Standardization of Polyherbal Extract for Type-2 Diabetes

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Abstract: *Aims*: This study was aimed to standardize the polyherbal extract containing *Annona squamosa, Phyllanthus emblica, Berberis aristata and Curcuma longa* for the management of type-2 diabetes. The standardization of polyherbal formulation is indispensable in order to achieve the quality, purity, safety and efficacy of drugs.

Study Design: Physico-chemical investigations, Physical characteristics, Qualitative phytochemical analyses, fluorescence analysis and HPLC analysis.

Materials and Methods: The Standardization of polyherbal extract was based on systematic organoleptic evaluation, physico-chemical investigation, physical characteristics, heavy metal analysis, fluorescence analysis, phytochemical screening, total alkaloid content, determination of viscosity, surface tension, density and HPLC analysis were carried out by official method.

Results: Organoleptic evaluation resulted that it was yellowish green in colour with characteristic odour, bitter, pungent taste and fine texture. All the applied Physico-chemical parameters like total ash, acid insoluble, water soluble ash, extractive values, observed pH, moisture content, crude fibre, foaming index were found to be within limit. The limits obtained from physical and other parameters could be used as reference in quality control. The phytochemical analysis indicated the presence of alkaloids, carbohydrates, flavonoids, volatile oils, tannins, saponins, phytosterols and mucilage. Absence of detectable levels of heavy metal confirmed that extract was non-toxic in nature. HPLC studies confirm the presence of marker compounds in each extract.

Conclusion: On the basis of observations and experimental results, the study can be used as reference standard for the further quality control research as it significantly ensures the use of genuine and uniform material and well-designed methodologies for standardization and development of poly herbal extract.

Keywords: Standardization, HPLC, Annona squamosa, Berberis aristata, Curcuma longa, Phyllanthus emblica.

1. INTRODUCTION

The prevalence of diabetes mellitus is escalating worldwide because of aging population structure, increasing obesity and stressing life style in the developed countries as well as in developing countries. Diabetes mellitus is a chronic metabolic disorder portray by degeneration of carbohydrate, protein and fat metabolism. These alterations results in increased blood glucose, which causes long-standing complications in many organs [1]. It is necessary to prevent acute symptoms and/or reduce the severity of upcoming chronic microvascular and macrovascular complications associated with diabetes [2]. Risk factors associated with diabetes include family history, age, obesity, abdominal fat, hypertension, ethnic background, lack of physical work out and food habit [3], this may be the reason of multiplying diabetic population worldwide and projected diabetes as main disablers/killers in coming next 25 year. Many herbal products and traditional plant medicines are recommended and used all over the world for the management of diabetes [4].

Herbs are customarily considered risk-free moreover increasingly being consumed by people without prescription. However, some herbs can cause health troubles, toxicity, less effective and may interact with other drugs. Health has been important always for mankind since ancient time so in market the health related product has been manufactured at different levels, therefore it is necessary to make sure with quality product with active constituents [5].

World Health Organization (WHO), record revealing 80% population of the world relying yet on herbal medicines [6]. Due to remarkable passion for medicinal plants people use them for many health related problems like common cold, memory enhancer, develop resistance and many more [7]. Medicinal plants as antidiabetic agents could be a good eminence source for drug design [8]. The significance of medicinal plants for public health care in developing countries appreciated by WHO and developed

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guidelines on traditional medication to study their prospective including evaluation, safety, purity and efficiency [9].

Herbal formulations standardization is vital in line to review the quality of drugs not only the bases of their active principal's concentration but also cover the complete field of study from birth of plant to its clinical applications. The quality appraisal of herbal preparation is an elementary prerequisite of industry/organization dealing with Ayurvedic and herbal products and make sure that every packet of medicine should have correct amount with therapeutic index [10].

The need of the hour is to standardize the herbal raw materials and herbal formulations with methodical approach and with well-designed methodologies [11].

The present research work was design to standardize a polyherbal extract containing *Annona squamosa, Berberis aristata, Curcuma longa, Phyllanthus emblica* as given in Table **1**.

