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Original Research Article (Experimental)

Bhallatakadi Ghrita: Development and evaluation with reference to *Murcchana* and *Shata-Dhauta* process

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ABSTRACT

Background: Ayurveda is primarily based upon use of herbs either singly or in combination (polyherbal). The cow ghee (clarified butterfat) is considered as a precious base for preparing medicines in Ayurveda. Processing of ghee with plant ingredients is renowned for enhancing their therapeutic efficacy.
Objective: In present research work, the attempt was made to develop cow ghee based Polyherbal *Bhallatakadi Ghrita* formulations and evaluate them with reference to '*Murcchana*' and '*Shata-Dhauta*' process.
Materials and methods: The research plants were identified, procured, authenticated and processed. The extracts of plant materials were prepared and used for development of Polyherbal *Bhallatakadi Ghrita* (PHBG), Polyherbal *Bhallatakadi Murcchita Ghrita* and Polyherbal *Bhallatakadi Shata-Dhauta Ghrita* formulations as per Ayurvedic procedures. The prepared *ghrita* formulations were subjected to organoleptic (colour, odour, taste, appearance and touch), physicochemical (pH, viscosity, moisture content, specific gravity, refractive index, acid value, saponification value, iodine value, peroxide value, Rechert Meissl value and Polenske value) evaluation, in-vitro antioxidant and GC-MS analysis. The accelerated and real time stability studies were carried out to determine shelf life of *ghrita* formulations.
Results: The results of evaluations indicate that, developed PHBG formulations retained the organoleptic and physicochemical characteristics of ghee. The shelf life of formulations was found to be in the range of 1.6 to 3.3 years at accelerated and 2.2 to 3.8 years at real time stability conditions. All *ghrita* formulations exhibited antioxidant activity in dose dependent manner.
Conclusion: The standardization or evaluation of Polyherbal *Bhallatakadi Ghrita* formulations was found to be crucial for the establishment of a steady biological, chemical or simply a quality assurance profile of the drugs.

1. Introduction

Historically, in 1300 A.D. the '*Sarangdhar Samhita*' has highlighted the concept of polyherbalism in Ayurvedic medicinal system, based upon therapeutic herbs either singly or in combination [1,2]. The active phyto-constituents of individual plants, generally present in minute amount, are insufficient to achieve the desirable therapeutic effects. The certain biological actions of active phytochemicals are substantial, only when potentiated by that of other plants, but not apparent when used alone [2]. In a polyherbal formulation (PHF), herbal ingredients may increase the potency of the formulation with reduced unwanted effects and make the formulation more palatable. Besides, it brings better patient compliance and therapeutic effect by eliminating the need of taking more than one different single herbal formulation at a time [3].

The word 'Ghee' is evolved from old *Sanskrit* word '*ghr*' (means bright or to make bright), usually prepared from cow, buffalo or mixed milk [4,5]. Because of unique ability to reach within the deepest tissues, ghee is considered as an ideal base for preparation of Ayurvedic formulations to target the specific body organs. The '*Ghrita*', also known as medicated ghee is the Ayurvedic medicinal preparation in which ghee is processed with some herbal decoctions and fresh paste of herbs, selected as per the formula mentioned in the Ayurvedic texts or Ayurvedic formulary of India

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[6,7]. Cow ghee (because of regenerative properties and promoting ability of growth of healthy cells) is generally prescribed for topical application for the treatment of wounds caused by heat or fire, painful ulcers, insect bites, herpes and leprosy [8].

In Ayurveda, '*Murcchana' samskara* (fat processing) is assumed as one of the crucial step in *ghrita* preparation entailing the use of '*Murcchita*' ghee i.e. ghee prepared with incorporation of *Murcchana* herbs (*Emblica officinalis* (Euphorbiaceae) fruits, *Cyperus rotundus* (Cyperaceae) rhizomes, *Curcuma longa* (Zingiberaceae) rhizomes, *Terminalia chebula* (Combretaceae) fruits and *Terminalia bellirica* (Combretaceae) fruits and juice of *Citrus medicus* in equal proportion in place of plain cow ghee. In some Ayurvedic scripts it is mentioned that before making any *ghritapaka* (*ghrita* preparation), ghee should undergo '*Murcchana*' *samskara* to enhance the medicinal potency of a ghrita and to get rid of bad odour and rancidity [9,10]. In our earlier studies, we have evidenced the effect of '*Murcchana'* process to ensure maximum acceptability, stability and better shelf life of ghrita preparation [11]. '*Shata-Dhauta'* is a process involves washing of purified ghee one hundred times with water which increases stability of ghrita and makes it elegant and suitable product for topical application [12].

Semecarpus anacardium L., *Argemone mexicana* L., *Cocculus hirsutus* L. and *Woodfordia fruticosa* K. are reported for their wound healing potential by the tribal or local community in traditional and folk medicinal practices [13–20]. The cow ghee increases the potency of certain herbal ingredients by carrying the lipid soluble active components to the interior of the cell [9]. Therefore, in present research work, the attempt was made to prepare cow ghee based polyherbal formulation of plant/herbal ingredients having wound healing properties. It was also aimed to study the effect of '*Murcchana'* and '*Shata-Dhauta'* processes on ghrita preparations and evaluate them on organoleptic, physicochemical and stability grounds for estimation of shelf life.

2. Materials and methods

2.1. Procurement, authentication and processing of plant materials

Research plants/herbal ingredients viz. *S. anacardium* L. (Anacardiaceae) (Bibba/Bhallataka) (fruits and leaves), *A. mexicana* L. (Papaveraceae) (Firangi Dhotara) (whole plant), *C. hirsutus* L. (Menispermaceae) (Vasanvel) (whole plant) and *W. fruticosa* Kurz. (Lytheraceae) (Dhatki) (leaves and flowers) were collected from western region of Maharashtra, India and deposited to Botanical Survey of India (B.S.I.), Pune, Maharashtra, India for identification and authentication (Reference number BSI/WRC/Tech./2013/SND-1 Dated 06/12/2013; BSI/WRC/Tech./2013/JRB-01 Dated 27/11/2013; BSI/WRC/Tech./2013/GVG-01 Dated 31/12/2013; BSI/WRC/Tech./2013/GG-01 Dated 31/12/2013). The identified and authenticated plant materials were processed to remove adhered dirt and toxic components [7,21], dried in shade and pulverized to get coarse powder (passed through sieve no. 40 and retained on sieve no. 60) [22–24] and then stored in airtight containers separately in crude form until it was used.

