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ORA- Analytical study

Pepsin modulation activity of *Truptighna Dashemani* drugs in correlation with total phenols: An in-vitro study

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ABSTRACT:

Background: *Trupti* is early satiety and likened to Dyspepsia which occurs as a symptom in many gastro-intestinal conditions. Functional dyspepsia (FD) presents with bothersome post prandial fullness, pain, burning in the epigastrium and early satiety. Pepsin enzyme has been recently identified as one of the potential targets in FD. Charaka Samhita mentions *Truptighna Dashemani*, for treating the condition *Trupti* (Dyspepsia). **Objectives:** The present study aimed to explore Pepsin enzyme modulation activity of *Truptighna Dashemani* drugs and relate this with the phenolic content present in them. **Methods:** Hydro alcoholic extract of each drug of *Truptighna dashemani* was prepared by cold maceration. Estimation of total phenols was performed by Folin-ciocalteu method using Gallic acid as standard. Pepsin enzyme assay was performed following internationally accepted standard INFOGEST protocol and was then correlated with total phenols in each drug. **Results:** *Chavya* showed the highest phenolic content (109.88 ±0.29 mg GAE/ gm of extract) while *Pippali* and *Vacha* showed the least. *Guduchi showed the highest Pepsin activity (23633 units/ mg) followed by Pippali (22633), Chitraka (17533 units/ mg) and Vidanga (12933). Patola* and *Nagara* showed negative values. Kruskal Wallis ANOVA comparing the results with positive control showed significant differences in Pepsin activity among eleven groups (control + ten extracts; H = 27.6, P = 0.0026, n = 3 per group). The association between phenol content and pepsin activity was assessed using nonparametric Spearman correlation, revealing moderate negative correlation (r = −0.56, P = 0.096, n = 10) indicating greater pepsin inhibition by extracts with lower phenolic content. **Conclusion:** *Truptighna Dashemani* drugs have Pepsin modulation action as evidenced by invitro studies. The total phenolic content present in the individual drugs might be having a role in Pepsin activity modulation. Drugs showing Pepsin activation can act as digestive stimulants while those inhibiting pepsin action act as gastro protectives.

KEYWORDS: Dyspepsia, Pepsin activation, Pepsin inhibition, Trupti, Phenolic content

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1. INTRODUCTION

Trupti is a feeling of early satiety and has been likened to Dyspepsia based on the symptoms. [1] As per Charaka Samhita, the classical text of Ayurveda, *Trupti* (Dyspepsia) is a condition resulting out of aggravated *Kapha dosha*, causing impairment of digestion and production of *Ama* (undigested food). This affects the *Rasavaha srotas* (clannels carrying primary products of digestion) and leads to symptoms like dislike for food (*Ashraddha*), tastelessness (*Aruchi*), bad taste in mouth (*Asyavairasya*), loss of sense of taste (*Arasajnata*), nausea (*Hrillasa*) and heaviness in body (*Gaurava*). [2, 3] Dyspepsia can be seen as a symptom in many gastro-intestinal conditions. Functional dyspepsia (FD) is one condition presenting with symptoms like bothersome postprandial fullness, pain and burning in the epigastrium, early satiation without any structural abnormality in the gastro-intestinal tract. [4] It causes significant impairment of the quality of life of patients and increases the burden on health care facilities. The causes for this disease are multiple while conventional treatment with proton pump inhibitors, serotonin reuptake inhibitors, antidepressants and probiotics have limitations. [5] FD is a disease of functional origin which usually occurs after a high protein or high fat diet. One of the prime causes for FD is gastric dysmotility which leads to delayed gastric emptying. After ingestion of food, protein digestion is initiated by Pepsin enzyme in the gastrium. Pepsin plays an important role in promoting the secretion of other digestive enzymes such as Cholecystochinin (CCK) and Gastrin which result in gastric emptying. Pepsin is also known to promote secretion of pancreatic enzymes by CCK which in-turn helps in pancreatic digestion and absorption. Due to this reason, today Pepsin is being considered as a potential target to tackle the functional gastrointestinal disorders which are manifested due to heterogenous factors. Modulation of pepsin may present a more effective approach to improve digestive function in many digestive disorders. Pepsin enzyme does the signaling of amino acids like phenylalanine and tryptophan and the peptides. This in-turn initiates the processes which promote digestion. The aromatic amino acids present in certain herbal drugs have been shown to stimulate the signal chains and secrete gastrin and cholecystochinin, mediated by gastrin and Ca2+ sensing receptors in the parietal cells, antral mucosa and duodenal mucosa. Due to the heterogeneous nature of the disease FD, Pepsin is being seen as a potential treatment modality due to its multiple pro-digestive effects. [6]

