aerobic microbial count, total yeast and mould count and the results are presented in Table 2.

All the prepared batches of Ashoka oral liquid formulation with sucrose failed the microbial challenge test, and also showed high Awof above 0.785. The Aw values of Ashoka oral liquid formulation batches with sucrose are given in Figure1.

The details of various batches of Ashoka oral formulations prepared with Aw-lowering agents like sorbitol, xylitol, glycerin and propylene glycol in various proportions are given in Table3. The same Ashoka liquid trial batches were evaluated for different physicochemical properties and microbiological attributes as mentioned previously and presented in Table4.

The Aw of Ashoka liquid formulations prepared with Aw-lowering agentsare given in Figure2. The formulation batches coded as TR-9 and TR-11 showed the lowest Aw and were found to be the best amongst all the tested optimized formulations in terms of various parameters analysed andhence were selected for stability studies.

Stability studies

Inclusion of Aw-lowering agents in Ashoka liquid formulations TR-1 to TR-11 yielded almost similar beneficial effects with respect to key analytical parameters.As expected, the formulation TR1, TR3, TR5 and TR7 failed to pass the MCT. TR2, TR4, TR6, TR8, TR9, TR10 and TR-11 passed MCT, out of them TR-9 and TR-11was selected for stability studies considering their superiority in stress study, pH, Aw, texture and taste and were recoded/renamed as AS-06 and AS-07 and studied for its stability (Table 5).

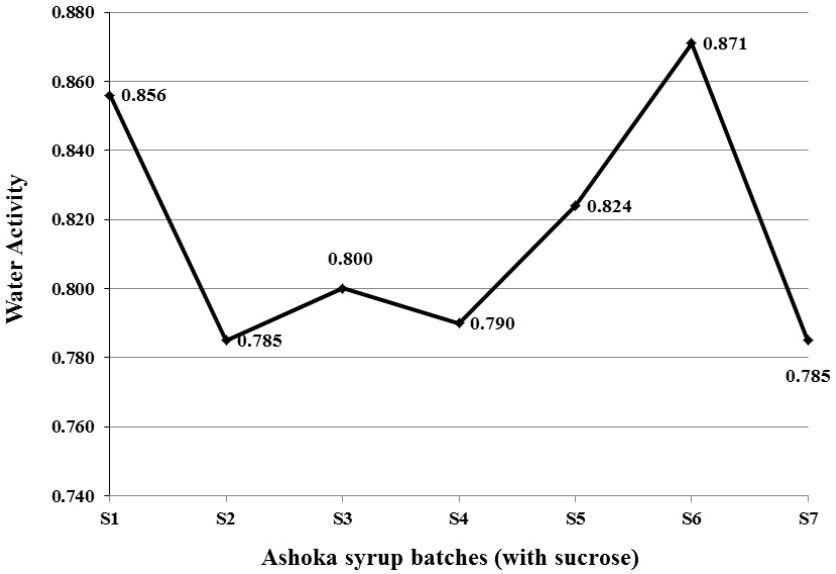

Fig. 1: Water activity of different batches of Ashoka syrup formulation formulated with sucrose Figure 2: Water activity of different batches of Ashoka liquid oral formulations prepared with water activity lowering agents)

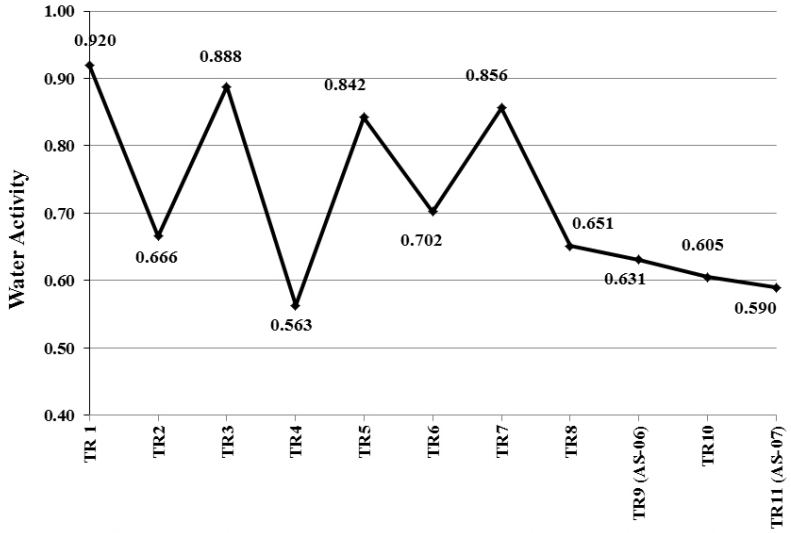

Fig. 2: Water activity of different batches of Ashoka liquid oral formulations prepared with water activity lowering agents)

Table 1: Composition of herbal preservative-free oral formulation containing Ashoka extract with sucrose.