2.2. Development of polyherbal Ghrita formulation

2.2.1. Procurement of cow ghee

The authentic cow ghee for the preparation of polyherbal ghrita formulations was procured from '*Govidyan Anusandhan Research Centre*', Deodapar, Nagpur, Maharashtra, India and stored in glass container in cool and dry place and away from light until it was used for further studies.

2.2.2. Preparation of plant extracts

The powdered plant materials were defatted for 2 h with petroleum ether (60–80 °C) in the soxhlet apparatus. The defatted plant materials were then air-dried, repacked in the soxhlet apparatus, and then extracted with alcohol [22–24]. The alcoholic extracts of research plants thus obtained were concentrated and stored in separate amber coloured containers until used for preparation of ghrita formulations.

2.2.3. Preparation of polyherbal Ghrita formulation

The concentrated extracts (*Kalka*) thus obtained were used for preparation of polyherbal *ghrita* formulation. Polyherbal *ghrita* formulation was prepared as per the standard Ayurvedic procedure of '*Ghrita Paka Kalpana*' [7,10]. The quantities of ingredients were calculated as per the Ayurvedic texts. Briefly, the stated quantity of ghee (*Sneha Dravya*) was poured in a large stainless steel vessel and allowed to melt under moderate flame. Further, the plant extracts (*Kalka*) in equal proportion, water (*Drava Dravya*) and molten ghee (*Sneha Dravya*) were combined in specified ratio of 1:16:4 respectively in same vessel and boiling was initiated till the complete evaporation of moisture and appearance of characteristic features of ghrita. The whole process of '*Ghrita Paka Kalpana*' was carried out on mild to moderate flame and continued until '*Sneha Siddhi Lakshana*' was obtained. The '*Sneha Siddhi Lakshana*' was characterized by burning of paste (*Varti*) without crackling sounds and disappearance of froth (*Phena*) in ghrita. The ghrita thus prepared was named as *'Polyherbal Bhallatakadi Ghrita'* and denoted as PHBG-I.

2.3. Optimization of 'Polyherbal Bhallatakadi Ghrita' formulation

To increase therapeutic quality, purity, efficacy and stability/shelf life, the prepared PHBG-I was optimized by processing with '*Murcchana*' and '*Shata-Dhauta*' *samskara* as per the ancient Ayurvedic procedures.

2.3.1. Preparation of 'Murcchita' ghee

The '*Murcchana' samskara* of ghee was carried out in a preliminary step and before '*Ghrita Paka Kalpana*'. *Murcchita* ghee was prepared as per the procedure described in reference texts [7,10]. Briefly, initially specified amount of plain cow ghee (768 g) was melted in a vessel with moderate heating. Coarsely powdered *Murcchana* herbs; pericarp of fruits of *haritaki* (*T. chebula* Retz., Combretaceae, 48 g), *amlaki* (*E. officinalis* Gaertn., Euphorbiaceae, 48 g) and *bibhitaki* (*T. bellirica* Roxb., Combretaceae, 48 g); rhizomes of *musta* (*C. rotundus* Linn., Cyperaceae, 48 g), *haridra* (*C. longa* Linn., Zingiberaceae, 48 g) were mixed and was ground with *matulunga* swarasa (*Citrus medica* var. *acidica*, 48 g) to form a smooth paste (*Kalka*). The *kalka* was added to the molten ghee along with water (3.072 L) and boiled on slow fire till complete evaporation of water. It was further strained through muslin cloth and stored in a well closed container.

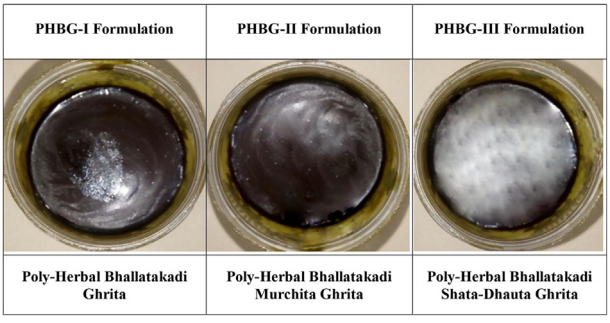

Fig. 1. - Prepared Poly-Herbal *Bhallatakadi Ghrita* (PHBG) formulations.

Table 1
In-vitro antioxidant evaluation of plain ghee and Polyherbal *Bhallatakadi Ghrita* (PHBG) formulations.

IC $_{50}$ Values ($\mu g.mL^{-1}$)					
Method	Plain *ghee*	PHBG-I	PHBG-II	PHBG-III	Ascorbic Acid
DPPH Method	38.72 ± 0.72 (0.9812)	19.56 ± 1.02 (0.9712)	18.02 ± 1.21 (0.9782)	18.32 ± 0.98 (0.9761)	13.82 ± 0.42 (0.9812)
NO Method	39.32 ± 1.34 (0.9842)	22.34 ± 1.23 (0.9831)	22.11 ± 1.08 (0.9723)	21.24 ± 0.78 (0.9672)	18.11 ± 0.52 (0.9762)
H_2O_2 Method	41.21 ± 1.22 (0.9791)	26.09 ± 1.63 (0.9843)	24.09 ± 0.92 (0.9803)	24.61 ± 1.11 (0.9733)	21.321 ± 0.43 (0.9733)

Where; PHBG-I- Polyherbal *Bhallatakadi Ghrita*, PHBG-II: Polyherbal *Bhallatakadi Murcchita* Ghrita, PHBG-III: Polyherbal *Bhallatakadi Shata-Dhauta* Ghrita.
All values are mean of three replications and expressed as Mean ± SEM, Values in bracket indicate r* i.e. regression coefficient.

2.3.2. Preparation of 'Polyherbal Bhallatakadi Murcchita Ghrita' formulation

'Polyherbal Bhallatakadi Murcchita Ghrita' was prepared by using '*Murcchita*' ghee instead of plain ghee following the same procedure [7,10] and denoted as PHBG-II.

2.3.3. Preparation of 'Shata-Dhauta' ghee

The '*Shata-Dhauta*' ghee was prepared by using reported and ancient procedure mentioned in Ayurvedic text [7,10]. Briefly, the mixture of specified quantity of cow ghee (2.5 kg) and distilled water (1.5 L) was triturated for 5–8min in previously cleaned copper vessel with the help of laboratory agitator (REMI). Thereafter the content of vessel was allowed to settle and water was decanted carefully to avoid loss of ghee. The fresh slot of same quantity (1.5 L) of distilled water was added in previously washed cow ghee and similar procedure was repeated for one hundred times to obtain '*Shata-Dhauta*' ghee which was further stored in a well closed container.

