Int J Ayurvedic Med,Vol 17 (1), 2026; 246-255

Ayurveda Sahiti Prabha's

International Journal of Ayurvedic Medicine

ISSN No: 0976-5921

Research Article

In-vitro static digestion and bio-accessibility analysis of selected Ayurvedic Drugs for the assessment of their *Guru-Laghu gunas* (heaviness and lightness) in terms of digestibility properties- An exploratory study

Divya Khare[1]*, Avinash Koradhanyamath[2]

1. Associate Professor, Department of Dravyaguna, KAHER's Shri B.M.Kankanawadi Ayurveda Mahavidyalaya, Shahapur, Belagavi- 590003, Karnataka, India.
2. Tutor/ Junior Research Officer, Dept. of Biochemistry, S R Patil Medical College, Hospital & Research Center, Badagandi, 587116. Karnataka, India.

Received: 21-07-2025 Accepted: 01-03-2026 Published: 31-03-2026

Abstract

Introduction: In Ayurveda *Guna* forms the basis for selecting drugs for treatment. Drugs possessing *Guru guna* are nourishing but require longer digestion whereas those with *Laghu guna* are lighter and digest faster. This exploratory study evaluated these traditional classifications using modern digestive parameters and bio accessibility. Material & Methods: An in-vitro static digestion model simulating oral, gastric and intestinal phases was used. Two representative drugs from each category were selected: *Shatavari* (*Asparagus racemosus* Willd.) and *Chandrashura* (*Lepidium sativum* L.) for *Guru guna; Amalaki* (*Phyllanthus emblica* L.) and *Usheera* (*Vetiveria zizanioides* (L.) Nash) for *Laghu Guna*. Carbohydrates and protein bio-accessibility were measured at defined intervals (G-60, G-120, I-60, and I-120 minutes). Results: *Shatavari (Guru)* demonstrated sustained and increased carbohydrate bio-accessibility with a moderate biphasic protein release- oral phase (139.56%) and intestinal phase at I-60 (87.73%) reinforcing its nourishing role. *Chandrashura (Guru)* showed delayed and limited carbohydrate bio-accessibility but markedly high intestinal protein bio-accessibility (802.05 % at I-60 and 1214.97 % at I-120) consistent with *Guru* characteristics. In contrast *Amalaki* (*Laghu*) exhibited sustained carbohydrate release with rapid, high gastric protein bio-accessibility (416.68%). *Usheera* (*Laghu*) showed consistently low carbohydrate availability and a poor gastric protein bio-accessibility (negative values), followed by mild intestinal increase (up to 497.93 %) aligning with its *Laghu* classification. Conclusion: The findings suggest that Ayurvedic *Guna* based drug classification has a measurable biochemical basis. The study highlights the potential of integrating feasible scientific models to interpret and validate traditional Ayurvedic pharmacological concepts.

Access this article online

Website: https://ijam.co.in

DOI: https://doi.org/10.47552/ijam.v17i1.6272

Keywords: Carbohydrate, Gastric phase, Guna, Intestinal Phase, Protein, Static digestion.

Introduction

Ayurveda as the Indian traditional stream of medicine, has a strong foundation of certain basic concepts which describe not only the physiological aspects of human body but also the properties of medicinal plants chosen for treatment of health conditions. These concepts are well studied and time tested. But they lack modern scientific validation.

*** Corresponding Author:**
Divya Khare
Associate Professor, Department of Dravyaguna, KAHER's Shri B.M.Kankanawadi Ayurveda Mahavidyalaya, Shahapur, Belagavi. India. 590003. A Constituent unit of KLE Academy of Higher Education and Research, Belagavi, India.
Email Id: divyakhare2008@gmail.com

One such concept, "*Guna"* (quality, attribute or property of drugs) described in the Ayurveda literature (1) is the basis for drug selection. Among 41 gunas described in Ayurveda, the *Gurvadi gunas* (a group of 10 pairs of *Gunas* starting with *Guru-Laghu*), (2) are a group of diverse physico-pharmacological properties which are very useful in diagnosis as well as treatment.

The first pair among these is *Guru-Laghu* (heaviness-lightness). (3) As per the definitions given in the classical literature of Ayurveda, the pharmacological action of *Guru guna* is described as *Brimhana*, (4) which means nourishing to the body. Further, *Guru dravyas* are *Chirapaki* (5) ie., they undergo digestion very slowly. And this indicates that the drug having *Guru guna* must be having complex proximate principles (Carbohydrates, Proteins and/ or Fats) which take longer duration to break down into simpler molecules and become available to the body for functioning. And the converse is true for *Laghu guna* drugs ie., their conversion might take place comparatively early. But we lack a standard methodology to test this property. There is a need to develop an acceptable tool to demonstrate the *Gunas* by employing modern analytical techniques. This will further help to fix the properties of new drugs for their addition to Ayurvedic pharmacopoeia.

Shatavari (*Asparagus racemosus* Willd.) which is commonly used in Ayurveda as an immune modulator, as a galactagogue and to treat female reproductive tract disorders, is mentioned as having *Guru guna* (heaviness, nourishing, delayed digestion). (6) The roots of Shatavari contain high amount of nutrients and bioactive compounds, such as fibers and phenolics. (7) Another drug for *Guru guna*, *Chandrashura* (*Lepidium sativum* L.) is having nourishing action on the body (8) and is commonly used as a dietary supplement in post-partum period. The two drugs chosen for *Laghu guna* (lightness, non-nourishing, early digestion) were *Amalaki* (*Phyllanthus emblica* L.) and *Usheera* (*Vetiveria zizanioides* (L.) Nash). *Amalaki* is described as *Ruksha* (Dry) and *Laghu* (Light) and is used in treating Diabetes (*Prameha*) and bleeding disorders (*Raktapitta*). (9) *Usheera* is *Sheeta* (cold) and *Laghu* (light) and is indicated in fever (*Jwara*) and other *Pitta* predominant disorders. It is non-nourishing, fibrous and aromatic. (10)

To assess *Guru* and *Laghu* properties of drugs, controlled replication of digestion process is beneficial. This would help in studying the bioaccessibility of Carbohydrates and Proteins at each stage. Thereby the *Guru* and *Laghu gunas* (heaviness and lightness) with respect to digestibility and bioaccessibility could be substantiated.

