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Characterization of Selected Polysaccharide Hydrogels as Pharmaceutical Excipients

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Abstract
Polysaccharide hydrogels from the seeds of *Tamarindus indica* and from the trunk of *Prunus amygdalus* were selected for physicochemical characterization and microbial load determination to establish them as pharmaceutical excipients. After isolation / purification, the yields of the hydrogels were 58.29 and 78% w/w. Both the hydrogels were tested for the purity by performing chemical tests for different phytochemical constituents. The result showed that the isolated / purified samples were pure carbohydrates. Both hydrogels exhibited excellent swelling index, good water uptake capacity and wetting property. The pH values of the hydrogels were near neutral, indicating non-irritant nature of the hydrogels to different mucous membranes of the body and skin. The pharmaceutical properties such as density, porosity, packing arrangement, flow, were found to be good for using them as pharmaceutical excipients. The Scanning electron microscographs (SEM) revealed rough surface of the partides, which indicated their possible use as sustaining agents. The Fourier transform infrared (FT-IR) spectra of the hydrogels confirmed their carbohydrate nature. Both the hydrogels had microbial load within specified limits for natural excipients and the pathogenic organisms were absent. Hence, it was conduded that the selected hydrogels had promising properties for application as multifunctional excipients.

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INTRODUCTION

Polysaccharides have been proposed as the first biopolymers to have formed on the earth.[1] They are complex carbohydrates containing one or more monosaccharides or their derivatives linked in a bewildering variety of linkages and structures.[2] They are condensation polymers. The polysaccharides are conventionally classified into two groups, viz., gums and mucilages. The term gum refers to polysaccharide hydrogels, which do not form a part of cell wall, but are exudates or slimes and are pathological products.[3] Mucilages are part of cell and are physiological products.[3] In recent years, these polysaccharides have evoked tremendous interest in pharmacy, medicine and food technology.

Polysaccharide hydrogels possess a complex, branched polymeric structure because of which they exhibit high cohesive and adhesive properties. Such properties are highly useful in pharmaceutical preparations. Hence, polysaccharide hydrogels find diverse applications in pharmacy. They are ingredients in dental and other adhesives and as bulk laxatives.[4] These hydrophilic polymers are useful as tablet binders[5], disintegrants[6], emulsifiers[7], suspending agents[8], gelling agents[9] and sustaining agents in matrix tablets.[10] They have also been used for colon targeting of drugs. Polysaccharides retain their integrity in stomach and intestine, because they are resistant to the digestive action of gastrointestinal enzymes. But once they reach colon, they are acted upon by the bacterial polysaccharidases, which degrades the matrix and releases the drug.[11] Naturally available polysaccharides are preferred to synthetic materials due to their non-toxicity, low cost and abundant availability.

Tamarind seed polysaccharide is a galactoxyloglucan, obtained from the kernels of *Tamarindus indica* (Family: Leguminosae). It possesses properties like high viscosity, broad pH tolerance and adhesivity.[12] Recently, its non-carcinogenicity,[13] mucoadhesivity, biocompatibility,[14] high drug holding capacity[15] and high thermal stability[16] have been reported. Due to these properties, it is being used as stabilizer, thickener, gelling agent and binder in food industry.

Almond gum is a polysaccharide exudate obtained from the trunk of the tree Prunus amygdalus (Family: Rosaceae). This gum hydrolyzes into L-arabinose (4 parts), D-xylose (2 parts), D-galactose (3 parts) and D-glucouronic acid (1 part). Aldobio uronic acid is also present in this gum.[17] The gum is non-toxic and is used in pharmaceutical and food industries as an alternative to gum tragacanth.[17] It is reported as a release modifier in sustained release spheroids.[18]

In the present study, the physicochemical characteristics and microbial load of these two polysaccharides were studied in an attempt to establish them as pharmaceutical excipients.

MATERIALS AND METHODS

Materials

The seeds of *Tamarindus indica* and gum of *Prunus amygdalus* were purchased from Belgaum, (India) during May-June 2002. All other materials and reagents used in the study were of AR grade and were purchased from SD Fine Chemicals, Mumbai (India) or Sigma Aldrich, USA.

