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RESEARCH ARTICLE

HPLC Quantification and Stability Study of Antilipidemic Polyherbal Formulation

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Abstract

Hyperlipidemia is one of the major risk factor for cardio vascular disorders, which accounts for the one third of total death in the world. In the present study an Antilipidemic polyherbal formulation was prepared and contains four herbal drugs namely *Murraya koenigi* leaves, *Acacia catechu* bark, *Emblica officinalis* fruits and leaves of *Gymnema sylvestra.* The study involved HPLC marker-based quantification and shelf-life evaluation of a traditional Antilipidemic formulation. The quantification method for the biomarkers present in the polyherbal formulation was developed using reverse phase method by using shim-pack HPLC C_{18} column (250 X 4.6 mm, 5 µm). The four phytoconstituents selected for the study are mahanine, quercetin, gallic acid and gymnemic acid respectively. The percentage content of quercetin, gallic acid, gymnemic acid and mahanine in the PHF was found to be 0.82%w/w,10.15%w/w, 2.77%w/w and 14.19%w/w respectively. Shelf life is an important component that is mandatory to be displayed on the label of all medicinal products. This is also applicable to Ayurveda. The shelf life of polyherbal formulations is also assessed in this study using an accelerated stability analysis. At a temperature of 40.2 °C and a relative humidity of 75%, physicochemical parameters were measured. The analysis was repeated at 1, 3, and 6-month intervals, with the average 10% deterioration time calculated and extrapolated to determine the shelf life. It was found that the **s**helf life of formulated Antilipidemic polyherbal formulation was 2 years 2 months. The polyherbal formulation prepared and evaluated can be effectively used for the treatment of hyperlipidemia and the preparation can be used without any deterioration for a period of 2 years.

Keywords: Hyperlipidaemia, HPLC Quantification, Polyherbal Formulation, Shelf-life Study

1. Introduction

Owing to its comparable efficacy, fewer side effects, and higher acceptability than allopathic medications, Ayurvedic Polyherbal Formulations (PHFs) have experienced a "renaissance" around the world. Polyherbalism has been mentioned in Ayurvedic book Sarangdhar Samhita to achieve better therapeutic efficiency. Because of its medicinal and therapeutic properties, polyherbal formulations have been utilised all over the world[1]. Hyperlipidemia is a common condition. It is a key component of metabolic syndromes such as stroke, type 2 diabetes, and cardiovascular disease. When combined with other diseases such as hypertension, diabetes, and cardiovascular disease, this disease increases ill health and mortality. The harmful impacts of currently available lipid-lowering medications have increased the desire to use traditional herbal remedies rather than pharmaceuticals[2]. Although polyherbal formulations are widely utilised in many regions of the world, there is still a dearth of scientific proof. Many herbal remedies are still being tested in animals and have not yet been subjected to clinical studies. Furthermore, no safety assessments such as toxicological or shelf-life tests have been conducted. The quality of medicines has become a major concern as their popularity has grown. The shelf life of a medicinal product is an important factor that must be displayed on the label. This is also true in

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Ayurveda; as a result, it is critical to determine the shelf life of formulations that are marketed and utilised in therapies[3].

The study aims to evaluate the shelf life and quantification of active ingredients by HPLC using the marker compounds. The Antilipidemic Polyherbal formulations used as a traditional medicine in the Malabar area, Kerala.

2. Material and Methods

2.1 Collection and Authentication

Contents of Polyherbal formulation were collected from Malappuram dist. Kerala, (March–April) during summer season. Dr. Sreekala, senior scientist, Drug standards section, Arya Vidhya Sala Kottakkal, Centre for medicinal plant research, Malappuram District, Kerala, attested to their authenticity.

2.2 Preparation of Formulation

Ingredients of the formulation are *Murraya koenigii* leaves (from 4 to 5 years old plant), *Acacia catechu* bark (from 18 to 20 old tree), *Emblica officinalis* fruits (from more than 15 years old tree) and leaves of *Gymnema sylvestra* (from more than four years old plant). All the contents are collected, dried in shade and powdered separately and then sieved through 80 mesh size sieve and are mixed with equal proportion. The powdered Polyherbal formulation is stored in an airtight container.

2.3 Quantification of Polyherbal Formulation using Four Biomarkers by HPLC Method[4, 5]

HPLC grade quercetin, gallic acid, gymnemic acid and mahanine (purity 99%) were procured from Natural Remedies Pvt. Ltd., Bangalore, India. All the solvents used were of HPLC grade. The HPLC analysis was performed at Care Keralam. The HPLC instrument employed in this method was of the model Agilent Technologies 1200 Infinity Series. The C18 (4.6250mm5m) analytical column was utilised to separate the analytes.

2.3.1 Chromatographic Conditions

The quantification method for the biomarkers present in the PHF1 was developed using reverse phase method and shim-pack HPLC C_{18} column (250 X 4.6 mm, 5 µm). The other chromatographic conditions were given in Table 1.

