Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
https://www.phytojournal.com
JPP 2023; 12(1): 219-225
Received: 12-10-2022
Accepted: 21-12-2022

Charles Dorni A Irudaya
Vijayani Nutraceuticals Pvt. Ltd, Ambattur, Chennai, Tamil Nadu, India

Umamaheswari Venkatesan
Vijayani Nutraceuticals Pvt. Ltd, Ambattur, Chennai, Tamil Nadu, India

Rokesh Srinivasan
Vijayani Nutraceuticals Pvt. Ltd, Ambattur, Chennai, Tamil Nadu, India

Sridhar Moorthy
Vijayani Nutraceuticals Pvt. Ltd, Ambattur, Chennai, Tamil Nadu, India

Kamalesh Raja
Vijayani Nutraceuticals Pvt. Ltd, Ambattur, Chennai, Tamil Nadu, India

Corresponding Author:
Charles Dorni A Irudaya
Vijayani Nutraceuticals Pvt. Ltd, Ambattur, Chennai, Tamil Nadu, India

Evaluation of stability and determination of shelf life of an herbal food supplement: Naturovita

Charles Dorni A Irudaya, Umamaheswari Venkatesan, Rokesh Srinivasan, Sridhar Moorthy and Kamalesh Raja

DOI: https://doi.org/10.22271/phyto.2023.v12.i1c.14580

Abstract
Herbal formulations are easily prone to deterioration of the active compounds during storage over a period of time. The stability study helps in understanding the physical and phytochemical changes associated with natural product formulation. The present study was conducted to determine the shelf life of the herbal formula, Naturovita which is used as a food supplement to support respiratory health. The accelerated stability study (Temperature: 40 ºC ± 2 and relative humidity (RH): 75% ± 5) was conducted as per ICH guidelines. The change in the organoleptic, physico-chemical properties, proximate, active phytochemical content and microbial load were verified over a period of 6 months at an interval of 0th, 2nd, 4th and 6th month. The results from various analysis performed were taken in consideration for the determination of extrapolated shelf life of Naturovita. The extrapolated shelf-life value was calculated with 10% degradation of the physico-chemical parameters at an accelerated condition. The shelf life of Naturovita formula was found to be 24.08 months (2.01 years).

Keywords: Traditional herbal medicines, stability study, Naturovita, accelerated condition, shelf life

1. Introduction
Traditional herbal medicines have been used by around 80% of the world's population for the primary needs of healthcare [1]. The production and supply of herbal based food supplement products have increased in the market, particularly in developing countries. India currently contributes to less than 1% of the global herbal market. Nevertheless, it is quickly emerging as a significant global supplier of herbal based formulations [2]. The herbal products are considered safe as they are derived from plants [3]. The plants as a whole or their individual parts are subjected to various treatments such as extraction, purification, distillation, and active ingredient concentration for the herbal formulations [4]. People from different countries rely on herbal products as standalone food supplements or in combination with allopathic medicines. Herbal food supplements are mostly a combination of herbs with different constituents. The shelf life of herbal food supplements is a critical factor for their efficacy and purported health and nutritional benefits during long term storage. The stability study for the determination of the shelf life of herbal food supplement formula is a challenging task as the entire product with different herbal constituents is considered the active compound and the storage condition plays a key role in the evaluation [5].
Shelf life can be defined as the extent with which a supplement formula or product retains, within specified limits, throughout its storage period and use, the same properties and benefits that it possessed at the time of its manufacture [6]. The shelf life of a product can be determined by two different types of stability studies, an accelerated study and a real time study. Pharmaceutical products are generally subjected to accelerated stability testing; the interpretation of the result provides the shelf life of the product [7]. The shelf life of the product depends on various parameters, such as the organoleptic properties, physico-chemical parameters, active compounds, and microbial analysis. The quality, effectiveness, and shelf life of the herbal product depends on the presence and concentration of the active phytochemical compounds. Alterations in the bioactive content of an herbal medicinal product with a known therapeutic constituent should not, unless or otherwise justified, exceed ±5% of the initially proposed concentration [2].
The Naturovita product consists of two ingredients, namely Indian frankincense (*Boswellia serrata*) and *Spirulina* spp. (Blue-green algae). *Boswellia serrata* (Family: *Burseraceae*; Genus: *Boswellia*) is a branching tree of moderate to large size. It is found in the dry mountainous regions of India, the Middle East, and Northern Africa. In India, they are commonly known as Indian frankincense [8]. Monoterpenes such as α-thujene, diterpenes such