2.3.4. Preparation of 'polyherbal Bhallatakadi Shata-Dhauta Ghrita' formulation

The *'Shata-Dhauta'* ghee thus obtained was used, instead of plain ghee for preparation of *'Polyherbal Bhallatakadi Shata-Dhauta Ghrita'* (PHBG-III) formulation using the same procedure.

The Polyherbal Bhallatakadi Ghrita formulations i.e. PHBG-I, PHBG-II and PHBG-III thus prepared were stored in well closed and air tight glass container away from light till further studies.

2.4. Organoleptic and physicochemical evaluation

As per the standard pharmacopeial procedures, PHBG-I, PHBG-II and PHBG-III were subjected for organoleptic (colour, odour, taste, appearance and touch) and physicochemical evaluation (pH, viscosity, moisture content, specific gravity, refractive index, acid value, saponification value, iodine value, peroxide value, Rechert Meissl value and Polenske value) [7,25–27].

2.5. Antioxidant evaluation

Antioxidant activity of freshly prepared PHBG-I, PHBG-II and PHBG-III on the same day of preparation was assessed using various *in-vitro* methods; DPPH radical scavenging assay, Nitric oxide radical scavenging assay and Hydrogen peroxide scavenging assay [22,28]. The antioxidant potential was expressed as IC_{50}, which was the concentration of test samples that inhibited the formation of free radicals by 50%. Ascorbic acid was used as reference standard in all methods.

2.5.1. DPPH radical scavenging assay

The radical-scavenging or hydrogen-donating ability of PHBG-I, PHBG-II and PHBG-III was estimated using the established 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) method. Briefly, 3.0 mL of 10–100 $\mu g.mL^{-1}$ of ghrita solutions and 1.0 mL of 0.1 mM solution of DPPH in ethanol was mixed together and after 30 min the

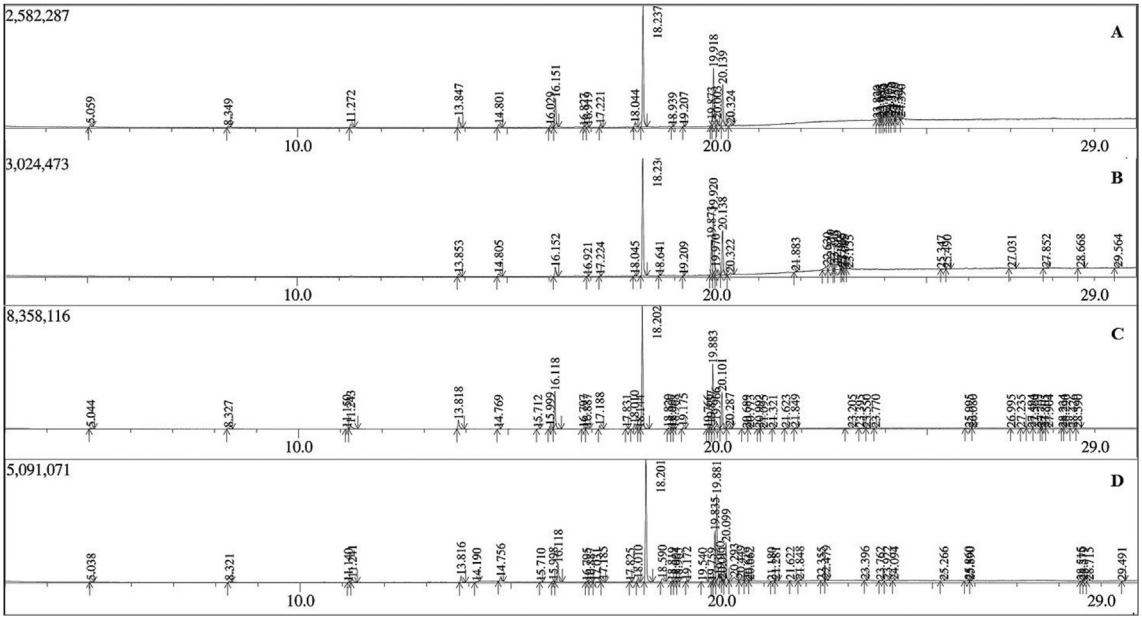

Fig. 2. GC–MS analysis of plain ghee and Polyherbal *Bhallatakadi Ghrita* (PHBG) formulations. Where **A-** Plain Ghee, **B-** Polyherbal *Bhallatakadi Ghrita* (PHBG-I), **C-** Polyherbal *Bhallatakadi Murcchita* Ghrita (PHBG-II) and **D-** Polyherbal *Bhallatakadi Shata-Dhauta* Ghrita (PHBG-III).

absorbance was measured at 517 nm. Lower absorbance of the reaction mixture specifies higher free radical-scavenging activity.

2.5.2. Nitric Oxide radical scavenging assay

Nitrite detection method i.e. Greiss reaction to measure Nitric Oxide (NO) generated from sodium nitroprusside was used to assess radical scavenging activity of test samples. Briefly, 3.0 mL of ghrita solutions at the concentration of 10–100 μg mL^{-1} were mixed with sodium nitroprusside (5 mM) in phosphate-buffered saline and allowed to incubate at 25 °C for 150min. Further these samples were reacted with Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% napthylethylenediamine dihydrochloride). The chromophore formed during the diazocoupling of nitrite with sulphanilamide and napthylethylenediamine was subjected for absorbance measurement at 546 nm. The reaction mixture without test sample but with equivalent quantity of distilled water served as control.

2.5.3. Hydrogen peroxide scavenging assay

The PHBG-I, PHBG-II and PHBG-III were subjected for Hydrogen peroxide (H_2O_2) scavenging assay based on replacement titration. Briefly, 1.0 mL of 0.1 mM H_2O_2, 1.0 mL of 10–100 μg.$mL^{-1}$of ghrita solutions, 2 drops of 3% ammonium molybdate, 10 mL of 2 M H_2SO_4, and 7.0 mL of 1.8 M KI were mixed together and the resultant solution was titrated with 5.09 mM $Na_2S_2O_3$ till complete disappearance of yellow color. Hydrogen peroxide scavenging potential was calculated as

$$\%\ \text{Inhibition} = (V_0 - V_1)/V_0 \times 100$$

Where, V_0 was volume of sodium thiosulphate solution used to titrate the control sample in the presence of hydrogen peroxide (without ghrita) and V_1 was the volume of sodium thiosulphate solution used in the presence of the ghrita.

