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EVALUATION OF PREBIOTIC POTENTIAL OF *HIBISCUS ROSA SINESIS* LEAF MUCILAGE

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ABSTRACT
In the present investigation, it was attempted to establish the prebiotic potential of *Hibiscus* mucilage and also perform the proximate analysis of the extracted mucilage. Hibiscus mucilage was extracted from the leaves in yield of 3.7% by weight of dry leaf powder and subjected to phytochemical screening and estimation of protein, fat, carbohydrates, ash and moisture using the method reported by AOAC. The mucilage was also evaluated for its DPPH radical scavenging potential and its resistance to hydrolysis by gastric juice (acidic hydrolysis) or by α-amylase (enzymatic hydrolysis). The prebiotic potential of the mucilage was studied by assessing its effect on the growth of various lactobacillus strains. The mucilage was found to contain 17.27% moisture, 5.13% ash, and 76.9% carbohydrates. Proteins and fats were totally absent in the mucilage. The total sugar in the *Hibiscus* mucilage sample was determined by Fehling's reagent method and was found to be 69 µg. The IC_{50} value of the mucilage in inhibiting DPPH radical was found to be 57.01µg/mL. Artificial gastric juice (pH 1) was used to hydrolyze the Hibiscus mucilage as well as inulin. The acidic hydrolysis of inulin was found to be 7.14% while that of the Hibiscus mucilage was found to be 13.89%. The enzymatic (α-amylase) hydrolysis of inulin was found to be 12.34% while that of the extracted Hibiscus mucilage was found to be 16.14%. The prebiotic promoted the growth of Lactobacillus strains in varying degree. The highest growth was obtained for *L. rhamnosus* (2.05 ± 0.02) followed by *L. acidophilus* (1.99 ± 0.03 Log_{10}CFU/mL) and the least for *L. fermentum* (1.81 ± 0.03 Log_{10}CFU/mL).

KEYWORDS: Mucilage, prebiotic, lactobacillus, amylase, hydrolysis.

INTRODUCTION

Aqueous extracts from leaves of plants have been found to contain carbohydrates and flavones in them as secondary metabolites.[1] Mucilages of seeds as well as the carbohydrates and flavones from leaves are mucopolysaccharides produced in early diverging non-vascular plant groups. They are composed of total, acidic or neutral polysaccharides or heteropolysaccharides. Mucilage is well recognized as a prebiotic functional food that can positively affect human intestinal microbiota, leading to the modulation of bowel habits concurrent with the reduction of several ailments, i.e., intestinal tumors.[2] The potential of mucilage as a prebiotic is attributed to its polysaccharide nature, where the high content of soluble heteropolysaccharides, the main progenitor of short chain fatty acids (SCFAs), in mucilage helps to promote the growth of beneficial gut probiotic bacteria. Prebiotics are not only modulators of gut microbiota, but their potential is being harnessed in a number of diseases such as colorectal cancer and inflammatory bowel diseases.[3] They also aid in the absorption of several minerals, and help in the prevention of obesity and relieving constipation.

The mucilage of *Hibiscus rosa sinensis* is widely used in sustained release matrices owing to its good swelling propertly. The mucilage is also reported to contain L-rhamnose, D-galactose, D-galactouronic acid, and D-glucuronic acid. These sugars are useful for several digestive and pharmaceutical uses.[4] The objective of the present investigation is to examine the prebiotic potential of mucilage obtained from the leaves of *Hibiscus rosa-sinensis* by studying its effect on the growth of Lactobacillus along with its ability to resist the hydrolysis by gastric juice and α-amylase.

MATERIAL AND METHODS

Collection of plant material

The leaves of *Hibiscus rosa sinensis* were collected from the plants found in locality of Bhopal city, Madhya Pradesh.

Preparation of the plant material for extraction

The leaves of the plant were washed with distilled water and dried in shade (preventing from direct sunlight). The dried leaf has been powdered using slow speed blender and is kept in closed airtight container.

Extraction of leaf mucilage[5,6]

The *Hibiscus rosa sinensis* leaves (100 g) were soaked for 12 hour in distilled water (1litre). Then mucilage was separated by passing through vacuum pump. After that remaining particulate matter separated by passing through muslin cloth. The separated clear material was treated with 15 mL acetone and allowed to stand for 30 min precipitate the mucilage. The mucilage was dried in hot air oven at 60°C for 16 h. Then powder was passed through 80 # mesh sieve and weighed to calculate the yield.