as incensole, incensole oxide, iso-incensole oxide, and serratol, triterpenes such as such as α- and β-amyrins, pentacyclic triterpenic acid such as boswellic acid, and tetracyclic triterpenic acids were all found in the resinous section of *Boswellia serrata* [8, 9]. Research studies performed *in vitro* with animal models revealed the inhibitory activity of boswellic acid against the synthesis of pro-inflammatory enzymes such as 5-lipoxygenase (5-LO), including 5-hydroxyeicosatetraenoic acid (5-HETE), and leukotriene B4 (LTB-4), which results in increased vascular permeability, chemotaxis, and bronchoconstriction [10-12].

Arthrospira platensis (Spirulina), which belongs to the family *Oscillatoriaceae* has been in use for decades as a source of proteins and vitamins [13]. Among other species, *Spirulina platensis* and *Spirulina maxima* are the most widely used species in the field of medicine and the food industry [14]. Apart from its protein and vitamins, Spirulina is also rich in terms of essential amino and fatty acids, minerals, and antioxidant contents [15]. In recent years, spirulina has taken a huge leap in terms of research studies. These studies reveal numerous health benefits of spirulina, such as anti-inflammatory, antioxidant, immunomodulatory, antiviral, antibacterial, and anticancer activities, and also shows positive attributes against diabetes, obesity, anemia and malnutrition [16, 17]. By controlling important cytokines like interleukin [IL-1, IL-2, IL-4, IL-6, IL-10], and tumor necrosis factor (TNF), spirulina also has a variety of immunomodulatory and anti-inflammatory effects [18-20]. The current study was conducted under accelerated conditions to determine the shelf life of the product Naturovita.

2. Materials and Methods

2.1 Test Product Details

Naturovita is a food supplement formulation, which comprises dried powders of two well-known herbs, Indian frankincense (*Boswellia serrata)* and Spirulina (*Arthrospira platensis*) in equal proportion in a capsule form. Each 120 ml polypropylene container contains 60 capsules. The average capsule weight of the product is 575.0- 625.0 mg. The capsules used are of vegetable origin and 000 size. Three different batches of Naturovita capsules were used to determine the shelf life of the product by an accelerated stability study conducted as per the ICH guidelines. (Batch Number: B-1: VNL 21-053; B-2: VNL 21-090; B-3: VNL 21-104).

2.2 Storage condition and evaluation parameters

The accelerated stability study was conducted as per the ICH guidelines Q1 A (R2) in the stability chamber (Remi Elektrotechnik Ltd, Model: SC-35 PLUS). Storage conditions were: Temperature: 40ºC ± 2 and Relative Humidity (RH): 75% ± 5. The change was observed for 6 months at a study interval of 0^{th}, 2^{nd}, 4^{th} and 6^{th} months. A 10% degradation was set to extrapolate the accelerated stability study as the acceptable point.

The following parameters were considered for evaluation of the stability study.

- Organoleptic properties such as appearance, colour, taste and odour.
- Physico-chemical parameters such as weight variation, disintegration time, pH, water soluble extractive and alcohol soluble extractive.
- Proximate content such as moisture, ash, protein, fat, carbohydrate and energy
- Active ingredient content and microbial load.

2.3 Evaluation of Organoleptic properties

The organoleptic properties such as the appearance, color, taste and odor were examined by well-trained panelists. The formulated product Naturovita was taken in a watch glass and were placed under the white background for observation of the appearance and color. The odor and waste were examined by smelling and placing the sample on the taste buds of the tongue.

