Quality control and antioxidant activity of Haritaki, an ayurvedic formulation

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Abstract

Standardization of herbal formulation is requisite to appraise the quality of drugs based on the concentration of their active principle. Haritaki possess a wide variety of activities like antimicrobial, antioxidant, and anti purgative. Market formulation named as haritaki were prepared in the lab using standard raw materials, for comparison purpose, with slight modifications from that of marketed formulation. To establish some standards for the marketed formulation quantitative analysis was carried out as per methods suggested in the WHO manual. Physical characteristic like bulk density, tap density, haussner ratio and Carr's index, weight variation test, friability, hardness test were determined for both formulations. Thus ash values, extractive value, fluorescence Analysis, loss on drying, of microbial limits were estimated. Evaluation was carried out on the total ash obtained, which represents the inorganic salts consists of carbonates, phosphates, silicates, iron, calcium and potassium. These apart preliminary phytochemical analysis, determination of anti bacterial activity and *Invitro* antioxidant activity were carried out. The amount of gallic acid and rutin as a marker compound, for Haritaki on LF and it was estimated in MF (MF_1, MF_2, MF_3, MF_4) by HPTLC.

Keywords: Haritaki, Carr's index, Tap density, Haussner ratio.

Introduction

Herbal formulations due to less side effects has led to an increase in demand, which resulted in the decline of their quality, primarily due to a lack of adequate regulations pertaining to this sector of medicine. Thus, standardization of herbal formulations is essential to assess the quality and purity of the active principles in the formulation through a systematic approach and to develop well-designed methodologies. *Terminalia Chebula* called as Haritaki is the "king of medicines" in Tibet and is always listed first in the Ayurvedic materia medica because of its extraordinary powers of healing[1]. The fruit *Terminalia Chebula* belonging to the family *Combretaceae* contains ellagic acid, gallic acid, chebulinic acid, chebugalic acid and corilagin, has been extensively used in ayurveda, unani & homoeopathic medicine and has become cynosure of modern medicine[2]. *Terminalia Chebula* was traditionally used to cure asthma, urinary disorders, heart disease and reported for cardiotonic, antioxidant, antibacterial, anticancer, hypocholesterolemic, purgative and antispasmodic[3-9]. Gallic acid and rutin are the most common flavonoids present in the medicinal plants which are reported to possess antioxidant, anti-inflammatory, anticancer, hepatoprotective, antiulcer, antiallergic and antimicrobial activities.[10-11]

Quantitative analysis was carried out as per methods suggested in the WHO manual[12]. Thus ash value, extractive value, **f**luorescence analysis and loss on drying, determination of antibacterial activity were determined. Qualitative analysis was also carried out on the total ash obtained, which represents the inorganic salts consists of carbonates, phosphates, silicates, iron, calcium and potassium. Laboratory formulation (LF) and marketed formulation (MF) have been standardized on the basis of organoleptic characters[13], and physico-chemical properties[14]. Physical characteristic like bulk density, tap density, Haussner ratio and Carr's index, weight variation test, friability, hardness test were determined for different formulation. The objective of this work is to make an in-house standards for powder and

compare it with different marketed samples using its fingerprint characteristics and to further quantify them with specific active principle. The amount of gallic acid and rutin as a marker compound, for Haritaki on LF and it is compared with different MF (MF_1, MF_2, MF_3, MF_4) by HPTLC.

Methodology

Collection of raw material

Terminalia chebula (Haritaki) were collected from the local market and physically authenticated in Pharmacognosy laboratory for its genuiness. The Marketed formulations Haritaki (MF_1, MF_2, MF_3, MF_4) were obtained from local Ayurvedic pharmacy, Coimbatore, Tamilnadu.

Reagents and chemicals

Analytical grade toluene, ethyl acetate, methanol, formic acid, chloroform, glacial acetic acid was obtained from SD fine chemicals Ltd, Mumbai. Pure gallic acid and rutin were obtained from the Natural Remedies Ltd, Bangalore. Precoated HPTLC aluminium sheets silica gels 60F254 (10 x10 cm, 0.2 mm thick) were obtained from E. Merck Ltd, Mumbai.

