Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 6, 2014

Original Article

EVALUATION AND CHARACTERIZATION OF PURIFIED HIBISCUS ESCULENTUS L. POLYSACCHARIDE AS A PHARMACEUTICAL EXCIPIENT AND MUCOADHESIVE AGENT

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Received: 23 Mar 2014 Revised and Accepted: 20 Apr 2014

ABSTRACT

Objective: The present study was undertaken with an objective to expound the physicochemical, and mucoadhesive properties of polysaccharide obtained from *Hibiscus esculentus* Linn (HEP). The extraction of polysaccharide was maximized employing design of experiment by using response surface methodology.

Methods: The crude polysaccharide was purified by gel filtration chromatographic method. Elemental analysis, Scanning electron microscopy (SEM), X-ray diffraction crystallography (XRD), Differential scanning calorimetry (DSC), Fourier-transformed infrared spectroscopy (FT-IR) and Nuclear Magnetic Resonance (NMR) spectroscopy techniques were employed to characterize the polysaccharide. The mucoadhesive property of the polysaccharide was determined by Wilhelmy plate and falling liquid film technique.

Results: Optimization of process variables leads to an experimental yield of 8.32% w/w with relative deviation of 0.56% between the experimental and the predicted values. Elemental analysis of the polysaccharide has shown the contents of carbon, hydrogen and nitrogen to be 29.5%, 5.67% and 1.7% (w/w) respectively. SEM micrograph analysis suggests that the polysaccharide has irregular particle size and mostly seen in aggregates. The XRD pattern of the polysaccharide indicates both crystalline and amorphous structure. The DSC and FT-IR data also confirmed the presence of polysaccharide structure. The ex-vivo mucoadhesive study by Wilhelmy plate Method showed detachment force of 1.7gm, 2.3gm and 3.1gm for 1%, 2% and 3% w/w HEP gel respectively.

Conclusion: The experimental work provides enough evidence to exploit this natural biopolymer in pharmaceutical food and cosmetic industry. Ex-vivo mucoadhesion study demonstrated the usefulness of HEP as a bioadhesive agent for pharmaceutical dosage forms.

Keywords: *Hibiscus esculentus*, Polysaccharide, optimization of extraction, Box-behnken design, Response surface methodology, Mucoadhesion, wilhelmy plate method.

INTRODUCTION

In recent decades, plant polysaccharides have attracted a great deal of attention in the biomedical field due to their broad spectra of therapeutic properties and relatively low toxicity. Polysaccharides are polymers of monosaccharide(s), and many of them are combined with uronic acids and on hydrolysis yield a mixture of sugars and uronic acids [1]. They have found wide application in pharmaceutical formulations such as polymer matrices in sustained release solid dosage forms, disintegrating agents, binders in tablets, stabilizers or suspending agents in liquid dosage forms and coating material for tablets [2-6]. Natural polysaccharide can also be modified to have tailor-made properties for efficient delivery of drugs [7]. Naturally available polysaccharides are preferred to synthetic materials due to their intrinsic compatibility, low cost, safety, easy availability, emollient and nonirritating nature [8].On the other hand as they are obtained from natural sources, batch to batch variation, inconsistent supply, microbial contamination, altered physicochemical characters on long storage limits their application [1]. Yield and quality of the natural polysaccharide is also known to the affected by method of extraction and degree of purification [9].

Hibiscus esculentus Linn is a tropical annual herb belonging to the family, Malvaceae. In our preliminary literature survey, we found that the crude mucilage of *H. esculentus* Linn fruit has been investigated as release retardant, binder, disintegrant, coating and suspending agent [10-12]. But the mucilage was reported to contain proteins, minerals and other impurities [13]. However, no research work has been conducted on the isolated and purified polysaccharide of the fruit of the plant. Therefore, the objective of this study was to isolate and purify the polysaccharide from *Hibiscus esculentus* Linn and examine the various pharmaceutical properties to assess its functionality as an excipient. Specifically, the study evaluated the physicochemical properties including flow characteristics, elemental composition, molecular weight and thermal properties. The isolated polysaccharide may provide an alternative to other natural polysaccharides or their synthetic counterparts to design and formulate suitable novel drug delivery systems.

MATERIALS AND METHODS

Materials

Matured fruits of *H. esculentus* were obtained from local market of Guwahati, India and authenticated by Department of Botany, Gauhati University, Assam, India (Vide Accession No.

017299). DEAE- Sephadex A-50 was purchased from Hi-media, Mumbai, India. Acetone GR was procured from Merck Specialities Pvt. Ltd, Mumbai, India and Ethyl Alcohol was purchased from Changshu Yangyuan Chemicals, China. All other chemicals and reagents were of analytical grade.

Extraction of polysaccharide

Fresh fruits of *Hibiscus esculentus* L. were collected and sorted from damaged or rotten fruits. They were washed thoroughly with distilled water and longitudinal slices were made using a sharp knife and seeds were removed. The pieces were then dried in a tray dryer maintained between 60 and 80°C for 72 h to obtain dry slices which were milled to get the dry powder. The dried powder was passed through sieve number 40 and stored in an airtight container. The powder (1 kg) was extracted with hot distilled water under reflux. The aqueous extract was filtered through clean muslin cloth. Filtrate was combined and deproteinated by Sevag method [14]. The filtrate was concentrated in a rotary evaporator under reduced pressure, and then centrifuged at relative centrifugal force (RCF) of1500×g for

15 min. The supernatant was precipitated with three volumes of acetone, and stored overnight at 4°C. The precipitate was collected and washed again three times with acetone to get crude polysaccharide

Optimization of Polysaccharide yield [15-16]