2.3.2 Preparation of Sample Extract

PHF 1 was properly weighed at 500 mg and extracted three times with a combination of methanol and water in a 70:30 ratio. The extracts were then mixed and condensed to 100 mL on a rotary evaporator (Equitron rotevar, Medica Instrument Mfg. Co.) at a lower temperature (50°C). The extract was filtered via a 0.45 mm nylon membrane filter before use. To get a clear solution, the sample extract was filtered. The stock

Table 1. Chromatographic conditions

Chromatographic condition	Quercetin	Gallic acid	Gymnemic acid	Mahanine
Column	C_{18} 4.6×250mm×5µm			
Flow rate	1.0mL/Minute	1.0mL/Minute	1.0mL/Minute	0.5mL/Minute
Injection Volume	20µl	20µL	10µL	10µL
Wave length	369nm	270nm	210nm	300nm
Run Time	10 minute	10 minute	20 minute	20 minute
Mobile phase	0.1% O-phosphoric acid in HPLC water: Methanol (35:65)	0.5% Acetic acid in water: ACN (80:20)	Methanol: Phosphate buffer pH 3.5 (70:30)	Acetonitrile: Ammoniumacetate buffer 5mM (85:15)

solution after suitable dilutions was used for further analysis. The standard solutions and sample extract were injected in to the HPLC instrument under the specified chromatographic conditions. The contents of the PHF was detected by UV detector in the chromatograph. Chromatogram was obtained to estimate the content.

2.4 Shelf-life Studies of Polyherbal Formulation — Storage Conditions and Evaluation Parameters

Accelerated stability tests were performed on the prepared formulation[6,7].

Packing

The polyherbal formulation was sealed in a food-grade plastic container to keep it fresh.

Sample Quantity

In an accelerated stability chamber, four containers holding 75 gms of polyherbal formulations were packed and stored.

Study Period

September 2020 to February 2020.

Storage Condition

This accelerated stability analysis was carried out in accordance with ICH guideline Q1 (R2). The accelerated stability test was carried out in accordance with ICH recommendations[12]. The temperature was 40 +2 °C during the study period, with a relative humidity of 75+ 5%.

Frequency of Withdrawal of Sample

At intervals of 0, 1, 3, and 6 months, the formulation was removed from the stability chamber and evaluated for key parameters.

2.4.1 Parameters for Evaluation

The variations in organoleptic characteristics like colour, odour, and taste were investigated at specific intervals in the polyherbal formulation. Physicochemical parameters like moisture content, water soluble extractive value, alcohol soluble extractive value, and ash values were assessed. Microbial content tests were performed at the start of the trial and again at the end of the sixth month. The presence of heavy metals in the formulation was first determined using an atomic absorption spectrophotometer, as indicated in WHO recommendations.

The intercept, slope, and 10% degradation of polyherbal churna were calculated using data collected at various phases of withdrawal (0, 1, 3, and 6 months). A 10% degradation is defined as the acceptable point for extrapolating the accelerated stability data. The real-time ageing factor was multiplied by the mean of these months. Because India is classified as a climatic zone III country, the real-time ageing factor used to extrapolate shelf life is 3.33. Extrapolated shelf life of any product can also be computed using accelerated stability data and a 10% degradation rate, as shown in the equation below[8].

$$\text{Month of 10 \% degradation} = \frac{[0 \text{ month assay value- (0 month assay value/10) x 100}] - \text{intercept}}{\text{Slope}}$$

2.4.2 FTIR Analysis of PHF 1

The extract of PHF 1 was subjected to analysis by Fourier transform infrared (FTIR) analysis. As a part of evaluation of stability, FTIR analysis was performed at 0-month, 1st, 3rd and 6th month. The application of IR helps in the identification of a compound based on the existence of different functional groups. Any type of degradation or deterioration can be detected by changes in the functional groups of the phytoconstituents which can be identified in the FTIR spectrum[9,10].

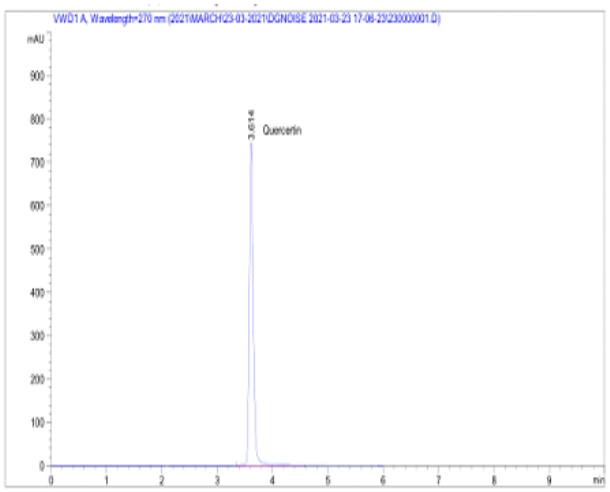
3. Results and Discussion

3.1 Quantification of Final Formulation using Four Biomarkers by HPLC Method

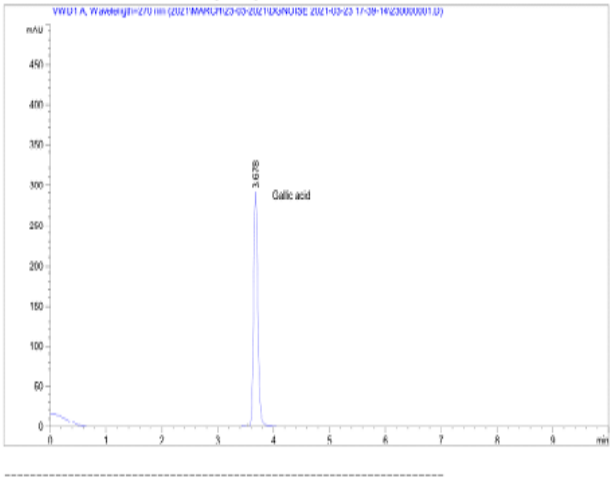
An appropriate method with optimal chromatographic conditions was adopted for the analytical determination of the selected biomarker namely Quercetin, Gallic acid, Gymnemic acid and Mahanine on a shim-pack HPLC C_{18} column (250 X 4.6 mm, 5 µm) in the PHF under study. Primarily, Standard biomarker solutions were run in the HPLC using the specified mobile phase. It resulted in the elution of quercetin at 6.57