2.4 Determination of disintegration time test

The disintegration time test was performed by using a digital microprocessor-based disintegration apparatus (Lab India, Model: Tablet Disintegration Tester DT 1000). One capsule was placed in each tube, a disc was added to each tube of the basket, and the apparatus was operated using water as the release medium maintained at 37 ± 2 °C. The setup was suspended in water in a 1000 ml beaker. The capsules were observed, and the time taken for complete disintegration of the capsules was determined [21].

2.5 Determination of pH

1 g of the formulated sample was weighed and placed in a beaker. The sample was mixed with 100 ml of distilled water. The solution was sonicated for about 10 minutes to achieve complete solubility of the sample. The pH of the solution was measured with the help of calibrated digital pH meter.

2.6 Determination of Alcohol-soluble Extractive

About 5 g of the product powder was macerated with 100 ml of ethanol in a closed flask for 24 hours, shaking frequently during 6 hours and allowed to stand for 18 h. After 24 h the solution was filtered and around 25 ml of the filtrate was transferred to a tared flat bottom evaporating plate and evaporated to dryness on a boiling water bath. The sample was dried at 105ºC, and then cooled and weighed [22]. The percentage of alcohol soluble extractive was calculated from the weight of the residue and were expressed in terms of percentage.

$$\text{Alcohol Soluble Extractive\% (w/w)} = [(W_f \times V_s \times 100) / (W_i \times V_f)]$$

Whereas,
W_i denotes the initial weight of the sample
V_f denotes the volume of filtrate taken
W_f denotes the final weight of the sample
V_s denotes the volume of the alcohol taken for soaking

2.7 Determination of Water-soluble Extractive

About 5 g of the product powder was macerated with 100 ml of Chloroform water (0.25 ml in 100 ml of distilled water) for 24 hours, shaking frequently during 6 hours and allowed to stand for 18 hours. After 24 h the solution was filtered and around 25 ml of the filtrate was transferred to a tared flat bottom evaporating plate and evaporated to dryness on a boiling water bath. The sample was dried at 105ºC, and then cooled and weighed [22]. The percentage of water-soluble extractive was calculated from the weight of the residue and were expressed in terms of percentage.

$$\text{Water Soluble Extractive\% (w/w)} = [(W_f \times V_s \times 100) / (W_i \times V_s)]$$

Whereas,
W_i denotes the initial weight of the sample

V_f denotes the volume of filtrate taken
W_f denotes the final weight of the sample
V_s denotes the volume of the alcohol taken for soaking

3. Proximate analysis

3.1 Moisture content

5 g of the product powder (Wi) was weighed and placed in a pre-weighed moisture box. The initial weight of the sample was recorded as W_i. The moisture box with a sample box was placed in a preheated hot air oven for 3 hours at 105 ± 2°C. The moisture box was cooled by placing in the desiccator. The final weight of the sample was measured and recorded as W_f [23]. The moisture content was calculated by using the formulae.

Moisture (%) = [{(W_i-W_f)/W_i)} *100]

3.2 Ash content

2g of the product powder (Wi) was weighed and placed in a pre-weighed crucible. The initial weight of the sample was recorded as W_i. the crucible containing the sample was placed in a muffle furnace for a period of 12 hours at 550 ± 25 °C. The sample was cooled and the final weight of the sample was recorded as W_f [23]. The ash content was calculated by using the formulae.
Ash (%) = [{(W_i-W_f)/W_i)} *100]

3.3 Protein content

The estimation of protein was carried out by Kjeldhal method using a semi-automated digestion and Kjeldhal distillation unit. About 0.2 g of product powder is digested with 40 g of K_2SO_4, 4 g of $CuSO_4$ and 10 ml of conc. H_2SO_4. The digestion is initiated by maintaining a temperature of about 250 °C. The protein digestor (Pelican Equipments, Model: Kelvac) programmed at 3 segments (250 °C for 10 mins, 350°C for 10 mins and 410°C for 90 mins). The sample undergoes color change from blue to green at the end of the digestion. The distillation (Pelican Equipments, Model: Classic-DX VA) starts with addition of 30 ml of water to the sample and the solution is made alkali with 40% NaOH. The liberated ammonia is absorbed in the solutions of 4% boric acid and the nitrogen contents are determined by titrating with 0.1 N HCL solution [24]. The percentage of protein (on dry weight basis) is determined by using the formulae:

$$\text{Protein, \% (w/w)} = \frac{14.01 \times N \times (T_S - T_B) \times 6.25 \times 100 \times 100}{W_S \times 1000 \times TS}$$