When food is consumed, only a small portion is actually available at the site where it is needed for normal physiological functions. The bioavailability of food involves three primary stages: its digestibility and solubility in the gastrointestinal tract, absorption by the intestines and subsequent transport into the bloodstream, and finally, its integration into the functional entity or target. (11) Static methods, also known as biochemical methods, are the most straightforward techniques in this context. They involve two or three digestion phases (oral, gastric, and intestinal), with the resulting products largely remaining stationary within a single static bioreactor. These methods replicate a limited set of parameters of physiological digestion and do not imitate physical processes such as shearing, mixing, hydration, temporal condition changes, or peristalsis. (12, 13)

In vitro techniques that replicate digestion processes are extensively employed to examine the gastrointestinal behavior of foods or pharmaceuticals. While human nutritional studies remain the "gold standard" for exploring diet-related inquiries, in vitro methods offer the benefits of being quicker, more cost-effective, less labor-intensive, and free from ethical constraints. They aim to replicate physiological conditions in vitro by considering factors such as the presence and concentration of digestive enzymes, pH levels, digestion duration, and salt concentrations, among others.

The selected drugs mentioned to be having *Guru* and *Laghu gunas* in the classics were analyzed in the laboratory using these techniques. The drugs were first subjected to proximate analysis which gave the quantity of proximate principles (moisture, ash, carbohydrates and proteins) present in the drug before hydrolysis. Then they were subjected to enzymatic hydrolysis. Later, the end product analysis (quantitative analysis of the end product after Enzymatic Hydrolysis) was done. The present study was an attempt made to assess if there is any difference in digestive profile and bioaccessibility of *Guru* and *Laghu* drugs based on which definite biochemical basis could be established for assessing the *Gunas*.

Methodology

Raw materials: Raw materials were procured from GMP Certified KLE Ayurveda Pharmacy, Belagavi.

Authentication: The drugs were authenticated by experts in the Central Research Facility, AYUSH Approved drug testing laboratory for ASU drugs, KAHER's Shri B.M. Kankanawadi Ayurveda Mahavidyalaya, Belagavi.

Analysis: All the analyses including in-vitro digestion and bioaccessibility were performed at the Central Research Facility, AYUSH Approved drug testing laboratory for ASU drugs, KAHER's Shri B.M. Kankanawadi Ayurveda Mahavidyalaya, Belagavi.

Preparation of sample: Drugs were powdered in Mixer (Mangal-CSPO-SK-01, 2400 Watt) and then sieved through mesh number 80.

Physical and Functional Properties:

1. **Proximate composition:**
   - Moisture content and ash were determined according to the standard methods of API.
2. **Physical properties:**
   - Appearance/ Color attributes,
   - Odour and
   - Taste was assessed by organoleptic examination and compared with API standards.
3. **Functional properties:**

a. Water absorption Index (WAI) and water solubility Index (WSI)

The Kadan et al. approach was used to determine the WAI and WSI. (14) A vortex mixer was used to combine the drug powders (1 g) for one minute after they had been suspended in 10 mL of distilled water. In a water bath, the suspensions were heated to 30°C for 30 minutes while being gently stirred. Later, they were centrifuged for 10 minutes at 3000 rpm. After being carefully transferred into an aluminium moisture dish, the supernatants were left to dry overnight at 105°C. Then the sediments were weighed.

The following formulas were used to determine the WAI and WSI.

WAI (g/g) = Weight of wet sediment (g)/ Dry weight of sample (g)

WSI (%) = Weight of dried supernatant (g)/ Dry weight of sample (g) * 100

b. Oil Absorption Index (OAI)

The method developed by Malomo et al. was used to analyze the OAI. (15) Ten milliliters of soybean oil were combined with one gram of the samples. For 20 minutes, the mixtures were centrifuged at 4,000 rpm. After that, the excess oil was decanted and the residues (weight of oil absorbed) were weighed. The OAI was calculated as follows:

OAI (g/g) = Weight of Oil absorbed (g)/ Weight of Sample (g)

c. Bulk density and Tapped density

Bulk density (BD) was calculated using the Coulibaly et al. method. (16) A 50 ml graduated measuring cylinder was filled with 10 grams of the sample. A record of the sample volumes was made. The following formula was then used to determine BD:

Bulk density (g/cm³) = Weight of sample (g)/ Volume of sample before tapping (cm ³).

After that, the samples were packed by gently tapping the cylinder ten times from a height of five centimetres on the bench top. A record of the sample's volume was made. The following formula was then used to determine the taped density:

Tapped density (g/cm³) = Weight of sample (g)/ Volume of sample after tapping (cm ³)

Total Carbohydrates and total Proteins

Estimation of total carbohydrates was done by Anthrone method (17, 18, 19) and estimation of total Proteins was done by Bradford method (20) for all the samples.