In preliminary investigations it was found that yield of polysaccharide is largely influenced by heating temperature, extraction time and water to *H. esculentus* (HE) fruits dry powder ratio. Box-Behnken Design was employed to determine their optimum levels to achieve maximum yield of polysaccharide. As shown in Table 1 the three factors chosen for this study were designated as X_1, X_2 and X_3 and prescribed into three levels, coded +1, 0,−1 for high, intermediate and low value, successively. Three test variables were coded according to equation – 1.

$$X_i = \frac{X_1 - X_0}{\Delta X} \ldots\ldots \text{Eq. (1)}$$

Where x_i is the coded value of an independent variable; Xi is the actual value of an independent variable; X_0 is the actual value of an independent variable at center point; and ΔX is the step change value of an independent variable. Table 2 shows the Box-Behnken design matrix of the experiment of 15 trials. All experiments were performed in triplicate and the averages of polysaccharide yield were taken as response. For predicting the optimal point, a second-order polynomial model was fitted to correlate relationship between independent variables and response (polysaccharide yield) as per equation – 2.

$$Y = \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2 \ldots \ldots \text{Eq. (2)}$$

Table 1: Levels and coded variables chosen for Box-Behnken Design.

Variables	Coded Symbols	Coded Level		
		−1	**0**	**+1**
Temperature (ºC)	X_1	40	60	80
Extraction Duration (Hours)	X_2	6	15	24
Water to*H. esculentus* powder ratio	X_3	2	3	4

Purification of extracted polysaccharide

Crude HEP was dissolved in distilled water (10 mg/mL) and subjected to DEAE-50 Sephadex column (2.6 cm×60 cm). The column was eluted with deionized water following gradient elution by 0.1, 0.2 and 0.3 mol/L NaCl respectively at a flow rate of 0.5 mL/min. Fractions(5 mL, each) were collected and combined according to the total carbohydrate content determined by the Anthrone-sulphuricacid method [17-18]. Most of the pigments were absorbed in the column. The elutes collected, were concentrated under reduced pressure to an appropriate volume, then dialyzed using dialysis membrane (Himedia LA387, molecular cut off 5000 – 10000, Himedia, India) against distilled water and dried using a Freeze drier (IIC Industrial Corp. Kolkata, India) maintaining chamber pressure at 15±2 torr and temperature at− 32ºC. Dried purified *H. esculentus* polysaccharide (HEP) was powdered and used for further investigations.

Total ash and soluble ash [5]

1.0gmHEP was weighed into a pre-ignited and pre-weighed crucible, and transferred into a muffle furnace (MSW-251, Micro Scientific Works, Delhi, India). The ignition temperature was 550ºCfor 24 hours. The recovered ash was transferred into a desiccator to equilibrate to room temperature before weighing. In order to determine soluble ash, the resultant ash from above was mixed with distilled water (25 ml), boiled and filtered through ash-less filter paper. The filter paper was then rinsed until the filtrate volume reached 60 ml. Both filter paper and residue were transferred to the crucible and ignited for 24 hours until a constant weight was reached. Thereafter, it was cooled in a desiccator and weighed. Percent total ash and insoluble ash were calculated.

Determination of total polysaccharide

Total polysaccharide content was determined by spectrophotometer using Anthrone method [19]. In brief, about 200 mg of sample was accurately weighed and dissolved in 10 ml of hot ethanol to remove sugars. The tube was centrifuged (PR-24, Remi Laboratory Instruments, Mumbai, India) at relative centrifugal force (RCF) of1500×gfor 10 minutes and the residue was collected and dried over water-bath. Dried residue was transferred to 5ml distilled water and 5ml of 2.5N hydrochloric acid was added and cooled to room temperature. The resultant solution was neutralized with sodium carbonate and the volume was made up to 100 ml and centrifuged at at relative centrifugal force (RCF) of1500×gfor 10 minutes. 0.5ml of supernatant was collected in a test tube and 4 ml of anthrone reagent was added and heated for 8 minutes over a boiling water bath. The resultant solution was cooled rapidly over an ice-bath, and the intensity of green color was measured at 630nm using UV-Vis spectrophotometer (UV-1800, Shimadzu Corp. Kyoto, Japan). The experiment was performed in triplicate. Calibration curve of glucosewas constructed for concentration range 10 - 100µg/ml with regression coefficient of 0.9984. Amount of polysaccharide present in the sample was calculated by using equation – 3.

$$\% \text{ Total polysaccharide} = \frac{\text{Weight of glucose} \times 2.5}{\text{Weight of Sample}} \times 100 \ldots \text{ Eq. (3)}$$

Loss on drying

1gm of HEP powder was placed in a previously weighed clean silica crucible. It was dried in a tray drier (Rolex Scientific Engineers®, Ambala Cant. India) at 105̊C till constant weight obtained. The crucible was removed from the drier and weighed again. The LOD was then determined as the ratio of weight loss to weight of sample expressed as a percentage.

pH

A 1 % w/v dispersion of the sample in distilled water was shaken for 5 min and the pH was determined by using Systronics 3335 digital pH meter (Systronics®, Ahmedabad, India). The measurements were made in triplicate.

Swelling Index [20]

Percentage swelling index (SI) was calculated as percentage ratio of swelled volume and volume of anhydrous material. This was done by taking 1.0 g quantity of HEP in a 15 ml polypropylene centrifuge tube and the volume occupied was noted. Ten milliliters of distilled water was added to it and the content was mixed on a vortex mixer (Labline Equipments®, India) for 2 min. The mixture was allowed to stand for 10 min and immediately centrifuged at at relative centrifugal force (RCF) of200×gon a Laboratory centrifuge. The supernatant was carefully decanted and the volume of sediment was measured. The experiment was performed in triplicate.