2.6. GC–MS analysis

Fatty acid analysis of ghee and ghrita formulations were done by modified Bligh and Dyer method [29,30]. Briefly, 0.1 mL of molten ghee/ghrita formulations were taken into screw capped glass test tube and dissolved in 1 mL of 0.6 N methanolic HCl. The tube was vortexed and heated at 100 °C for 2 hr for rapid reaction. After cooling to room temperature, samples were extracted with hexane three times for extraction of fatty acid methyl esters (FAME). Hexane layers were mixed together and concentrated under vacuum evaporation. FAME analysis of test samples were done using GC Shimadzu TQ8030 GC–MS (Shimadzu, Kyoto, Japan) equipped with an AOC-20i (CTC Combipal, CTC Analytics, Zwingen, Switzerland) autosampler configured for solid phase microextraction (SPME). The fatty acids in the samples were identified by comparing peaks with those standards available in the spectral library attached to GC–MS instrument.

2.7. Stability studies

The accelerated and real time stability studies of PHBG-I, PHBG-II and PHBG-III formulations were carried out as per the ICH guidelines, Q1A (R2) [31]. The storage conditions in humidity chamber (NEWTRONIC, NEC 212 ET) were set as 40±2 °C temperature and 75 ± 5% RH for accelerated stability studies whereas 25±2 °C temperature and 60 ± 5% RH for real time stability studies. The changes in physicochemical properties of the formulation samples were observed for 6 months for accelerated (interval of 0, 1, 3 and 6 months) and for 12 months for real time stability study (interval of 0, 1, 3, 6, and 12 months) [31–34].

Table 2
Physico-chemical evaluation of Polyherbal *Bhallatakadi Ghrita* (PHBG) formulations during accelerated stability study.

Sr.No.	↓ Parameter Month →	PHBG-I				PHBG-II				PHBG-III			
		0	1	3	6	0	1	3	6	0	1	3	6
1	pH	4.9 ± 0.06	4.8 ± 0.09	4.5 ± 0.1	4.20 ± 0.1	5.4 ± 0.1	5.4 ± 0.1	5.1 ± 0.1	4.8 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	6.5 ± 0.1	6.2 ± 0.1
2	Viscosity (cp)	8697 ± 57.4	8565 ± 64.8	8301 ± 72.5	8047 ± 82.4	10,590 ± 101.3	10,447 ± 98.4	10,218 ± 99.1	9947 ± 37.9	9691 ± 41	9564 ± 52.2	9377 ± 27.3	9146 ± 73.7
3	Moisture Content (%)	27.14 ± 1.1	27.46 ± 1.2	28.47 ± 2.1	29.86 ± 1.8	12.84 ± 0.6	13.01 ± 0.7	13.36 ± 1.1	13.71 ± 0.9	18.59 ± 1.1	18.74 ± 1.1	19.11 ± 1.3	19.49 ± 1.5
4	Specific Gravity	0.92 ± 0.03	0.89 ± 0.08	0.83 ± 0.09	0.77 ± 0.07	1.00 ± 0.09	0.97 ± 0.05	0.92 ± 0.07	0.87 ± 0.06	0.92 ± 0.09	0.90 ± 0.08	0.85 ± 0.07	0.79 ± 0.06
5	Refractive Index	1.42 ± 0.01	1.43 ± 0.01	1.47 ± 0.01	1.51 ± 0.01	1.45 ± 0.01	1.47 ± 0.01	1.52 ± 0.01	1.59 ± 0.02	1.43 ± 0.01	1.44 ± 0.02	1.47 ± 0.02	1.50 ± 0.01
6	Acid Value	1.44 ± 0.07	1.46 ± 0.09	1.52 ± 0.08	1.59 ± 0.07	0.76 ± 0.01	0.77 ± 0.01	0.80 ± 0.02	0.83 ± 0.01	0.67 ± 0.02	0.68 ± 0.01	0.70 ± 0.01	0.73 ± 0.02
7	Saponification Value	173.2 ± 17.3	175.0 ± 7.5	186.7 ± 11.2	199.5 ± 21.6	208.0 ± 127.3	210.4 ± 120.1	227.6 ± 117.9	247.1 ± 25.3	147.9 ± 7.9	148.5 ± 9.1	154.1 ± 18.3	160.6 ± 122.8
8	Iodine Value	29.74 ± 4.3	29.98 ± 2.4	31.06 ± 2.2	32.45 ± 1.8	20.47 ± 2.9	20.53 ± 2.2	21.16 ± 1.4	21.81 ± 1.1	14.92 ± 1.3	14.99 ± 1.2	15.61 ± 1.3	16.31 ± 2
9	Peroxide Value	1.41 ± 0.02	1.43 ± 0.06	1.49 ± 0.12	1.56 ± 0.11	1.33 ± 0.07	1.34 ± 0.17	1.39 ± 0.11	1.43 ± 0.13	1.19 ± 0.10	1.20 ± 0.12	1.24 ± 0.11	1.28 ± 0.13
10	Reichert Meissl Value	20.95 ± 1.02	20.83 ± 1.11	19.95 ± 0.9	18.86 ± 1.17	22.27 ± 1.23	22.18 ± 1.04	21.55 ± 1.11	20.91 ± 1.19	24.02 ± 0.8	23.94 ± 1.14	23.21 ± 1.21	22.45 ± 1.11
11	Polenske Value	1.42 ± 0.12	1.43 ± 0.11	1.49 ± 0.15	1.56 ± 0.11	0.94 ± 0.07	0.95 ± 0.05	0.99 ± 0.02	1.02 ± 0.11	0.79 ± 0.01	0.80 ± 0.08	0.83 ± 0.07	0.87 ± 0.05

Where; PHBG-I- Polyherbal *Bhallatakadi Ghrita* Formulation, PHBG-II: Polyherbal *Bhallatakadi Murcchita* Ghrita, PHBG-III: Polyherbal *Bhallatakadi Shata-Dhauta* Ghrita.
All values are mean of three independent repeated experiments and expressed as Mean ± S.D.

2.7.1. Shelf life estimation

The stability data of PHBG formulations was used to extrapolate the graphs for each physico–chemical parameter. On the basis of physicochemical values, intercept and slope were calculated using 'GraphPad Prism 8' software and followed by expected time for 10% degradation for each parameter. The accelerated stability data was extrapolated by taking 10% degradation as acceptable point. The number of months at which 10% degradation occurred was calculated by the use of formula;

$$\text{Number of months at which 10\% degradation occurs} = [0\ \text{Month Assay Value} - \{[0\ \text{Month Assay Value X } 10/1000\}] - \text{Intercept/Slope}$$

The average months for 10% degradation were used to calculate the shelf life of ghrita formulations by using real time aging factor 5 for climatic zone I and II and 3.3 for climatic zone III and IV [35].