Annona squamosa belong to family Annonaceae is commonly known as Custard apple/sugar apple, and fruit is known custard apple [12]. *Berberis aristata* DC. (Family Berberidaceae) is one of the herbs mentioned in various used for the treatment of numerous illnesses. *Berberis aristata* is popularly known as Daruhaldi and Indian barberry [13] *Curcuma longa* L. (Family Gingeberaceae) is a medicinal plant extensively known as turmeric used as home remedy for various diseases and well documented for its therapeutic potential [14]. *Phyllanthus emblica* belongs to family Euphorbiaceae. It is also named as Amla, *Emblica officinalis* or Indian gooseberry [15].

2. MATERIALS AND METHODS

All the matured curde drugs were collected and authenticated by botanist from Botanical Serve of India (BSI) and Forest Research Institute (FRI) Uttarakhand. It helps in identification of crude drugs for further evaluation.

2.1. Preparation of Homogenous Mixture

All the dried extracts (*Annona squamosa, Berberis aristata, Curcuma longa, Phyllanthus emblica)* weighed combined together and passed through sieve and mixed together in specified proportion to get homogenous mixture used for standardization.

2.2. Standardization Parameters

The poly herbal extract was standardized for organoleptic evaluation, physico-chemical investigation, physical characteristic of powder, heavy metal analysis, fluorescence analysis, phytochemical analysis and quantitative analysis.

2.2.1. Physico-Chemical Investigations

Physicochemical parameters such as ash values, extractive values, loss on drying, pH, foaming index and crude fibre were determine method given by WHO [16] and Khandelwal [17].

2.2.2. Physical Characteristics

Physical characteristics include bulk density, tap density, angle of repose were analysed as per given method by Lackman [18] and hausner's ratio were carried out as suggested by Chandel [5].

2.2.3. Qualitative Phytochemical Analyses

For identifying various phytoconstituents present in combined extract were performed by following standard method in Kokate and Khandelwal [19, 17].

2.2.4. Fluorescence Analysis

Powder polyherbal extract was exposed at ordinary and ultraviolet light. 1 mg of sample was placed on a glass slide and treated with various chemical reagents for observed their fluorescence characters under UV and visible light [20].

2.2.5. Viscosity, surface tension and density determined with 1% aqueous solution of sample according to given method in Martin 1991 [21].

Table 1: Composition of Polyherbal Extract

S. No.	Common Name	Botanical Name/Family	Part used	Quantity taken (mg/kg)
1	Indian Gooseberry	*Phyllanthus emblica/ Euphorbiaceae*	Fruit	50
2	Custard Apple	*Annona squamosa/ Annonaceae*	Leave	50
3	Indian Barberry	*Berberis aristata/ Berberidaceae*	Root	100
4	Turmeric	*Curcuma longa/ Zingiberaceae*	Rhizome	100

2.2.6. Determination of Total Alkaloid Content in Annona squamosa

Weigh powdered leaves of sample was extracted with ethanol in soxhlet apparatus, extract was filtered and filtrate was concentrated. Ethanolic extract of plant material was socked with 15 ml of NH_4OH and extracted at room temperature with ethyl acetate for three days. Extract was filtered and solvent was evaporated under reduced pressure. The residue dissolved in H_2O and acidified with H_2SO_4 to bring pH 3-4, and then extracted with petroleum ether (40-60°) to remove fats, lipids, waxes, acids and neutral material. Defatted marc obtained and made basify with NH_4OH and extracted with $CHCl_3$, then extract washed with distilled water to obtained neutral pH, dried with Na_2SO_4, and concentrated under reduced pressure to obtain crude alkaloids [22]. The residue was performed TLC by using solvent system toluene: ethyl acetate diethyl amine (70:20:10), detection of alkaloids was done with Dragendorff's sodium nitrite reagent in UV (254nm) was done by following method of Wagner [23].