Hydration capacity [21]

1gm of dry HEP powder was taken in a 15ml tarred centrifuge tube, 10ml of distilled water was added to it, allowed to stand for 1 hour and centrifuged at relative centrifugal force (RCF) of1000×gfor 10min. after the centrifugation supernatant was decanted. The tube was then weighed and the percentage hydration capacity was calculated as percentage ratio of weight of hydrated sample and weight of dry sample. The experiment was performed in triplicate.

Viscosity and rheological behavior

The viscosity of a 1.0% w/v dispersion of the HEP was read at shear rates between 0.1 to 10.0 reciprocal seconds and at 23ºC using a Brookfield viscometer (LVDV-E, Brookfield Engineering Labs®, Stoughton-USA). Spindle 2 was used and 3 minutes was allowed for stabilization of the readings before the viscosity was read.

Effect of ageing on viscosity

To study the effect of ageing on viscosity of HEP, the above was stored in a 15ml test tube, capped with aluminum foil and placed in environment chamber (CHM-6S, Remi Laboratory Instruments, Mumbai, India) 75 % RH and 40°Cfor 180 days after which the viscosity experiment was repeated and the average results recorded.

Microbial content

The total aerobic viable count and fungal count of HEP was determined by the pour-plate method [22-23]. In determining the presence of pathogenic bacteria and fungus in the polysaccharide, 0.1 g of the powder was dissolved separately in 10 ml of sterile double distilled water and 1ml of the solution was inoculated into a previously sterilized casein soya bean digest agar and Sabauraud plus streptomycin agar respectively. Four specific pathogens viz. *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella* spp. were checked for their presence along with total aerobic bacterial count and combined yeast and mould count. The inoculated agars were incubated at 37°C for 48 hours and growth of specific organisms depending on the selective media that was used was read as present or not present. All the experiments were carried out in triplicate.

Determination of heavy metals

Limit test for arsenic, lead and iron was determined as per WHO guidelines for quality control of herbal materials [24].

Density, compressibility and flowability [25]

The HEP powder (10 g) was accurately weighed into a 100 ml measuring cylinder and without disturbing the cylinder the volume of powder was read to give the bulk volume. The measuring cylinder was then clamped to the USP I tapper of a USP tap density tester (ETD-1020, Electrolab®, Mumbai, India).The volume of the powder was read after every 25 taps up to a total of 300 taps when volume of powder was constant. This represents the tapped volume of the powder.

Hausner ratio

Hausner ratio is the ratio of the tapped tothe bulk density and it indicates the flowability of a powdered material. A low Hausner ratio means that the powder has a high flowability. Hausner ratio above 1.25 indicates poor flow.

Determination of the Maximum Volume Reduction and the Compressibility Index [26]

The compaction nature was analyzed to understand the behavior of powder from the bulk density state to the tap density state. The reduction in volume after tapping (using tap density tester) was noted. Values of the reciprocal slope and the intercept of the plots of N/C against N were obtained and used for the calculation of constants 'a' (maximum volume reduction) and 'b' (compressibility index) which are properties characterizing the powders.

$$\frac{n}{c} = \frac{n}{a} + \frac{1}{ab} \quad \text{..... Eq. (4)}$$

Where, *'n'* is number of tapings and *'c'* is the degree of volume reduction. 'c' can be expressed as $C = V_0 - V_\infty / V_0$ where, V_0 is the initial volume before tapping and V_∞ is the final tapped volume after 'n' number of tapings.

Angle of repose

The HEP powder (10 g) was accurately weighed and carefully introduced into a funnel clamped to a stand with its tip 10 cm from a plane paper surface. The powder was allowed to flow freely unto the paper surface. The height of the cone, *H* formed after complete flow and the radius of the cone, *R* were measured and angle of repose was calculated.

Fourier Transformed Infra-Red spectrometric study

The Fourier transform-infra red (FT-IR) spectrum of the sample was recorded in an IR spectrometer (Bruker Alpha-E FTIR, Bruker®, Germany)equipped with zinc-selenide ATR accessory with preset resolution of 4 cm^{-1} and 16 scans per sample to reduce the noise. FT-IR data was analyzed using OPUS® Software.

Differential Scanning Calorimetry

A differential scanning calorimetry (JADE DSC, Perkin Elmer, USA) was used to study the thermal properties of the HEP. The polysaccharide was scanned in the temperature range of 50-220°C under an atmosphere of nitrogen. The heating rate was 20°C /min, followed by a cooling cycle back to 30°C at the same rate.

Elemental Analysis [27]

Elemental Analysis of carbon, hydrogen, and nitrogen was carried out using a Perkin Elmer 2400 Series II CHN analyzer (Perkin Elmer®, USA). Accurately weighed 0.5mg of sample was heated to 1150 °C and the corresponding element was determined by using thermal conductivity detector.

X-Ray crystallography [28]

X-ray diffraction (XRD) patterns of the HEP were analyzed using a Siemens D5000 X-ray diffractometer (Siemens®, Munich, Germany). Powder sample, packed in rectangular aluminium cells, illuminated using CuKα radiation (λ = 1.54056Å) at 45 kV and 40 mA. Samples were scanned between diffraction angles of 5° to 40° 2θ. Scan steps of 0.1 were used and the dwell time was 15.0 Sec. A nickel filter was used to reduce the Kβ contribution to the X-ray signal. The'd' spacing was computed according to Bragg's law of diffraction.

Nuclear Magnetic Resonance Spectroscopy

^{1}H and ^{13}C NMR spectra of isolated polysaccharide were recorded in a Bruker Ultrasheild 300 NMR spectrophotometer (Bruker®, USA); chemical shifts are expressed in ppm downfield from tetramethylsilane.