Whereas
T_S denotes the volume (ml) of 0.1 N HCl used for sample titration
T_B denotes the volume (ml) of 0.1 N HCl used for blank titration
N denotes the Normality of HCl
W_S denotes the weight (g) of the sample
14.01 denotes the atomic weight of Nitrogen
6.25 denotes the Protein – Nitrogen conversion factor for food and feed
TS denotes the Total solids (100 -% Moisture content)

3.4 Fat content

The fat content was determined by performing Soxhlet extraction using a semi-automated Soxhlet Apparatus (Pelican Equipments, Model: SCS-6). About 2 g of the product powder was weighed (W_S) and placed into an extraction thimble. The thimble was placed in a beaker to which 80 ml of petroleum ether was added and the setup is fixed on a heating mantle. The extraction of the sample in the Soxhlet apparatus is initiated with an already programmed segment (100°C – 90 mins and 160°C- 20 mins). The beaker containing the sample is dried at 80-90°C until complete drying is achieved. The final weight of the sample in the beaker is weighed and recorded as W_2 [25]. The percentage of fat (on dry weight basis) is determined by using the formulae:

$$\text{Fat, \% (w/w)} = \frac{(W_2 - W_1)}{W_S} \times \frac{100}{TS} \times 100$$

Whereas,
W_1 denotes the initial weight of the beaker (g)
W_2 denotes the final weight of the beaker (g)
W_S denotes the weight of the sample (g)
TS denotes the Total solids (100 -% Moisture content)

3.5 Carbohydrate and Energy

The percentage of carbohydrate content was estimated by using "By difference" method and Kcal value of energy were calculated by using the below given formulas [26].
Total Carbohydrate content (%) (w/w) = 100 – [% of (Moisture+ Ash+ Fat+ Protein)]
Energy (Kcal) = (4 ×% Carbohydrate) + (4 ×% Protein) + (9 ×% Fat)
The values 4, 4 and 9 used in the formula for energy calculation are the general factors for carbohydrate, protein and fat in the sample.

3.6 Estimation of Active compound (Boswellic acid)

The HPLC system (Shimadzu-Prominence), consisting of pump (LC-20AD), UV detector (SPD-20 A), autosampler (SIL-20AC) with Column Solar C-18 (250 × 4.6 mm ID, 5 µm) was used. The flow rate (1 ml/min), sample injection of 10 µL and the oven temperature (40ºC) were kept standard for the sample and the standards. For boswellic acid, mobile phase consisted of 0.1% acetic acid and acetonitrile (15: 85 v/v) was used. 20 mg of the sample was weighed and transferred in to a 10 ml volumetric flask, to which 6 ml of methanol was added and allowed to sonicate for 30 mins. The solution was cooled to room temperature and filtered using 0.45 µm nylon syringe filter [27].

3.7 Microbial Load

The microbial load was carried out as per standard procedure mentioned in US Pharmacopoeia [28]. This includes Total bacterial plate count, yeast and molds and presence of Staphylococcus *aureus*, *Escherichia coli* and *Salmonella spp*. and verified as per the limits of FSSAI Nutraceutical & Food Supplement regulations, 2022.