Charaka Samhita, the classical text of Ayurveda mentions *Truptighna Dashemani*, a group of ten drugs useful for treating the condition *Trupti* (Dyspepsia). Ayurveda mentions direct action of these drugs on digestion depicted by pharmacological actions like enhancer of appetite (*Dipana*) and promoter of digestion (*Pachana*). Some of the drugs mentioned in *Truptighna dashemani* have been proven to be effective in dyspepsia and other gastrointestinal conditions through preclinical and clinical trials. [7- 11] But there are no studies so far assessing the role of these drugs on the Pepsin enzyme activity which can modulate gastric digestion.

The herbal drugs are rich in polyphenolic compounds and there are evidences suggesting that phenols can alter the pepsin enzyme activity, mostly through inhibition, causing reduced protein digestion and gastroprotective action. [12] The present study aimed to explore the pepsin enzyme modulation activity of *Truptighna Dashemani* drugs by employing the internationally accepted INFOGEST protocol [13] for Pepsin activity assay and relate this activity with the phenolic content present in the individual drugs of the group. INFOGEST protocol is the internationally accepted and widely used method for enzyme activity assays in different fields like pharmacology, food science, nutraceutical research and evaluation of functional food. It gives a method which is standardized by multiple trials, which is physiologically relevant and also can be reproduced through invitro digestion model. This method simulates human gastric conditions by

maintaining the conditions of pH (acidic around 2-3), body temperature (37° C), composition of electrolytes and concentration of enzymes. These conditions closely resemble the human gastric environment suitable for pepsin activity and thus improves the biological relevance of the assay. This helps to minimize the experimental variability and ensures a reliable measurement of proteolytic activity and release of peptides by Pepsin. The INFOGEST protocol recommends that pepsin should be used based on enzyme activity (Units/ mL) rather than based only on mass concentration. This is very important to ensure consistency.

Though ten drugs have been mentioned under the same group, each drug might have different modes of action. The assessment and comparison of pepsin modulation action of each drug would help to choose them logically in a gastro-intestinal disorder for better action. With this background the present study tried to analyze and compare the pepsin enzyme modulation action of *Truptighna Dashemani* drugs to understand their possible mode of action.

The objective of this study was to quantify phenols in the hydroalcoholic extracts of individual drugs of *Truptighna Dashemani gana* and assess their pepsin modulation activity through standard INFOGEST protocol and then to correlate the pepsin modulation activity with phenolic content in each drug.

2. MATERIALS AND METHODOLOGY

Materials: All chemicals of analytical grade were procured from licensed dealers. The following chemicals were used: Aluminium chloride ($AlCl_3$, anhydrous, Analytical grade, Sigma-Aldrich/ Merck, catalog number 206911-100G) , Hydrochloric acid (HCl, Analytical grade, NICE chemicals, CAS number 7647-01-0), Sodium chloride (NaCl, Analytical grade, Sigma-Aldrich/ Merck, catalog number S7653), Sodium Hydroxide (NaOH, Analytical grade, Sigma-Aldrich/ Merck, catalog number S5881), Porcine Hemoglobin (Hemoglobin from Porcine blood, suitable for biochemical assays/ reagent grade, Sigma-Aldrich/ Merck, Catalog number G4131-1G), Porcine Pepsin (Pepsin from porcine gastric mucosa, Sigma-Aldrich/ Merck, Catalog number P7012-250 mg).

Procurement and authentication of drugs: *Truptighna Dashemani* crude drugs were procured from GMP certified KLE Ayurveda Pharmacy, Khasbag, Belagavi. The herbs included in this group are *Nagara* (*Zingiber officinale* Roscoe.) rhizome*, Chavya* (*Piper Chaba* Trel. & Yunck.) stem*, Chitraka* (*Plumbago zeylanica* L.) root*, Vidanga* (*Embelia ribes* Burm.f.) fruit*, Murva* (*Marsdenia tenacissima* (Roxb.) Moon.) root, *Guduchi* (*Tinospora cordifolia* (Thunb.) Miers.) stem*, Vacha* (*Acorum calamus* L.) rhizome*, Musta* (*Cyperus rotundus* L.) rhizome*, Pippali* (*Piper longum* L.) fruit and *Patola* (*Trichosanthes dioica* Roxb.) leaves. Voucher specimens of these were deposited in the Central Research Facility (CRF), AYUSH approved drug testing laboratory for ASU drugs, KAHER's Shri B.M. Kankanawadi Ayurveda Mahavidyalaya, Shahapur, Belagavi. The voucher specimen number were given as CRF/Auth/88/2025-26, CRF/Auth/89/2025-26, CRF/Auth/90/2025-26, CRF/Auth/91/2025-26, CRF/Auth/92/2025-26, CRF/Auth/93/2025-26, CRF/Auth/94/2025-26, CRF/Auth/95/2025-26, CRF/Auth/96/2025-26 and CRF/Auth/97/2025-26 and were authenticated by experts at CRF.