***In-vitro* mucoadhesion study**

Wihelmy Plate Method

The mucoadhesion strength of HEP in terms of detachment weight required to break the mucus-polymer bond against adhesion was determined by Wihelmy's plate method [29]. Small glass plates of dimension 2×5cm were coated by dipping them in 1%, 2 % and 3% w/v gel of HEP and 1% w/v carbopol 934P gel. Coated plates were dried at 40ºC. Thread was attached at one end of coated glass plate. Coated glass plate was dipped into a small beaker containing 0.1N hydrochloric acid (ionic strength I = 0.1) while other side of thread was passed through pulley and end of thread was attached to small plastic sac. The schematic diagram of the assembly is shown in Figure: 1A. Weights were added into the sac with small increments of water till the glass plates get pull out and separated vertically from mucus gel. Glass plate weight was subtracted from the final detachment weight. Weight in gram required to pull out the glass plate from the mucus gel represents the force required to break the mucus-polymer bond against adhesion.

Effect of pH on mucoadhesion strength

Effect of pH on mucoadhesion property of HEP was assessed by falling liquid film method [30-31]. The goat stomach mucosa was obtained from local slaughter house and kept in a Krebs solution during transportation and was used within 6 hours after sacrifice of the animal. A piece of isolated goat gastric mucosa was cut longitudinally (2 × 25cm), spread and held in position by cyanoacrylate glue on a hemispherical plexi-glass support as shown in Figure 1B. 30mg (W_1) of dry powdered HEP were dispersed on the mucosal tissue and left for 20 minutes for interaction. At the end of this period, the mucous membrane was washed with either 0.1N

hydrochloric acid(Ionic strength I = 0.1)or 6.8 phosphate buffer (Ionic strength I = 0.714) for 5 minutes at a flow rate of 20ml/min. the washing solution was collected in a beaker and the weight of the detached particles was determined and average of three determination calculated (W_2). The experiment was repeated with a non-adhesive material (negative control, powdered glass, average particle size 84µ) to compare the mucoadhesion behavior of HEP powder. The mucoadhesive capacity was determined using equation – 5

$$\text{Mucoadhesion capacity} = \frac{W_1 - W_2}{W_1} \quad \text{..... Eq. (5)}$$

RESULTS & DISCUSSION

Statistical analysis and influence of the process variables on polysaccharide yield

The experimental data and the process variables for the yield of polysaccharides at different extracted conditions are presented in Table 2. The average yield (n = 3) of polysaccharides varied from 4.32% to 8.23%. Data analysis was performed using Design Expert 8.0.7.1 (Stat-Ease Inc. Minneapolis) software.

After the response surface regression procedure, the results of F-test ($P < 0.005$) showed a good fitness with the model. A low value of coefficient of the variation (CV = 3.66) clearly indicated a high degree of precision and a good reliability of the experimental data. By applying multiple regression analysis on the experimental data, the dependent variable and independent variable are related by using equation – 14.

$$Y = 5.82 + 0.79\,X_1 - 0.28\,X_2 + 0.36\,X_3 - 0.52\,X_1X_2 + 0.55\,X_1X_3 - 0.09\,X_2X_3 + 0.93\,X_1^2 - 0.77\,X_2^2 - 0.01\,X_3^2 \quad \text{..... Eq. (6)}$$

Three-dimensional response surface (Figure – 2A) and contour plot (Figure – 2B) for the response are plotted to study the effects of independent variables and their interactions on polysaccharide yield according to the results of regression equations. The results indicated that all the three variables tested, i.e. extraction temperature, duration of time and water to HE powder ratio were positively related to the response, and the optimal level of them was determined as 79.71ºC, 6 hours and 3.94respectively, with a predicted extraction rate of 8.45%. In order to confirm the predicted results, further experiment using the optimum extraction parameters determined above was performed and the yield of 8.32 of polysaccharides was obtained, with relative deviation of 0.56% between the experimental and the predicted values.

Fehling's reagent and iodine-potassium iodide reactions indicated that the purified fraction of polysaccharide did not contain reducing sugar and starch type polysaccharide. Heavy metal content in terms of arsenic (< 1 mg/kg), lead (< 1mg/kg) and iron (< 5mg/kg) was well within the prescribed limits [24]. The total polysaccharide content was estimated to be 93 %. The UV-Visible spectra showed that, the polysaccharide had an absorption peak at 190 nm only, which is the characteristic UV absorption peak for a polysaccharide. There was no absorption at 260 and 280 nm, indicating absence of aromatic ring containing proteins and polypeptides [33]. The physicochemical properties of purified polysaccharide of *H. esculentus*are summarized in Table 3.

The polysaccharide is sparingly soluble in water and practically insoluble in ethanol, acetone and chloroform. The results of the swelling characteristics show that HEP has moderate swelling index suggesting that the polysaccharide may perform well as suspending agent/ disintegrant/ matrixing agent [5]. The swelling was highest in water followed by phosphate buffer and least in 0.1 N HCl. The lower swellability in acidic environment may contribute to it polymeric backbone that consists of uronic acid derivatives [34].This marginal difference in pH responsive swelling may affect the drug release behavior from the polymer matrices in different parts of gastrointestinal tract. The results indicate that the polysaccharide is a pH responsive polymer and may find application in controlled release formulations [35].