Table 2. Estimation of biomarkers in PHF 1 by HPLC analysis

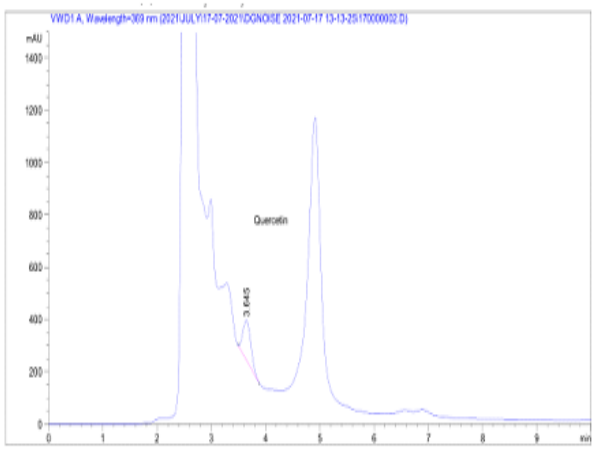
Formulation	Biomarker	Amount estimated (ppm) (n=3)	Content in %
PHF 1 (1000ppm)	Quercetin	8.21	0.821
	Gallic Acid	101.52	10.1.52
	Gymnemic Acid	27.72	2.772
	Mahanine	141.97	14.19



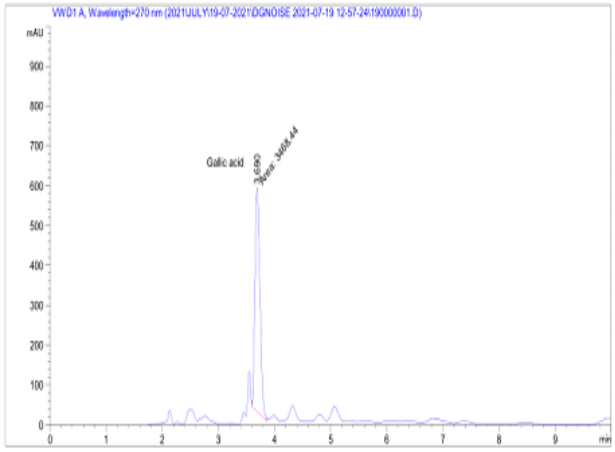
Chromatogram of quercetin standard



Chromatogram of gallic acid standard



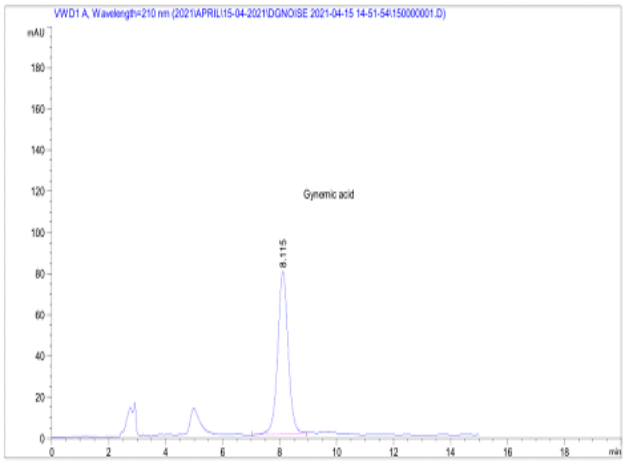
Chromatogram of quercetin sample



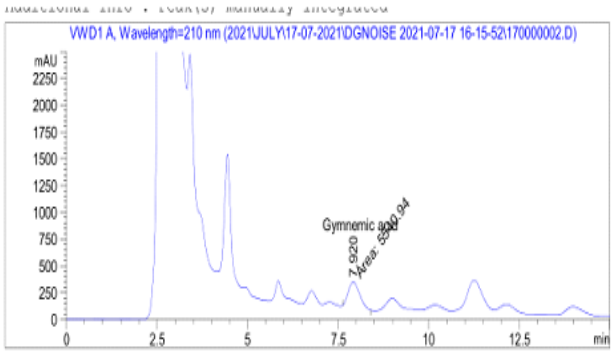
Chromatogram of gallic acid sample

Figure 1. Quercetin Std and quercetin in PHF

Figure 2. Gallic acid Std and Gallic acid in PHF.