Phytochemical screening of mucilage[7]

The mucilage obtained was tested for the presence or absence of phytoconstituents using different qualitative tests. Mayer's test (alkaloids), froth test (saponins), Salkowski test (triterpenoids), ferric chloride test (Phenolics), gelatin test (tannins), Shinoda test (flavonoids), Millon's test (amino acid) and Molish's test (carbohydrates) was performed.

Proximate Analysis of mucilage

Determination of Moisture content[8]

An accurately weighed sample (approximately 1 g) was placed in an aluminum pan and the sample was dried in a previously heated hot air oven at 105°C to a constant weight.

Determination of Amount of Ash[9]

Accurately weighed sample (approximately 0.1 g) was placed in a ceramic crucible (previously heated and cooled until constant weight was obtained) and was subjected to ashing in a muffle furnace maintained at 550°C until a constant final weight for ash was achieved.

Determination of Amount of Fat[10]

A known weight of the sample (approximately 10 g/thimble) was defatted in a Soxhlet apparatus using petroleum ether (boiling point range = 40-60°C) as the solvent (product-to-ratio of 1:10 w/v) for 8 hours. Defatted samples were dried overnight (approximately 10-12 h) in a fume hood to remove residual traces of petroleum ether and the samples will be weighed to calculate the lipid content.

Lipid (%) = [initial wt. of full fat product (g) – final weight of defatted product (g)] / [initial weight of full fat product (g)] x100

Determination of Amount of Protein[11]

The micro-Kjeldahl method was used to determine total proteins. Briefly, 0.1 g of the sample was placed in a micro-Kjeldahl flask. A catalyst (mixture of 0.42 g of $CuSO_4$ + 9.0 g of K_2SO_4), a few glass beads (to prevent sample bumping), and 15mL of concentrated H_2SO_4 (36 N) was added to each sample. Sample digestion was done at 410 °C for 45-75 min. (until a clear green solution was obtained, which ensured complete oxidation of all organic matter). The digest was then diluted with 50 mL of distilled water and the micro-Kjeldahl flask was attached to the distillation unit. After the addition of 45 mL of 15 N NaOH, sample distillation was commenced and released ammonia was collected into boric acid solution containing the indicators methylene blue and methyl red (blue: red :: 1:2). Borate anion (proportional to the amount of nitrogen) was titrated with standardized 0.1 N H_2SO_4. A reagent blank was run simultaneously. Sample nitrogen content was calculated using the formula.

% N = [(mL of H_2SO_4 for sample – mL of H_2SO_4 for blank) x Normality of H_2SO_4 x 1.4007] / weight of sample (g)

Protein % = total N (%) x approximate factor for sample.

Determination of Amount of Carbohydrates

Total carbohydrates were determined by difference of weights. (Carbohydrates = 100 - % proteins - % fats - %Ash - % moisture).

Determination of DPPH radicals scavenging activity[12]

The free radical scavenging activity of the test solution was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH.

Determination of DPPH radicals scavenging activity was performed by the previously reported method. Separately, 1mM solution of DPPH and test solution (50-250 µg/mL) were prepared in ethanol. 1.5ml of the test solution was added to 1.5 ml of DPPH solution. The absorbance was measured at 517 nm against the corresponding blank solution which was prepared using 3 mL ethanol. The control sample used was 3 mL of DPPH. The assay was performed in triplicates. Percentage inhibition of free radical DPPH was calculated based on control reading by following equation.

$$\text{DPPH scavenged (\%)} = \frac{(A_{con} - A_{test})}{A_{con}} \times 100$$

A_{con} - is the absorbance of the control reaction

A_{test} - is the absorbance in the presence of the test solution.

Prebiotic Activity

The prebiotic action of the gum obtained was examined for its prebiotic and related actions using previously reported methods.

Test organisms

A whole of three strains of lactobacilli species were examined including *Lactobacillus acidophilus* MTCC 10307, *Lactobacillus rhamnosus* MTCC 1423 and *Lactobacillus fermentum* MTCC 903 and the strains were purchased from Institute of Microbial Technology, Chandigarh in the form of lyophilized culture.

Assessment of total sugar

The total amount of sugar contained in the Hibiscus mucilage was determined using copper reduction method utilizing Lane and Eynon technique that involves titration of Fehling's reagent.[13]

Reducing sugars in sample

1 g of Hibiscus mucilage should be weighed and shifted to a 50 mL volumetric bottle. To the phenolphthalein end point, add approximately 100 ml of water and neutralise with NaOH solution. Ten minutes after adding 10 ml of neutral lead acetate solution, shake the mixture. Add modest volumes of potassium oxalate solution until precipitation stops. Make the solution up to volume, thoroughly mix it, and then run it through a Whatman No. 1 sieving circle. The filtrate was transferred to a 50 ml burette, and then follow the steps in 5.8.6.2.2 to titrate. Calculate the percent reducing sugar in the solution using the formula

$$\%\ reducing\ sugar = \frac{0.0025\ X\ V1\ X\ V2\ X\ 100}{V3\ X\ W}$$

Where W – weight of sample; V_1 – Titre volume for standardization; V_2- Dilution volume of sample; V_3 – Volume of sample solution used.