2.2.7. Heavy Metal Determination

Contamination of medicinal plant materials with heavy metals (Pb, As, Cd, Hg) can be recognized by many reasons including environment pollution and traces of pesticides. Sample of dried plant extract were placed in clean silica crucibles, digested with a mixture of acid having a ratio of conc. HNO_3: $HClO_4$ (1:1) and heated until a white residue was obtained. The dry residue in each crucible was dissolved in 20 ml of distilled water and used for estimation of heavy metal concentration. Digested sample were analysed for Cd and Pb using graphite furnace atomic absorption spectrophotometer (AAS), concentration of Hg was determine through AAS using cold vapour technique and air acetylene flame was used for determination of As concentration. The metal quantification was based on calibration curve by using chemical standard with 1000mg/L concentration. In the samples, concentration of the particular metal was expressed as mg of metal per kg (ppm) [24].

2.2.8. HPLC Analysis

It is simple rapid and precise method for identification. Standard prepared by dissolving 2 mg of each standard was weighed and dissolved in 1 ml of solvent (methanol), from which 100 µl was taken and made up to 1 ml with solvent (methanol), from this stock solution 200 µl was injected.

10 mg of each sample was weighed separately and dissolved in 10 ml solvent (methanol) and filtered. 20 µl of each filtered sample were injected in to Column RP C_{18}, (250 X 4.6mm, 5microns) High Performance Liquid Chromatography system (Shimadzu LC-10 ATVP) and with Flow rate 1 ml/min, connected to detector UV 275 nm by using mobile phase Methanol: Phosphate buffer (70:30) pH: 4.5 (0.005M).

3. RESULTS

Organoleptic evaluation revealed yellowish green coloured powder with characteristic odour, bitter pungent taste and fine texture as shown in Table **2**.

Table 2: Organoleptic Properties of Polyherbal Extract

Organoleptic Properties	Characteristic
Appearance	Powder
Colour	Greenish yellow
Odour	Characteristic
Taste	Pungent bitter
Texture	Fine
Particle size	100 mesh

Physicochemical investigation have resulted that total ash, acid insoluble and water soluble ash values were 11.58%, 2.23% and 8.47% respectively. Water soluble extractive value (15.23% w/w) was found lower than alcohol soluble extractive value (50.53% w/w). Observed pH values of 1% and 10% solution were 5.61 and 4.81 % w/v, moisture content 4.11%, crude fibre 0.22 gm and foaming index less than 100 shown in Table **3**.

Table 3: Physico- Chemical Characteristic of Polyherbal Extract

S. No.	Parameters	Mean ± SEM
1	Total ash % (w/w)	11.58 ± 0.62
2	Acid insoluble ash % (w/w)	2.23 ± 0.11
3	Water soluble ash % (w/w)	8.47 ± 0.32
4	Water soluble extractive % (w/w)	15.23 ± 0.68
5	Alcohol soluble extractive % (w/w)	50.53 ± 1.52
6	Moisture content % (w/w)	4.11 ± 0.13
7	pH 1% w/v and 10 % w/v	5.16 ± 0.10, 4.81 ± 0.06
8	Crude fiber (gm)	0.22 ± 0.01
9	Foaming index	<100

Physical parameters like bulk density (0.43), angle of repose (38^o), hausner's ratio (1.13) indicating good

compressibility, the rheological properties of 1 % solution have shown density 0.79, viscosity 0.99 cp and surface tension 48.6 mentioned in Tables **4** and **5**.

Table 4: Physical Characteristic of Polyherbal Extract

S. No.	Parameters	Mean ± SEM
1	Bulk density (gm/ml)	0.43 ± 0.020
2	Tapped density (gm/ml)	0.51 ± 0.014
3	Hausner's ratio	1.13 ± 0.142
4	Angle of repose (°)	38° ± 2.020

Table 5: Determination of Density, Viscosity and Surface Tension

Parameters	Values
Density 1% solution	0.79
Viscosity 1% solution	0.99cP (centipoise)
Surface tension 1% solution	48.6 dyne/cm

Phytochemical analysis of polyherbal extracts have shown the presence of alkaloids, carbohydrates, flavonoids, volatile oils, tannins, saponins, phytosterols and less amount of mucilage as shown in Table **6**.