Authentication of Plant Material

The authentication of plant material was done by the Department of Pharmacognosy, JSS College of Pharmacy, Ootacamund. The specimen samples are preserved in the laboratory.

Isolation of Hydrogel from Tamarind Seed (TSP)

The seeds of *Tamarindus indica* were washed thoroughly with water to remove the adhering materials. Then, the reddish testa of the seeds was removed manually and the seeds were crushed lightly and used for isolation of mucilage. The isolation of the hydrogel was carried out using modification of reported method.[5,10,19,20]

The crushed seeds of *Tamarindus indica* were soaked in water for 24 h, boiled for 1 h, and kept aside for 2 h for the release of hydrogel into water. The soaked seeds were taken and squeezed in a muslin bag to remove mark from the filtrate. Then, to the filtrate, equal quantity of acetone was added to precipitate the

hydrogel. The hydrogel was separated by filtration. The marc was not discarded but it was sent for multiple extractions with decreasing quantity of extracting solvent, i.e., water with the increase of number of extractions. The isolation was continued until the material was free of hydrogel. The separated mucilage was dried in incubator at temperature 40 °C. The dried hydrogel was powdered and stored in airtight containers at room temperature.

Purification of Hydrogel from Almond Gum (AG)

The gum of *Prunus amygdalus* was thoroughly cleaned to remove all the adhered foreign materials and then was dissolved in warm water, reprecipitated using ethanol (1:1), dried at 40 °C, powdered and stored in airtight container at room temperature.

Identification Tests for Hydrogels

The identification of the isolated polysaccharide hydrogels was carried out by using the following tests:[21]

a. The powder was mounted on a slide with ruthenium red solution and covered with a cover slip. After a few seconds, it was irrigated with lead acetate and the excess stain was sucked off with a blotting paper. (Lead acetate solution was added to prevent undue swelling of the test solution). The color of the particles was noted.
b. The powder sample was mounted on a slide with freshly prepared corallin soda solution and covered with a cover slip. After a few seconds it was irrigated with 25% sodium carbonate solution. The color of the particles was noted.
c. Hydrogel was heated with distilled water for some time and then cooled. Formation of gelatinous mass was noted.
d. To 2 ml of hydrogel solution, 2-3 drops of N/50 iodine solution was added and the color of the particles was noted.

Determination of Purity of Hydrogels

To determine the purity of hydrogels, tests for alkaloids, carbohydrates, flavonoids, steroids, amino acids, terpins, saponins, oils and fats, and tannins and phenols were carried out.[22]

Organoleptic Evaluation of Hydrogels

The isolated hydrogels were characterized for organoleptic properties such as color, odor, taste, fracture and texture.

Ash Values of Hydrogels

Ash values such as total ash, acid insoluble ash and water-soluble ash were determined by methods described in Indian Pharmacopoeia.[23] For determination of different ash values, the hydrogel was powdered and the powder was passed through British Standard Sieve (BSS) no. 40. The following procedures were used for determination of different ash values.

Total Ash

Accurately weighed hydrogel (3 g) was taken in a silica crucible, which was previously ignited and weighed. The powder was spread as a fine, even layer at the bottom of the crucible. The crucible was incinerated gradually by increasing temperature to make it red hot until free from carbon. The crucible was cooled and weighed. The procedure was repeated to get constant weight. The percentage of total ash was calculated with reference to air-dried drug.

Acid Insoluble Ash

The ash obtained as described above was boiled with 25 ml of 2 N hydrochloric acid for 5 min. The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into a silica crucible, ignited and weighed. The procedure was repeated to get a constant weight. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

Water Soluble Ash

The ash obtained as described in the determination of total ash was boiled for 5 min with 25 ml of water. The insoluble matter was collected on ashless filter paper and washed with hot water. The insoluble ash was transferred into silica crucible, ignited for 15 min, and weighed. The procedure was repeated to get a constant weight. The weight of insoluble matter was subtracted from the weight of the total ash. The difference of weight was considered as water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried drug.

Solubility Behavior of Hydrogels

One part of dry hydrogel powder was shaken with different solvents and the solubility was found out.

pH of Hydrogels

The hydrogels were weighed and dissolved in water separately to get a 1%w/v solution. The pH of each solution was determined using digital pH meter.