3. Results and discussion

In present research work, attempt was made to prepare cow ghee based Polyherbal Ghrita formulation as per Ayurvedic texts. Based on therapeutic (wound healing) potential, present herbs of Western Ghats, India were selected to prepare herbal extracts (*Kalka*) [12,13]. The name 'Bhallatakadi' was assigned from the synonymous name of *S. anacardium*. The possible decomposition of fatty acids during storage can lead to bad odour and rancidity which affects stability and shelf life of ghrita therefore prepared polyherbal ghrita formulation was processed with ancient Ayurvedic procedures viz. '*Murcchana*' and '*Shata-Dhauta*' *samskara* [12,36,37].

Few Ayurvedic scripts say that before making any ghrita, ghee should be processed with *Murcchana* herbs i.e. *Murcchana kriya* to remove '*Durgandha*' and '*Ama Dosha*' properties of ghee which may be due to rancidity problem of ghrita formulations [9,10]. The '*Murcchana*' *samskara* of ghee not only maintain the ratio of unsaturated and saturated fats but also modify the solubility pattern and absorbability of ghrita formulation [37]. Effect of antioxidant herbs on oxidative stability of cow ghee, thus their better role in preservation of food system over butylated hydroxyanisole (BHA) has been reported [38–43]. The herbs used in '*Murcchana*' process, reported with their potent antioxidant and anti-lipid peroxidation properties, play significant role in protection of ghrita from oxidative damages. These herbs were also known to boost the palatability of the ghrita formulation in terms of colour, odour and therapeutic value.

In '*Shata-Dhauta*' (*Shata*-one hundred, *Dhauta*-washing) process, trituration of ghee with aqueous phase was eventually results in formation of w/o type of emulsion as lipid (cow ghee) phase is major. Further washings with trituration (associated with pressure) reduce the particle size of fat granules. Eventually with successive washings, aqueous phase dominates over lipid phase and results in the phase inversion i.e. formation of o/w emulsion. The washings of ghee for one hundred times could led to the formation of a complex system of emulsion i.e. w/o/w [12].

The prepared PHBG-I, PHBG-II, PHBG-III formulations (Fig. 1) were standardized on the basis of qualitative (organoleptic/sensory) and quantitative (physicochemical) evaluation.

The sensory analysis is an integral part and pilot basis for quality control as well as quality assurance of ghee-based products. In general, the palatability of the product is found to depend upon sensory characteristics [44]. The cow ghee used for preparation of ghrita formulation was of golden yellow colour, oily, granular with characteristic odour and taste, therefore the PHBG-I prepared by '*Ghrita Paka Kalpana*' was found to be retained some characters of cow ghee i.e. oily consistency and granular appearance. However, it

Table 3
Estimation of shelf-life of Polyherbal *Bhallatakadi Ghrita* (PHBG) formulations by accelerated stability study.

Sr. No.	Physico-chemical Parameter	PHBG-I				PHBG-II				PHBG-III			
		Graphical Results		10% Degradation	Month at Which 10% Degradation Occur	Graphical Results		10% Degradation	Month at Which 10% Degradation Occur	Graphical Results		10% Degradation	Month at Which 10% Degradation Occur
		Intercept	Slope			Intercept	Slope			Intercept	Slope		
1	pH	4.9	0.12	4.40	4.50	5.40	0.11	4.80	5.43	6.80	0.10	6.10	7.21
2	Viscosity (cp)	8673	108.3	7827.3	7.81	10,565	105.8	9531	9.77	9668	89.24	8721.90	10.60
3	Moisture Content (%)	27.08	0.46	29.85	5.74	12.87	0.15	14.12	9.05	18.60	0.15	20.45	12.32
4	Specific Gravity	0.91	0.03	0.83	3.39	0.99	0.02	0.90	4.60	0.92	0.02	0.83	4.21
5	Refractive Index	1.42	0.02	1.56	8.97	1.45	0.02	1.59	6.00	1.43	0.01	1.57	12.84
6	Acid Value	1.44	0.03	1.58	5.61	0.76	0.01	0.84	6.40	0.67	0.01	0.74	6.70
7	Saponification Value	172.2	4.6	190.5	3.6	206.2	6.8	228.8	2.8	147.2	2.2	162.7	6.3
8	Iodine Value	29.64	0.47	32.71	6.17	20.40	0.24	22.52	8.39	14.85	0.24	16.41	5.85
9	Peroxide Value	1.41	0.03	1.55	5.49	1.33	0.02	1.46	7.59	1.19	0.02	1.31	7.62
10	Reichert Meissl Value	21.06	0.36	18.86	6.06	22.32	0.24	20.04	9.59	24.09	0.28	21.62	8.98
11	Polenske Value	1.41	0.02	1.56	5.60	0.94	0.01	1.03	6.84	0.79	0.01	0.87	5.72
Mean		PHBG-I			5.7203	PHBG-II			6.9523	PHBG-III			6.9523
Climate Zone I & II (Real Time Aging Factor 5)		28.60 [2.4 Years]				34.76 [2.9 Years]				45.98 [3.8 Years]			
Climate Zone III & IV (Real Time Aging Factor 3.3)		18.88 [1.6 Years]				22.94 [1.9 Years]				30.35 [2.5 Years]			

Where; PHBG-I- Polyherbal Bhallatakadi Ghrita, PHBG-II: Polyherbal Bhallatakadi *Murcchita* Ghrita, PHBG-III: Polyherbal Bhallatakadi *Shata-Dhauta* Ghrita.

Table 4
Physico-chemical evaluation of Polyherbal *Bhallatakadi Ghrita* (PHBG) formulations during real time stability study.