Preparation of extracts

The dried fruit pulp of *Terminalia chebula* the Laboratory Formulation (10 g) and different Marketed formulation sample (10 g) of Haritaki were extracted by Maceration process in 100 mL of methanol for 7 days. The methanolic extract was filtered through Whatmann Filter paper and then evaporated using rotary vacuum evaporator at 40°C. The stock solutions of the extracts were prepared using methanol to get the final concentration of 1g/10ml for LF and MF.

Physico-Chemical Investigations

Physico-chemical investigations of formulations were carried out, including the determination of extractive values and ash values (Table 1)

Phytochemical Analysis

Phytochemical screening procedures were conducted following the procedure of Harborne[15] and kokate [16]

Tests for flavonoids- The extracts were dissolved in alcohol and a piece of magnesium was added followed by a drop of concentrated hydrochloric acid and heated. Appearance of crimson red or occasionally green to blue colour infers the presence of flavonoids.

Test for saponins- To the extract, 20 mL of distilled water was added and agitated on a graduated cylinder for 15 mins. The formation of about 1 cm layer of foam indicates the presence of saponins.

Tests for phenols- One ml of alcoholic solution of extract was diluted with 5 mL of distilled water and to this few drops of 1% aqueous solution of lead acetate was added. A yellow colour precipitate was formed which indicates the presence of phenols.

Test for alkaloids- A small portion of the solvent free extract was stirred separately with a few drops of dilute hydrochloric acid and filtered. To a few mL of the filtrate, 1 mL of Dragendroff's reagent was added. Formation of orange red precipitate indicates the presence of alkaloids.

Tests for tannins- In a test tube containing about 5 mL of an aqueous extract, few drops of lead acetate was added. A yellow or red colour precipitate was formed indicates the presence of tannins and the data were recorded in Table 2.

Determination of Physical Characteristics of the Formulations[17]

Physical characteristic like bulk density, tap density, Haussner ratio and Carr's index, weight variation test, friability, hardness test were determined for different formulation (Table 3)

Bulk Density and Tapped Density

The term bulk density refers to packing of particles or granules. The volume of packing can be determined in an apparatus consisting of graduated cylinder mounted on mechanical tapping device (Thermonik Tap Density Apparatus). The Twenty five gram of weighed formulation powder was taken and carefully added to cylinder with the aid of a funnel.

The initial volume was noted and sample was then tapped until no further reduction in volume was noted. The initial volume gave the bulk density value and after tapping the volume reduced, it gives the value of tapped density. The equation for determining bulk density (D_b) is $D_b=M/V_b$ where M is the mass of particles and V_b is the total volume of packing. Haussner ratio is related to inter particle friction and as such can be used to predict the powder flow properties. The equation for measuring the Haussner ratio is D_f/D_o, where D_f is the tapped density and Do is the bulk density. *Carr's index* is another indirect method of measuring the powder flow from bulk density. The equation for measuring Carr's index is $I= (D_f-D_o)/ D_f$ x100 Where D_f is the tapped density and D_o is the bulk density.

Weight Variation Test- Weight of 20 tablets was taken and then average weight of tablet was found and further take the individually weight of each tablets. Compare the individual weight of each tablet with average weight of tablet. The percentage deviation was calculated.

Hardness Test- The hardness of the tablets is measured by using Monsanto hardness tester. It indicates the tensile strength and it is measured in terms of kg / cm^2. The friability of the tablet is found to determine the capacity of the tablets to withstand the stress/shocks that may develop during its transport. This generally refers to loss in weight of tablets in container due to removal of fine particle from their surface.

Fluorescence Analysis-Four different CF of haritaki and LF of haritaki were exposed to UV radiation at wavelength of 254 nm and in day light. The fluorescence analysis was carried out in accordance with the procedure reported by Kokoshi *et al*[18]and Chase & Pratt[19]. One milligram of was placed on a micro slide and treated with 1 mL of 1M HCl, 1M NaOH, Conc. H_2SO_4, Conc. HNO_3, Con.HCl, Glacial acetic acid and Iodine and observed under UV 254 nm and in day light and the results were recorded in Table 4.