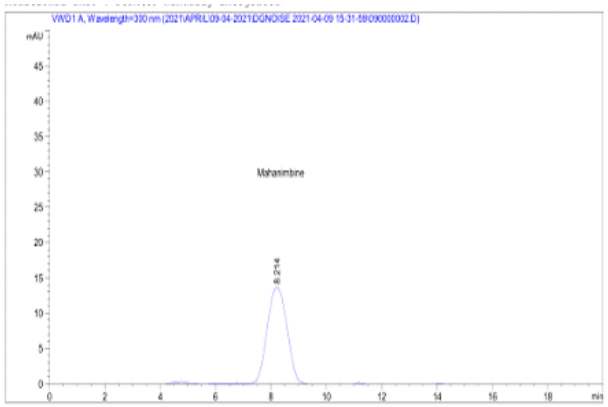


Chromatogram of gymnemic acid standard

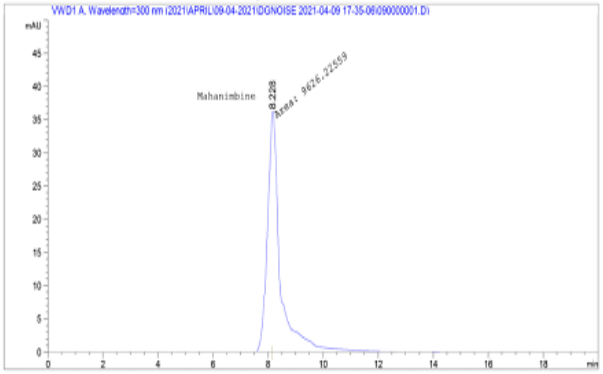


Chromatogram of gymnemic acid sample

Figure 3. Gymnemic acid Std and Gymnemic acid in PHF.



Chromatogram of mahanimbine standard



Chromatogram of mahanimbine sample

Figure 4. Mahanine Std and Mahanine in PHF.

min, Gallic acid at 7.32 min, Gymnemic acid at and Mahanine at 8.57 min. The sample of PHF churna was extracted using the specified solvent and run in the HPLC using the specified mobile phase system. The biomarkers eluted out. The retention time of the biomarkers in the sample extract was compared with that of the respective standard biomarkers and found to be similar to that of the standard drugs. The area under curve (AUC) was observed for both sample extracts and the standard biomarkers. The AUC were compared and used to quantify the percentage of the selected biomarkers present in the PHF. The same was repeated thrice. The standard chromatograms and the sample chromatograms are shown in the Figures 1–4. The mean peak area determined for the sample using three determinations which gave the concentration of quercetin in the crude to be 8.21ppm, gallic acid to be 101.52 ppm, gymnemic acid to 27.72ppm and mahanine to be 141.97ppm. The percentage content of quercetin, gallic acid, gymnemic acid and mahanine in the PHF was found to be 0.82%w/w,10.15%w/w, 2.77%w/w and 14.19%w/w respectively as shown in the Table 2.

3.2 Evaluation of Stability of Polyherbal Formulation

3.2.1 Organoleptic Parameters

Observation of organoleptic parameters of the PHF at initial storage conditions i.e at 0 month followed at 1st,

Table 3. Organoleptic parameters

Organoleptic parameters	Initial	1st Month	3rd Month	6th Month
Taste	Slightly sour	Slightly sour	Slightly sour	Slightly sour
Colour	Pale yellowish green	Pale yellowish green	Pale yellowish green	Pale yellowish green
Odour	Characteristic pleasant	Characteristic pleasant	Characteristic pleasant	Characteristic pleasant

Table 4. Physciochemicalparameters of PHF 1 on storage

Physcio-Chemical parameters	Initial	1st Month	3rd Month	6th Month
LOD (%w/w)	2.56	2.88	2.98	3.12
Total Ash value (%w/w)	8.05	8.11	9.24	9.01
Acid insoluble ash value (%w/w)	2.52	2.67	2.90	2.89
Water soluble ash value (%w/w)	5.21	5.30	6.43	7.01
Water soluble extractive value (%w/w)	9.34	9.45	10.24	10.67
Alcohol soluble extractive value (%w/w)	2.65	2.78	3.67	4.24
pH Value	5.6	5.7	6	6.2

Table 5. Heavy Metal Analysis of PHF

Heavy metal	Results	Permissible limit as per WHO guidelines in ppm*
Arsenic	BDL	Not more than 3 ppm
Lead	2.26 ppm	Not more than 10 ppm
Mercury	BDL	Not more than 1 ppm
Cadmium	BDL	Not more than 0.3 ppm

*parts per million

3rd and 6th month is depicted in Table 3. No change in the organoleptic parameters was observed at the end of the study.

3.2.2 Physicochemical Evaluation

Polyherbal formulation was studied for physicochemical parameters at the initial, 1, 3, and 6-month intervals are shown in Table 4.

3.2.3 Heavy Metal Evaluation

Heavy metals in the formulation were estimated based on preliminary analysis results given in Table 5.

3.2.4 Microbial Load

The microbial analysis of the PHF was studied initially at 0 month and at 6th month as per API 2007[11]. Microbial growth was determined to be below the API 2007 limits, and harmful organisms such as *E. Coli, Staphylococcus aureus Pseudomonas aeruginosa* and *Salmonella* spp. were completely absent. The results of microbial analysis is given in Table 6.