Sr. No.	↓ Parameter Month →	PHBG-I				
		0	1	3	6	12
1	pH	4.9 ± 0.07	4.8 ± 0.1	4.5 ± 0.1	4.2 ± 0.1	3.8 ± 0.1
2	Viscosity (cp)	8697 ± 62.4	8594 ± 67.4	8399 ± 31.8	8145 ± 75.8	7801 ± 29.7
3	Moisture Content	27.14 ± 2.1	27.37 ± 2.2	28.12 ± 2.1	29.04 ± 2.1	30.15 ± 2.3
4	Specific Gravity	0.92 ± 0.09	0.91 ± 0.09	0.89 ± 0.07	0.85 ± 0.07	0.81 ± 0.06
5	Refractive Index	1.42 ± 0.01	1.44 ± 0.01	1.48 ± 0.01	1.52 ± 0.01	1.57 ± 0.01
6	Acid Value	1.44 ± 0.06	1.52 ± 0.09	1.61 ± 0.08	1.71 ± 0.09	1.83 ± 0.09
7	Saponification Value	173.2 ± 11.7	174.6 ± 12.3	176.9 ± 14.2	185.8 ± 21.7	197.7 ± 24.8
8	Iodine Value	29.74 ± 2.7	30.35 ± 1.6	31.54 ± 0.9	33.22 ± 2.1	35.46 ± 1.9
9	Peroxide Value	1.41 ± 0.07	1.44 ± 0.06	1.51 ± 0.03	1.6 ± 0.02	1.71 ± 0.17
10	Reichert Meissl Value	20.95 ± 0.92	20.79 ± 1.17	19.88 ± 1.1	18.75 ± 0.84	17.31 ± 0.84
11	Polenske Value	1.42 ± 0.04	1.44 ± 0.10	1.51 ± 0.13	1.6 ± 0.11	1.71 ± 0.10

Where; PHBG-I- Polyherbal *Bhallatakadi Ghrita,* PHBG-II: Polyherbal *Bhallatakadi Murcchita* Ghrita, PHBG-III: Polyherbal *Bhallatakadi Shata-Dhauta* Ghrita.
All values are mean of three independent repeated experiments and expressed as Mean ± S.D.

was observed that the palatability of PHBG-II and PHBG-III formulations was increased after processing with '*Murcchana*' and '*Shata-Dhauta*' *samskara* respectively. The *Murcchana* process altered organoleptic properties (colour, odour and taste) of PHBG-II formulation whereas *Shata-Dhauta* process resulted in complete disappearance of characteristic odour, granular and oily consistency of cow ghee and made PHBG-III formulation homogeneous and smooth (Supplementary Table 1).

Being a fatty product, PHBG-I, PHBG-II and PHBG-III formulations were evaluated for physicochemical properties like pH, viscosity, moisture content, specific gravity, refractive index and acid value, saponification value, iodine value, peroxide value, Rechert Meissl value and Polenske value (tests for fats and oils) (Supplementary Table 2).

The pH value, measure of hydrogen activity in the formulation conventionally represents the acidity or alkalinity [25]. The pH variations may have impact on flavor, consistency and shelf life of the ghee-based formulations. The pH of PHBG-I, PHBG-II and PHBG-III formulations was found to be 4.9 ± 0.06, 5.3 ± 0.10 and 6.7 ± 0.17 respectively. It can be noted that the shift in pH from acidic to neutral makes it beneficial to prevent skin irritation and useful in case of open wound application/treatment.

The viscosity may affect the appearance and the consistency as it measures a resistance of ghrita formulations to the motion under an applied force. The viscosity of the PHBG-II formulation i.e. 10528 ± 33.06 was found to be increased after *Murcchana* process. The viscosity of PHBG-III formulation was found to be high i.e. 9685 ± 87.21 as compared to the PHBG-I i.e. 8724 ± 74.91.

Moisture is an important factor in food quality, preservation, and resistance to deterioration. The amount of water content can affect texture, taste, appearance and stability of formulations [45]. The moisture content of the PHBG-I formulation was found to be 27.22 ± 1.91. The heat applied during *Murcchana* process of PHBG-II formulation decreases its moisture content to 13.60 ± 0.99. However, during *Shata-Dhauta* process due to hundred times washing with water, moisture content of PHBG-III formulation was found to be higher (20.65 ± 1.85) which may be useful for skin hydration and cooling effect in case of topical application [12].

The specific gravity gives an idea about solid to liquid ratio in the formulations and eventually density with respect to water which can be significantly used to enhance the consistency of formulations. The less liquid content in formulation increases the life span and thus its therapeutic value [36]. The upward shift in specific gravity of PHBG-II formulation (1.0796 ± 0.07) was observed which may be due to the solid extractives, originated from the added herbs during the formulation process whereas the specific gravity of PHBG-III (0.9278 ± 0.01) was found to be greater, than that of PHBG-I (0.9191 ± 0.03).

The refractive index represents the behavior of light in the prepared medium, which can be used to determine the concentration of solutes in an aqueous solution. The refractive index increases with decrease in the chain length whereas a double bond elevates the refractive index [46]. The refractive index of PHBG-II (1.4484 ± 0.01) and PHBG-III (1.4314 ± 0.01) formulations was found to be increased when it was compared with the PHBG-I (1.4207 ± 0.01) formulation.

The acid value is the measure of free fatty acids. As oil and fats start to rancidify on storage, triglycerides are converted into fatty acids and glycerol, causing an increase in acid value. The less acid value denotes the less chance of decomposition of ghrita formulation thus increasing life span and therapeutic value [26,36,47]. The free fatty acid content of PHBG-II (0.88 ± 0.09) and PHBG-III (0.77 ± 0.09) formulations was found to be decreased when it was compared to the PHBG-I (1.46 ± 0.06) formulation. It can be noted that washing of cow ghee with water by hundred times might have led to splitting of triglycerides into glycerol and fatty acids which are removed along with aqueous phase [12].

The saponification value gives an indication of the number of fatty acids and their average molecular weight in the ghrita formulations. More the fatty matter content or more the carboxylic functional group per unit mass, there will be more chances of rancidity factor and less will be the shelf life and therapeutic value [26,36,37]. The saponification value of PHBG-I formulation was found to be 167.37 ± 23.86 whereas it was 156.61 ± 5.31 in PHBG-III. It can be noted that increase in saponification value of PHBG-II formulation could be attributable to interactions between different ghee components and phytoconstituents.

The iodine value indicates quantity of iodine absorbed at unsaturation which signifies the degree of unsaturation of the ghrita formulations. The formulation with higher iodine value is more reactive and susceptible to the oxidation [26,32]. The PHBG-I formulation was found to be with highest degree of unsaturation (34.86 ± 5.14) so more susceptible to oxidation. Decrease in iodine value of PHBG-II (19.80 ± 1.52) and PHBG-III (13.37 ± 1.63) formulations eventually reduce chances of rancidity and increase the stability, which could be the protective effect of antioxidant herbs.