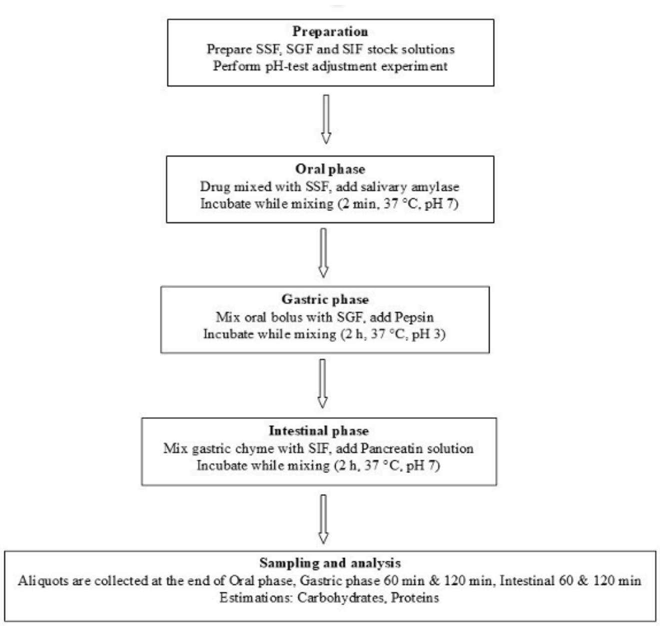
Enzyme assay

Alpha-amylase and Pepsin enzyme assay was conducted as per the INFOGEST method to ensure the enzyme activity and to know the optimum concentration of the enzyme to be used for in-vitro digestion. (21)

Invitro-digestion

A standardized static in vitro digestion technique that has been universally agreed to be appropriate for food, was followed for the selected drugs in the next step. Static digestion procedures were carried out according to Minekus et al., with brief modification. (22)

Chart 1: Flow chart indicating the stages of in vitro digestion



All chemicals of standard analytical grade were procured. The electrolyte stock solutions were prepared. Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared from the corresponding electrolyte stock solutions in advance and stored in -20 deg C. Calcium chloride was prepared fresh on the day of performing in vitro digestion.

Sampling procedure

For each drug aliquots were collected at the end of the Oral phase, the Gastric phase at 60 min & 120 min, Intestinal phase at 60 minutes & 120 minutes. Snap freezing of samples in ice packs was done immediately after the collection for further analysis and the sample's aliquots were stored at -20 ºC until further analysis. The samples collected at different stages of digestion were given suitable codes.

End Analysis of Protein and Carbohydrate

After in-vitro digestion, the sampled aliquots of each stage were analyzed to determine the quantities of micro-molecules yielded after the process of in-vitro digestion. All the stored samples of different stages were analyzed for total carbohydrate and total proteins in solution by using the same procedure mentioned above.

Bio accessibility assay

Bio accessibility of Carbohydrates and Proteins at different stages of digestion was calculated by using the formula:

Bio accessibility = (Concentration in respective phase/ Concentration in undigested sample)*100. (23)

Statistical Analysis

All analyses were done in triplicates. Mean values and standard deviation were calculated and tabulated in excel sheet. The data were analyzed using Graph Pad Prism. A multifactorial analysis of variance (ANOVA) and Tukey's multiple range test were carried out to determine if there is any significant difference at p-value < 0.05.

Results and discussion

Proximate composition

All the physico-chemical test values were compared with the API standards. Ash values of all the test drugs were within the prescribed limits. Moisture content was highest in *Amalaki* and least in *Usheera*. *Amalaki* has the highest alcoholic extractive value, whereas *Shatavari* showed the highest Hydro alcoholic extractive value. In *Chandrashura*, due to the presence of mucopolysaccharides, most of the solvent got absorbed into the seed coat, making very less amount of filtrate available for determination of alcohol extractive value, so the percentage is less compared to API standard. Further, extractive value differs with the source and habitat of sample collected. This might be the reason for lesser extractive value in *Amalaki*. Fibres present in *Usheera* roots absorb the solvent and swell, resulting in slightly less extractive value.

Table 1: Proximate composition of drugs

Parameters	*Shatavari*		*Chandrashura*		*Amalaki*		*Usheera*	
	Observed values	API standards	Observed values	API standards	Observed values	API standards	Observed values	API standards
Ash in %	5.955	Not more than 5%	5.013	Not more than 8%	2.775	Not more than 7%	3.452	Not more than 9%

Moisture content in %	5.88	Not mentioned	5.213	Not mentioned	9.073	Not mentioned	4.049	Not mentioned
Alcohol Extractive value in %	15.15	Not less than 10%	6.69	Not less than 13%	20.08	Not less than 40%	3.90	Not less than 4%
Hydro-alcoholic Extractive value in %	23.58	Not mentioned	23.50	Not mentioned	17.259	Not mentioned	0.479	Not mentioned