The crude drugs were powdered and sieved through sieve of 60 mesh size and used for subsequent steps.

Extract preparation

Each drug of *Truptighna dashemani* was taken in powder from (60 mesh size) and hydro alcoholic extract was prepared with ethanol and distilled water in 7:3 ratio. The method of extraction used was agitation in orbital shaker at room temperature for 6 hours followed by cold maceration for 18 hours. The filtered extract was then concentrated in rota evaporator and dried on a water bath at 50 °C. This extract was further used for estimation of total phenols and pepsin activity assay.

Estimation of Total Phenols

Estimation of total phenols was performed by Folin-ciocalteu method [14] using Gallic acid as standard. Gallic acid was dissolved in methanol and serial dilutions from 6.25 µg/mL to 100 µg/mL were prepared. Each drug extract was dissolved separately in ethanol to get 1 mg/ mL concentration stock solutions. Sample extract and 10-fold dilute Folin- Ciocalteu reagent were taken in the test tube along with sodium carbonate (7.5%) to adjust the pH, covered and incubated at room temperature for 30 minutes. Later the absorbance was recorded in UV- spectrophotometer (Shimadzu UV-1800) at a wavelength of UV- 760 nm. Blank consisted of methanol. The absorbance was plotted against concentration to get the calibration curve. Using slope equation of the calibration curve, total phenols in samples were calculated and were expressed as mg gallic acid equivalent (GAE)/gm dried extract.

Pepsin activity assay

Pepsin activity assay was performed according to INFOGEST method. [15] The principle behind this method is that Pepsin breaks down the protein in hemoglobin and results in the production of peptide residues. This reaction takes place under controlled temperature and pH. The TCA added later combines with larger proteins and get precipitated/ sedimented. The peptide residues remaining in the supernatant were quantified Spectrophotometrically. The method of Pepsin assay is depicted in flow chart 1.

1. Pepsin stock solution was prepared by dissolving 25 mg Porcine Pepsin in 25 mL Tris buffer
2. Serial dilutions of Pepsin in 10 mM HCl (from 10 µg/mL to 60 µg/mL) (10 µL of Pepsin stock solution mixed in 990 µL of 10 mM HCl to get 10 µg/mL concentration, 20 µL of Pepsin stock solution mixed in 980 µL of 10 mM HCl to get 20 µg/mL concentration and so on)
3. 2 % weight by volume Hemoglobin solution was prepared by dissolving 1 mg Porcine hemoglobin in 40 mL ultrapure water & 5 mL of 300 mM HCl, pH was adjusted to 2 with 1 M NaOH and total volume was made to 50 mL
4. Hemoglobin 500 µL + corresponding pepsin solution 100 µL (10 mM HCl in blank) were incubated at 37 °C at 650 RPM for 10 minutes in orbital shaker incubator (Bench-top shaking incubator, OSI-650, RIA instruments, India)
5. Add 1 mL of 5% TCA solution rapidly to stop the reaction
6. Centrifuge at 6000 RPM for 30 minutes (High speed Centrifuge, model R-24, REMI Elektrotechnik Ltd., Mumbai, India)
7. Absorbance of supernatant was recorded by UV Spectrophotometer at UV 280 nm (Shimadzu UV Spectrophotometer, Model UV-1800 240V, Toshvin Analyst Pvt. Ltd, Mumbai, India)
8. The pepsin concentration exhibiting optimum activity (30 µg/mL) was selected for assay of test drugs (25 µg/mL) following the same steps
9. To minimize the substrate and drug interference, substrate blank and drug blank were tested keeping the volume constant

Flow chart 1: Steps for Pepsin enzyme assay

Substrate blank consisted of 500 µL of Hemoglobin solution, 100 µL of 10 mM HCl, 25 µL Solvent used for extract (Distilled water: Ethanol in the ratio 3:7) and TCA. Drug blank consisted of 500 µL of 300 mM HCl, 100 µL of 10 mM HCl, 25 µL Sample extract and TCA.