Determination of microbial limits

Microbial analysis was carried for the microbial contamination as per procedures of Indian pharmacopoeia[20] (1996) and WHO guidelines (1998). The test included total bacterial count, total yeast and mould count, identification of specified organism such as *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus and Pseudomonas aeruginosa.*

Chromatographic Conditions

Methanolic extracts of LF and MF of haritaki and standard gallic acid and rutin were spotted on a Precoated TLC aluminium sheets (silica gel 60 F_{254} 10x10cm, 0.2 mm thickness) as 6 mm wide band width by using automatic TLC applicator Linomat V,10 mm from the bottom. Chromatogram was developed using Toluene:ethyl acetate:glacialacetic acid : formic acid (2:6:1:1) The plates were kept for development in twin trough chamber for 15 min until the solvent front reaches 8 cm. After development the plates were dried in air and scanned at 280 nm for gallic acid and 254 nm for rutin by using CAMAG Scanner III. The plates were photographed at 254 nm by using Camag Reprostar instrument.

Quantification of Markers Present in Haritaki Formulations

The contents of the active compounds were quantified using calibration curve of each marker individually The two markers used for quantification showed well resolved peaks by the proposed HPTLC method in Haritaki formulations.

***In vitro* Antioxidant Assay**

Oxidative stress is an important contributor to the pathophysiological conditions including inflammation, carcinogenesis and cardiovascular dysfunction. Human body has multiple mechanism especially enzymatic and non enzymatic anti oxidant system to protect the cellular molecules against Reactive oxygen species (ROS), induced damage. However innate defense may not be enough for severe (or) continued oxidative stress. Therefore certain amounts of exogenous anti oxidant are constantly required to maintain an adequate level of anti oxidant in order to balance the ROS in human body. Hence compounds especially from natural sources capable of protecting against ROS mediated damage may have potential application in preventing or curing of diseases [21]

The free radical scavenging activity of the extract is investigated by employing the following *in-vitro* models

DPPH (α, α-diphenyl-β-picryl hydrazyl) Radical scavenging[22]

FRAP (Ferric Reducing/ Antioxidant Power assay)[23,24]

TRAP (Total Peroxy Radical trapping antioxidant potentials)[25,26]

ABTS Radical scavenging activity[27,]

Results and Discussion

The present study reveals that comprehensive study between the lab formulation and marketed formulation. Physicochemical parameters like loss on drying, extractive value of fruit powder, ash value, pH of the substance was examined and it was compared with the standard value listed out in Ayurvedic Pharmacopeia (Table 1)**.** Haritaki was qualitatively analyzed with flavonoids, saponins, tannins, phenols, alkaloids content in it (Table 2). Physical characteristic evaluation viz. bulk density, tap density, Haussner ratio and Carr's index for powder formulation and weight variation test, Friability, Hardness test for tablet formulation determined were found to be within the limits of USP (Table 3). The powdered samples were exposed to Ultra violet light at wavelength of 254 nm and in day light (Table 4). Examination of microbes in the formulation revealed that they were within the limits (Table 5). The finger print analysis was carried out to determine the active constituents present in it. Standard Gallic acid (RF: 0.59) and Rutin (RF: 0.27) showed single peaks in HPTLC chromatogram. The amount of gallic acid and rutin present in the both formulations were computed from the calibration curve (Table 6) The linearity of gallic acid and rutin were found to be 1000-5000 ng/spot and 1000-5000 ng/spot.

The compounds such as flavonoids and polyphenols contains hydroxyl group, which are responsible for the radical scavenging effects in plants. The amount of flavonoids and phenolic contents present in the haritaki extract were calculated. The amount of flavonoid content was found to be increased with increase in concentration of haritaki extract, which may be responsible for the natural antioxidants activity.

The *in-vitro* antioxidant activity of Haritaki extract were carried out using scavenging of DPPH, FRAP, TRAP and ABTS radical scavenging methods. The DPPH Radical scavenging activity increased with increasing percentage of the free radical inhibition. Significant radical scavenging activity was observed for all the tested concentrations (Table 7, Fig 1). The scavenging activity was found to be increased with increase in concentration of haritaki extract.

Antioxidant activity of FRAP has been reported to be concomitant with the development of reducing power. The reducing power of haritaki and the reference compound gallic acid increased steadily with increasing concentration. Higher absorbance of the reaction mixture indicated greater reducing power(Table 8). The reducing capacity of a

compound may serve as a significant indicator of its potent ial antioxidant activity. An azo initiator (TRAP) was used to produce peroxyl radicals, and the scavenging activity of the haritaki extracts was monitored. The scavenging activity was found to be increased with increase in concentration of haritaki extract (Table 9, Fig 3). The haritaki extract consisted of hydrophilic polyphenolic compound that caused greater reducing power.