Table 6: Qualitative Phytochemical Analysis

S. No.	Phytoconstituents	Observation
1	Alkaloids	+
2	Carbohydrates	+
3	Glycosides	-
4	Phytosterols	+
5	Proteins	-
6	Flavonoids	+
7	Tannins	+
8	Volatile oils	+
9	Gums and mucilage	less
10	Saponins	+

(+) present, (-) Absent.

Fluorescence analysis have been presented in Table **7** and heavy metal analyses of polyherbal extract were found with in permissible limit. Total alkaloid content was found 0.82% w/w *in Annona squamosa* extract.

Each extract was subjected to reverse phase chromatography as mentioned in material and methods. Retention time for standard berberine was 4.41 min (Figure **1**) and for sample was 4.50 min (Figure **2**). The retention time for standard Curcumin was 7.33 min (Figure **3**) and sample was 7.28 min (Figure **4**). The retention time for standard Gallic acid was 2.84 min (Figure **5**) and sample was 2.88 min (Figure **6**). All the major peaks with retention time were analysed and shown the presence of marker compound in each extract and percentage of each constituents in Table **8**.

Table 7: Fluorescence Analysis

Powdered drug	Visible/ordinary light (254 nm)	Ultra violet light (366nm)
Powder as such	Light green	Yellow green
Powdered + $Fecl_3$	Dark green	Light green
Powder + Conc 50% HCL	Orange green	Brown yellow
Powder + 1 N NaOH	Light green	Green
Powder + Conc50% H_2So_4	Dark yellow	Yellowish green
Powder + Methanol	Dark green	Light green
Powder + CH_3COOH	Orange green	Yellow green
Powder + I_2	Black green	Dark green

4. DISCUSSION

Standardization of poly herbal extract has been carried out according to WHO guidelines which involves various parameters like organoleptic properties (appearance, colour, odour, taste, texture and particle size), physico-chemical characteristics (ash value, extractive value, moisture content, pH, crude fibre and foaming index), physical characteristics (bulk density, tapped density, hausner's ratio, angle of repose), density, viscosity, surface tension, qualitative phytochemical, fluorescence, heavy metal and quantitative analysis as well. All these parameters are essential for selection and handling of crude material, safety, efficacy and stability assessment of finished product.

Organoleptic characteristic of powdered drug showed identity of the herbs. Results of quantitative analysis of the polyherbal extract were within limit confirming that extract have less impurities and help in determining authenticity and purity of drug. Total ash indicate amount of minerals and earthy minerals and acid insoluble ash indicate presence of siliceous matter in the plant. Other parameter like extractive value indicates solubility and good quality of powder. The

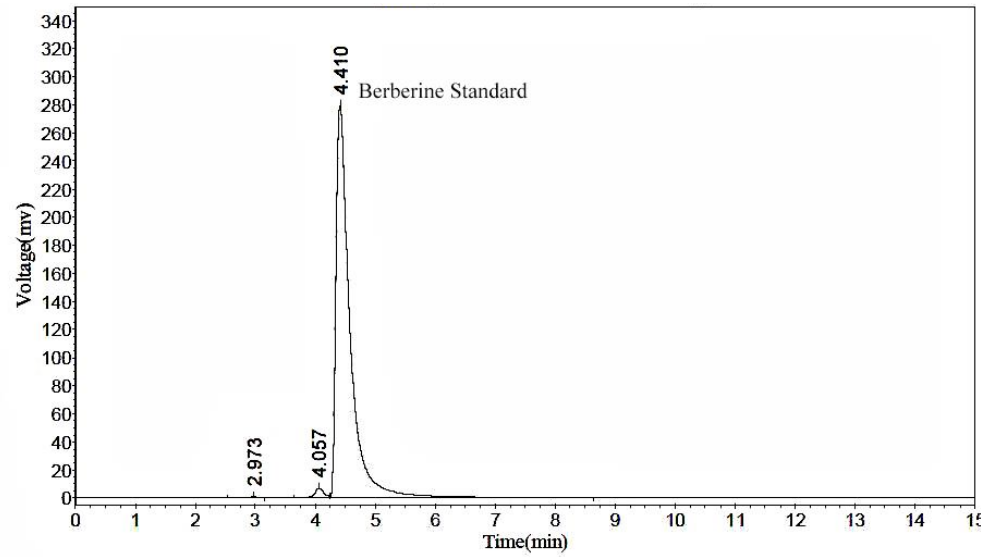

Figure 1: HPLC Chromatogram of standard Berberine.

