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Effect of *Gunas* (physico-pharmacological properties) of selected Ayurveda drugs on carbohydrate and protein bio-accessibility of *mudga* [*Vigna radiata* (L.) R. Wilczek] in an *in vitro* digestion model

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A *Dravya* (drug) can perform its action due to its *Gunas* (*properties*). *Gunas* act as a medium for predicting or assessing *Karma* (*drug action*). Understanding *Guna* will play a leading role in the application of *Dravyas* in treatment. According to *Acharya Sushruta*, *Ushna guna* is having a prominent action on *Pachana* (*digestion*). This concept was utilized to assess the action of *Ushna* and *Sheeta guna* on *Agni* by the application of an *in vitro* digestion model. A standardized static *in vitro* digestion (INFOGEST) method was followed for three groups (i) *Mudga* alone (S) (ii) *Mudga* with *Ushna* drug (*Maricha* - *Piper nigrum* Linn.) (SM) and (iii) with *Sheeta* drug (*Usheera* -*Vetiveria zizanoides* Linn.) (SU), separately. The concentration of carbohydrates and proteins in different stages of digestion was estimated and bio-accessibility in each group was calculated and compared. Both the test groups showed significant effects on the bio-accessibility of Carbohydrates of *Mudga*. The test drugs do not have any effect on protein digestion in *Mudga*. But at the intestinal phase at 60 min, a significant difference was noted between the trial groups at p=0.0391. *Ushna guna* of *Maricha* and *Sheeta guna* of *Usheera* showed their effects on the carbohydrate bio-accessibility of *Mudga* during *in vitro* static digestion. The effect was more pronounced in the *Maricha* group. *Maricha* being an *Ushna dravya* must have modulated the enzymatic activity in a better way and improved the bio-accessibility of CH in *Mudga* as found in this study.

Keywords: Bio-accessibility, Carbohydrate, *Guna*, *In vitro* digestion, Protein, *Sheeta*, *Ushna*

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The principles of Ayurveda have been formulated by a process of continuous observation with the help of special senses. There is a need to re-assess these principles by applying the tools of contemporary science for a better understanding. *Guna* of a *dravya* (*Drug*) is one such principle. A drug can perform its action due to its *Gunas* (a group of properties) and hence *Gunas* have greater importance in Ayurveda.

Different types of *Gunas* have been elaborated in Ayurveda. *Gurvadi guna*[1] (a category which depicts a combination of physical and pharmacological properties of drugs), have found wide application in treatment. *Gunas* help in the prediction or assessment of drug actions.

Health or otherwise is dependent on the state of *Agni* (digestion and metabolism) and the main cause for any disease is said to be *Mandagni* (weakened digestive fire)[2]. According to classical texts of Ayurveda, *Ushna guna* has a major action on *Agni* and it helps in *Pachana* (digestion)[3]. Although multiple factors are involved in digestion, enzymatic process forms the major part and *gunas* of drugs might play a role in modulating these enzymes. This concept was taken as the basis to determine the action of *Ushna* and *Sheeta Guna* on digestion by applying *in-vitro* digestion model. *Ushna guna* is the result of the predominance of *Agni Mahabhuta* in a drug, giving it a hot and pungent taste (*katu rasa*) which is known for its hot and penetrating properties and promoting digestion[4]. Hence *Ushna guna* plays a major role in deciding the course of digestion and metabolism. The contrary of *Ushna* is *Sheeta guna* which is responsible for actions like diuresis, controls sweating, controls purgation and pacifies other symptoms of aggravated *Pitta* such as giddiness (*Murcha*), thirst (*Trishna*) and burning sensation (*Daha*). Drugs that are commonly selected for treatment for the related conditions are chosen based on this pair of *gunas*.

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During the process of digestion, complex carbohydrates, proteins and fats are broken down into simple components by the action of enzymes. Digestive enzymes such as proteases, lipases, amylases and nucleases break down the polymeric macromolecules into smaller units that facilitate their absorption by the body. From the ingested food, only a fraction is available for utilization at the action site to assist normal physiological functions. This bioavailability of food is the result of three important steps *i.e.*, digestion, absorption and incorporation from circulation into target site[5].

Human trials and *in vivo* studies are still considered as gold standard for assessing metabolism, absorption, excretion and bio-accessibility studies of food. They have their disadvantages concerning exploratory studies. *In vitro* digestion (IVD) tries to imitate the physiological conditions of *in-vivo* model. These are realistic and promising designs that facilitate development of food and oral formulations depending on their fate of digestion in the stomach and small intestine. These methods are increasingly being applied to assess the fate of digestion and potential toxicity of the ingested materials which are either natural or bioengineered (nanomaterials). Static methods (also called biochemical methods) are the simplest techniques that include two or three steps of digestion (oral, gastric and intestinal). These methods simulate certain parameters of physiological digestion. They are rapid, not so expensive, require less labor and do not have any ethical restrictions. So they have an advantage over other methods like *in-vivo* and clinical studies. The presence of digestive enzymes, their pH, concentrations, time taken for digestion and concentrations of different salts are taken into account during these procedures[6].