Physical properties

Table 2: Appearance/ Color attributes, Odour and Taste of the drugs

Physical	*Shatavari*	*Chandrashura*
Appearance	Consists of tuberous roots of *Asparagus recemosus* Willd. (Fam. Liliaceae). Root tuberous, tapering at both ends with longitudinal wrinkles. Consists of *Asparagus recemosus* Willd. tuberous roots (Fam. Liliaceae). Root are with longitudinal creases and taper at both ends.	Consists of *Lepidium sativum* L. (Fam. Cruciferae) dried seeds. The seeds are tiny, oval-shaped, triangular at one end, and have a smooth surface. There is a tiny wing-like extension on both of the seed's edges, and a furrow that extends up to two thirds downward on both surfaces. The seed coat swells and becomes coated in a clear, colorless mucilage when it is immersed in water.
Dimensions of crude drug	10 to 30 cm in length and 0.1 to 0.5 cm thick	About 2-3 mm long, 1-1.5 mm wide
Color	Cream	Reddish brown
Odour	No characteristic odour	Mild characteristic odour
Taste	Sweetish	Mucilaginous
Physical	***Amalaki***	***Usheera***
Appearance	Consists of pericarp of dried mature fruits of *Phyllanthus emblica* L., (Fam. Euphorbiaceae). Drug is seen as curled pieces of pericarp of dried fruit occuring either as separated single segment or multiple segments. The fragments display a transversely wrinkled lateral surface and a large, heavily shrivelled and wrinkled external convex surface. There are a few white spots on the outside, and sometimes chunks have some rocky testa visible (which should be removed before processing). Tough, cartilaginous, and rough in texture.	Consists of dried, aromatic, fibrous roots from *Vetiveria zizanioides* (L.) Nash (Poaceae family). The wiry root clusters are tiny, longitudinally grooved, fractured, short, and splintery, with a maximum diameter of 2 mm.
Dimensions of crude drug	1-2 cm long or united as 3 or 4 segments	2-3 mm in diameter and 10-15 cm long
Color	Bulk color grey to black	The color ranges from pale yellow to brown, cream, or grey.
Odour	No characteristic odour	Strong aromatic
Taste	Sour, Astringent	Slightly bitter

Functional properties

Table 3: Functional Properties of the drugs

Parameters	*Shatavari*	*Chandrashura*	*Amalaki*	*Usheera*
Water Solubility Index %	48.319	3.891	40.506	3.130
Water Absorption Index g/g	5.297	7.529	3.043	4.643
Oil Absorption Index g/g	3.290	2.101	2.143	3.175
Bulk Density	0.2042	0.3853	0.5128	0.1809
Tapped Density	0.2944	0.5894	0.6896	0.3576

Water solubility index in highest in *Shatavari* (48.319) and next to that *Amalaki* (40.506) shows the highest value. Both *Chandrashura* (3.891%) and *Usheera* (3.130%) have shown less solubity in water. *Chandrashura* contains mucopolysaccharides which do not dissolve in water but absorb moisture and swell. *Usheera* is rich in fiber and hence insoluble in water. WSI didn’t show results in accordance to *Guru- Laghu gunas*, rather it depends on the nature and composition of drugs. If WSI is high, it will aid in digestion as the drug is hydrolysable easily.

Water Absorption Index is highest in *Chandrashura* (7.529 g/g) followed by *Shatavari* (5.297g/g), *Usheera* (4.643g/g) and least in *Amalaki* (3.043g/g). The outer layer of *Chandrashura* seeds is made up of mucopolysaccharides which is hydrophilic in nature. When put in water, the seeds absorb water and swell, forming a slimy mass. So, WAI is more in this. Shatavari is rich in starch which have water absorbing nature. *Usheera* has fibers which again absorb water but less in comparison to that of starch. *Amalaki* is rich in pectin. (24) Pectin is known for its gelling property and is good alternative to starch. (25)

The WAI and WSI describe how the extruded product interacts with water and are frequently used significant markers that influence the feed's subsequent processing (drying, storage, etc.). Additionally, they monitor the amount of starch that is transformed or converted during the extrusion process. Since native starch does not absorb water at ambient temperature, the WAI index can be used to assess the level of gelatinization.

Oil absorption index is highest in *Shatavari* (3.290 g/g), followed by *Usheera* (3.175 g/g), *Amalaki* (2.143 g/g) and *Chandrashura* (2.101 g/g). This is again because of the composition of individual drugs and is not in consistency with the *Guru-laghu gunas*.

Bulk and Tapped density are found to be highest in *Amalaki* and then in *Chandrashura*. *Shatavari* showed higher bulk density value while *Usheera* showed higher value of tapped density. Thus, density values of trial drugs are also not in consistency with *Guru-laghu gunas*.

Since the physical properties showed variable values in comparison to classically mentioned *Gunas* (physical properties), we can conclude that the *Gunas* do not indicate the physical properties of heaviness (density) or lightness but infact they are more in coherence with the pharmacological properties.

Amylase Enzyme assay

To assess the enzyme activity, Amylase enzyme assay was carried out. Amylase enzyme was dissolved in suitable solvent and the activity was calculated based on the release of Maltose from starch and compared with the standard Maltose graph. The Amylase activity was recorded as 9.41 ± 2.80 Units/ mg at amylase concentration of 1 mg/ml. The maltose standard curve was linear.

Pepsin activity assay

Pepsin enzyme assay was carried out to assess its activity by dissolving in suitable solvent. The Pepsin activity was recorded as 300 ± 25.01 Units/ mg at pepsin concentration of 0.03 mg/ml.

Carbohydrate and Protein in crude drug

Estimation of Total Carbohydrates in raw and digested samples was done by Anthrone method using Glucose as the standard. Estimation of Proteins was done by Bradford method using Bovine serum albumin as the standard.