Swelling Index of Hydrogels

The swelling index is the volume (in milliliters) taken up by the swelling of 1 g of test material under specified conditions. The swelling indices of the selected hydrogels were determined by the method described by WHO.[24]

Accurately weighed quantity of the hydrogel (1 g), previously reduced to the required fineness, was introduced into a 25 ml glass-stoppered measuring cylinder. Twenty five milliliters of water was added and mixture was shaken thoroughly every 10 min for 1 h. It was then allowed to stand for 3 h at room temperature. Then the volume occupied by the hydrogel, including any sticky mucilaginous portion was measured. The same procedure was repeated thrice and the mean value was calculated.

Surface Tension

The surface tension of the selected polysaccharides was determined by drop count method, using a stalagmometer.[25] The stalagmometer was filled with purified water above the upper mark. Using the screw pinch cork, the flow rate was adjusted to 10-15 drops/min. Then, number of drops of water was counted between the marks of the stalagmometer (n_1). The water was removed and the stalagmometer was filled with the polysaccharide solution (0.1% w/v) and number of drops was counted (n_2). The surface tension of the polysaccharide (γ_2) was determined using formula given below:

$$\text{Surface tension } (\gamma_2) = n_1 \rho_2 \gamma_1 / n_2 \rho_1 \quad (1)$$

Where, n_1 - number of drops of water; n_2 - number of drops of sample; ρ_1 - density of water (0.9956 g/ml); ρ_2 - density of sample, and γ_1 - surface tension of water (71.18 dyne/cm).

The density of the sample was determined using specific gravity bottle. First, weight of an empty specific gravity bottle was determined. Then, the polysaccharide solution was filled in it and the total weight was determined. The liquid was removed, the bottle was washed and dried and filled with purified water and the weight was determined. From these, the weight of polysaccharide solution and water was determined. The density was calculated using the formula given below:

$$\text{Density of liquid } (\rho) = [\text{Weight of liquid} \times \text{Density of water}] / \text{Weight of water} \quad (2)$$

Loss on Drying

Loss on drying is the loss in weight in percentage w/w, resulting from water and volatile matter of any kind that can be driven off under specified conditions. The test was carried out according to the procedure described in Indian Pharmacopoeia.[23]

One gram of hydrogel powder was weighed accurately in a tared glass stoppered bottle and was dried in a hot air oven at 105 °C and the weight was checked at intervals of 1 h, until a constant weight was

obtained. The weight difference was noted and the percentage of weight lost by the powder was calculated.

Moisture Absorption

The hydrogel sample (10 g) was placed in an open glass dish of 50 mm diameter and 30 mm height in a desiccator over sulfuric acid (14%) and it was allowed to remain for 24 h. The increase in weight was noted and expressed as percentage moisture absorption.[26]

Bulk Density and Bulkiness

Bulk density is defined mathematically as:[27]

$$\text{Bulk density } (\varrho_b) = \text{Mass of powder (w)} / \text{Bulk volume } (V_b) \quad (3)$$

The inverse of bulk density is called as bulkiness. The bulk density and bulkiness values are used as standards for quality control of pure chemicals. It also determines the volume occupied by the powder the lower the bulk density (or higher the bulkiness), the higher the volume occupied by the powder. It also influences the size of packing container, apparatus used in manufacture of dosage forms.

Accurately weighed quantity of hydrogel powder (50 g) was introduced into a graduated measuring cylinder. The cylinder was fixed on the bulk density apparatus and the volume occupied by the powder was noted. Then, the powder was subjected to tapping in a bulk density apparatus until constant volume was obtained. The final volume (bulk volume) was noted. Then the bulk density was calculated using above equation.