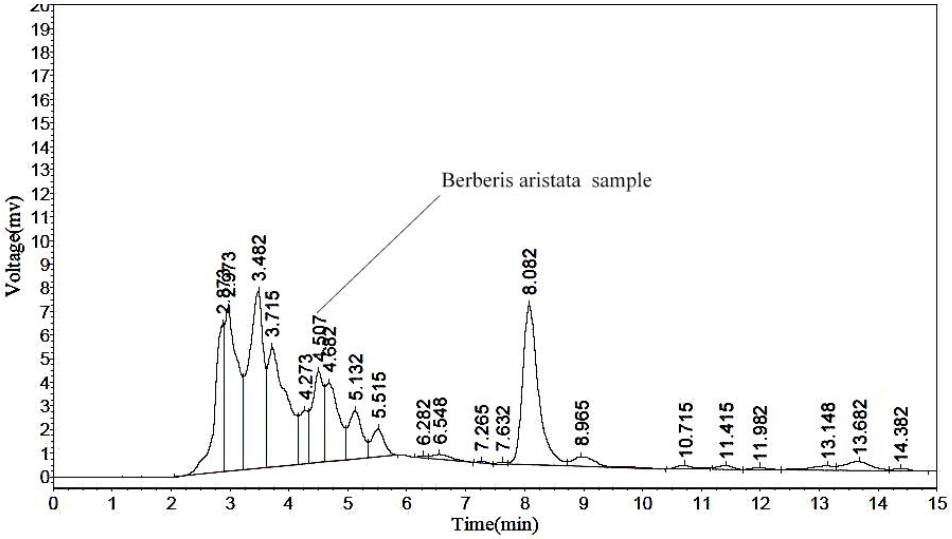

Figure 2: HPLC Chromatogram of ethanolic extract of *Berberis aristata* sample.

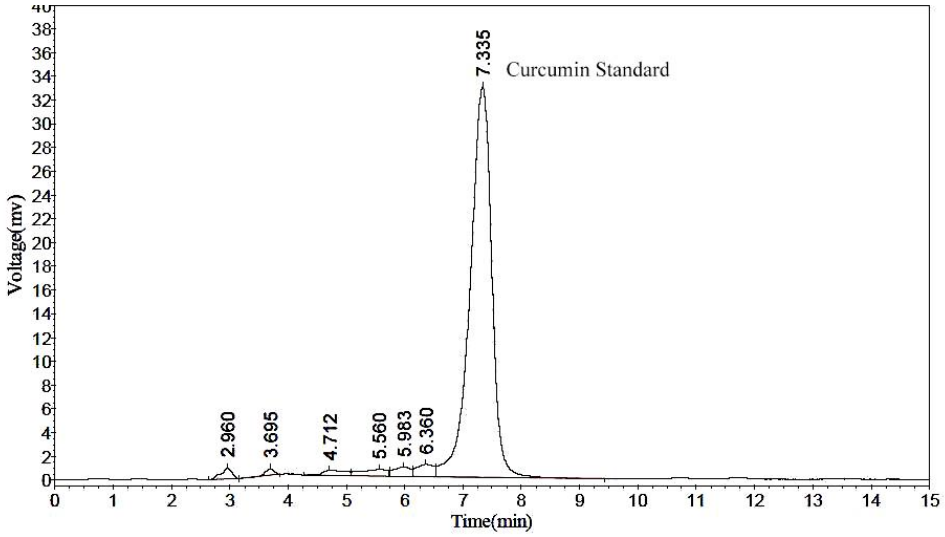

Figure 3: HPLC Chromatograph of standard Curcumin.