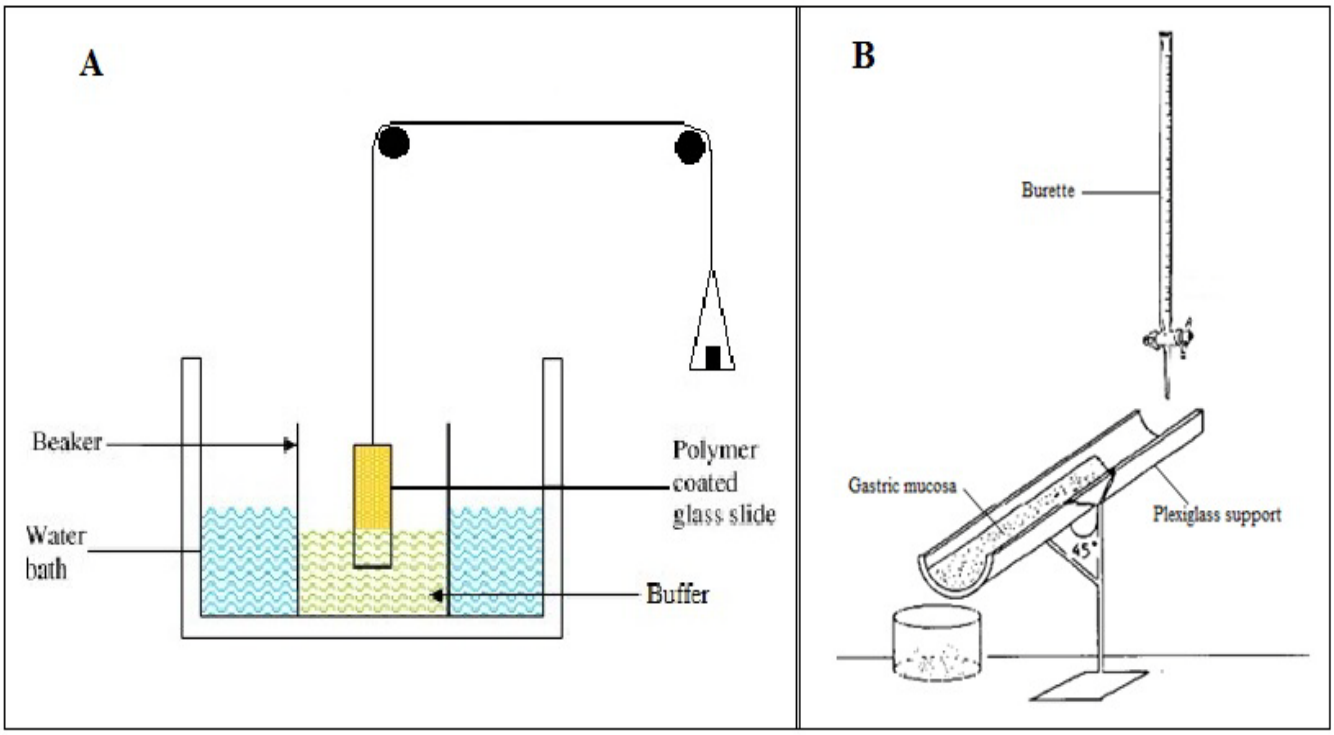

Fig. 1: Schematic diagram of Wilhelmy Plate Assembly (A) and falling liquid film technique (B).

Table 2: Effect of the process variables in polysaccharide yield

Run	X_1	X_2	X_3	Y (% $^{w}/_{w}$ Polysaccharide yield)	% Standard Deviation
1	0	+1	-1	4.32	3.82
2	0	-1	+1	5.95	4.17
3	-1	+1	0	5.41	2.70
4	-1	0	-1	6.35	3.04
5	+1	0	-1	6.74	5.61
6	0	-1	-1	4.73	2.75
7	-1	-1	0	4.88	3.08
8	0	0	0	5.82	2.65
9	+1	+1	0	6.04	3.62
10	0	0	0	5.79	4.05
11	0	0	0	5.86	3.80
12	+1	0	+1	8.23	2.88
13	0	+1	+1	5.18	3.05
14	+1	-1	0	7.6	4.73
15	-1	0	+1	5.64	2.30

Table 3: Physicochemical and microbial properties of purified polysaccharide of *Hibiscus esculentus* L.

Test	Value (Mean ± SD)	Unit
Total ash	0.014 ± 0.002	% $^{w}/_{w}$
Acid insoluble ash	0.004 ± 0.001	% $^{w}/_{w}$
Swelling Index		
Water	330 ± 3.61	% $^{v}/_{v}$
0.1N Hydrochloric acid	245 ± 2.17	% $^{v}/_{v}$
Phosphate buffer (pH 7.4)	310 ± 1.93	% $^{v}/_{v}$
Hydration capacity	194 ± 0.73	% $^{v}/_{v}$
pH (at 25°C)	6.5 ± 0.02	--
Viscosity	376 ± 0.06	mPa.s
Loss on drying	3.4 ± 2.07	% $^{w}/_{w}$
Bulk density	0.69 ± 1.31	gm/ml
Tapped density	1.04 ± 0.73	gm/ml
Hausner ratio	1.507 ± 0.88	--
Angle of Repose	34 ± 2.33	°
Microbial quality	1.31×10^3	cfu/gm
Total viable aerobic count	0.47×10^3	cfu/gm
Total fungi count	0.84×10^3	cfu/gm
Escherichia coli	Absent	--
Staphylococcus aureus	Absent	--
Pseudomonas aeruginosa	Absent	--
Salmonella Spp	Absent	--

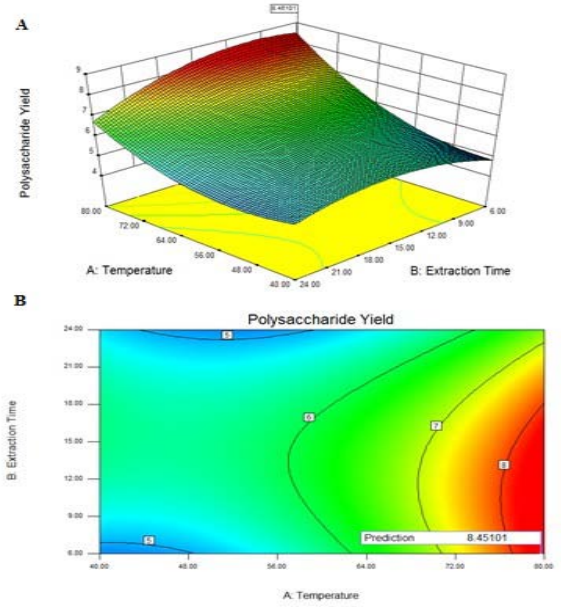

Fig. 2: 3D Response surface plot (A) and Contour plot (B) of polysaccharide yield.