Usually lipid peroxidation is assumed as a major deteriorative change commonly found in fats and the extent of lipid peroxidation depends upon different attributes viz. unsaturation level, packaging material and storage conditions [32,48]. The peroxide value was determined to obtain initial evidence of rancidity in

PHBG-II					PHBG-III				
0	1	3	6	12	0	1	3	6	12
5.4 ± 0.1	5.2 ± 0.1	5 ± 0.1	4.8 ± 0.1	4.4 ± 0.1	6.8 ± 0.1	6.6 ± 0.1	6.4 ± 0.1	6.1 ± 0.1	5.8 ± 0.1
10,590 ± 123.4	10,503 ± 102.1	10,331 ± 112.5	10,125 ± 97.9	9897 ± 57.4	9691 ± 62.2	9592 ± 32.6	9406 ± 52.2	9179 ± 81.3	8914 ± 43.2
12.84 ± 0.8	13.03 ± 0.6	13.65 ± 0.2	14.46 ± 0.4	15.54 ± 0.7	18.59 ± 1.1	18.74 ± 0.9	19.22 ± 0.9	20.01 ± 0.9	21.07 ± 0.9
1.00 ± 0.09	0.99 ± 0.07	0.98 ± 0.09	0.95 ± 0.08	0.92 ± 0.07	0.92 ± 0.07	0.92 ± 0.07	0.90 ± 0.06	0.87 ± 0.04	0.83 ± 0.04
1.45 ± 0.01	1.47 ± 0.01	1.53 ± 0.01	1.59 ± 0.01	1.67 ± 0.01	1.43 ± 0.01	1.45 ± 0.01	1.48 ± 0.01	1.52 ± 0.01	1.57 ± 0.01
0.76 ± 0.01	0.79 ± 0.02	0.83 ± 0.02	0.89 ± 0.02	0.97 ± 0.02	0.67 ± 0.01	0.7 ± 0.01	0.74 ± 0.02	0.79 ± 0.02	0.85 ± 0.01
208 ± 316.5	209.7 ± 21.4	212.5 ± 31.7	224.3 ± 13.2	239 ± 211.4	147.9 ± 9.7	149 ± 11.7	151.1 ± 18.7	160.5 ± 7.7	172.9 ± 21.7
20.47 ± 1.6	20.89 ± 1.3	21.54 ± 2.2	22.25 ± 2.3	23.17 ± 2.1	14.92 ± 0.7	15.29 ± 0.9	15.85 ± 1.1	16.54 ± 1.2	17.35 ± 1.3
1.33 ± 0.11	1.35 ± 0.02	1.39 ± 0.03	1.45 ± 0.04	1.53 ± 0.12	1.19 ± 0.09	1.22 ± 0.17	1.26 ± 0.11	1.32 ± 0.09	1.39 ± 0.04
22.27 ± 0.96	22.17 ± 1.11	21.52 ± 1.18	20.8 ± 0.89	19.6 ± 1.12	24.02 ± 1.04	23.96 ± 0.72	23.36 ± 0.34	22.65 ± 0.62	21.54 ± 0.81
0.94 ± 0.08	0.95 ± 0.06	0.99 ± 0.04	1.05 ± 0.14	1.14 ± 0.4	0.79 ± 0.03	0.81 ± 0.06	0.84 ± 0.07	0.89 ± 0.03	0.95 ± 0.05

formulations. The peroxide value of PHBG-II and PHBG-III formulations was found to be lower i.e. 1.33 ± 0.06 and 1.23 ± 0.15 respectively when it was compared with PHBG-I formulation (1.57 ± 0.15). Antioxidant *Murcchana* herbs and catalytic effect of copper (from copper vessel used in *Shata-Dhauta* process) in fat splitting seemed to be offering protective effect against rancidity of processed ghrita formulations [49]. The storage of ghrita formulations in well closed containers and away from light may provide the maximum protection against lipid peroxidation [32].

The Rechert Meissl and Polenske values are important indices and principally used for determination of quality of fats of ghee-based formulations. The fats of cow ghee can be distinguished from other fats by the presence of glyceryl esters of relatively low molecular weight fatty acids, especially butyric as well as caproic acids [27]. The Rechert Meissl values of PHBG-II and PHBG-III formulations were found to be 22.18 ± 1.21 and 23.10 ± 0.94 respectively whereas the less content of low molecular weight, volatile and water-soluble compounds in PHBG-I formulation (21.74 ± 1.29) was observed.

The Polenske value measures the steam volatile and water insoluble fatty acids, chiefly caprylic, capric and lauric acids present in ghrita formulations. The butter fat contains less amount of steam volatile but water insoluble caprylic and capric acid glycerides [27]. The Polenske value of PHBG-I formulation (1.43 ± 0.06), was found to be high as compared to the PHBH-II (0.97 ± 0.12) and PHBG-III (0.80 ± 0.10) formulations, indicates the high concentration of water insoluble and volatile low molecular weight compounds in PHBG-I formulation.

In case of *in-vitro* antioxidant evaluation, all test samples i.e. plain ghee, PHBG-I, PHBG-II and PHBG-III exhibited concentration dependent (10–100 μg.mL−1) free radical scavenging activity (Table 1). The IC_{50} of PHBG-II by the DPPH method was found to be 18.02 ± 1.21 μg mL^{-1}, whereas plain ghee, PHBG-I and PHBG-III showed IC_{50} values as 38.72 ± 0.72, 19.56 ± 1.02 and 18.32 ± 0.98 μg mL^{-1} respectively. In Nitric Oxide method, IC_{50} for PHBG-II was found to be 22.11 ± 1.08 μg mL^{-1}, whereas plain ghee, PHBG-I and PHBG-III showed IC_{50} value as 39.32 ± 1.34, 22.34 ± 1.23 and 21.24 ± 0.78 μg mL^{-1} respectively. Plain ghee, PHBG-I, PHBG-II and PHBG-III demonstrated dose dependent H_2O_2 scavenging activity with the IC_{50} of 41.21 ± 1.22, 26.09 ± 1.63, 24.09 ± 0.92 and 24.61 ± 1.11 μg mL^{-1} respectively. Ascorbic acid revealed excellent antioxidant activity in all in-vitro methods. Antioxidant potential of plain ghee and ghrita formulations by all *in-vitro* methods was found in increasing order i.e. plain ghee < PHBG-I < PHBG-III < PHBG-II. Various tannin-rich herbs used in preparation of *Murcchita* ghee might be responsible for potent antioxidant activity of PHBG-II. There could be synergistic effect of antioxidant herbs from *Murcchita* ghee and PHBG-I which resulted in highest antioxidant potential of PHBG-II.