Enzyme activity in the presence of drug extract was calculated and compared with positive control for the similar concentration of enzyme. Positive control consisted of 500 µL of Hemoglobin solution, 100 µL of Pepsin solution, 25 µL Solvent used for extract (Distilled water: Ethanol in the ratio 3:7) in place of drug extract and TCA. Negative control consisted of 500 µL of Hemoglobin solution, 100 µL of 10 mM HCl in place of Pepsin solution, 25 µL Sample extract and TCA.

Subsequently enzyme modulation activities of all the drugs were compared and interpreted to understand the actions.

Enzyme activity was calculated using the formula:

Units/mg = $[(A_{test} – A_{blank}) * 1000] / [\Delta t * X * 0.001]$

Pepsin activity is expressed as units per mg where one unit is defined as the amount of enzyme required to release 1 µg of tyrosine per minute under assay conditions (pH 2, temperatute 37 °C and Hemoglobin as substrate).

A_{test} = Absorbance of test sample

A_{blank} = Absorbance of blank

Δt = time of reaction = 10 minutes

X = amount of pepsin in the final reaction mixture in the cuvette (mg) assuming 1 mL pepsin solution is added= 1 (1 mg/ml concentration of enzyme)

0.001 = constant = absorbance value attributed to 1 unit of enzymatic activity

The concentration of drug selected was 25 µg/mL. Pepsin is active only in acidic condition (pH 2.5-3.5). If higher concentration of drug is introduced into the assay system, it might alter the pH and affect the enzyme activity. So, a minimum concentration of 25 µg/mL was chosen after pilot trials.

Statistical analysis: Statistical analysis was performed using GraphPad Prism software (version 8.0.2). Three statistical tests were employed: one way ANOVA, Kruskal Wallis ANOVA and non-parametric Spearman correlation tests for analysing the results. Total phenolic content was determined in triplicates and the mean along with standard deviation were calculated. One way ANOVA test was applied to compare and understand the statistical significance of phenolic content between the groups. The Pepsin activity of all the samples was compared against the positive control by applying Kruskal Wallis ANOVA as the values of pepsin activity were not normally distributed. The relation between total phenolic content and Pepsin modulation activity among different groups was statistically assessed by using the non-parametric Spearman correlation test.