3.8 Determination of Shelf Life

The parameters considered for the evaluation of the shelf life are pH, water soluble extract, alcohol soluble extract, moisture, ash and active ingredient. The data obtained from the three batches at the interval of 0^{th}, 2^{nd}, 4^{th} and 6^{th} months were compiled and the average value has been calculated for these parameters. The data graphs are plotted with respect to the four time points for each parameter separately to evaluate the slope and the intercept.
The shelf-life was calculated with 10% degradation of these parameters. The number of months taken for 10% degradation to occur was calculated using the following formula [7].

$$Months\ when\ 10\%\ degaradtion\ occurs = \frac{\left[\left[0th\ month\ assay\ value - \left\{\frac{0th\ month\ assay\ value * 10}{100}\right\}\right] - Intercept\right]}{Slope}$$

Using the formula, the shelf life of each parameter was calculated and the average shelf life of the product was obtained. The obtained shelf life of the product was extrapolated using the real time aging factor 3.3 for climate zone III & IV countries (India) [29].

4. Results and Discussion

In the accelerated stability study conducted for Naturovita, an herbal food supplement, temperature: 40 ºC ± 2 and relative humidity (RH): 75% ± 5 were maintained for 6 months in a calibrated stability chamber and the product batches were withdrawn and analyzed during 0th, 2nd, 4th and 6th month intervals. There is no significant change was noticed in the organoleptic properties such as the appearance, color, taste and odor of the Naturovita product up to the 6th month (Table 1). The change in color and appearance of Naturovita upto the 6th month were illustrated in Figure 1. The results of the analysis of physio-chemical parameters were represented in the table.1. The results of the proximate, active compounds at accelerated condition showed the stability of the product Naturovita up to the 6th month are provided in Table 2. The results of microbial load of Naturovita at accelerated condition were represented in Table. 3.

Table 1: Organoleptic properties and Physico-chemical properties of Naturoiva.

Test Parameters	Batch	0th Month	2nd Month	4th Month	6th Month	Criteria	Result
Organoleptic Properties							
Appearance	B-1	Red coloured capsules	Red coloured capsules	Red coloured capsules	Red coloured capsules	No significant change	No significant change
	B-2	Red coloured capsules	Red coloured capsules	Red coloured capsules	Red coloured capsules		
	B-3	Red coloured capsules	Red coloured capsules	Red coloured capsules	Red coloured capsules		
Colour	B-1	Green colour	Green colour	Green colour	Green colour	No significant change	No significant change
	B-2	Green colour	Green colour	Green colour	Green colour		
	B-3	Green colour	Green colour	Green colour	Green colour		
Taste	B-1	Characteristic	Characteristic	Characteristic	Characteristic	No significant change	No significant change
	B-2	Characteristic	Characteristic	Characteristic	Characteristic		
	B-3	Characteristic	Characteristic	Characteristic	Characteristic		
Odour	B-1	Characteristic	Characteristic	Characteristic	Characteristic	No significant change	No significant change
	B-2	Characteristic	Characteristic	Characteristic	Characteristic		
	B-3	Characteristic	Characteristic	Characteristic	Characteristic		
Physico-Chemical Parameters							
Disintegration Time (Min.)	B-1	20.00	14.20	10.00	7.31	Not more than 30.0 mins	Complies
	B-2	20.00	14.20	10.50	8.54		
	B-3	20.00	14.20	10.50	10.22		
pH	B-1	5.26	4.81	5.16	4.98	3.0 – 5.0	Complies
	B-2	5.42	4.60	5.09	5.06		
	B-3	5.38	5.31	4.36	4.23		
Water Soluble Extractive (%)	B-1	55.13	49.37	54.71	53.77	± 25.0%	Complies
	B-2	49.08	49.35	48.35	44.75		
	B-3	55.44	49.96	45.73	44.27		
Alcohol Soluble Extractive (%)	B-1	17.14	15.51	15.56	16.22	± 25.0%	Complies
	B-2	18.09	17.35	17.35	15.34		
	B-3	23.54	16.70	16.39	13.70		