Ingredients(% w/v)	S1	S2	S3	S4	S5	S6	S7
Ashoka dry extract	3	3	3	3	3	3	3
Sucrose	60.0	66.5	65.0	70.0	60.0	75.0	66.5
Glycerol	25	25	25	25	20	20	20
Citric acid monohydrate	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sodium citrate	0.05	0.5	0.05	0.05	0.05	0.05	0.05
Purified water				QS to make 100%			

Table 2: Physicochemical/microbiological parameters of different batches of Ashoka liquids(with sucrose) oral formulations.

Parameters	S1	S2	S3	S4	S5	S6	S7
Water Activity(Aw)	0.856	0.785	0.800	0.790	0.824	0.871	0.785
pH	03.98	04.00	03.80	3.85	03.88	03.86	03.60
MCT	Fails	Fails	Fails	Fails	Fails	Fails	Fails
RMR	59.14	67.82	65.79	67.70	65.78	70.70	59.93
Specific gravity (g/ml)	1.216	1.304	1.292	1.305	1.091	1.317	1.275
TAMC (cfu/g)	100	<10	<10	<10	100	<10	<10
TYMC (cfu/g)	<10	<10	<10	<10	<10	<10	<10

MCT: Microbiological challenge test; RMR: Refractive meter reading; TAMC: Total aerobic microbial count; TYMC: Total yeast and mould count.

Table 3: Composition for optimization of Ashoka liquid formualtion with water activity-lowering substances such as glycerin, sorbitol and xylitol

Ingredients	TR1	TR2	TR3	TR4	TR5	TR6	TR7	TR8	T 9(AS-06)	TR 10	TR 11 (AS-07)
Ashoka dry extract	3	3	3	3	3	3	3	3	3	3	3
Glycerol	10	55	10	70	10	45	10	40	40	60	60
Sorbitol 70% solution	16	16	45	16	45	30.5	16	20	20	16	16
Xylitol	5.00	20.00	5.00	5.00	20.00	12.50	20.00	21.75	21.75	8.75	8.75
Propylene glycol	-	-	-	-	-	-	-	-	10	-	10
Citric acid	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sodium citrate	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Purified water	QS to make100%										

Table 4: Physicochemical/microbiological parameters of different batches of Ashoka liquids with water activity-loweringsubstances such as glycerin, sorbitol and xylitol.

Para-meters	TR1	TR2	TR3	TR4	TR5	TR6	TR7	TR8	TR9 (AS-06)	TR10	TR11 (AS-07)
Taste	0	3	3	2	4	4	5	5	4	5	4
Water activity	0.920	0.666	0.888	0.563	0.842	0.702	0.856	0.651	0.631	0.605	0.590
pH	03.98	04.13	03.99	04.18	03.94	4.10	3.98	3.94	3.90	4.01	3.90
MCT	Fails	Complies	Fails	Complies	Fails	Complies	Fails	Complies	Complies	Complies	Complies
RMR	-	65.70	-	70.60	-	42.0	-	54.39	54.39	51.39	51.39
Specificgravity (g/ml)	-	1.180	-	1.017	-	1.190	-	1.060	1.060	1.220	1.260
TAMC (cfu/g)	-	<10	-	<10	-	<10	-	<10	<10	<10	<10
TYMC (cfu/g)	-	<10	-	<10	-	<10	-	<10	<10	<10	<10

MCT: Microbiological challenge test; RMR: Refractive meter reading; TAMC: Total aerobic microbial count; TYMC: Total yeast and mould count.

Table 5: Summary of accelerated stability studies of Ashoka liquid formulations.

Time of Interval		Initial		3rd Month/ 40°C 75%RH		6thMonth/ 40°C 75%RH	
Parameters	**Limits**	**AS-06**	**AS-07**	**AS-06**	**AS-07**	**AS-06**	**AS-07**
Description	Brown coloured syrup	Complies	Complies	Complies	Complies	Complies	Complies
pH	3.7 to 4.3	3.98 ± 0.03	4.00± 0.11	3.97± 0.05	4.02± 0.07	3.96± 0.04	3.93± 0.05
RMR	47 to 53	50.21± 0.09	50.09± 0.05	50.31± 0.29	50.27± 0.13	50.19± 0.05	50.28± 0.08
Water activity	0.57 to 0.65	0.63± 0.01	0.59± 0.01	0.63± 0.00	0.59± 0.01	0.64± 0.01	0.59± 0.01
Assay for total polyphenols(%w/w)	NLT 0.60	0.66± 0.01	0.68± 0.01	0.69± 0.01	0.66± 0.02	0.68± 0.01	0.67± 0.02
Assay for catechins(%w/w)	NLT0.027	0.03± 0.00	0.03± 0.00	0.03± 0.00	0.03± 0.00	0.03± 0.00	0.03± 0.00
TVMC(cfu/g)	NMT10^3	10.00± 0.00	10.00± 0.00	10.00± 0.00	10.00± 0.00	10.00± 0.00	10.00± 0.00
TYMC (cfu/g)	NMT 10^2	10.00± 0.00	56.67± 80.83	40.00± 51.96	10.00± 0.00	10.00± 0.00	10.00± 0.00
MCT	Complies	Complies	Complies	Complies	Complies	Complies	Complies