The ABTS assay was based on the inhibition of the absorbance of the radical cation $ABTS^{+}$. The results depicts that a steady increase in the ABTS radical scavenging activity of extract haritaki and standard gallic acid(Table 10, Fig4).. Hence, the scavenging activity was found to be increased with increase in concentration of haritaki extract. These studies revealed that the lab formulation and marketed formulations of haritaki possesses antioxidant activity. Recent reports indicated that there was an inverse relationship between the dietary intake of antioxidant rich foods and the incidence of human diseases[28].

Table 1. Pharmacognostical Evaluation of Haritaki

Parameters	Standard Value as per Ayurvedic Pharmacopeia	Crude Drug	Marketed Formulations			
			I	II	III	IV
Loss On Drying	#NMT 5%	3%	1.8%	2%	0.6%	0.93%
Alcohol Soluble Extractive	#NLT 40%	57.4%	51.2%	53.3%	52.4%	56%
Water Soluble Extractive	# NLT 60%	64.8%	62.8%	67.4%	64.6%	63.5%
pH of 5%w/v Solution	2.5-5.5	4	3.8	5	4.3	5
Ash Value	#NMT 5	3	4	3	2.7	3.5

NMT- Not More Than, NLT- Not Less Than

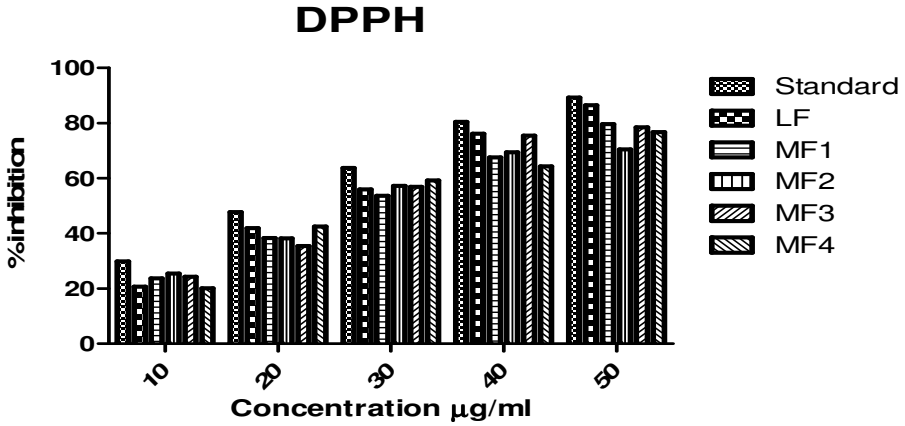

Fig.1. DPPH Radical scavenging activity

Table 2. Preliminary Phytochemical Evaluation of Haritaki

Constituents	Lab formulation	Marketed formulation			
		I	II	III	IV
Flavonoids	+ve	+ve	+ve	+ve	+ve
Tannins	+ve	+ve	+ve	+ve	+ve
Alkaloids	+ve	+ve	+ve	+ve	+ve
Glycosides	+ve	+ve	+ve	+ve	+ve
Saponins	-ve	-ve	-ve	-ve	-ve
Phenols	+ve	+ve	+ve	+ve	+ve

+ve- Present; -ve-Absent

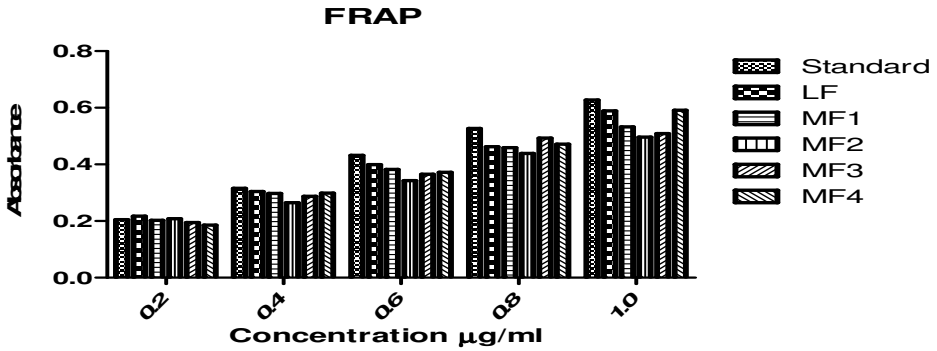

Fig.2. FRAP Radical scavenging activity

Table 3. Determination of Physical Characteristics of Ayurvedic Formulation-Haritaki