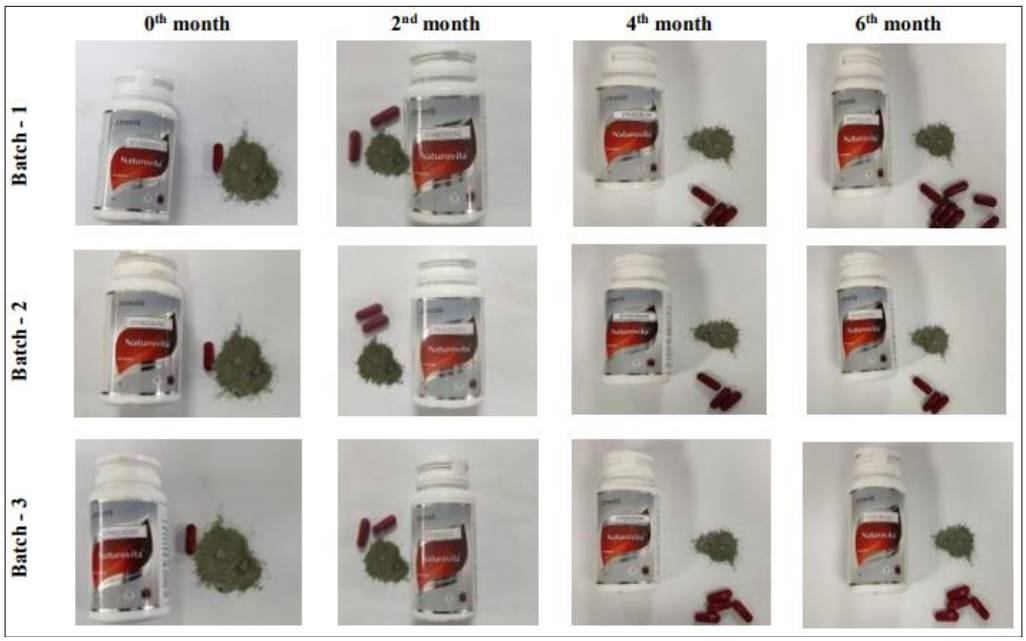

Fig 1: Monitoring of change in the color and appearance of Naturovita up to the 6th month of study

Table 2: Proximates and active ingredient of Naturovita

Test Parameters	Batch	0th Month	2nd Month	4th Month	6th Month	Criteria	Result
Proximate							
Moisture (%)	B-1	5.03	5.46	5.67	6.23	Not more than 5.0%	Complies
	B-2	4.97	5.62	5.81	6.37		
	B-3	3.82	4.74	5.04	5.30		
Ash (%)	B-1	5.96	6.28	5.97	5.77	± 25.0%	Complies
	B-2	5.34	5.45	5.39	5.32		
	B-3	5.63	5.92	5.60	5.41		
Protein (%)	B-1	29.74	25.97	32.14	32.50	± 25.0%	Complies
	B-2	30.74	20.71	32.98	32.20		
	B-3	31.46	19.13	33.07	31.70		
Fat (%)	B-1	4.52	5.22	4.88	4.20	± 25.0%	Complies
	B-2	4.84	5.42	5.18	4.42		
	B-3	7.38	8.38	6.37	6.80		
Carbohydrate (%)	B-1	54.75	57.07	51.34	51.30	± 25.0%	Complies
	B-2	54.11	62.80	50.64	51.69		
	B-3	51.71	61.83	49.92	50.79		
Energy (Kcal/100 g)	B-1	378.64	379.14	377.84	373.00	± 25.0%	Complies
	B-2	382.96	382.82	381.1	375.34		
	B-3	399.10	399.26	389.29	391.16		
Active Compound							
Boswellic acid (%)	B-1	66.00	65.93	65.54	64.61	± 15.0%	Complies
	B-2	64.00	65.00	64.88	62.63		
	B-3	69.10	69.30	64.55	62.56		