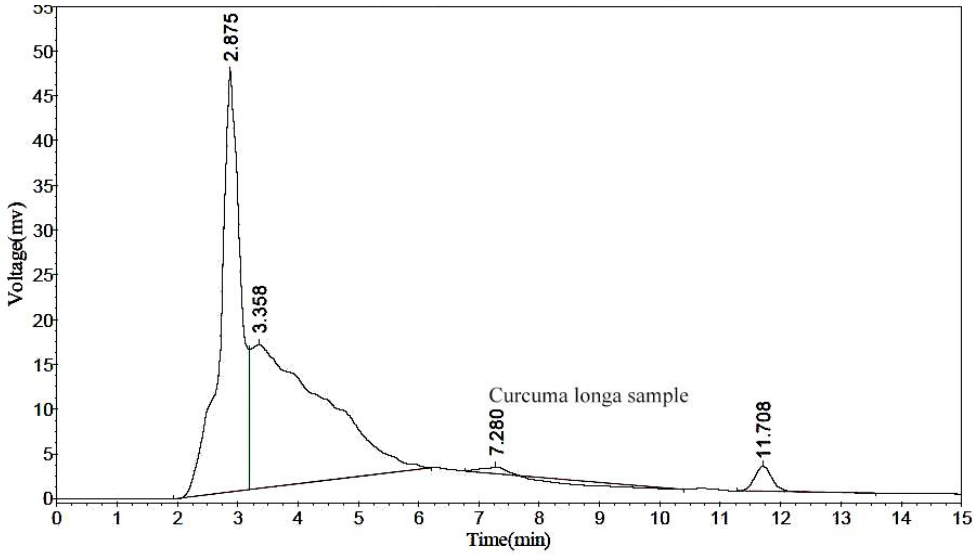

Figure 4: HPLC Chromatograph of methanolic extract *Curcuma longa* sample.

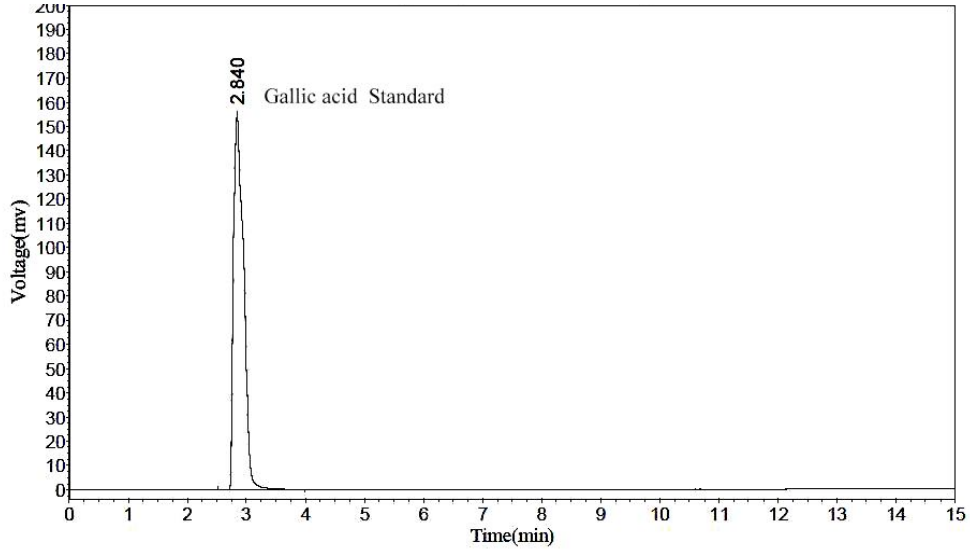

Figure 5: HPLC Chromatograph of standard Gallic acid.