A low LOD of HEP indicates low moisture content. It is important to investigate the moisture content of a material because the economic importance of an excipient for industrial application lies not only on the cheap and ready availability of the biomaterial but the optimization of production process such as drying, packing and storage [36]. Given suitable temperature moisture will lead to the activation of enzymes and the proliferation of microorganisms, thereby affecting the shelf life of most routine formulations. Therefore, the low moisture content value of HEP indicates its suitability in formulations containing moisture sensitive drugs [37].

The total ash and acid insoluble ash value of HEP were found to be 0.014 and 0.004% w/w respectively. Ash values reflect the level of adulteration or handling of the drug. Adulteration by sand or earth is immediately detected as the total ash is normally composed of inorganic mixtures of carbonates, phosphates, silicates and silica. Therefore, the low values of total ash and acid insoluble ash obtained in this study indicate high level of purity [36].

The bulk and tapped density give an insight on the packaging and arrangement of the particles and the compaction profiles of the material. Compressibility values up to 15% usually results in good to excellent flow properties and indicate desirable packing characteristics. Compressibility index above 25% are often sources of poor tableting qualities. Between these two value less than optimum performance might be anticipated and require

modification of the formulation during process development [37]. Results shown in Table 3 of HEP would be accepted to have moderate flow and compressibility properties.

Figure – 3 illustrates the compaction behavior of HEP powder. The values of maximum volume reduction due to tapping 'a' and index of compressibility 'b' were found to be 34.1% and 0.101 respectively. Hence, the results indicate higher compactability of HEP powder. According to the published results, the compactability and cohesiveness values obtained indicate fair flowability and moderate cohesiveness [38].

The viscosity of the polysaccharide dispersion decreases with an increase in shear rate (Figure4). This is indicative of pseudoplastic or shear thinning behavior. At high shear rates, the decrease in viscosity can be attributed to a decreasing number of chain entanglements [39]. There is no significant difference in the viscosity after the colloidal solution of the polysaccharide was stored in a humidity chamber.

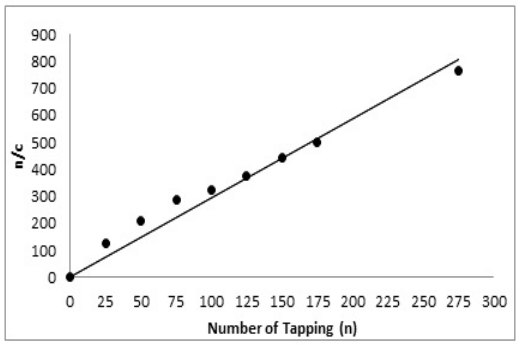

Fig. 3: Kawakita Plot of purified *H. esculentus* polysaccharide powder.

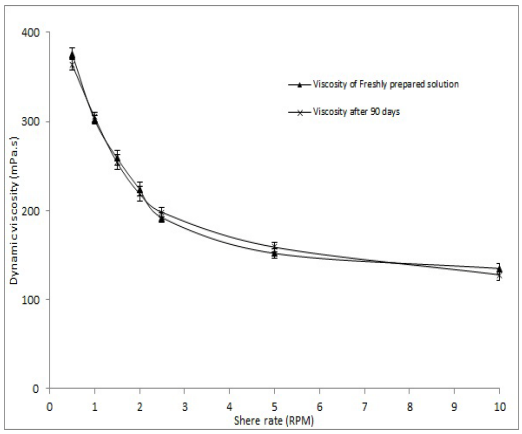

Fig. 4: Effect of ageing on apparent viscosity of purified *H. esculentus* polysaccharide.

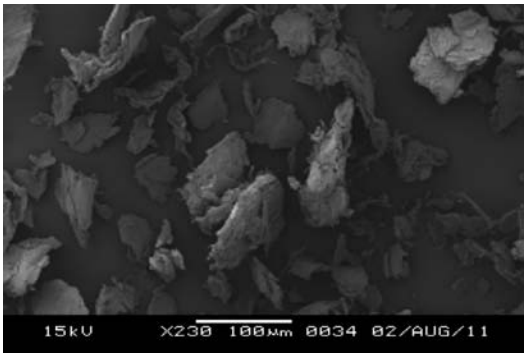

Fig. 5: Scanning electron micrograph of purified *H. esculentus* polysaccharide.

The microbial quality of the polysaccharide was assessed to determine its acceptability for use as a pharmaceutical excipient. The total viable aerobic count and fungal count of the HEP was lower than that of the prescribed limit of European pharmacopoeia. The purified polysaccharide did not contain *E. coli, Pseudomonas aeruginosa, Salmonella spp. and pathogenic Staphylococci.* Microbial quality of purified polysaccharide was satisfactory and met the specifications of European Pharmacopoeia [21].

Scanning electron micrographs of HEP are shown in Figure - 5. The micrographs of the HEP revealed the surface morphology of the polysaccharide particles. The particles are mostly seen in aggregates of irregular shapes and dimensions which are fibrous in nature.

FTIR spectra of HEP presented in Figure-6 show two characteristic peaks in fingerprint region at 1018 cm^{-1} and 1244 cm^{-1}attributingto C–O stretching vibration. The peak at 1593 cm^{-1} indicates presence of O–H bending vibration. Absence of characteristic sharp peaks at 1650 cm^{-1} to 1700 cm^{-1} indicates a modest degree of amino cross-linking in the molecule. The sharp band at 2914 cm^{-1} is characteristic of methyl C–H stretching associated with aromatic ring system. The thick band at 3245 cm^{-1} is due to intra- and inter-molecular hydroxyl groups that make up the gross structure of polymeric carbohydrates. All these characteristic bands confirmed a non-starch and non-cellulose polysaccharide with modest degree of cross-linking.