Table 3: Microbial load of Naturovita

Test Parameters	Batch	0th Month	2nd Month	4th Month	6th Month	Criteria	Result
Total Plate Count (CFU/g)	B-1	< 10	-	-	< 10	1 X 10^5 / g	Complies
	B-2	< 10	-	-	< 10		
	B-3	< 10	-	-	< 10		
Yeast & Moulds (CFU/g)	B-1	< 10	-	-	< 10	1 X 10^3 / g	Complies
	B-2	< 10	-	-	< 10		
	B-3	< 10	-	-	< 10		
Staphylococcus (per g)	B-1	Absent	-	-	Absent	Absent	Complies
	B-2	Absent	-	-	Absent		
	B-3	Absent	-	-	Absent		
E. coli (per g)	B-1	Absent	-	-	Absent	Absent	Complies
	B-2	Absent	-	-	Absent		
	B-3	Absent	-	-	Absent		
Salmonella (per 25 g)	B-1	Absent	-	-	Absent	Absent	Complies
	B-2	Absent	-	-	Absent		
	B-3	Absent	-	-	Absent		

The results of the different physico-chemical parameters and the active compound was taken into consideration to evaluate the intercept and slope (Table 4). The extrapolated shelf life of Naturovita was calculated with 10% degradation at accelerated condition 40 ºC ± 2 and Relative Humidity (RH): 75% ± 5 (Table 5).

Table 4: The intercept and slope of different physico-chemical parameters and active compound of Naturovita

Intercept and slope of different physico-chemical parameters						
Parameters	**0th month**	**2nd month**	**4th month**	**6th month**	**Intercept**	**Slope**
pH	5.35	4.91	4.87	4.76	5.2457	-0.0913
Water Soluble Extractive (%)	53.22	49.56	49.6	47.6	52.516	-0.8412
Alcohol Soluble Extractive (%)	19.59	16.52	16.43	15.09	18.947	-0.6798
Moisture (%)	4.61	5.27	5.51	5.97	4.6913	0.2157
Total Ash (%)	5.64	5.88	5.65	5.5	5.769	-0.033
Active Ingredient (Boswellic acid) (mg/capsule)	66.37	66.74	64.99	63.27	67	-0.5527

Table 5: Extrapolated shelf life of Naturovita from different physico-chemical parameters and active compound

Stability data @ 40 °C ± 2 and 75% ± 5 RH						
Parameters	**0th month**	**10% of 0th month**	**At 10% degradation**	**Intercept**	**Slope**	**Months at 10% degradation**
pH	5.35	0.535	4.815	5.2457	-0.0913	4.72
Water Soluble Extractive	53.22	5.322	47.898	52.516	-0.8412	5.49
Alcohol Soluble Extractive	19.59	1.959	17.631	18.947	-0.6798	1.94
Moisture	4.61	0.461	4.149	4.6913	0.2157	-2.51
Total Ash	5.64	0.564	5.076	5.769	-0.033	21.00

Active Ingredient (Boswellic acid)	66.37	6.637	59.733	67	-0.5527	13.15
Average mean (Months)			7.30			
Extrapolated value (Months)			24.08			
Shelf life (Years)			2.01			

5. Conclusion

The goal of the present stability study is to verify and determine the product shelf life with respect to the specification limits of parameters such as its organoleptic, physio-chemical characteristics, proximate and microbial load. The main industrial purpose behind determining the shelf life of the product is to claim the expiration period in the label and also to ensure the quality and efficacy of the food supplement product, Naturovita. The stability study for any product should be designed based on the knowledge of the behavior and characteristics of the drug substance and the dosage form used [30].

On the basis of the data attained from the accelerated study, the shelf life of the product Naturovita is determined to be 24.08 months (2.01 years) for the countries under the climatic zone III & IV (India) based on the ICH guidelines [29]. It has been calculated with the consideration of 10% degradation rate for the objective physico chemical parameters and active ingredient. No significant change has been observed in the organoleptic, physiochemical properties and the microbial load of the product till the 6th month of the accelerated study which confirms the guaranteed shelf life of minimum 2 years for the food supplement product, naturovita

6. Acknowledgment

The authors wish to sincerely thank the Directors of Vijayani Nutraceuticals Pvt. Ltd Chennai- Mr. Karthikeyan. P (CEO), Mr. Moulishankar. S., (COO) and Mr. Suman S, (CFO) for providing us the laboratory facilities necessary to carry out this study.

7. Conflicts of Interest

The authors declare that they have no conflicts of interest.

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