The INFOGEST network of scientists, with their efforts and studies, has now developed a static *in vitro* digestion (IVD) model based on conditions that are relevant physiologically and have been collected from human samples. This protocol has undergone validation through wide inter-laboratory trials. There are several scientific publications focusing on IVD systems. This has opened a new path towards developing neutraceuticals and therapeutic agents specifically designed as per the needs of populations. This also helps in the rational designing of foods to suit specific groups of populations and can act as preventive or curative for a disease[7].

Though the classical literature of Ayurveda extensively describes the *Gunas* of herbal drugs and their actions, there are limited evidences for experimental validation of *Ushna-Sheeta gunas* using modern scientific models and instrumentation. Since the effect of *Ushna- Sheeta gunas* is predominantly on the digestive system, analyzing this action through the application of invitro digestion model would help in validation of *Guna* theory in Ayurveda. With this aim, the present study explores the novel application of *in vitro* digestion technique for assessing *Ushna-Sheeta Guna*.

To see the effect of *Ushna & Sheeta* drugs on digestion and bio-accessibility in an *in vitro* digestion model, we took *Mudga* (*Vigna radiata* L.) as the source of carbohydrates and proteins, which was subjected to IVD in the presence of *Ushna* & *Sheeta* drugs. As per the Classics, *Mudga* (green gram) is the best among the pulses and is advocated for regular consumption[8]. Green gram has a high nutritional value. Among the various nutrients, it is a rich source of carbohydrates (59.9) and proteins (24.5). 100 g of *Mudga* produces 348 kcal of energy and is a good source of minerals and vitamins and contains essential amino acids[9]. The *Ushna* & *Sheeta* drugs selected for the study are most commonly used in daily Ayurveda practice for treating various systemic illnesses. This is an attempt to find out the possibility of employing IVD experimental technique to assess *Ushna-Sheeta Gunas* in the selected drug substances.

Materials and Methodology

Materials

Raw drugs collection and processing: Drugs were collected, identified and confirmed by a Taxonomist and the herbariums of the drugs were deposited at the Central Research Facility, AYUSH approved laboratory for ASU drugs, KAHER's Shri B.M. Kankanawadi Ayurveda Mahavidyalaya, Belagavi. The drugs were stored in aseptic conditions.

Anthrone reagent: 200 mg of Anthrone reagent was dissolved in 100 mL of ice-cold 95% H_2SO_4, prepared fresh before use. *Standard Glucose:* 100 mg dissolved in 100 mL water. Bovine serum albumin (BSA) was procured from Sigma-Aldrich. Coomassie brilliant blue G-250 was procured from Sigma-Aldrich, catalog number: 27815). The enzymes for *in-vitro* digestion, alpha-amylase from human saliva (A1031-1KU), pepsin from porcine gastric mucosa (P7012-250 mg), pancreatin (P7545-25 mg) and hemoglobin porcine (H4131-1 g) were procured from Sigma Aldrich. Other reagents of analytical grade

were used such as 2.5 N HCl, methanol, phosphoric acid (H_3PO_4), Bradford reagent and salts for electrolyte namely $CaCl_2(H_2O)_2$, NaOH (sodium hydroxide), HCl (hydrochloric acid), KCl (potassium chloride) KH_2PO_4 (potassium dihydrogen phosphate), $NaHCO_3$(sodium hydrogen carbonate), NaCl (sodium chloride) $MgCl_2(H2O)_6$ (magnesium chloride hexahydrate), $(NH_4)_2CO_3$, (ammonium carbonate) $CaCl_2$ $(H_2O)_2$ (calcium peroxide).

Methodology

Aim of the study was to determine whether *in vitro* digestion technique could be applied to assess the *Ushna-Sheeta gunas*. For this, *Maricha- Piper nigrum* L. was selected for *Ushna Guna* and *Usheera- Vetiveria zizanoides* (Linn.) Nash / *Chrysopogon zizanoides* (L.) Roberty. was selected for *Sheeta guna* and *Mudga* (green gram) as the substrate.

The objective was to determine whether selected *Ushna* and *Sheeta* drugs have any effect on the enzymatic digestion process. Theoretically *Ushna guna* should enhance the enzymatic digestion resulting in quick breakdown of complex nutrients into simpler molecules (carbohydrates and proteins into glucose and amino acids respectively).

Proximate analyses of all the samples along with total carbohydrate and proteins in solution was done followed by *in vitro* digestion of the substrate (cooked *Mudga*) in the presence of *Ushna* and *Sheeta guna* drugs separately. Samples were collected at different stages of digestion (oral, gastric and intestinal phases) at different time points. These samples were subjected to end analysis to determine the extent of conversion and then compared.