Graph 1: Maltose standard curve

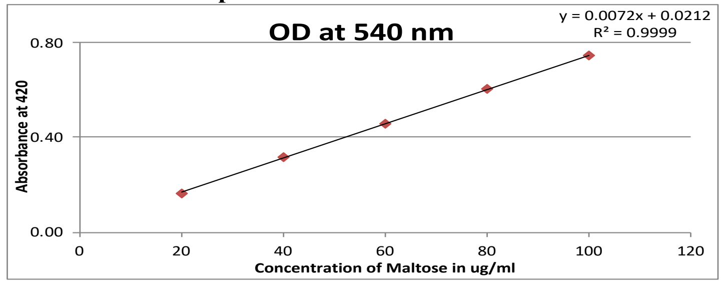


Table 4: Total carbohydrates and proteins in solution in raw drugs

Sl No.	Drug	Total carbohydrates (ug in 100 mg sample)	Total Proteins in solution (ug in 1 ml WSE)
1	*Shatavari*	93.8	542.32
2	*Chandrashura*	183.91	333.13
3	*Amalaki*	58.35	112.26
4	*Usheera*	62.25	79.75

Chandrashura contains highest carbohydrate content (183.91 ug in 100 mg sample), next highest is found in *Shatavari* (93.8 ug/ 100 mg sample), both being *Guru*. *Shatavari* showed highest protein content (542.32 mg/ml WSE) and next to that is *Chandrashura* (333.13 mg/ml ASE). So the nutritive nature of these two *Guru guna* drugs (*Guru- Brimhane shaktih*) (2) is substantiated.

Amalaki and *Usheera* (both *Laghu*) showed lesser carbohydrate and protein content when compared to that of *Shatavari* and *Chandrashura*. So, their property (*Laghu*- less nutritive) is substantiated.

Estimation of Carbohydrates, Proteins and Bioaccessibility after invitro digestion

The supernatant samples collected during in-vitro digestion were analyzed for total carbohydrates and total proteins in solution. The results are as follows:

Estimation of Carbohydrates and Bioaccessibility

Table 5: Mean Concentration of Carbohydrates (ug/ml) in different samples at different stages of digestion

Stages of digestion	***Shatavari*** **(S)** ***(ug/ml)***	***Chandrashura*** **(C) (ug/ml)**	***Amalaki*** **(A)** ***(ug/ml)***	***Usheera*** **(U)** ***(ug/ml)***
Blank	9199.262	298.40115	335.9689	21.53469
S	33172.46	**No supernatant**	857.4056	49.81199
G-60	50419.51	1033.8899	1072.553	75.38378
G-120	47209.3	2533.2451	1343.067	142.0221
I-60	45158.82	5686.9488	1776.298	204.4617
I-120	21246.19	3234.5702	1263.981	166.2202

Shatavari showed less carbohydrate content before digestion which increased profoundly during different stages of digestion. It shows very significant and sustained increase in carbohydrate concentrations throughout the digestion phase (Table 5). This is in line with the *Guru Guna* (heaviness), which is associated with a slow-digesting and nutrient-rich substance in Ayurveda. Scientifically, this is explained by its higher content of complex polysaccharides and starch-like compounds, which, after enzymatic degradation, produce a high amount of sugars that can be consumed over a period of time. (26)

Comparable mean carbohydrate concentrations are observed for *Chandrashura*, *Amalaki* and *Usheera* without statistically significant differences (Table 5). However, this does not imply a functional similarity, since *Chandrashura* contains predominantly mucopolysaccharides and hence not digestible my amylase. (27) Further these mucopolysaccharides absorbed all the liquid part. So no supernatant was obtained in Oral (S) stage. Whereas in other stages, it showed higher concentration of carbohydrates, highest being in intestinal phase at 60 minutes.

However, in the two *Laghu guna* drugs *Amalaki* and *Usheera*, Carbohydrate concentration was less compared to that in *Guru drugs*. *Amalaki* contains simple phenols and tannins which facilitate rapid but low-yield release of energy. (28) *Usheera* is aromatic and fibrous and has negligible availability of carbohydrates. (29) This substantiates the *Laghu Guna* attributed to the drug in Ayurveda. Digestion with enzymes was not able to change the nature of this drug.

Table 6: Comparison of concentration of Carbohydrates in different samples at different stages of digestion

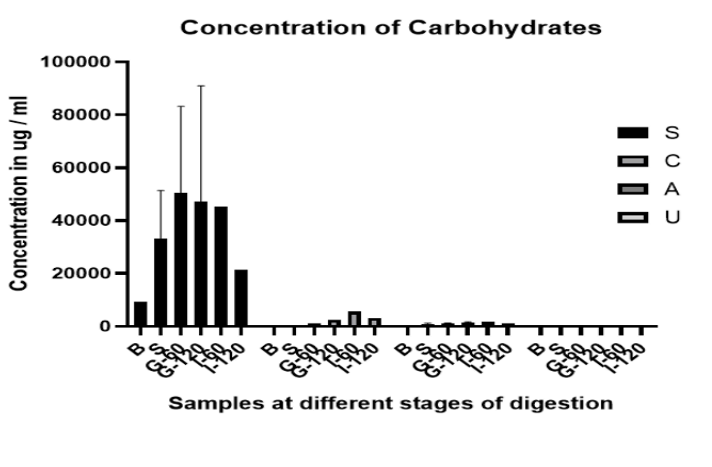
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
S vs. C	32166	18930 to 45403	Yes	****	<0.0001
S vs. A	33293	20056 to 46529	Yes	****	<0.0001
S vs. U	34291	21054 to 47528	Yes	****	<0.0001
C vs. A	1126	-12110 to 14363	No	ns	0.9953
C vs. U	2125	-11112 to 15361	No	ns	0.9704
A vs. U	998.3	-12238 to 14235	No	ns	0.9967

All the three samples *Chandrashura*, *Amalaki* and *Usheera* showed statistically significant difference ($p<0.0001$) in carbohydrate concentration, when compared to that of *Shatavari*. But differences between *Chandrashura*, *Amalaki* and *Usheera* were statistically not significant.