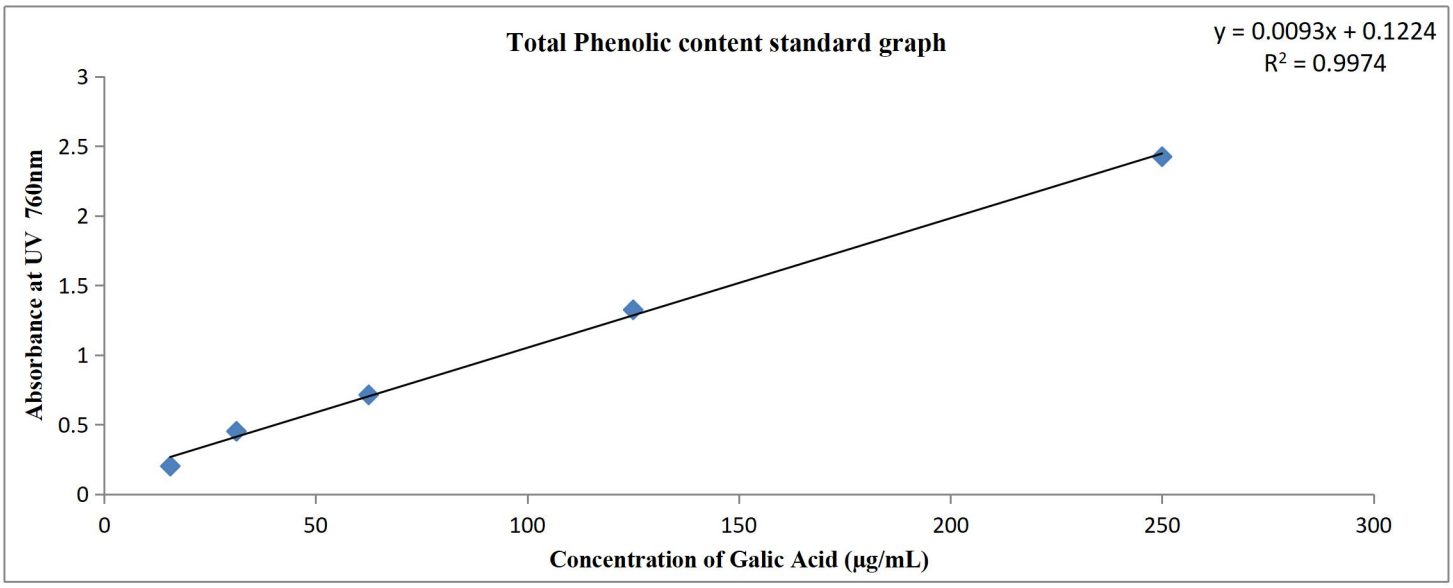
3. RESULTS

After authentication, the drugs of *Truptighna Dashemani* were subjected to extraction by cold maceration using ethanol and distilled water in 7:3 ratio as the solvent. This solvent mixture was used as it would help to extract the phenolic compounds soluble in both water and ethanol. In Ayurveda, water-based formulations are used more, in the form of *Kashaya* (decoction) or water as a media (*Anupana*) with drug powder (*Churna*). Further preparations like *Asava* and A*rishta* containing self-generated alcohol are also in practice. Hence, a hydro-alcoholic extract appears more appropriate for extraction. Further, it is an established fact that phenolic compounds are known to be thermolabile (highly sensitive to heat). [16] If hot extraction methods like Soxhlet extraction was used, it would result in the degradation of the chemical structure, leading to a loss in both concentration and biological activities like antioxidant or enzyme inhibitory properties.

All the drugs yielded average extractive value of 60.6±1.02 mg per gm of sample. These extracts were further dissolved in suitable solvent and used for further analysis.

Total phenolic content: The standard graph of gallic acid exhibited linearity (R^2 = 0.99). The graph is depicted below (Graph 1).

Total phenolic content was found to be highest in *Chavya* (109.88 ±0.29 mg GAE/ gm of extract), next to that *Murva* (82.93 ± 0.73 mg GAE/ gm of extract) followed by *Musta* (78.23 ± 1.07) and *Nagara* (76.36 ± 0.29 mg GAE/ gm of extract). *Vidanga* and *Patola* also showed good phenolic content whereas *Chitraka* and *Guduchi* showed moderate phenolic content. *Pippali* and *Vacha* showed the least phenolic content (table 1).



Graph 1: Standard graph of Gallic acid

Table 1: Total Phenolic content in hydro-alcoholic extracts of all the drugs

Sl No.	Sample name	Conc 1	Conc 2	Con 3	Mean (mg GAE/ gm of extract)	STD Dev
1.	*Nagara*	76.53	76.53	76.02	76.36	0.29
2.	*Chavya*	109.85	110.77	109.03	109.88	0.86
3.	*Chitraka*	50.26	49.85	49.34	49.82	0.46
4.	*Vidanga*	72.85	74.59	72.75	73.40	1.03
5.	*Murva*	82.46	83.79	82.56	82.93	0.73
6.	*Guduchi*	47.71	46.17	47.60	47.16	0.85
7.	*Vacha*	14.29	13.67	14.29	14.08	0.35
8.	*Musta*	77.14	78.27	79.29	78.23	1.07
9.	*Pippali*	21.34	21.54	22.56	21.81	0.65
10.	*Patola*	62.53	63.96	62.63	63.04	0.79

Table 2: Statistical analysis of Total Phenols by One way ANOVA

ANOVA summary	
F	3914
P value	<0.0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R square	0.9994

Statistical analysis by one way ANOVA showed an overall significant difference in the total phenolic content between the drugs at p value of <0.0001 (table 2). Tukey's multiple comparison test showed significant difference between the individual drugs (p value < 0.0001) except in case of *Nagara* and *Musta* where the difference was not significant (p= 0.1730) and less significant between *Nagara* vs. *Vidanga* (p= 0.0046) and between *Chitraka* vs. *Guduchi* (p= 0.0137).

Pepsin enzyme modulation assay

Results of Pepsin enzyme modulation assay are interpreted as higher absorbance value relates to higher pepsin activity while lower absorbance corresponds to enzyme inhibition. Pepsin enzyme activity was calculated by applying the suitable formula {Units/mg = [($A_{test} - A_{blank}$) * 1000] / [Δt * X

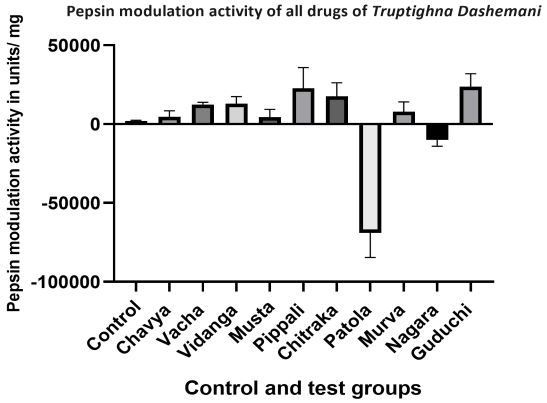
* 0.001]}. In the results of Pepsin activity calculation, positive values indicate enzyme activation and negative values indicate enzyme inhibition. During the study *Guduchi* showed the highest Pepsin activity (23633 units/ mg) followed by *Pippali* (22633), *Chitraka* (17533 units/ mg) and *Vidanga* (12933). *Murva* and *Musta* showed least Pepsin activity. *Patola* and *Nagara* showed negative values. The pepsin activity in different samples in relation with total phenolic content of samples is depicted in Table 3.