The intercept and slope for the physicochemical parameters were obtained from the respective charts (Figure 5). The intercept and slope (Table 7) were used

Table 6. Total microbial growth analysis report in PHF

Microbial test	Initial	6th Month	Permissible limit as per WHO
Total bacterial count (cfu/g)	37000 CFU/g	38500 CFU/g	NMT 10^5 /g
Total fungal count (cfu/g)	220 CFU/g	256 CFU/g	NMT 10^3 /g
Escherichia coli	Absent	Absent	Absent
Pseudomonas aeruginosa	Absent	Absent	Absent
Staphylococcus aureus	Absent	Absent	Absent
Salmonella Spp	Absent	Absent	Absent

cfu: colony forming units, g: grams

Table 7. PHF intercept and slope of various parameters

Physcio-chemical parameters	Initial	Intercept	Slope
LOD (%w/w)	2.56	2.68	0.080
Total Ash value (%w/w)	8.05	8.142	0.184
Acid insoluble Ash value (%w/w)	2.52	2.595	0.06
Water soluble Ash value (%w/w)	5.21	4.355	0.653
Water soluble extractive value (%w/w)	9.34	9.336	0.235
Alcohol soluble extractive value (%w/w)	2.65	2.635	0.28
pH Value	5.6	5.619	0.102

Table 8. Period for 10% Degradation (approximate)

Physcio-chemical parameters	Initial	Result 10% Degradation	Months required for 10% degradation
LOD	2.56	2.3	4.73
Total Ash value	8.05	7.245	4.87
Acid insoluble ash value	2.52	2.268	5.45
Water soluble ash value	5.21	4.689	0.51
Water soluble extractive value	9.34	8.406	3.96
Alcohol soluble extractive value	2.65	2.385	0.89
pH Value	5.6	5.04	5.68
Mean months when 10% degradation occurs			3.7

Table 9. Extrapolation of shelf life

Formulation	Mean Months for 10% degradation	Multiplication Factor	Shelf Life	
			Months	Years
PHF 1	3.7	3.3	12.21	More than 1 year

Table 10. Observed IR peaks of PHF (0 Month) and their interpretation

Observed peak (cm-1)	Group present	Interpretation
3267	Ar-OH (H bonded)	Phenols
2920	$-CH_2-$ (C-H stretching)	Alkanes
1607	-C=C stretch	Alkenes
1718	-C=O stretch	Ketone

Table 11. Observed IR peaks of PHF (1st month) and their interpretation

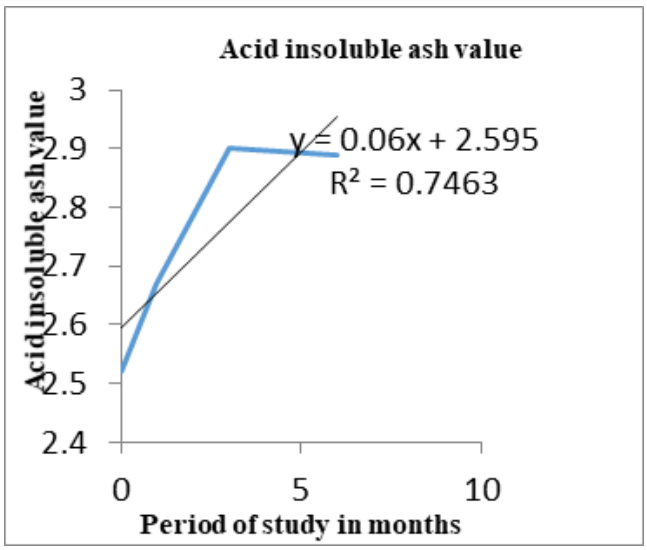
Observed peak (cm-1)	Group present	Interpretation
3274	-C=C-CO-OH	Carboxylic acid
2914	$R-CH_2CH_3$	Alkane
1703	-C=O Stretch	Aldehyde /ketone
1612	-C=C-	Conjugated alkenes
1422	Ar C-C	Aromatic ring
1210	Ar-O-R	Ether (C-O stretch)
1026	-C-CO-R	Ether Bend

Table 12. Observed IR peaks of PHF (3rd month) and their interpretation

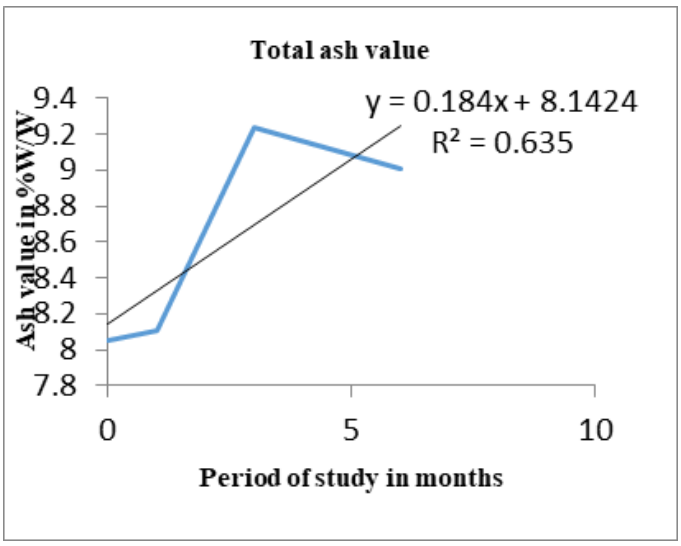
Observed peak (cm-1)	Group present	Interpretation
3460	R-OH	Alcohol
3265	-C=C-CO-OH	Carboxylic acid
2915	$R-CH_2CH_3$	Alkane
1704	-C=O Stretch	Aldehyde/ketone
1603	-C=C-	Conjugated alkenes
1525,1447	Ar C-C	Ar C-C Stretch
1208	Ar-O-R	Ether (C-O stretch)