Parameters	Standard value as per USP	Lab formulation	Marketed formulation*			
			I	II	III	IV
Tap Density (g/cc)	0.20-0.90	0.5609	0.6923	0.5864	-	-
Bulk Density (g/cc)	0.20-0.60	0.4609	0.5321	0.4632	-	-
Haussner's ratio	0.50-1.5	1.2169	1.301	1.265	-	-
Carr's index	0.2-0.60	0.1782	0.2314	0.2101	-	-
Weight Variation Test	±5%	-	-	-	4	4.5
Hardness test	3-5 kg/cm^2	-	-	-	4	4.5
Friability	Within 1%	-	-	-	0.65	0.5

***I,II is haritaki churanam where as III and IV is tablet formulation**

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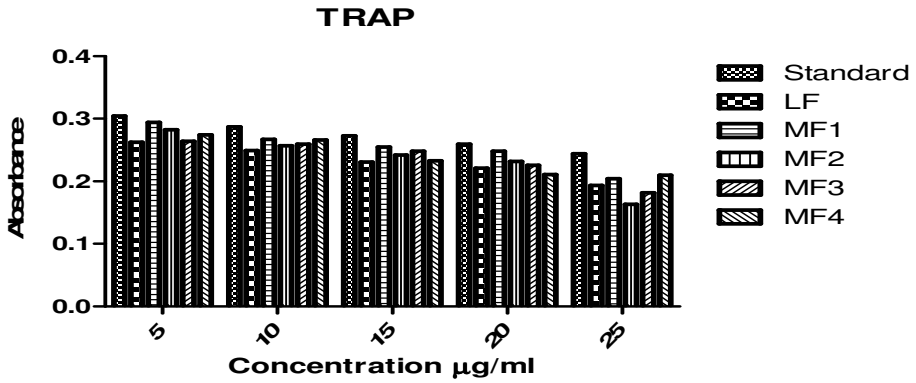

Fig.3. TRAP (Total Peroxy Radical trapping Antioxidant potential)

Table 4. Fluorescence Analysis of Ayurvedic Formulation-Haritaki

Treatment	Lab Formulation		Marketed Formulation	
	Day light	UV 254 nm	Day light	UV 254 nm
Powder as such	LY	LY	LY	LY
Powder + 1M NaOH	YR	YR	RB	RB
Powder + 1M HCl	PG	PG	LG	LG
Powder + Con. HNo_3	BY	BY	BY	BY
Powder + Con. H_2SO_4	LB	LB	LB	LB
Powder +Con. HCl	DG	DG	PG	PG
Powder + CH_3COOH	No Change	LB	LB	LB
Powder + Iodine in water	YR	YR	YR	YR

LY-Light Yellow; YR- Yellowish Red; RB-Reddish Brown; PG-Pale Green; LG-Light Green; DG-Dark Green; LB-Light Brown; YR-Yellowish Red; BY-Bright Yellow

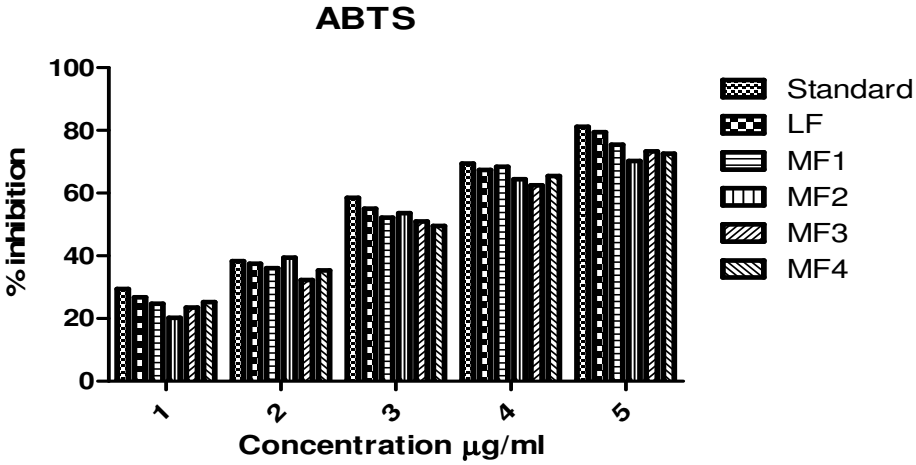

Fig.4. ABTS Radical scavenging activity

Table 5 Determination of microbial limits

Organisms	LF AND MF (M 1,M2,M3,and M4)
Escherichia coli	Nil
Salmonella typhimurium	Nil
Staphylococcus aureus	Nil
Pseudomonas aeruginosa	Nil
Total bacterial count	Less than 1000 cfu/gm
Total yeast and mould count	Less than 1000 cfu/gm