True Density

True density is the density of the material itself. It is defined as:[27]

$$\text{True density } (\varrho) = \text{Weight of powder (w)} / \text{True volume of powder } (V_t) \quad (4)$$

Liquid displacement method is the simplest method and was used in the present study. Acetone was selected as the liquid for displacement, because, both the hydrogels were insoluble and heavy in acetone. A specific gravity bottle was used for the displacement study. The true density was determined as described below:

Weight of specific gravity bottle = w1
Weight of specific gravity bottle + sample = w2
Weight of sample = w3 = w2 – w1
Weight of specific gravity bottle + sample powder + liquid = w4
Weight of liquid displaced by solids (related to volume of liquid displaced) = w4 – w2
True density (ϱ) = [w2 – w1] / [w4 – w2]

Total Porosity

Total porosity of a substance is expressed as percentage, and is denoted by 'ε'.[27] It can be calculated from true density and bulk density values as given in the equation 5.

$$\varepsilon\ (\%) = [(\varrho - \varrho_b)/\varrho] \times 100 \quad (5)$$

Powder Flow

The flow characteristics were measured by angle of repose.[27] Improper flow of powder is due to frictional forces between the particles. These frictional forces are quantified by angle of repose. It can be calculated by following formula:

$$\text{Tan } \theta = h/r \text{ or } \theta = \tan^{-1} h/r \quad (6)$$

Where, h= height of pile; r= radius of the base of the pile and θ = angle of repose.

A dry and clean funnel was fixed on to a burette stand at particular height (2-3 cm). A graph paper was placed on the flat surface and a sufficient quantity of the powder (10 g) was allowed to flow slowly through the funnel until the heap touched the tip of the funnel. The circumference of the heap was drawn and the midpoint was located and its radius was measured. The experiment was repeated thrice and the average height and radius were calculated. Using these readings and the above formula, the angle of repose was calculated.

Powder Compressibility (Carr's Consolidation Index)

Carr's indices of the isolated hydrogels were determined by the reported method.[27] The hydrogel powder (5 g) was transferred into a 10 ml measuring cylinder with the help of a funnel and the measuring cylinder was placed on the bulk density apparatus. The initial volume occupied by the powder was noted (fluff volume, V_0). The measuring cylinder was then tapped until a constant volume was obtained. After completing the tapping the final volume was noted (tapped volume, V_t) and the compressibility was calculated using the below formula:

$$\text{Consolidation Index} = [(\text{Tapped density} - \text{Fluff density})/\text{Tapped density}] \times 100 \quad (7)$$

Surface Characteristics of Hydrogels

To study the surface characteristics of hydrogels, scanning electron micrographs (SEM) of the powders of *Tamarindus indica* and *Prunus amygdalus*, after passing through British Standard Sieve no. 80 were taken. The powder was evaporated with carbon and then sputtered with gold to make the samples electrically connected. Carbon was layered to a thickness of approximately 10 nm and gold was layered to approximately 25 nm. The SEM was taken in Hitachi S-2400 electron microscope.

Infrared Spectra of Hydrogels

Hundred milligrams of the hydrogel powder was mixed with potassium bromide (400 mg) and was compressed in a hydraulic press to form a pellet at 15 tons pressure. The pellets were scanned from 4000 to 400 cm^{-1} in a Perkin Elmer FTIR spectrophotometer.

Total Microbial Load of Hydrogels

The microbial load in terms of cfu/g of bacteria and fungi for the selected hydrogels was carried out according to the procedure described in Indian Pharmacopoeia.[23]

Tests for Presence of Specific Microorganisms

The substances which are to be used as excipients in dosage forms need to be free from *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, according to Pharmacopoeias. International Conference for Harmonization (ICH) has also supported this criterion. The tests for these microorganisms were carried out as described in Indian Pharmacopoeia.[23]

RESULTS AND DISCUSSION

After hot water extraction and acetone treatment, *Tamarind* seeds and *Prunus* gum yielded 58.29 and 78% w/w of hydrogel, respectively. The isolated hydrogels were subjected to identification tests using ruthenium red, corallin soda and by dissolving them in hot distilled water. With ruthenium red and corallin soda, the particles stained pink and a gelatinous mass was formed when the powder was heated with distilled water. All these tests indicated that the isolated hydrogels were polysaccharide mucilages. In the iodine test, the particles did not stain blue, indicating the absence of starch.