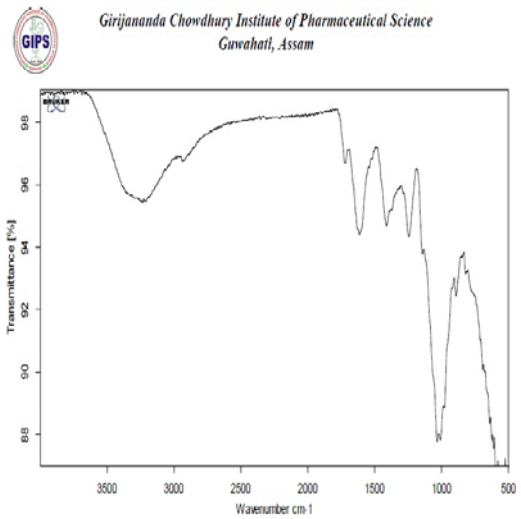

Fig. 6: FT-IR spectra of purified *H. esculentus* polysaccharide showing various absorption peaks.

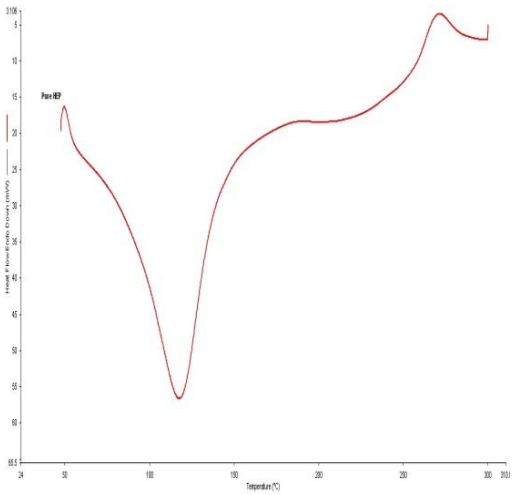

Fig. 7: DSC thermogram of purified *H. esculentus* polysaccharide.

Differential scanning calorimetry (DSC) was used to measure the occurrence of exothermal or endothermal changes with an increase in temperature. Figure-7 shows the DSC curve of the polysaccharide. The DSC thermogram illustrated a wider endothermic peak at 117ºC which may be attributed to desorption of moisture. An exothermic peak at 240ºC indicate the pyrolytic degradation of polysaccharide backbone. Degradation over 200ºC demonstrate acceptable thermal stability as pharmaceutical excipient. Absence of sharp endothermic peak indicative of amorphous nature of the isolated polysaccharide.

The X-ray diffraction pattern of HEP is shown in Figure 8. The sample shows only few identifiable peaks at approximately 15º, 27º and 30º. However, other peaks are very weak and unresolved or are shoulders on more intense peaks. The result of the XRD confirms that of the DSC which shows that, HEP exhibits mostly amorphous nature with short range of crystallinity within the polysaccharide chain.

Elemental Analysis of HEP shows presence of 29.5% carbon, 5.67% hydrogen and 1.7% nitrogen. The ratio of carbon to hydrogen in the sample indicates the presence of carbon ring sugar monomeric units. Also moderate proportion of nitrogen attributes to the amino-linkage in the polysaccharide chain.

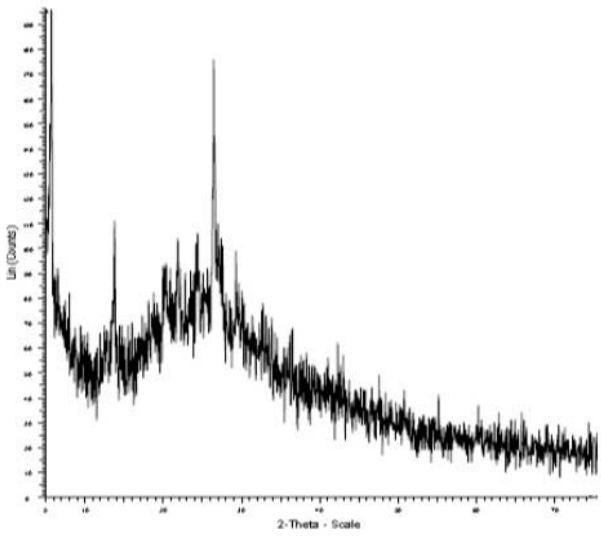

Fig. 8: X-Ray Diffraction Pattern of purified *H. esculentus* polysaccharide.