Table 3: Pepsin modulation activity in units/mg and its relation with total phenols

Samples	Pepsin activity in units/ mg	Total phenols in mg GAE/ gm of extract
Positive control (Enzyme & substrate present, no drug)	1750	--
Chavya (Both enzyme and drug present)	4533	109.88
Vacha (Both enzyme and drug present)	12467	14.08
Vidanga (Both enzyme and drug present)	12933	73.40
Musta (Both enzyme and drug present)	4467	78.23
Pippali (Both enzyme and drug present)	22633	21.81
Chitraka (Both enzyme and drug present)	17533	49.82
Patola (Both enzyme and drug present)	-69000	63.04
Murva (Both enzyme and drug present)	7767	82.93
Nagara (Both enzyme and drug present)	-10033	76.36
Guduchi (Both enzyme and drug present)	23633	47.16

Patola and *Nagara* showed negative values of Pepsin activity. These negative values might result due to multiple reasons. This can occur due to assay interference by substrate and by the sample (drug) extract. Negative values may also result when the absorbance of substrate is higher than that of the trial drug. The drug extracts themselves being colored solutions, can result in interference. In order to nullify these interferences, “only substrate” and “only drug” absorbances were noted initially. The absorbance values noted for “only drug”, “only substrate” and “positive control” (enzyme only) helped to validate the assay system. During this validation, absorbance value of “only drug” (0.106) indicated no enzymatic activity. Absorbance of “only substrate” is taken as the baseline value/ reference value. Positive control depicted normal pepsin activity.

The Pepsin activity of all the samples was compared against the positive control by applying Kruskal Wallis ANOVA (n=3) as there were two outliers (negative values) and normal distribution criteria was not met by the data. Each trial drug group was compared with the control. The test showed significant differences in pepsin activity among eleven groups (control + ten extracts; H = 27.6, P = 0.0026, n = 3 per group). Activities ranged from -69000 to 23633 units/mg. indicating both stimulative and inhibitory activity across all groups. The same is graphically depicted in graph 2.



Graph 2: Pepsin enzyme modulation activity of different samples in units/mg

Strong pepsin enzyme activation is seen in *Guduchi*, *Pippali* and *Chitraka* moderate activation in *Vacha*, *Vidanga* and *Murva*, mild activation in *Chavya*, weak activation in *Musta*, moderate inhibition in *Nagara* (-10033 units/ mg) and very strong inhibition in *Patola* (-69000 units/ mg). The overall difference was statistically significant at p=0.0026.

The assay validation parameters were assessed and it was found that Vacha shows moderately good precision (10.63%) which is acceptable based on assay type. Repeatability assessment could not be done as it requires at least 6 replicates whereas in this study triplicates were analysed.

The relation between Pepsin modulation activity and total phenolic content was assessed by applying Spearman correlation test (non-parametric). This test uses ranks instead of absolute values, so useful in case of outliers (negative values). The results demonstrated moderate negative correlation between total polyphenols content and pepsin activity (r = -0.56), although the relation did not have any statistical significance (p=0.096, n = 10).

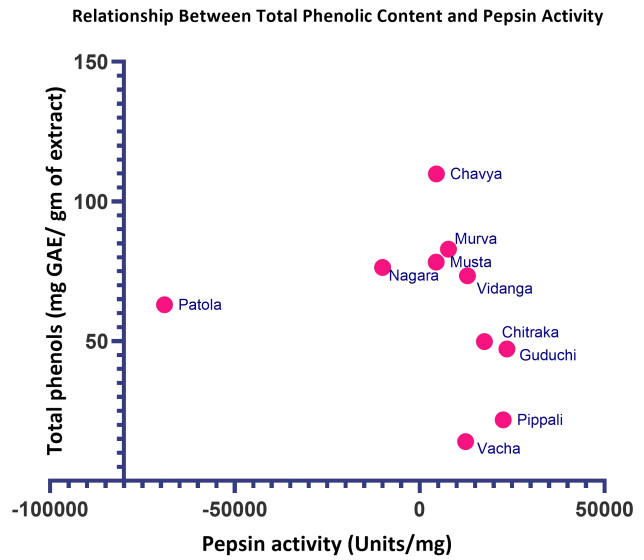
The observed trend indicates that drugs possessing higher phenolic content exhibited lower pepsin activity under experimental condition. However, absences of statistical significance indicate that total phenols alone cannot fully explain the variation in pepsin activity among study drugs. Rank comparison analysis (Table 4) further supports this observation. *Chavya, Musta, Nagara* and *Patola* exhibit relatively high phenolic raking but comparative lower pepsin activity ranking, whereas *Pippali, Guduchi* and *Chitraka* demonstrated higher pepsin activity despite lower phenolic rankings. These findings suggest additional phytochemical constituents and complex interaction among multiple compounds may contribute to the observed response in the study.