Proximate analysis as per AOAC guidelines[10] was carried out. The parameters included moisture (Loss on Drying), ash values, total carbohydrates, total proteins (in solution) and crude fat. The procedures mentioned in Ayurvedic Pharmacopoeia of India were followed.

Assay for estimation of proteins in solution

All the test drugs (*Mudga*, *Maricha*, *Usheera* and cooked *Mudga*) were taken in a quantity of 5 g separately in conical flasks, 100 mL of distilled water was added to each. Then they were subjected to agitation for 6 h followed by maceration for 18 h (cold extraction method) and then filtered. The filtrates were used for the estimation of soluble proteins by Bradford method[11].

Bovine Serum Albumin (BSA) in the range of 31.25 to 1000 ug/mL (1 mg/mL solution) was used as the standard for estimation of proteins in the samples. Briefly Bradford reagent 2 mL was added to each test tube containing 40 uL of drug or standard and the contents were mixed properly. The samples were incubated at room temperature (RT) for 5 min. The absorbance was recorded at 595 nm with the help of a UV spectrophotometer. The concentration was calculated using absorbance values and the standard graph was plotted.

Estimation of total carbohydrates by Anthrone method

The total carbohydrates of raw drugs (*Mudga*, *Maricha* and *Usheera*) and cooked *Mudga* were performed. Briefly, 100 mg of the sample were hydrolyzed in a boiling water bath for 3 h with 5 mL of 2.5 N HCl and later cooled to room temperature. The hydrolysed samples were then neutralized with solid sodium carbonate until effervescence ceased. The total volume of each sample was made up to 100 mL with distilled water and centrifuged and supernatant was collected. 10 uL of the supernatant sample was taken for further analysis. Glucose in the range of (20 ug/mL, to 100 ug/mL) was used as standard and distilled water was used as blank. The volume was made up to 500 uL in all the tubes including the sample tubes by adding distilled water.

To all the tubes 2 mL of Anthrone reagent was added slowly on the ice bath. Later the tubes were heated for eight minutes in a boiling water bath and cooled rapidly with the help of an ice pack. It gave green to dark green color. The absorbance was noted at 630 nm in the UV Spectrophotometer[12].

Preparation of samples and grouping

Five grams of *Mudga* was added with 80 mL of water (1:16 ratio mentioned for *Yusha*- a classical method for cooking green gram)[13] and boiled (temperature 100 deg C) on medium flame until the grains were properly cooked and soft. The total carbohydrates and total protein estimation of cooked *Mudga* was done. Cooked *Mudga* was then stored in a refrigerator until the digestion process.

The common dosage of *Churna* (drug powder) is 6 g BD and the quantity of food we take at a time is approximately 600 g. The ratio of food and drug is 100:1. To ensure better observations in *in vitro* conditions, the ratio of food and drug was modified to 20:1.

Cooked *Mudga* 5 g was taken in the first group as a standard substratum. 250 mg each of fine powders (60 no. mesh) of the test drugs *Maricha* and *Usheera*

were used in the other 2 groups along with cooked *Mudga* (Table 1).

A standardized static *in-vitro* digestion method, by international consensus,[7] was followed for the digestion of *Mudga* alone and *Mudga* with drugs separately in different groups.

Enzyme assay

Alpha-amylase enzyme and pepsin enzyme assay were performed as per standard procedures[14].

Amylase activity was recorded as 9.41±2.80 Units/ mg at amylase concentration of 1 mg/mL. Pepsin activity was recorded as 300±25.01 Units/ mg at a pepsin concentration of 0.03 mg/mL.

Static digestion

Digestion procedures were carried with brief modifications.

Simulated digestion fluids

Simulated salivary fluid (SSF), Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were made with corresponding electrolyte stock solutions. The electrolytes and simulated fluids were prepared in advance as per the composition mentioned[14] and stored at -20 degrees C. Calcium chloride was prepared fresh on the day of performing *in vitro* digestion.

Digestion procedure for three phases

For each group, samples were taken in 3 conical flasks. The sample code is described in Table 1.

The orbital shaker was switched on 2 h before starting the digestion procedure. The temperature of the orbital shaker was set to 37°C and a thermometer was kept inside the shaker to check whether the temperature inside had reached 37°C. Each sample was subjected to *in vitro* digestion in three phases- oral, gastric and intestinal phase. In each phase of digestion, suitable simulated fluids, $CaCl_2$ and distilled water were added, pH was adjusted and enzyme solution was added. The procedure was performed according to the flow chart 1.

Table 1 — Groups for *in-vitro* digestion

Sl. No.	Groups	Sample Code	Blank (without enzyme)	With enzyme
1	Group 1- Standard	S	SB- Cooked *Mudga* 5 g	S- Cooked *Mudga* 5 gm with enzymes
2	Group 2- Test	SM	SMB- Cooked *Mudga* 5 g + *Maricha* 250 mg	SM- Cooked *Mudga* 5 g + *Maricha* 250 mg with enzymes
3	Group 3- Test	SU	SUB- Cooked *Mudga* 5 g + *Usheera* 250 mg	SU- Cooked *Mudga* 5 g + *Usheera* 250 mg with enzymes

Sampling

Aliquots were collected at the end of the oral phase, the Gastric phase at 60 min & 120 min, intestinal phase 60 & 120-min. Snap freezing of samples in ice packs was done immediately after the reaction and were stored at -20 degrees C 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 *in-vitro* digestion. All the stored samples of different stages were analyzed for total carbohydrate and total proteins in solution by using the same procedures 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)x 100[15].