Table 7: Mean Bioaccessibility of Carbohydrates in different samples at different stages

Stages of digestion	*Shatavari* (S) (%)	*Chandrashura* (C) (%)	*Amalaki* (A) *(%)*	*Usheera* (U) *(%)*
S	1157.231	**No supernatant**	53.77269	2.724051
G-60	1758.899	4.1705389	67.26581	4.122486
G-120	1646.91	10.218687	84.23126	7.766714
I-60	1575.379	22.940199	111.4016	11.18133
I-120	741.1795	13.047715	79.27133	9.090025

Graph 2: Mean Concentration of Carbohydrates (ug/mg) in different samples at different stages of digestion



Graph 3: Mean Bioaccessibility of Carbohydrates (%) in different samples at different stages

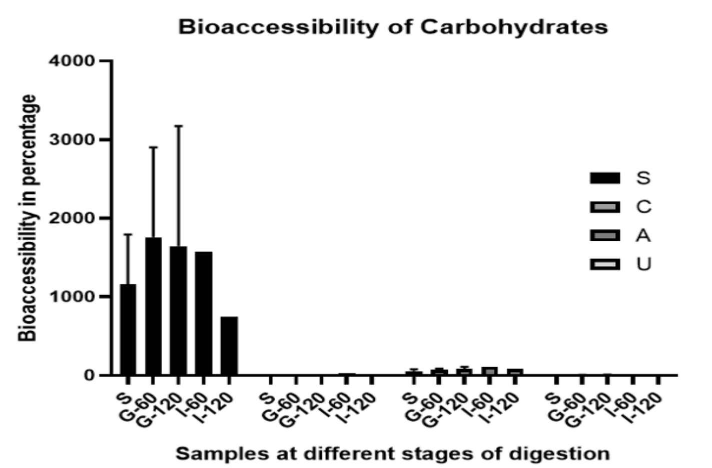


Table 8: Comparison of Bioaccessibility of Carbohydrates in different samples

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
S vs. C	1365	803.1 to 1928	Yes	****	<0.0001
S vs. A	1297	734.4 to 1859	Yes	****	<0.0001
S vs. U	1369	806.7 to 1931	Yes	****	<0.0001
C vs. A	-68.63	-630.9 to 493.7	No	ns	0.9859
C vs. U	3.581	-558.7 to 565.9	No	ns	>0.9999
A vs. U	72.21	-490.1 to 634.5	No	ns	0.9836

The difference in bioaccessibility of carbohydrates between *Shatavari* and the remaining three drugs is statistically significant (at $p<0.0001$). Among the three drugs, *Chandrashura*, *Amalaki* and *Usheera*, the difference is statistically not significant.

Shatavari shows Carbohydrate Bioaccessibility peak at G-60 (1758.90) (Table 7) and a gradual decline thereafter, indicating initial degradation of the starch-rich fractions and subsequent depletion or limited solubility of the remainder of the contents. This supports the Ayurvedic view of *Chirapaki* (slow digestion) which is typical of the *Guru Guna*. The high initial bioaccessibility is probably due to the enzymatic swelling and gelatinization of the starch grains, which results in a high solubility. (30)

The statistically higher carbohydrate bio-accessibility of *Shatavari* in comparison with others (table 5) reinforces its identity as a *Guru Dravya* with rich, complex, yet digestible starches.

Amalaki shows a gradual and sustained increase in Carbohydrate bioaccessibility. It shows a moderate initial bioaccessibility, reaching 111.4 percent (I-60) and then decreasing. This is in line with the characteristics of *Lagu guna* - rapid digestion and efficient release of nutrients. It contains hydrolysable tannins and acids (e.g. ascorbic acid) that contribute to rapid breakdown and absorption. (31)

Chandrashura showed delayed and limited Carbohydrate bioaccessibility. Extremely low accessibility initially, increasing only slightly at later stages (maximum 22.94 percent at I-60). Although the total carbohydrate content is high, the mucopolysaccharides are poorly hydrolysed by amylase, which explains the difference in quantity versus availability and reflects the dense and compact matrix that is the hallmark of the *Guru guna* characteristics. Although *Chandrashura* has the highest total carbohydrate content, it exhibits a low enzymatic bioaccessibility, probably due to the presence of mucopolysaccharides (non-starch polysaccharides) that are resistant to hydrolysis by amylase.

Usheera showed consistently low carbohydrate bioaccessibility which is negligible. This is significant at all stages (maximum: 11.18 percent at I-60), indicating a very low digestible carbohydrate content. This is consistent with its fibrous, non-nutritive, and aromatic composition, which contributes to its *Sheetala* and *Laghu guna* characteristics, but does not serve as a source of carbohydrates.

Estimation of Proteins and Bioaccessibility

Table 9: Mean Concentration of Proteins (ug/ml) in different samples at different stages

Stages of digestion	*Shatavari* (S) *(ug/ml)*	*Chandrashura* (C) (ug/ml)	*Amalaki* (A) *(ug/ml)*	*Usheera* (U) *(ug/ml)*
Blank	293.12	180.1168	143.2002	101.68
S	756.8559	**No supernatant**	189.5256	1.533701
G-60	57.07331	74.56797	467.7611	-123.304
G-120	43.72458	671.5896	298.3405	-110.106
I-60	475.8155	2671.876	251.2866	253.3601
I-120	200.3083	4047.446	145.9886	397.1003

The in vitro digestion model used in this study revealed significant differences in protein concentration and bioaccessibility between the four selected Ayurvedic medicinal products - *Shatavari*, *Chandrasharma*, *Amalaki* and *Usheera* - each of which has the characteristics of the classical *Guru* and *Laghu*. These differences were consistent with the Ayurvedic conceptual framework and provided biochemical evidence to support classification of these substances under *Guru- Laghu gunas*.