Abbreviations: RMR: Refractometer Reading, MCT: Microbial challenge test, cfu: Colony forming units,RH: Relative Humidity; NMT: Not more than; NLT: Not less than; TAMC: Total aerobic microbial count; TYMC: Total yeast and mould count

DISCUSSION

We have adopted an approach to develop aself-presserving, preservative-free Ashoka liquid formulation, which offers both chemical and microbiological stability. Aw of the formulation was taken into consideration to achieve our set objectives. Initially, two approaches were used, with and without sucrose by employing Aw reducing polyols. Ashoka liquid formulation with sucrose alone was ineffective to reduce the Aw below 0.77 and failed in MCT, where as in the second approach with different cosolvent systems, the desirable properties were obtained. The purpose of the study was to develop a preservative free Ashoka herbal liquid stable formulation by reducing water activity. After various trials and optimization the final shortlisted formulations had Aw less than 0.7. The mouth feel of these formulations were comparable to sucrose formulation, and also the formulations were stable. Ashoka liquid without sugar and with other solutes exhibited low water activity than with that of sugar. Product was found to be stable with water activity lowering agents which was proved by its intactness during stability studies.

According to Figure 1, Ashoka liquids with sucrose showeda Aw of above 0.785, revealing that more water is available in the formulation not only for biochemical reaction (casuing breakdown of macromolecules) but also for the proliferation of microorganisms like bacteria and fungi. These factors may result in the deterioration of formulation during its shelf-life, which was well demonstrated by the failure of these formulation in the MCT with respect to *Aspergillus niger* (Aw required for the growth of *A. niger* is 0.77). As per Figure 2, the Ashoka liquid formulations with solutes and Polyols replacing sugar, the Aw was found to be less than 0.700 indicating the addition of polyols such as glycol and glycerin resulted in the formulations with a lower water activity. Water activity is low due to addition of solutes which makes water to be in bound state

making it unavailable to the microorganisms and reduces the proliferation of microorganisms in the product(Martin and Linwoood, 1957; Beuchat, 1983).

Water activity, defined as "ratio of the vapor pressure of water in a material to the vapor pressure of pure water at the same temperature" plays an important role in the growth of microorganisms. Microorganisms requires a minimum water activity for its growth and when they are placed in an low water activity environment, the required unbound or free water is not available to them, causing osmotic stress resulting in the decrease in microbial growth, denaturation of its enzymes, sporulation and toxin production..

Theosmotic pressure of a solution is related to its Aw. The bacterial cell wall provides certain tolerance to the changes in the osmotic pressure of the external environment, by developing turgor pressure.

However, owing to the permeability of plasma membrane to the water, when microbes encounter hypotonic surroundings, water diffuses inside the cell. Osmotic lysis or cell rupture may happen if the cell is unable to develop the required counter pressure to prevent the water inflow. When the external environment encountered is hypertonic, which means external osmolarity is higher than within the cell, water moves out from the cells causing dehydration and plasmolysis resulting in cell death. The osmotic pressure tends to increase significantly in formulations with lower Aw, which explains the reason behind the self-preserving nature of liquid formulation with low Aw.

The assay values of all the formulation variants with respect to markers for herbal substances were found to comply well within the predefined limits, i.e., 95 to 105 % for both polyphenols and catechins to their initial values (Table 5).

Accelerated stability studies performed for 6 months as per ICH guidelines (ICH Harmonized Tripartite Guidelines, 2003) revealed that the Ashoka liquid oral formulation with Aw-lowering agents did not exhibit any physical, chemical and microbiological deterioration/change during the study period and that the active contents were found to be more than 95% at the end of 6 months of accelerated conditions.

Ashoka liquid formualtion with Aw-lowering agents not only reduced the bioburden to acceptable limits of pharmacopoeial standards, but also exhibited compliance with other parameters, such as, stability and quality of the product. The Ashoka oral liquid formulations prepared by the approach of reducing Aw exhibited stable behaviour even at accelerated stability conditions (6 months).

CONCLUSION

The present study showed design and development of stable formulation which is preservative free, Ashoka oral liquid formulation with combined effects of low pH, low Aw and osmotic changes. Our series of studies successfully yielded vehicle systems that could be used commercially for manufacturing stable preservative-free herbal liquid orals. An added advantage of such a system is that it is sugar-free and comes with a greater pharmacological/commercial interest in the contemporary formulation/pharmaceutical development.

Reduction in the Aw without compromising the organoleptic properties significantly reduced the deterioration of Ashoka liquid oral preparation during storage and thus, shelf-life was extended. The outcome of this study shows that using Aw-lowering agents in herbal liquid dosages is an effective, reliable and assured approach for microbial reduction and can be employed as a distinctive method for reducing bio-burden in the formulation. The approach becomes more relevant in the development of preservative-free formulations.

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