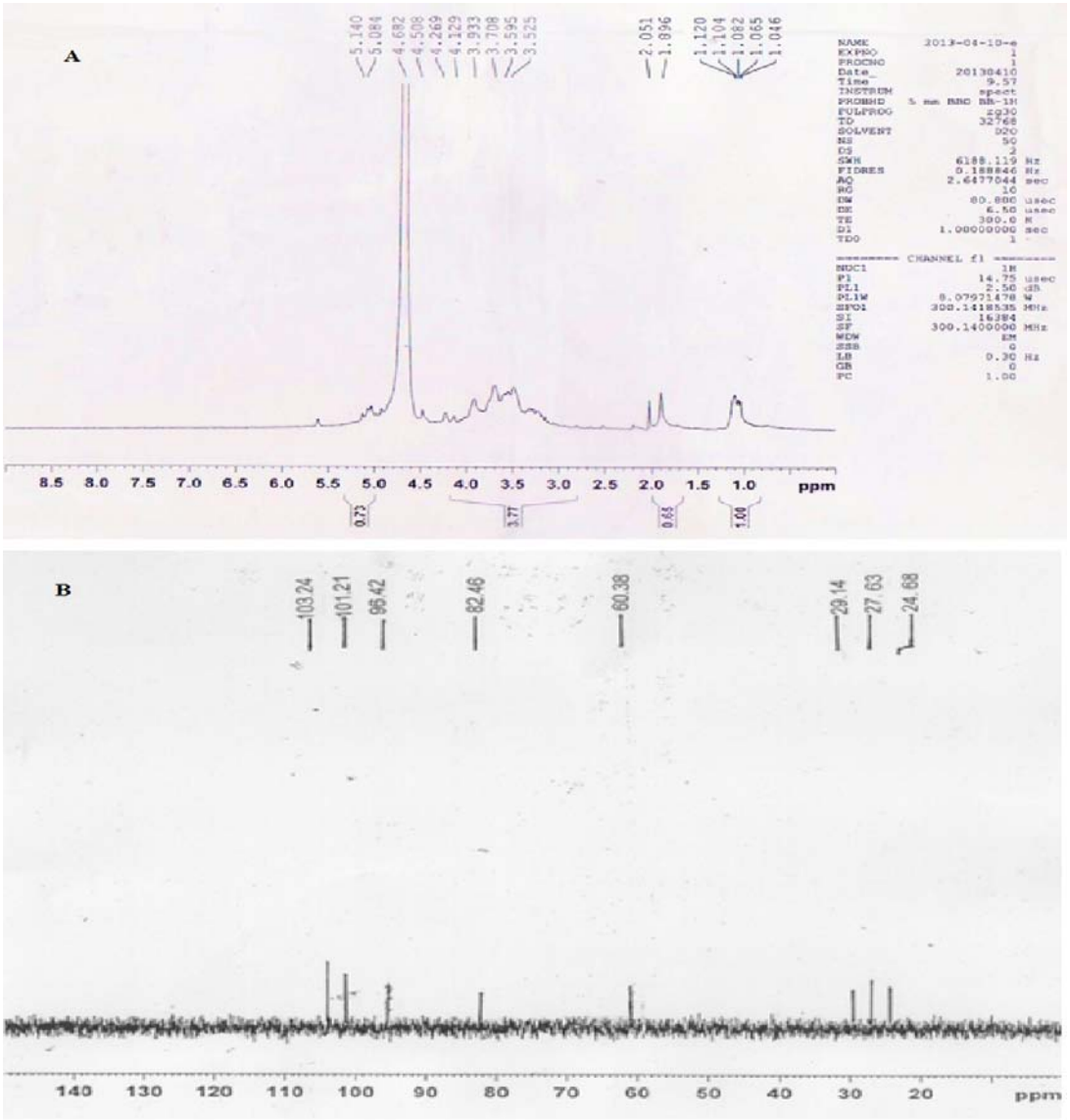

Fig. 9: ^{1}H NMR (A) and ^{13}C NMR (B) spectrum of purified *Hibiscus esculentus* polysaccharide.

The ^{1}H and ^{13}C NMR spectra of HEP are shown in figure 9. ^{1}H spectrum is crowded in a narrow region between 3 and 5 ppm typical of polysaccharides and confirms the presence of many similar sugar residues. The anomeric protons have been assigned to β-sugar and α-sugar residues due to presence of signals between 4.50 and 4.68 ppm and 5.08 and 5.14 ppm respectively [32].The signals between 3.20 and 3.70 ppm have also been assigned to –O-CH_3.The ^{1}H NMR spectrum of the HEP showed signal corresponding to presence of galactose and rhamnose moieties. The ^{13}C NMR spectrum of the polysaccharide showed the presence of sugar residues. The resonance peak at 103.24 and 101.21ppm unambiguously confirms the β-configuration of glycosidic bond. The linkage (1→2) is proved by the resolved peak at 82.46ppm. Signal observed at 60.38ppm is corresponding to O-substituted $_{D}$-galactopyranose unit [40]. The result of ^{1}H and ^{13}C NMR study suggests the presence of galactose, rhamnose and galactouronice acid.

Ex-vivo mucoadhesion study by Wilhelmy Plate method showed sufficient mucoadhesive strength of 1.7±0.31gm, 3.3±0.66gm and 5.1±0.84gm for 1%, 2% and 3% w/w HEP gel respectively which is comparable to the result of 1% carbopol gel 5.17±0.88gm. pH of the environment found to affect the mucoadhesion capacity of HEP. The falling liquid film method shown significant difference ($p< 0.05$) in mucoadhesion capacity i.e. 58.76% (S.D. ±2.69) at pH 6.8 phosphate buffer (negative control 9.34±0.32%)compared to50.39% (S.D. ±2.95) at 0.1N hydrochloric acid (negative control 10.02±0.81%).

CONCLUSION

The results obtained in this study were established for the first time regarding the physicochemical and structural characteristics of the purified polysaccharide obtained from the fruit of *Hibiscus esculentus* L. In order to optimize the polysaccharide yield, a Box-Behnken design was applied to investigate the influence of three critical variables viz. extraction temperature, extraction time and water to *H.* esculentus powder ratio. Through the response analysis, a maximum yield of 8.32% w/w was obtained. The study revealed that polysaccharides with such properties have therefore been used as stabilizers and suspending agents in foods, cosmetics and as release retardant polymer in novel drug delivery system. But low viscosity and moderate swellability limits its application as swellable gel systems. The high thermal stability of the HEP indicates that it can be used as a pharmaceutical excipient even under conditions of high thermal stress. Further, the polysaccharide obtained from *H. esculentus* has good potential to be used as a bioadhesive agent in mucoadhesive pharmaceutical drug delivery systems. The relative abundance and easy availability of HEP may serve as an alternative to currently available pharmaceutical excipients.

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