Statistical analysis

All analyses were done in triplicates. Mean values and standard deviation were calculated and tabulated in excel sheet. The data were analysed 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-values < 0.05.

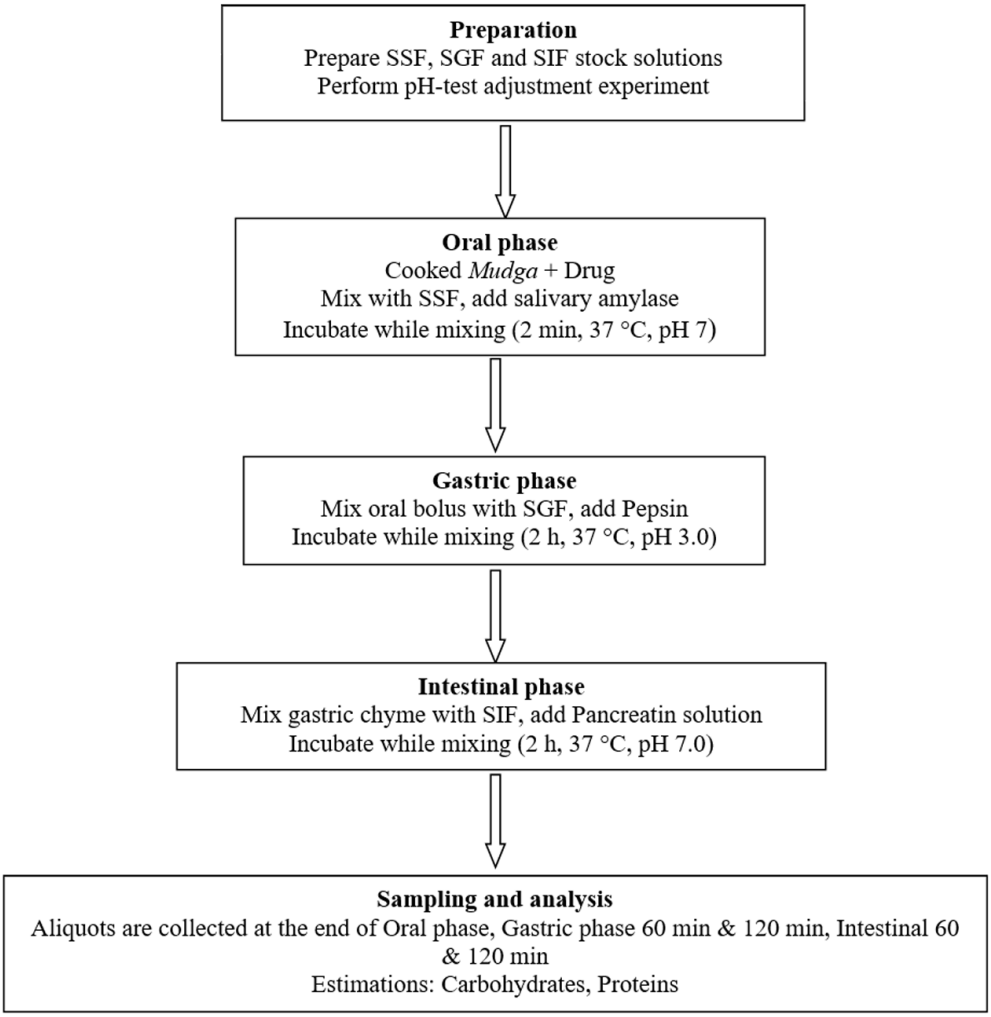
Results

Results of proximate analyses

Proximate analyses show that both the study drugs have parameters within the limits of API. Moisture content was more in *Usheera* (*Sheeta*) while Ash was found to be more in *Maricha* (*Ushna*). *Maricha* showed higher values of Water extract, Alcoholic extract and fat content when compared to that of *Usheera*. The results of the proximate analysis of trial drugs are depicted in (Table 2).

Results of total carbohydrate and total protein estimation in crude drug

Cooked *Mudga* showed the highest carbohydrate (3920.52 µg/ 100 mg of sample) and protein content



Flow Chart 1 — Procedure of *in vitro* digestion

(5726.77 µg/ mL WSE). Among the trial drugs, *Maricha* showed the higher carbohydrates (177.24 µg/ 100 mg of sample) and protein content (344.97 µg/ mL WSE). Total carbohydrates and total proteins in solution for all the samples are depicted in (Table 3).