Table 4: Rank comparison between total phenolic content and pepsin activity among the studied drugs

	Pepsin activity(U/mG)	Rank Pepsin	Total Phenols (mg GAE/ gm of extract)	Rank Phenols	Rank Difference*
Chavya	4533	7	109.88	1	6
Vacha	12467	5	14.08	10	-5
Vidanga	12933	4	73.40	5	-1
Musta	4467	8	78.23	3	5
Pippali	22633	2	21.81	9	-7
Chitraka	17533	3	49.82	7	-4
Patola	-69000	10	63.04	6	4
Murva	7767	6	82.93	2	4
Nagara	-10033	9	76.36	4	5
Guduchi	23633	1	47.16	8	-7

*Rank Difference = Pepsin Rank − Phenolic Rank. (Negative value indicate higher pepsin activity relative to phenol ranking, whereas Positive value indicates higher phenol content relative to pepsin ranking).

The graphical representation of correlation is given in Graph 3. Negative values even on repeated testing indicate compounds with optical density exerting stronger interference in the absorbance at the specific wavelength. Still these values are retained in the study as these drugs are a part of the *Truptighna dashemani gana*.



Graph 3: Correlation between Pepsin modulation activity and total phenolic content

4. DISCUSSION

The present study demonstrated highest total phenolic content in *Chavya* (109.88 ±0.29 mg GAE/ gm of extract). *Murva* and *Musta* also showed good Phenol content while *Pippali* and *Vacha* showed the least (Table 1). The difference was statistically significant at p value of <0.0001 (Table 2). In Pepsin modulation assay, *Guduchi* showed the highest Pepsin activity (23633 units/ mg) while *Patola* and *Nagara* showed negative results (Table 3). Among ten drugs of *Truptighna Dashemani,* two drugs (*Patola* and *Nagara*) showed negative values whereas the remaining eight drugs demonstrated positive pepsin activity (indicated in Graph 2). The correlation analysis between phenols and pepsin modulation results was statistically not significant (Graph 3). But the Rank comparison analysis (Table 4) between total phenols and pepsin assay results of all the drugs gave a negative correlation indicating that the drugs with higher phenolic content exhibit lower pepsin activity under experimental condition and vice-versa. This indicates the presence of additional phytocompounds other than phenols which might be responsible for such actions.

The present study tried to visualize pepsin enzyme as one of the targets in addressing the pathology in functional gastro-intestinal disorders (FGIDs). In FGIDs, there aren't any structural abnormalities rather there is functional derangement of the system, resulting in indigestion and the other symptoms related to it such as lack of interest in food, loss of taste, nausea, heaviness, stupor, debility etc. The drugs mentioned to be having *Deepana- Pachana* (digestive stimulant action) as told in Ayurveda can act as potent pepsin activator, which was shown in this study. The observed increase in pepsin activity might possibly contribute to the digestive stimulant action (*Pachana*) which was seen in the form of strong activation of Pepsin action in *Guduchi, Pippali and Chitraka*. While the other drugs showed mild to moderate pepsin activation, inhibition of pepsin action was noted in *Nagara* and *Patola*, indicating possible gastroprotective and anti-ulcer properties.