Table 6 Determination of amount and percentage of gallic acid and rutin in Ayurvedic Formulation-Haritaki

Parameters	**Lab formulation**		**Marketed formulation**							
			I		**II**		**III**		**IV**	
	mg/G	**%**	**mg/G**	**%**	**mg/G**		**mg/G**		**mg/G**	
Gallic acid	10.6mg	1.06	3.86mg	0.38	3.23mg	0.32	3.16mg	0.32	1.51mg	0.15
Rutin	8.03mg	0.8	6.78mg	0.67	8.62mg	0.86	11.13mg	1.13	13.1mg	1.31

Table7 DPPH (α, α-diphenyl-β-picryl hydrazyl) Radical scavenging activity

Concentrations (µg/ml)	**Percentage Inhibition**					
	Standard	**LF**	**MF_1**	**MF_2**	**MF_3**	**MF_4**
10	29.89	20.68	23.71	25.43	24.27	20.14
20	47.73	41.96	38.28	38.18	35.42	42.49
30	63.80	55.95	53.71	57.23	56.85	59.20
40	80.43	76.23	67.61	69.47	75.45	64.27
50	89.28	86.54	79.69	70.43	78.54	76.75

Table.8 FRAP Radical scavenging activity

Concentrations (µg/ml)	Absorbance					
	Standard	LF	MF_1	MF_2	MF_3	MF_4
0.2	0.2048	0.2176	0.2032	0.2085	0.1953	0.1854
0.4	0.3155	0.3045	0.2976	0.2653	0.2874	0.2982
0.6	0.4326	0.3990	0.3814	0.3430	0.3659	0.3717
0.8	0.5261	0.4626	0.4592	0.4392	0.4924	0.4713
1	0.6274	0.5892	0.5321	0.4961	0.5093	0.5916

Table.9 TRAP (Total Peroxy Radical trapping antioxidant potential)

Concentrations (µg/ml)	Absorbance					
	Standard	LF	MF_1	MF_2	MF_3	MF_4
5	0.3043	0.2626	0.2943	0.2821	0.2642	0.2743
10	0.2869	0.2492	0.2669	0.2568	0.2594	0.2657
15	0.2728	0.2309	0.2548	0.2420	0.2481	0.2328
20	0.2594	0.2209	0.2478	0.2315	0.2256	0.2109
25	0.2439	0.1936	0.2043	0.1632	0.1814	0.2097

Table.10. ABTS Radical scavenging activity

Concentrations (µg/ml)	Percentage Inhibition					
	Standard	LF	MF_1	MF_2	MF_3	MF_4
1	29.42	26.79	24.68	20.24	23.47	25.23
2	38.27	37.43	36.07	39.42	32.26	35.36
3	58.50	55.08	52.24	53.59	50.95	49.57
4	69.49	67.40	68.35	64.47	62.43	65.46
5	81.20	79.43	75.47	70.26	73.26	72.65

Conclusion

The present work was carried out to standardize and quantify the amount of Gallic acid and Rutin present in both the lab formulation and different marketed formulation of Haritaki. Preliminary phtyochemical studies and *in vitro* antioxidant studies revealed that all formulations can be used for therapeutic activity. The pharmacognostical study and preliminary phytochemical screening reveals that the Haritaki possesses a set of qualitative and quantitative parameter as that of standard. These studies revealed that the haritaki extract contains huge amount of polyphenolic and flavonoid component such as gallic acid and rutin, which are specifically responsible for radical scavenging activity. It serves as a significant indicator for its potential natural antioxidant activity.Recent reports indicated that there was an inverse relationship between the dietary intakes of antioxidant rich haritaki formulation which will incidentally decrease the human diseases. This standardization tool will help in maintaining the quality of important of ayurvedic preparation with the growing demand of herbal drugs in the world market.

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