Estimation of carbohydrates and bio-accessibility of *in vitro* digestion samples

The supernatant samples collected during different stages of *in-vitro* digestion were analyzed for total carbohydrates and their bio-accessibility. The results are as follows:

Compared to blank samples *i.e.,* 'SB, SMB & SUB', there is more release of glucose in the presence of enzymes during all stages of digestion in the 'S', 'SM' and 'SU' groups (Fig. 1a-c).

Statistical analysis showed that there is a significant difference between the standard substratum 'S' and the trial drug groups 'SM & SU' at p=0.0002 & 0.0133 respectively and also between the two trial groups 'SM & SU' (at p=0.0454) w.r.t Carbohydrate concentration (Table 4).

Table 2 — Proximate analyses of samples

Parameters	*Maricha*	*Usheera*
LOD (in %)	2.95	3.55
Total Ash (in %)	4.563	3.984
Acid insoluble ash (in %)	0.495	2.112
Water extractive value (in %)	12.44	7.735
Alcohol ext value (in %)	9.598	4.88
Fat content (in %)	9.097	0.535

Table 3 — Total carbohydrate and protein in solution of samples

Sl No.	Drug	Total carbohydrates (µg/ 100 mg of sample)	Total Proteins in solution (µg/ mL WSE)
1.	*Mudga*	114.49	2178.45
2.	*Maricha*	177.24	344.97
3.	*Usheera*	62.25	53.92
4.	Cooked *Mudga*	3920.52	5726.77

Bio-accessibility of carbohydrates is highest in the 'SM' group during all phases of digestion (Table 5 & Fig. 1d). While bio-accessibility is still detected in the 'SU' group, it is minimal in the 'S' group.

Statistical analysis showed that both the test drug groups 'SM & SU' have significant effects on the bio-accessibility of carbohydrates when compared to the standard substratum 'S' group. However, the difference between the trial drug groups 'SM & SU' is statistically not significant.

Table 4 — Two way ANOVA for concentration of carbohydrates between the groups

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted p-value
S vs. SM	-80.17	-120.7 to -39.64	Yes	***	0.0002
S vs. SU	-41.8	-75.40 to -8.196	Yes	*	0.0133
SM vs. SU	38.38	0.7081 to 76.04	Yes	*	0.0454

Table 5 — Bio-accessibility of Carbohydrates during different stages of Digestion

Stage number	Stages	S%	SM%	SU%
1	S	111.9619	168.4944	138.01
2	G-60	40.07637	279.5601	180.56
3	G-120	64.58319	197.9082	173.16
4	I-60	163.9361	398.0521	274.03
5	I-120	162.5266	497.3569	279.39

Results of total proteins in samples

Compared to blank samples *i.e.*, 'SB, SMB, SUB', there is more digestion of proteins in the presence of pepsin enzyme during the gastric stage of digestion in all the three groups *i.e.*, 'S, SM & SU' (Fig. 2a-c).

Statistical analysis showed that there is no significant difference in protein digestion among the groups (Table 6). The test drugs do not have any effect on protein digestion in *Mudga*. But at I-60, a significant difference is noted between SM & SU at p=0.0391.

Bio-accessibility at I-60 appears to be highest in 'S', then in 'SU' and least in the 'SM' group. This represents the residual (undigested) protein detected by the Bradford method (Table 7 & Fig. 2d).

Statistically, protein bio-accessibility was similar among the groups. The test drugs do not have any

Table 6 — Two way ANOVA for concentration of proteins between the groups

Tukey's multiple comparisons test	Mean diff.	95.00% CI of diff.	Significant?	Summary	Adjusted p-value
S vs. SM	-76.3	-1885 to 1732	No	Ns	0.9938
S vs. SU	-189.3	-1986 to 1607	No	Ns	0.9621
SM vs. SU	-113	-1782 to 1556	No	Ns	0.9842