Table 9 summarizes the Protein concentration patterns. The initial protein concentration in samples was highest in *Shatavari* (293.12 micrograms per ml), which is in line with the known nutritional value and the body-building effect (*Guru guna*) described in the ayurvedic literature. Interestingly, *Chandrashura* showed a dramatic increase in protein concentration only in the intestinal phase (4047.45 µg per ml in I-120), whereas it was almost undetectable in the oral and early intestinal phases. This indicates delayed release of proteins, probably due to the rich mucilage of the seed coat, which may prevent premature enzymatic hydrolysis.

Amalaki, classified as *Laghu*, demonstrated a rapid protein release with a peak in the gastric phase (467.76 µg per ml in G-60), indicating the presence of readily digestible, low molecular weight proteins or peptides that are sensitive to pepsin degradation. *Usheera*, on the other hand, showed the lowest protein concentration in all phases, reflecting its fibrous and non-nutritional root composition, which is in line with the aromatic character of *Sheeta* and *Laghu gunas*.

Table 10: Overall Comparison of concentration of Proteins in different samples

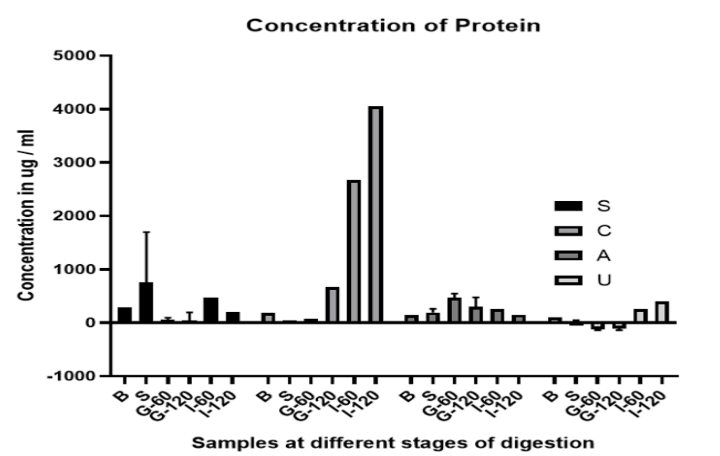
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
S vs. C	-978.1	-1202 to -754.0	Yes	****	<0.0001
S vs. A	55.13	-169.0 to 279.2	No	ns	0.9042
S vs. U	217.8	-6.342 to 441.9	No	ns	0.0590
C vs. A	1033	809.1 to 1257	Yes	****	<0.0001
C vs. U	1196	971.8 to 1420	Yes	****	<0.0001
A vs. U	162.6	-61.47 to 386.8	No	ns	0.2152

The difference in protein concentration between *Chandrashura* and the other three drugs was statistically significant ($p<0.0001$). Among the three drugs *Shatavari*, *Amalaki* and *Usheera*, the difference was statistically not significant. *Shatavari* and *Amalaki* showed protein content in similar ranges during different stages of digestion. This might be because of post digestive effect (*Vipaka*).

Table 11: Mean Bioaccessibility of Proteins (%) in different samples at different stages

Stages of digestion	*Shatavari* (S) *(%)*	*Chandrashura* (C) (%)	*Amalaki* (A) *(%)*	*Usheera* (U) *(%)*
S	139.5589	**No supernatant**	168.8274	1.923136
G-60	10.52392	22.38405	416.6765	-154.614
G-120	8.062505	201.5999	265.7585	-138.064
I-60	87.73703	802.052	223.8434	317.6929
I-120	36.93545	1214.975	130.0451	497.9314

Graph 4: Mean Concentration of Proteins (ug/ml) in different samples at different stages



Graph 5: Mean Bioaccessibility of Proteins (%) in different samples at different stages

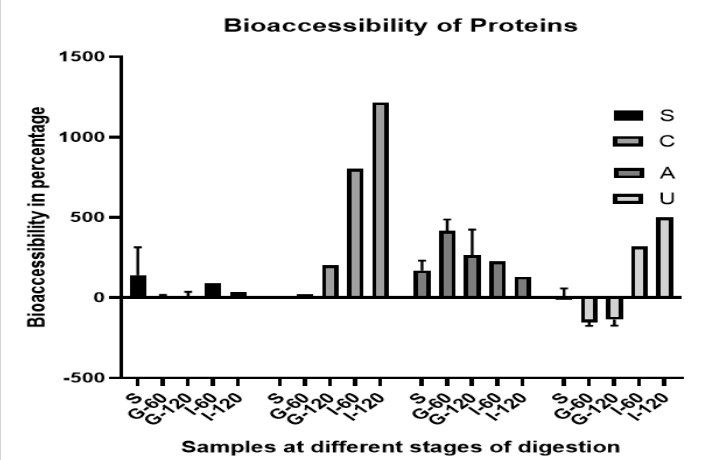


Table 11 shows bioaccessibility of protein. The values for bioaccessibility reflected this trend. *Chandrashura* showed a large increase in intestinal phase (802.05 percent at I-60 and 1214.97 percent at I-120) indicating that it is only after pancreatin exposure that protein is hydrolysed and dissolved. This delayed but high release is characteristic of *Guru guna* drugs in Ayurveda, which are *Chirapaki* (slow to digest) and *Brimhana* (nourishing) in action. (32)

Shatavari showed a moderate but two phase bioaccessibility, with a peak in the oral phase (139.56%) followed by a rise at intestinal phase I-60 (87.73%). This pattern indicates both early partial release and late digestion, which reflects the dual nature of complex nutritional content and digestibility.