Table 13. Observed IR peaks of PHF (6th month) and their interpretation

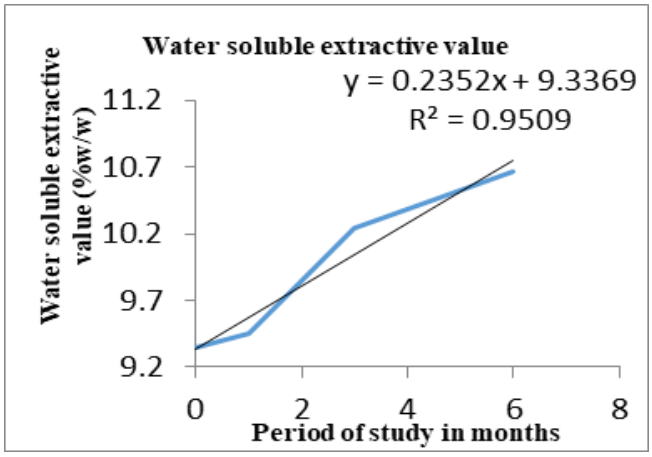
Observed peak (cm-1)	Group present	Interpretation
3293	-C=C-CO-OH	Carboxylic acid
2913	$R-CH_2CH_3$	Alkane
1718	-C=O Stretch	Ketone
1604	-C=C-	Conjugated alkenes
1515	Ar C-C str	Aromatic ring
1230	Ar-O-R	Ether (C-O stretch)


Acid insoluble ash value
y = 0.06x + 2.595
R² = 0.7463
Acid insoluble ash value
Period of study in months

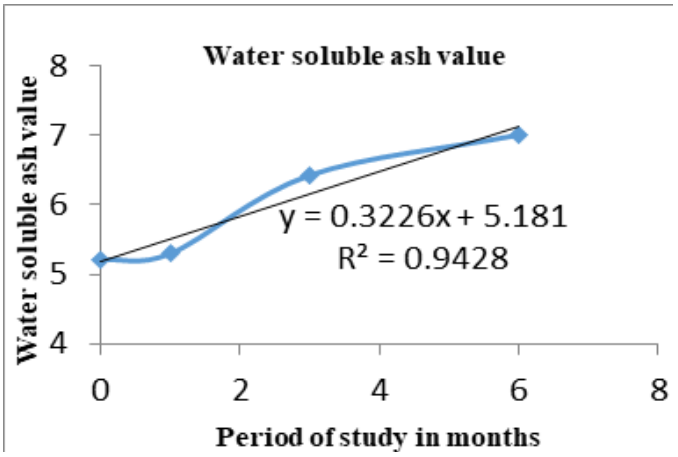
A


Total ash value
y = 0.184x + 8.1424
R² = 0.635
Ash value in %W/W
Period of study in months

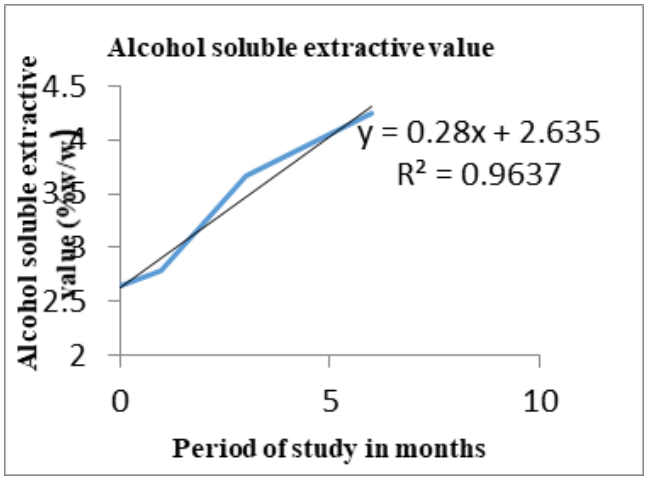
B


Water soluble extractive value
y = 0.2352x + 9.3369
R² = 0.9509
Water soluble extractive value (%w/w)
Period of study in months

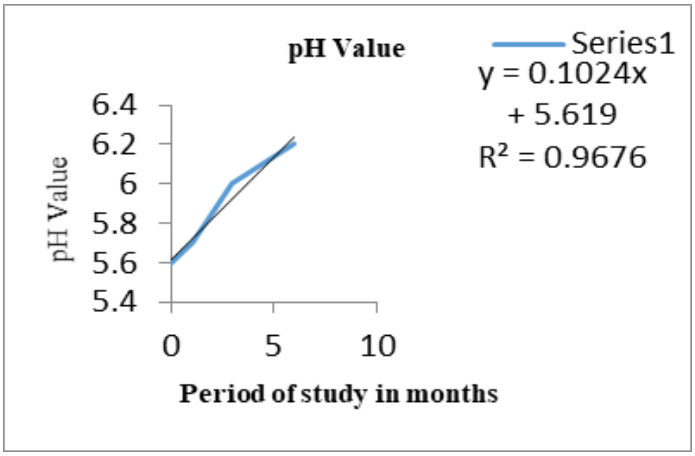
C


Water soluble ash value
y = 0.3226x + 5.181
R² = 0.9428
Water soluble ash value
Period of study in months