Many previous studies have emphasized the use of enzyme assay in various biomedical applications. [17] One such study by Singh KP et al., (2013) demonstrated the use of Pepsin assay as a substitute for low-cost HIV- protease screening, as both enzymes belong to the same Aspartyl family of enzymes & share same signature sequence at the active site. [18] Another study by Uthayakumar, C. and Rupert, S. (2020) emphasized the ability of *Phyllanthus amarus* leaf extract in exerting potential inhibitory effect on α-amylase, pepsin and trypsin enzymes. [19]

Phenols are a class of secondary metabolites produced in plants, also found in various naturally available foods (fruits and vegetables). They have the ability to interact with molecules in our body producing various pharmacological actions. [20] Previous studies have shown that polyphenols and phenolic acids present in herbal drugs can interact with digestive enzymes through hydrogen bonding, hydrophobic interactions or conformational changes, thereby altering the enzymatic activity. The enzyme activity can be either

accelerated or diminished by these interactions in consistency with the structural characteristics of phenolic compounds, their concentrations and the specific substrate – enzyme environment. [21]

Many evidences are available to support the effect of polyphenols on enzyme modulation. A study by Vyas K et al., 2024, offers molecular dynamics insights into the mechanisms by which the selected polyphenols inhibit the enzymes and also show the potential to enhance the application of curcumin, diosmin and other compounds as natural inhibitors of digestive enzymes. [22] Certain polyphenolic compounds like quercetin, catechin and resveratrol have shown the ability to augment catalytic activity of Pepsin during gastric digestion by elevating the initial reaction velocity of pepsin mediated digestion. This indicates that phenolic compounds can enhance protein digestion under specified conditions. [23] Whereas some other phenolic compounds such as phenolic acids and tannins inhibit pepsin activity through direct binding and conformational changes of the enzyme. A previous study shows that phenolic acids like chlorogenic acid, gallic acid and ferulic acid have the ability to bind to pepsin via non-covalent interactions. Thus, they can alter the structure of enzyme and reduce the catalytic activity. [24] Polyphenols present in tea are found to inhibit pepsin activity by about 32% invitro. [25] Earlier research activities on some medicinal plants have emphasized upon the direct correlation between higher phenolic content and suppression of pepsin enzyme activity. *Crotalaria longirostrata* showed highest phenolic content which was correlated to its lowest IC50 value (most potent inhibition) for pepsin. [26] This indicates that modulation of gastric proteolysis is possible by phenolic compounds present in many drugs of herbal origin. On one hand phenols inhibit pepsin whereas on the other hand the pepsin enzyme can also affect the phenols. The acidic environment which predominates during gastric phase of digestion and the activity of pepsin enzyme can bring about modifications in the phenolic compounds. This in turn leads to reduction in total phenol content, thereby affecting their bioavailability. [27]

The study suggests a possible inverse relation between total phenolic content and pepsin activity modulation of herbal drugs in *Truptighna dashemani*, however the association did not reach statistical significance. This also aligns with the classical digestive and gastroprotective indications of *Truptighna dashemani* drugs. *Nagara* which has shown Pepsin inhibition is mentioned to be having *Katu rasa* (pungent taste) and *Madhura Vipaka* (sweet post digestive effect) in Ayurveda. [28] *Katu rasa* (pungent taste) enhances appetite and helps in digestion (Agni *Dipana, Bhukta Shoshana, Rochana*) action which changes post digestion, producing enzyme inhibition and gastroprotective action. *Patola* is having *Tikta rasa* (bitter taste) [28] which is classically mentioned as *Pitta-shleshma upashoshana* (reduces bile and enzymatic secretions). [29] While choosing the drugs for any digestive disorders, these observations can be taken into consideration and the most suitable drugs among *Truptighna dashemani* can be selected and combined for the desired specific action.

Thus, the observed variation between total phenolic content and the enzyme modulation activity in these drugs may be considered in the selection of suitable drugs. Inhibition of pepsin activity can reduce damage to the gastric mucosa whereas activation of pepsin can result in enhanced digestive function in FGIDs. Further in-silico studies with molecular docking might help to visualize the exact binding nature and conformational changes bringing about pepsin inhibition or otherwise.

Limitations of the study:

The present study has some limitations in that the sample size was small, the method applied was invitro assay which has its limitations in terms of assay interferences and clinical validation of Pepsin activity is not possible due to multiple interfering factors in a living system.

5. CONCLUSION

The present study demonstrates variation in pepsin activity among the individual *Truptighna Dashemani* drugs under in-vitro conditions. Our study found that Pepsin activity differed significantly between individual *Truptighna Dashemani* drugs (p=0.0026) when compared to control showing higher pepsin activity in *Guduchi*, *Pippali* and *Chitraka,* supporting the traditional use of these individual drugs. Total phenols content varied among the drugs (p <0.0001). However, correlation demonstrated a non-significant negative association between total polyphenol content and pepsin activity. Over all the present study provides preliminary evidence for pepsin modulation action of *Truptighna Dashemani* drugs which may be due to additional phytochemicals along with polyphenols warranting further mechanistic and phytochemical investigations.

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