Estimation of reducing sugar

The extent of reducing sugar contained in the Hibiscus mucilage was estimated using Nelson—Somogyi method.[14] Briefly, accurately measured aliquots of 0.1 or 0.2 ml and 1.0 ml of the *Hibiscus* mucilage were taken in separate labelled test tubes. Working standards (0.2, 0.4, 0.6, 0.8 and 1.0 ml) were taken into separate tubes and labelled. Using distilled water topped up the volume to 2 ml in both tubes of sample and reference. Blank in another tube with 2 ml water was taken. All the tubes were placed in a boiling water bath for 10 minutes after adding 1.0 ml of alkaline copper tartarate to each one. Arsenomolybdic acid is added to all of the tubes once they have been cooled. Fill each tube to a volume of 10 ml with filtered water. After 10 minutes, read the absorbance of the developed blue colour at 620 nm. Plot a graph with µg of sugar against absorbance and calculate the amount of reducing sugar present in the Hibiscus mucilage.

Estimation of gastric juice hydrolytic action

Research on the ability of different dried plant extracts to withstand acidity was done using inulin and FOS as prebiotic standard materials. The sample was made by dissolving 1% (w/v) of Hibiscus mucilage in water. The sample solution (5 ml) was combined with artificial gastric juice (5 ml), which was then incubated for an additional 6 hours at 37±2°C in a water bath. At both 0 and 6 hours, total and reducing sugars were estimated. The quantity of reducing sugar released and the sample's overall sugar content were used to estimate the percentage of gum hydrolysis

$$\text{Hydrolysis}\ (\%) = \frac{\text{Reducing sugar}}{\text{Total sugar} - \text{Initial reducing sugar}} \times 100$$

Estimation of α-amylase hydrolysis action

2 units mL-1 of α-amylase were produced for enzymatic hydrolysis in sodium phosphate buffer (20 mM), which was then pH-adjusted to 6.9 using 6.7 mM of sodium chloride.[15] The sample was created by dissolving 1% (w/v) of Hibiscus mucilage in the buffer. The sample solution was combined with 5 ml of enzyme solution and incubated for a further 6 hours at pH 6.9 and 37 2 °C. By measuring the quantity of total and reducing sugar in the sample, enzymatic hydrolysis was determined. Estimates of the hydrolysis rate were made

$$\text{Hydrolysis}\ (\%) = \frac{\text{Reducing sugar}}{\text{Total sugar} - \text{Initial reducing sugar}} \times 100$$

Prebiotic potential of *Hibiscus* mucilage

To encourage the augmentation of various probiotic strains, Hibiscus mucilage was used as the source of carbon with inulin as the benchmark. Different probiotic strains, including *Lactobacillus fermentum* MTCC 903, *Lactobacillus rhamnosus* MTCC 1423, and *Lactobacillus acidophilus* MTCC 10307, were cultured on MRS broth for 24 hours at 37 2°C. The prebiotics were sterilised by passing through a membrane filter with a pore size of 0.45 m before being tested against the 5 ml of Hibiscus mucilage solution (0.5 & 1%, w/v) (Millipore). As a base growth media, MRS broth (carbohydrate-free) was employed. Hibiscus mucilage and common prebiotics (0.5 & 1% w/v) were added to the basal growth media along with the activated bacterial culture (1%). The broths were incubated anaerobically for 48 hours at a temperature of 37 2 °C. The sample (0.1 ml) was taken out of this broth solution and a further cell count was acquired using a hemocytometer. Three copies of each sample extract were used in the investigation.[16,17] Basal growth medium served as the positive control, and 2% glucose supplemented basal growth medium served as the negative control.

RESULTS AND DISCUSSION

Extraction of mucilage

The mucilage from *Hibiscus rosa sinesis* was extracted using the reported method and the yield of mucilage was 3.7 % w/w of the dry leaf powder. The mucilage was yellowish in color, tasteless and amorphous. The phytochemical screening revealed the presence of flavonoids, phenolic and tannins, triterpenoids and carbohydrates in the mucilage.

Proximate Analysis

The content of moisture, fat, ash, protein and carbohydrate in Hibiscus mucilage was established using the previously reported methods and the results obtained are presented in Table 1.

Table 1: Proximate composition of mucilage.