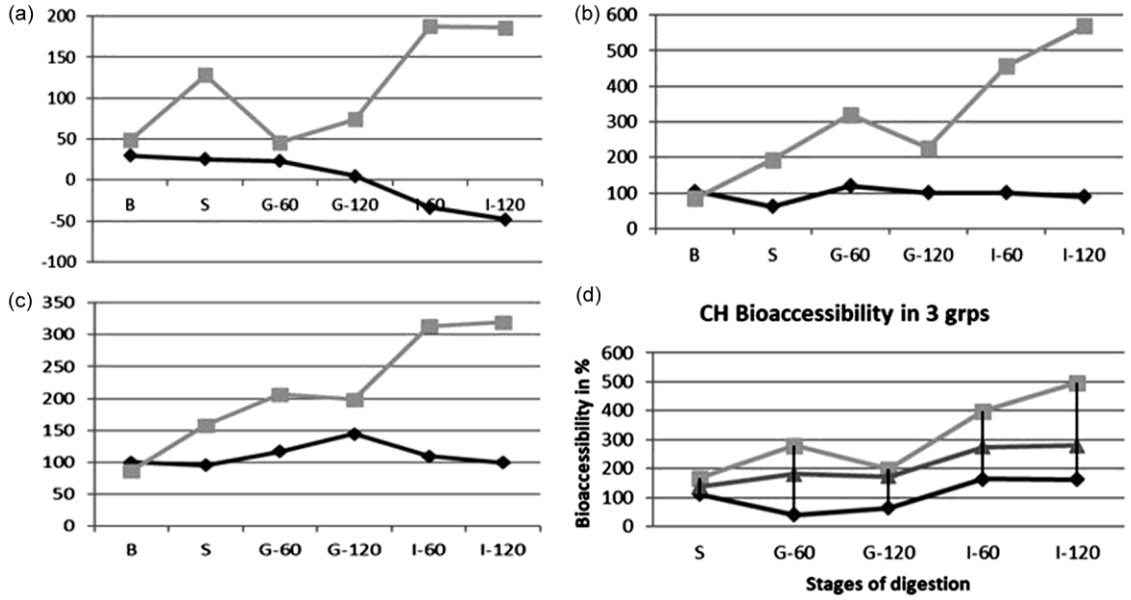


Fig. 1 — (a-c) Concentration and (d) Bio-accessibility of carbohydrates in different groups at different time points
B-Baseline, S-Salivary, G-60-Gastric phase at 60 min, G-120-Gastric phase at 120 min, I-60-Intestinal phase at 60 min, I-120-Intestinal phase at 120 min
(a)- *Mudga* Blank (without enzyme)SB and *Mudga* with Enzyme(S) ♦- *Mudga* Blank(SB), ■- *Mudga* with enzymes(S), (b)- *Mudga+Maricha* Blank(SMB) & with Enzyme(SM) ♦- *Mudga+Maricha* Blank(SMB), ■- *Mudga+Maricha* with enzymes(SM), (c)- *Mudga+Usheera* Blank & with Enzyme(SUB & SU) ♦- *Mudga+Usheera* Blank(SUB), ■- *Mudga+Usheera* with enzymes(SU), (d)- Bio-accessibility of Carbohydrates – comparison between the groups ♦-S, ■-SM, ▲-SU

significant effect on protein bio-accessibility of *Mudga*. But at I-60, there is a significant difference between SM & SU at p=0.0391 w.r.t bio-accessibility of proteins.

Discussion

The present study aimed to compare the effect of *Ushna* and *Sheeta guna* on digestive enzymes through an *in vitro* digestion model. Both the test drugs *Maricha* and *Usheera* were able to modify the digestion of Carbohydrates and Proteins present in *Mudga* and enhance their bio-accessibility. The action of *Maricha* was more pronounced during different stages of digestion, which might be because of its *Ushna guna.*

As per the classical literature of Ayurveda, *Ushna guna* has its major action on *Agni* which is responsible mainly for digestion and metabolism. *Maricha* (*Piper nigrum* L.) which has been taken as a representative of *Ushna guna* in this study has been mentioned as *Deepana-Pachana* (Carminative, digestive). *Usheera*, though *Sheeta*, is having the action of *Pachana* as in the context of *Shadangapaniya* in *Jwara chikitsa* due to its *Tikta rasa* (bitter taste). As per Ayurveda, both the test drugs have action on *Agni* vis-à-vis digestion, which was proven during the study.

Very few studies have been conducted so far on *Gunas* of drugs to prove their pharmacological activity. Deepa Anserwadekar et al. followed the *in vitro* digestion model to assess the *Snigdha* and *Ruksha guna* in normal and roasted food grains. It was found that roasted grains (*Ruksha*) were effective as antioxidants[16].

Present study followed the widely accepted *in-vitro* digestion model by INFOGEST with minor modifications[7]. After the digestion process, end analyses of carbohydrate assay revealed that there is more release of glucose in the presence of enzymes during all stages of digestion in trial groups. The carbohydrates present in *Mudga* got digested in the presence of enzymes in different stages of digestion, releasing glucose which was detected by the Anthrone method. Statistical analysis showed that there is a

Table 7 — Bio-accessibility of proteins during different stages of digestion

Stage number	STAGES	S %	SM %	SU %
1	S	168.7673	156.1963	169.2045
2	G60	-1.03765	9.331576	0.182262
3	G120	-2.17259	6.828054	10.35005
4	I60	23.90269	8.504675	20.53784
5	I120	7.273911	8.029755	13.92391