D


Alcohol soluble extractive value
y = 0.28x + 2.635
R² = 0.9637
Alcohol soluble extractive value (%w/w)
Period of study in months

E


pH Value
Series1
y = 0.1024x + 5.619
R² = 0.9676
pH Value
Period of study in months

F

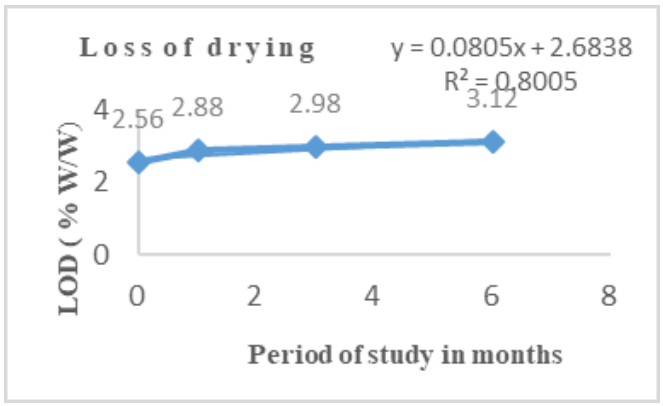


Figure 5. Chart for Acid insoluble ash **(A)** total ash values **(B)** Water soluble extractive value **(C)** Water soluble ash value **(D)** Alcohol soluble extractive value **(E)** pH value **(F)** Loss on drying **(G)** at 0,1,3 and 6th months.

to calculate the months required for 10% degradation. The months required for 10% degradation on storage under accelerated condition was calculated to be at 3.56 months (Table 8).

3.2.5 Extrapolated Shelf-life Calculation

Extrapolation of shelf life for climatic Zone I & II nations and climatic Zone III & IV countries was done using real-time ageing factors 5 and 3.3, respectively. India is classified as climatic zones III and IV[12]. As a result, the estimated shelf life of the formulation under accelerated conditions was calculated using a multiplication factor of 3.3. It was found that extrapolated shelf life of PHF would be stable 12.21 months or approximately 1 year 2 months (Table 9) under the specified storage conditions. The accelerated study period's predictive factor for zone III was 3.3[12,13]. It means that if a product is stable for 6 months at 40°C and 75% RH, its shelf life will be 20 months at 30°C and 70% RH (climatic zone IV). As a result of the above interpretations, it is safe to conclude that PHF has a shelf life of 12.21 months at room temperature.

3.3 Stability Study by FTIR Analysis of PHF

The PHF was subjected to FTIR studies at 0, 1, 3 and 6th month of accelerated stability testing procedure. During the storage period, chances of deterioration of formulation due to temperature, light and humidity, which affects the chemical constituents, this variation can be traced by IR spectroscopic studies. The respective IR spectra are shown in Figures 6 to 9 and interpretation data given in Tables 10–13.

The FTIR spectrum of PHF is shown in figure, where the characteristic bands of individual components of the formulation were identified and were detected. The OH groups stretching were detectable at 3460 -3200 cm^{-1}. was observed at all times of IR analysis of PHF. This could be attributed to the presence OH in gallic acid/ gymnemic acid/quercetin as well as in mahanine. All the three spectra show the presence of unsaturated monocarboxylic acid functional group of gymnemic acid. The C=O aryl ketonic stretch absorption was evident at 1700 cm^{-1} in all the three spectra. C=C-C=C conjugated alkene stretching vibrations were detectable at 1603, 1612, and 1604 cm^{-1} respectively in all the three spectra. Bands at 1210, 1208, and 1230 cm^{-1} were attributable to the C–O stretching in the aryl ether ring, Aromatic C-C stretching vibrations can be observed at 1447, 1525 and 1515 cm^{-1} in the three spectra. These identical IR spectral bands of the PHF 1 at 1st month, 3rd and 6th month indicate that the PHF has not undergone any significant deterioration on storage.

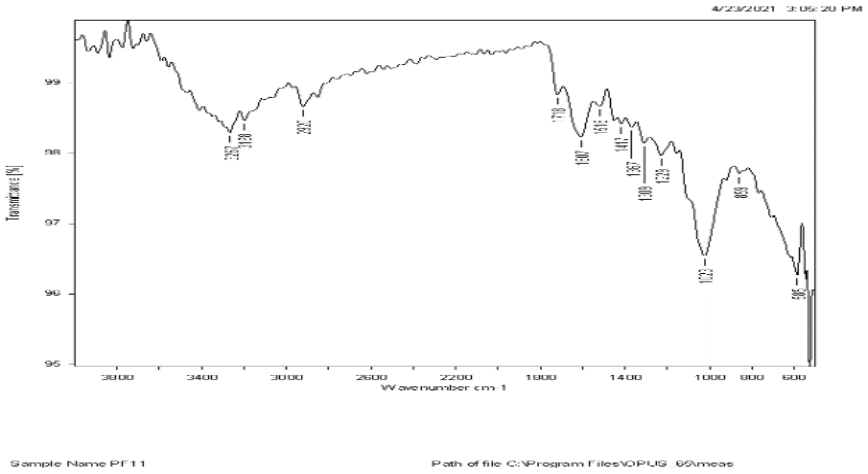


Figure 6. IR spectrum of PHF at 0 month.