S. No.	Parameter	Percentage
1	Moisture	17.27
2	Ash	5.13
3	Fat	0
4	Protein	0
5	Carbohydrate	76.9

DPPH radicals scavenging activity

The test solution exhibited a dose depended DPPH scavenging action with a dose of 150µg/mL producing protection against DPPH higher than the standard drug ascorbic acid (50µg/mL). The IC_{50} value of the Hibiscus mucilage against DPPH was found to be 57.01µg/mL.

Reducing Sugar

The total sugar in the Hibiscus mucilage sample was determined by Fehling's reagent method and was found to be 69 µg.

Hydrolytic effect of gastric juice

Artificial gastric juice (pH 1) was used to hydrolyze the Hibiscus mucilage as well as inulin. The acidic hydrolysis of inulin was found to be 7.14% while that of the Hibiscus mucilage was found to be 13.89%. The Hibiscus mucilage was able to resist the acidic hydrolysis. The incubation time of 6h was also in part responsible to hydrolysis of the Hibiscus mucilage as well as inulin allowing for conversion of polysaccharides to mono and di-saccharides. Since the Hibiscus mucilage was able to withstand about 90% hydrolysis, it could be assumed that it might reach the intestine surpassing the hydrolytic effect exhibited by the gastric juice in stomach.

Hydrolytic effect of α-amylase

Apart from the acidic degradation, enzymatic hydrolysis in the stomach plays a vital role in conversion of the complex polysaccharides to simple carbohydrates. An active food ingredient that is not degraded in the upper gastrointestinal tract might be a good prebiotic candidate. The percent hydrolysis of the Hibiscus mucilage in presence of α-amylase was determined by quantifying the reducing sugar. The enzymatic (α-amylase) hydrolysis of inulin was found to be 12.34% while that of the extracted Hibiscus mucilage was found to be 16.14%. The Hibiscus mucilage was found to be resistant to enzymatic hydrolysis almost equivalent to the standard prebiotic. Hence, the extracted Hibiscus mucilage presents a great potential to be a source of carbon in the gut microflora and establishing itself as a prebiotic.

Prebiotic potential of Hibiscus mucilage

The effect of the prebiotic on the growth of the probiotic strain was studied by counting the number of cells as colony forming units per mL of the prebiotic. The effect of the concentration of the prebiotic on growth of probiotic was also observed. The results indicate that at all the concentrations, the Hibiscus mucilage was able to significantly promote the growth of the Lactobacillus strains in comparison to the basal growth medium ($P<0.05$). It was also observed that the prebiotic promoted the growth of Lactobacillus strains in varying degree. The highest growth was obtained for *L. rhamnosus* followed by *L. acidophilus* and the least for *L. fermentum*.

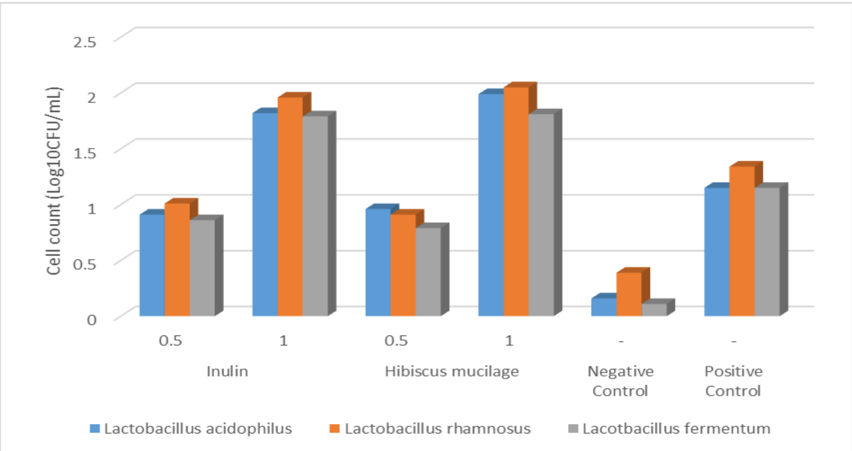

Figure 1: Effect of prebiotic on growth of lactobacillus.

The significantly improved growth of the probiotic strains could be attributed to the presence of sugars in the prebiotic (Figure 1). Higher levels of sugar variably cause a significant growth to probiotic.

CONCLUSION

In this study, the proximate composition of Hibiscus mucilage was evaluated and the effect of *Hibiscus* mucilage on microbiota of gut was studied using various strains of lactobacillus in vitro. The ability of Hibiscus mucilage to resist the acidic and enzymatic hydrolysis was also established. The results obtained led to the conclusion that Hibiscus mucilage exhibits prebiotic effects comparable to inulin.

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