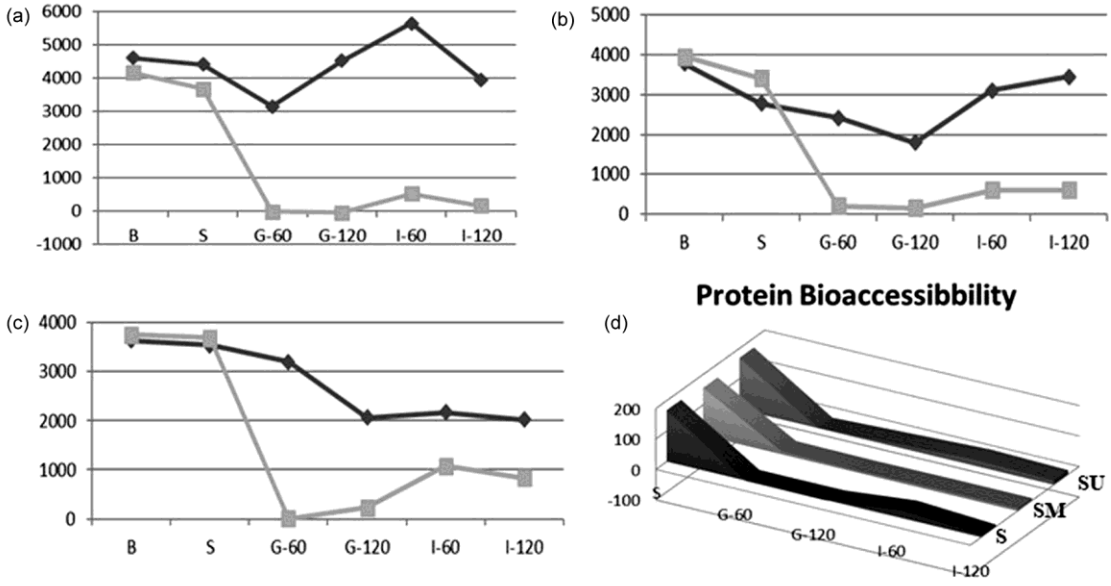


Fig. 2 — (a-c) Concentration and (d) Bio-accessibility of Proteins in different groups at different time points
B-Baseline, S-Salivary, G-60-Gastric phase at 60 min, G-120-Gastric phase at 120 min, I-60-Intestinal phase at 60 min, I-120- Intestinal phase at 120 min
(a)- *Mudga* Blank(SB) & *Mudga* with Enzyme(S) ♦- *Mudga* Blank(SB), ■- *Mudga* with enzyme(S), (b)- *Mudga+Maricha* Blank (SMB) & with Enzyme(SM) ♦- *Mudga+Maricha* Blank(SMB), ■- *Mudga+Maricha* with enzymes(SM), (c)- *Mudga+Usheera* Blank(SUB) & with Enzyme(SU) ♦- *Mudga+Usheera* Blank(SUB), ■- *Mudga+Usheera* with enzymes(SU), (d)- Bio-accessibility of Proteins – comparison between the groups

significant difference between the standard substratum *Mudga* 'S' and the trial groups 'SM & SU' at p=0.0002 & 0.0133 respectively and also between the two trial drug groups 'SM & SU' (at p=0.0454). These differences indicate that both the drugs might have enhanced enzymatic digestion of carbohydrates in *Mudga* providing an insight into their possible mode of action in digestion.

Protein digestion was more in the presence of pepsin enzyme during gastric stage of digestion in all the three groups. Statistical analysis showed no significant difference in protein digestion among the groups in the oral and gastric phases but significant difference between the test drug groups 'SM & SU' at the Intestinal phase at 60 min (p=0.0391).

Digestive enzymes can be modulated with any biomolecule (polyphenols, flavonoids, protein fragments, peptides, steroids etc.,). These modulators can either increase or decrease the activity of enzymes. The drugs chosen in this study might contain these modulators and hence they can modify the release of carbohydrates and proteins from *Mudga* during different stages of digestion.

Maricha (*Piper nigrum*) is rich in phenols, lignan derivatives, terpenes, chalcones, flavonoids, alkaloids and steroids. 4% alkaloids are present in berries. Piperine, an amide alkaloid, is a proven bioavailability enhancer and has a role in gastrointestinal disorders, can modulate the action of enzymes that metabolize drugs and improve bioavailability of several drugs. *P. nigrum* stimulates rat gastric acid secretion and its active agent piperine decreases the secretion of small intestine activated by castor oil. It also increases rat pancreatic enzyme activity[17].

Percentage bio-accessibility of any nutrient biomolecule (Carbohydrates and proteins) after digestion indicates the possibility of proper and increased assimilation of the compounds in the body for normal growth and development. In the present study, the bio-accessibility of carbohydrates was found to be highest in the 'SM' group during all phases of digestion.

Statistical analysis showed that both the test drug groups 'SM & SU' have a significant effect on the bio-accessibility of carbohydrates when compared to the 'S' group. Also the difference between the test drug groups 'SM & SU' is statistically significant and further we can infer from the figure (Fig. 1d) that during all stages of digestion, the *Maricha* group showed the highest bio-accessibility of carbohydrates.