The objective of GC–MS analysis was to analyze the changes in terms of fatty acids in prepared Polyherbal Bhallatakadi Ghrita (ghee based) formulations and to provide additional evidence of *Murcchana* and *Shata-Dhauta* process by comparing the results of plain ghee and prepared formulations. GC/MS spectrum for FAME of plain ghee and PHBG formulations were represented (Fig. 2) and compared with reference to the area per cent of major peaks (Supplementary Table 3). Total number of components of Cow ghee detected in GC–MS study was 18. Cow ghee contains 12 saturated and 6 unsaturated fatty acids. The major saturated fatty acids of cow ghee were capric, caproic, caprylic, hexadecanoic, lauric, margaric, palmitic and stearic and unsaturated fatty acids were linoleic, elaidic, phthalic. Linoleic acid is an essential fatty acid and one of the most abundant polyunsaturated fatty acids (PUFAs). The % area of linoleic acid methyl ester was found to be increased significantly i.e. from 1.70 to 12.32 in case of PHBG-III formulation. The % area of phthalic acid in cow ghee was 1.64 and it was found to be decreased up to 0.15 in PHBG formulations especially in PHBG-II. Elaidic acid is the trans isomer of oleic acid. The minor change in % area of elaidic acid methyl ester was observed in PHBG formulations as compared to cow ghee.

The % area of capric acid methyl ester of PHBG-II formulation (2.49) was found to be increased as compared to cow ghee (1.91) and % area of lauric acid methyl ester was found to be more in PHBG-II formulation (3.50) when compared with PHBG-I (1.22) and PHBG-III (1.91). But the minute change in % area of caprylic acid methyl ester was observed in case of PHBG formulations. The absence of hexadecanoic acid methyl esters area or their decreased % of area was observed in case of PHBG formulations. Heptadecanoic acid is also known as margaric acid. The % area of margaric acid methyl ester was found to be increased from 0.36 to 0.54 in case of PHBG-II formulation. The relative percentage of unsaturated fatty acids in case of PHBG-II and PHBG-III formulations were found to be decreased with relative increase in saturated fatty acids.

The quality or efficacy of herbal formulations varies with time under the influence of environmental factors such as temperature, humidity and light. Therefore, as per the ICH guidelines, prepared PHBG formulations were subjected for accelerated and real time stability study [50]. The shelf life of all *ghrita* formulations in climatic zone I/II and III/IV was determined by using real time aging factors 5 and 3.3 respectively. The changes in physicochemical parameters were recorded (Tables 2 and 4) at regular intervals and used to calculate the shelf life of PHBG formulations. The shelf life of PHBG-I was recorded as 2.4 years for climatic zone I/II and 1.6 years for climatic zone III/IV at accelerated conditions whereas at real

Table 5
Estimation of shelf-life of Polyherbal *Bhallatakadi Ghrita* (PHBG) formulations by real time stability study.

Sr. No.	Physico-chemical Parameter	PHBG-I				PHBG-II				PHBG-III			
		Graphical Results		10% Degra-dation	Month at Which 10% Degra-dation Occur	Graphical Results		10% Degra-dation	Month at Which 10% Degra-dation Occur	Graphical Results		10% Degra-dation	Month at Which 10% Degra-dation Occur
		Intercept	Slope			Intercept	Slope			Intercept	Slope		
1	pH	4.85	0.09	4.40	4.73	5.30	0.08	4.90	5.68	6.70	0.08	6.10	7.14
2	Viscosity (cp)	8654	74.31	7827.3	11.13	10,542	57.43	9531	17.60	9639	64.26	8721.9	14.27
3	Moisture Content	27.24	0.26	29.85	11.04	12.90	0.23	14.12	5.87	18.59	0.21	20.45	8.78
4	Specific Gravity	0.92	0.01	0.83	9.93	1.00	0.01	0.90	14.86	0.92	0.01	0.83	11.50
5	Refractive Index	1.43	0.01	1.56	12.50	1.46	0.02	1.59	8.33	1.44	0.01	1.57	12.93
6	Acid Value	1.49	0.03	1.58	6.14	0.77	0.02	0.84	5.13	0.69	0.01	0.74	5.68
7	Saponification Value	172.4	2.11	190.5	7.84	206.9	2.68	228.78	7.35	146.8	2.17	162.7	6.31
8	Iodine Value	29.96	0.48	32.71	6.70	20.70	0.22	22.52	10.38	15.12	0.20	16.41	8.52
9	Peroxide Value	1.42	0.03	1.55	6.13	1.34	0.02	1.46	8.30	1.20	0.02	1.31	8.11
10	Reichert Meissl Value	20.91	0.31	18.86	6.57	22.28	0.23	20.04	9.81	24.05	0.21	21.62	11.39
11	Polenske Value	1.43	0.02	1.56	6.08	0.94	0.02	1.03	5.43	0.80	0.01	0.87	6.52
Mean		PHBG-I			8.0698	PHBG-II			8.9771	PHBG-III			9.1956
Climate Zone I & II (Real Time Aging Factor 5)		40.35 [3.4 Years]				44.89 [3.7 Years]				45.98 [3.8 Years]			
Climate Zone III & IV (Real Time Aging Factor 3.3)		26.63 [2.2 Years]				29.62 [2.5 Years]				30.35 [2.5 Years]			

Where; PHBG-I- Polyherbal *Bhallatakadi Ghrita*, PHBG-II: Polyherbal *Bhallatakadi Murcchita* Ghrita, PHBG-III: Polyherbal *Bhallatakadi Shata-Dhauta* Ghrita.

time stability conditions it was found to be 3.4 years for climatic zone I/II and 2.2 years for climatic zone III/IV. In case of PHBG-II, at accelerated and real time stability conditions for climatic zone I/II shelf life was recorded as 2.9 and 3.7 years respectively and 1.9 and 2.5 years respectively for climatic zone III/IV. PHBG-III confirmed with the shelf life of 3.3 and 2.2 for climatic zone I/II and III/IV respectively for accelerated conditions whereas at real time stability conditions it was found to be 3.8 years for climatic zone I/II and 2.5 years for III/IV (Tables 3 and 5).

4. Conclusion

Present research work was an attempt to develop and evaluate ghee based Polyherbal *Bhallatakadi Ghrita* formulation with reference to '*Murcchana*' and '*Shata-Dhauta*' process. Homogeneous, smooth, non-granular and non-oily polyherbal formulation processed with *'Shata-Dhauta'* is easier for topical application and will improve patient compliance. *'Shata-Dhauta'* samskara increased shelf life of Polyherbal *Bhallatakadi Ghrita* formulation by almost 1.5 times for Indian environment (climatic zone III/IV) for accelerated stability conditions. Wound healing potential of prepared and processed ghrita formulations by *in-vivo* methods need further exploration in future.

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Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2020.05.005.

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