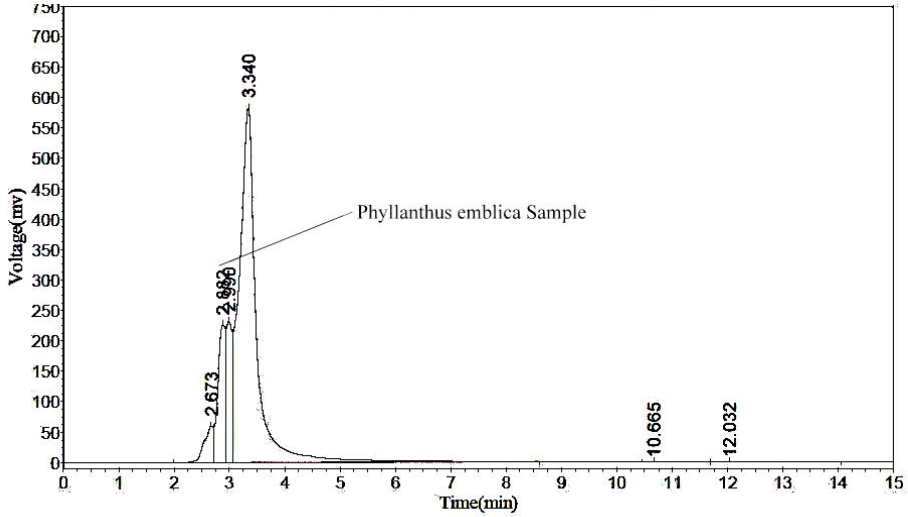

Figure 6: HPLC Chromatograph of ethanolic extract of *Phyllanthus emblica* sample.

Table 8: HPLC Estimation of Marker in each Extract

S. No.	Type of Sample	RT	Presence of marker in Extract (%)
1.	Berberine	4.410	0.045
	Berberis aristata	4.507	
2.	Curcumin	7.335	0.499
	Curcuma longa	7.280	
3.	Gallic acid	2.840	4.643
	Phyllanthus emblica	2.882	

water soluble extractive value indicated the presence of sugar, acids and inorganic components and alcohol soluble extractive value indicated presence of polar constituents [25].

Loss on drying was less than 5% w/w indicated the less chances of microbial growth like bacterial, fungal and yeast because low moisture content is constantly advantageous for higher stability of drugs [26]. Physical properties like bulk density, Tap density, Hausner's ratio, Angle of repose used as an indirect method to predict powder for bulkiness, flow properties and interparticle cohesion [5]. The observation of pH values were in the range from 5.2 to 5.9 indicating suitability for human use [5].

Phytochemical analysis revealed the presences of phytoconstituents and quantification of important chemical constituents in powdered drugs were tabulated in Table **6** [27]. It has been observed powdered herbal drugs get easily adulterated, but fluorescence analysis help in distinguishing feature for determination of a drug. In present study powdered drug exhibited various shade of green its mean parameter ascertain its purity and standard [28].

Polyherbal extract contains good amount of alkaloids because it contains *Annona squamosa* and *Berberis aristata* which are rich in alkaloid contents [29, 30]. Total alkaloid content found in *Annona squamosa* after extraction was 0.82% w/w.

Heavy metals may be present in crude drugs through atmospheric pollution and soil; moreover minerals and metals are also used in preparing Ayurvedic formulations. However, heavy metals have been associated with various adverse effect including status epilepticus, fatal infant encephalopathy, hepatotoxicity, congenital paralysis and deafness, and development delay. Because of heavy metals, many serious adverse reactions have been reported therefore heavy metal needs to be detected in herbal drugs [31]. In present study polyherbal extract showed with in permissible limit of heavy metal, therefore extracts are non-toxic in nature.

Each extract was subjected to HPLC as given in material and methods and showed chromatogram for standard and sample; also found the retention time and percentage of each marker compound. HPLC is main analytical and most frequently used technique for identification and quantification of marker compounds in more complex herbal mixture [7] with in less time. HPLC chromatograph not only provides information for correct identification but also serve as future standard data for quality assessment of pharmaceutical samples.

5. CONCLUSION

WHO guidelines and parameters are now very essential for developing herbal products for various diseases and ailments because it strengthens the process and minimize the sub-standardization. The current study have been subjected to various standardization procedures, may be quite useful for quality control of herbal remedies. These results indicate polyherbal extract are therapeutically safe. Standardization is the sum of all factors with associated with quality, effectiveness, purity, stability and acceptability of the product.

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Author MM designed the study, wrote the

protocol and first draft of the manuscript. Author VJ and AS supervised the research and wrote part of the manuscript. All authors read and approved the final manuscript.

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