Conversely, *Amalaki* demonstrated rapid and high bioaccessibility in the stomach (416.68%), in line with its *Laghu* classification. This highlights the rapid enzymatic hydrolysis of the protein content, possibly facilitated by the acidic pH and the simple structure of the protein. *Usheera* showed poor bioaccessibility in the gastric phase (negative values), but a slight increase in intestinal phase (up to 497.93 %), indicating delayed release from fibrous matrix, although the total protein content was low.

Table 12: Comparison of Bioaccessibility of Proteins in different samples in different stages

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
S vs. C	-393.6	-467.2 to -320.1	Yes	****	<0.0001
S vs. A	-184.5	-258.0 to -110.9	Yes	****	<0.0001
S vs. U	-48.41	-122.0 to 25.15	No	ns	0.2839
C vs. A	209.2	135.6 to 282.7	Yes	****	<0.0001
C vs. U	345.2	271.7 to 418.8	Yes	****	<0.0001
A vs. U	136.1	62.50 to 209.6	Yes	***	0.0002

ANOVA and Tukey post-hoc tests showed significant differences ($p<0.0001$) between *Shatavari* and *Chandrashura* (Table 12) and between *Chandrashura* and other samples, which confirmed that *Chandrashura* has the highest protein digestibility, especially in the intestinal phase. Despite the high initial content, the bioaccessibility of *Shatavari* was lower than that of *Amalaki* and *Chandrashura*, underlining the importance of release kinetics in understanding nutrient potential.

In Table 12, the bioaccessibility comparison showed a significant difference between *Shatavari* and *Chandrashura* ($p<0.0001$), *Shatavari* and *Amalaki* ($p<0.0001$) and *Amalaki and Usheera* ($p=0.0002$). This confirms that protein is more functionally available in *Laghu* drug *Amalaki* during the early digestion and in *Guru* drug *Chandrashura* during the later digestion. *Usheera* remained statistically and functionally inferior in protein bioaccessibility, which is aligning with its traditional role as a cooling and non-nutritive.

Table 13: Correlating Ayurveda concept of Guna with digestibility properties

Drug	*Guna*	Carbohydrate Content	Protein Content	Carbohydrate Bioaccessibility	Protein Bioaccessibility	Inference in relation with Ayurvedic concept
Shatavari	*Guru*	High	High	Peak at G-60, declines thereafter	Moderate. Dual-phase release, slow	Correlates with the *Brimhana* (nutritive) and *Chirapaki* (slow digestion), supports *Guru guna*
Chandrashura	*Guru*	Moderate	High in intestinal phase	Delayed and limited Carbohydrate bioaccessibility reflecting dense and compact matrix	Delayed but high release	Correlates with the *Chirapaki* (slow digestion) and *Brimhana* (nutritive), supports *Guru guna*
Amalaki	*Laghu*	Low	Low–Moderate	High early bioaccessibility	Rapid and high bioaccessibility in the gastric phase	Quick but limited nutrient availability, supports *Laghu guna*
Usheera	*Laghu*	Low	Low	Consistently low carbohydrate bioaccessibility which is negligible	Poor bioaccessibility	Correlates with non-nutritive nature, supports *Laghu guna*

From table 13 it is evident that the drugs categorized under *Guru gunas* showed specific digestibility properties like high nutrient content and delayed release. Whereas the drugs having Laghu guna showed medium or low nutrient content but quick release. This study helped us to establish the relation between digestive behavior of drugs and properties like *Brimhana* or *Langhana* (nutritive or non-nutritive) and *Chirapaki* or *Shighrapaki* (slow or rapid digestibility) of drugs categorized under specific *Gunas*. Thus, it provides evidence to understand the *Guna* (properties) of herbal drugs told in Ayurveda on the basis of digestibility and bioaccessibility.

Conclusion

The in vitro digestion model used in this study revealed significant differences in carbohydrate and protein concentration and bioaccessibility between the four selected Ayurvedic medicinal plants - *Shatavari*, *Chandrashura*, *Amalaki* and *Usheera* - each of which has the characteristics of the classical *Guru* and *Laghu* gunas. These differences were consistent with the Ayurvedic conceptual framework and provided biochemical evidence to support classification of these substances on the basis of *gunas* considering digestibility and nutritive actions. The difference established with these drugs should be tested with multiple samples. It would help in future characterization and classification of drugs based on these parameters.

Significance and Further scope

This was a preliminary exploratory study to determine whether the Invitro digestion technique and bioaccessibility of Carbohydrate and protein can form a parameter to understand the *Gunas* (digestibility properties) of herbal drugs. Only two drugs were taken as representatives for each *guna*.

The study gave promising results and can be further employed on a greater number of drugs for validation. With this basic understanding we can implement the same method to assess *Guru-Laghu gunas* (properties) of new (extrapharmacopoeial) drugs also. Further a scale can be established to define the extent of *Guru* and *Laghu gunas* in terms of bioaccessibility.

Funding and support

This project was funded by the KLE Academy of Higher Education and Research (KAHER) (Deemed-to-be University), Nehru nagar, Belagavi, Karnataka. The authors thank KAHER for funding the project. The project was conducted in the Central Research Facility (CRF), AYUSH approved drug testing laboratory for ASU drugs, KAHER's Shri B M Kankanawadi Ayurveda Mahavidyalaya, Belagavi. The authors thank CRF for providing the facilities.

Acknowledgement

Authors take this opportunity to acknowledge KAHER's Shri B M Kankanawadi Ayurveda Mahavidyalaya, Belagavi, for providing an opportunity to apply and complete the project.

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