The difference in bio-accessibility of proteins between the test drug groups 'SM & SU' is statistically significant only in the intestinal phase at 60 min (Table 7 & Fig. 2d). Protein digestion shows no major difference among the groups which might be because of the technique used for the determination of proteins ie., the Bradford method which determines only the residual protein (higher molecules) but not the smaller peptides and amino acids[18]. During the gastric phase of digestion, the pepsin enzyme breaks all the bigger protein molecules into smaller peptides, which further get converted into amino acids during the pancreatic phase.

In a previous study, Santosh Mane *et al*.[19] studied the application of *Ushna* and *Sheeta gunas* w.s.r to *Amlapitta* through a clinical trial. Patients who showed aggravated *Pitta* w.r.t *Drava guna* benefited significantly from *Dravyas* containing *Ushna guna*. Those with *Ushna guna* symptoms showed recovery with the *Sheeta* drug combination. The study concluded that some objective parameters at physico-chemical, pharmacological and therapeutic levels can help us to assess *Sheeta* and *Ushna gunas*.

In another study, Mehmood and Gilani (2020)[20] analyzed the effect of pepper crude extract on isolated guinea pig ileum and found that the drug has concentration dependent stimulant effect. In mice, it showed laxative effect at low dose whereas antisecretory and antidiarrhoeal effects at high dose, thus proving its role in gastrointestinal motility disorders.

In the present study, the difference in carbohydrate and protein content between the groups concluded that both the trial drugs had stimulation action on enzymes. Though in this study, we have explored the increased bio-accessibility by modulation of enzyme activity by drugs, there are other modes through which these drugs can act on the digestive system. One of the modes of action of these drugs was explored in a study by Chou *et al.*,[21] where they identified 25 compounds in the essential oil of *V. zizanoides* and tested the potential of these compounds in suppressing inflammatory response of LPS stimulated RAW macrophages. It was found in this study that *V. zizanoides* has the ability to act as an anti-inflammatory agent by regulation of inflammation related enzyme expression and inflammatory cytokines. Further, the anti-oxidant action was also proven, supporting its traditional use in inflammatory conditions of GI tract.

In physiotherapy, some herbal drugs are used for treating Functional Gastro-Intestinal disorders based on their active principles and pharmacological actions. These are categorized into Amara, Aromatica and Amara-Aromatica. Drugs coming in the "Amara category" basically act by stimulating gastric secretion. Those in "Aromatica" category show different modes of action. Among these, some contain spasmolytic and carminative essential oils or spasmolytic alkaloids. Others are rich in mucilage which smoothen the mucosa in GI tract or contain flavonoids with anti-inflammatory action. Herbs with different kinds of action can be combined for maximum efficacy and also specific action. *Maricha* (*P. nigrum*) might be acting like Amara while *Usheera* (*V. zizanoides*) might be exerting its action like an Aromatica drug on the digestive tract[22].

Thus, both the trial drugs can modulate the digestion by the effect of their active components. The present study supports the Ayurveda theory of *Ushna guna* of *Maricha* and *Sheeta guna* of *Usheera* aiding digestion and improving bio-accessibility. At the same time, it is evident from the study that *Ushna guna* of *Maricha* has more pronounced action on the digestive enzymes which correlates with the classical reference indicating the action of *Ushna guna* predominant drugs on digestion.

Though both the trial drugs showed their action on digestion, we don't have data on the exact molecular mechanism involved. The present study is just an exploration into finding a molecular basis based on biochemical sciences. The degree of hydrolysis and the amount of Amino Acids released were not calculated in this study, which could have aided in understanding the extent of hydrolysis of protein during different stages of digestion. Only residual protein concentrations were used to determine the bio-accessibility.

The study can be elaborated with more numbers of *Ushna* and *Sheeta* drugs to see how different drugs modulate the enzymatic action. The results from a bigger study can help us in characterizing the different *guna* (properties) of drugs based on their bio-accessibility.

Conclusion

Ushna guna of *Maricha* and *Sheeta guna* of *Usheera* showed their effect on the carbohydrate bio-accessibility of *Mudga* during *in vitro* static digestion. The effect was more pronounced in *Maricha* group. The difference between two trial groups was statistically significant, *Maricha* being an *Ushna Dravya*, modulated the enzymatic activity in a better way and improved the bio-accessibility of carbohydrates in *Mudga* as found in this study. But since *Usheera* is a *Sheeta guna* drug, the effect was less compared to that of *Maricha*. Our results support the Ayurveda concept that *Ushna dravya* facilitates digestion.

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Conflict of Interest

Authors declare that there is no conflict of interest related to the manuscript.

Author Contributions

We the authors of this manuscript undertake that we have made substantive intellectual contributions to the content of this manuscript in the following areas: AnM- Conceptualization, formal analysis, writing - original draft. DK- conceptualization, formal analysis, resources, writing - original draft, review & editing. AvM- Conceptualization, formal analysis, software, supervision, writing - review & editing.

Data Availability

Raw data related to the study is recorded and available in the Central Research Facility, AYUSH approved drug testing laboratory, KAHER's Shri B.M. Kankanawadi Ayurveda Mahavidyalaya, Belagavi, Karnataka.

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