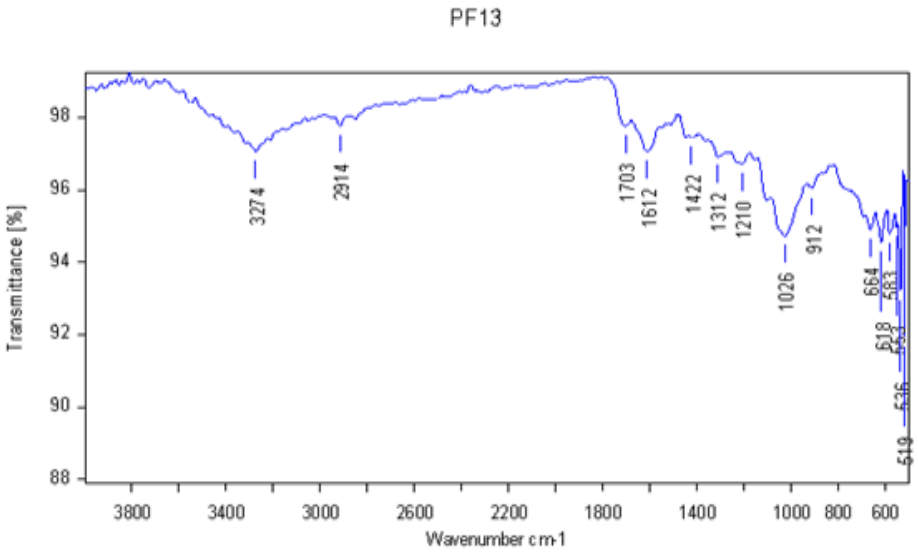


Figure 7. IR spectrum of PHF at 1st month.

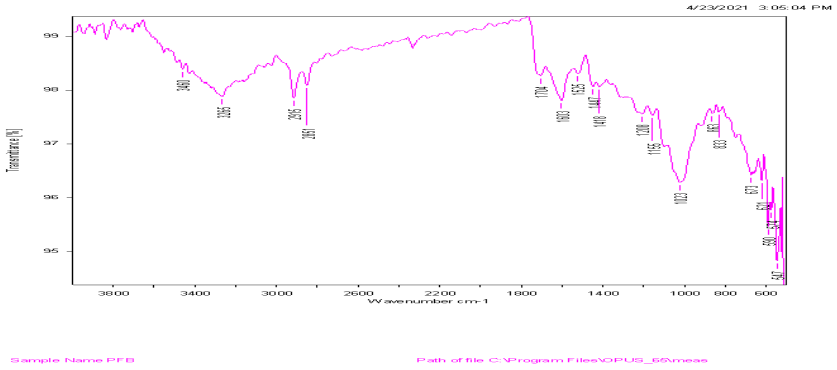


Figure 8. IR spectrum of PHF at 3rd month.

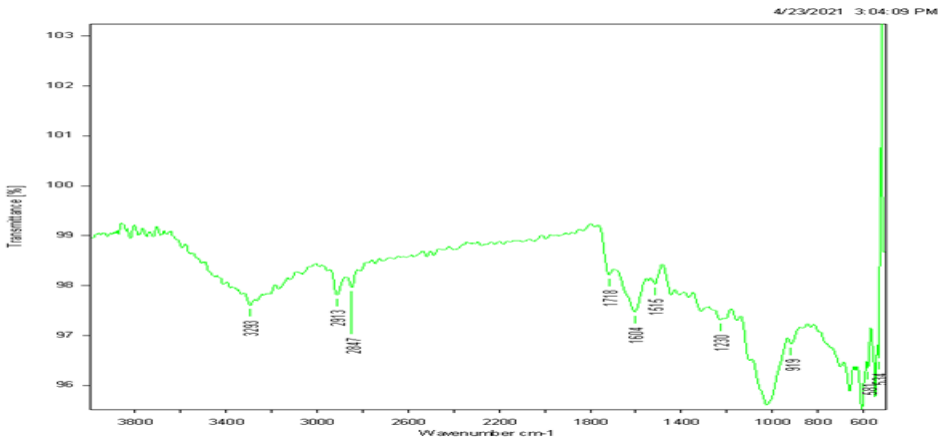


Figure 9. IR spectrum of PHF at 6th month.

4. Conclusion

This research work deals with the quantification of active principles in the Polyherbal formulation under study. The PHF constituents are Amla fruit, Gymnema leaves, Acacia bark and Curry leaves. The active principles responsible for antihyperlipidemic activity was selected as biomarkers namely Gallic acid, Quercetin, Gymnemic acid and Mahanine. The PHF was quantified by RP-HPLC method and found to contain appreciable amount of the selected biomarkers. The stability and shelf life of the drug was evaluated under accelerated stability conditions of storage parameters like storage at 40°C ± 2, Relative Humidity (RH): 75 % ± 5. The samples were analysed at 0-, 1-, 3- and 6-months interval. The formulation passed the organoleptic parameters and few physico-chemical parameters except extractive value. The mean months of 10% degradation was found as well the extrapolated shelf life of the PHF1 was determined to be more than 1 year. API and WHO has given 2 years for stability of churna formulations. But it depends on the phytochemicals present. The stability study was also confirmed by IR spectral analysis at 0-, 1-, 3- and 6-months interval. So under accelerated condition the drug is stable for 6 month and extrapolated shelf life could be 12.21 months.

5. Acknowledgement

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6. Conflict of Interest

No Conflict of Interest

7. References

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