The results of purity tests of hydrogels showed the presence of carbohydrates. Other phytoconstituents were absent in the isolated powder. This can be considered as proof for purity of the isolated hydrogels (Table 1). Further characterization of hydrogels was done by determining the organoleptic properties like color, odor, taste, fracture and texture. The TSP was brownish white in color and AG had slightly off white color. Both the hydrogels did not possess any odor and the taste of AG was bland, whereas, TSP hydrogel had characteristic taste. The fracture was rough and texture was irregular for both the isolated hydrogels (Table 2).

Ash values can be used as standards for the quality control of plant based materials. For the isolated hydrogels, total ash, acid-insoluble, and water-soluble ash values were determined and are shown in Table 3. All these values were within the limits for natural polysaccharide polymers.[23]

The solubility test for hydrogels showed that both the hydrogels formed viscous colloidal dispersions with warm water, and were insoluble in organic solvents such as ethanol, benzene, butanol, chloroform, and ether (Table 4).

The pH values of 1% solution of the hydrogels were found to be slightly acidic or near neutral (Table 5), which indicated that both the hydrogels were non-irritating to the mucous membrane of buccal cavity and gastrointestinal tract, and both could be used for the development of buccal and oral drug delivery systems.

Table 1 Determination of purity of selected polysaccharide hydrogels

Tests	TSP	AG
Alkaloids	-	-
Carbohydrates	+	+
Flavanoids	-	-
Steroids	-	-
Amino acids	-	-
Terpenes	-	-
Glycosides	-	-
Oils and fats	-	-
Phenols and tannins	-	-

+ Present; - Absent

Table 2 Organoleptic properties of selected polysaccharide hydrogels

Hydrogel	Color	Odor	Taste	Texture	Fracture
TSP	Brownish white	Odorless	Characteristic	Irregular	Rough
AG	Buff	Odorless	Tasteless	Irregular	Rough

Table 3 Ash values of selected polysaccharide hydrogels (percentage w/w)

Hydrogel	Total ash	Acid insoluble ash	Water soluble ash
TSP	3.72	1.5	0.82
AG	4.20	2.5	0.93

The TSP and AG hydrogels were found to swell to 18.26 and 21.73 mL, respectively, which is an indication of good water absorption, and hence, formation of a hydrated three-dimensional network from which the drug release might follow diffusion. The surface tension values of 0.01% w/v solutions of TSP

and AG hydrogels are shown in Table 4.5. AG gum exhibited lower surface tension than TSP. The surface tension of the polymer has been reported to influence the binding quality of the polymer in tablets. The effect of surface properties such as wetting and spreading of binder over substrates, binder-substrate adhesion and binder cohesion in determining the optimum granulation with polymer binders has been reported.[28,29] Surface tension of the polymer is known to influence its applications in dosage forms. For example, the surface tension of a binder solution is reported to influence its penetration into granulation mass.[30] Lower surface tension promotes better penetration and spreading of polymer solution over the drug during wet granulation and hence leads to formation of better granules.[30] Hence, AG might produce better granules than TSP.

The results of test for loss on drying and moisture absorption are shown in Table 6. The absorption of moisture by any substance represents hygroscopic nature of the substance. If excipient is hygroscopic, it can alter many properties of the dosage forms. Hence, it is necessary to determine the hygroscopic nature of the excipient and the amount of moisture that can be absorbed by the excipient. The result of the present study indicated that both the hydrogels were hygroscopic and need to be stored in air-tight containers.

Table 4 Solubility profile of selected polysaccharide hydrogels

Solvent	Solubility Behavior of hydrogels	
	TSP	AG
Cold water	Insoluble	Insoluble
Warm water	Forms a viscous colloidal dispersion immediately	Forms a viscous colloidal dispersion immediately
Benzene	Insoluble	Insoluble
Ether	Insoluble	Insoluble
Chloroform	Insoluble	Insoluble
n- Butanol	Insoluble	Insoluble
Ethanol	Insoluble	Insoluble

Table 5 pH, Swelling index and surface tension of selected polysaccharide hydrogels

Hydrogel	pH of 1% solution	Swelling index (mL)	Surface tension* (dynes/cm^2)	Specific gravity of 0.01% w/v solution (g/cm^2)
TSP	6.85	18.26	74.55 ± 0.69	1.01
AG	5.25	21.73	73.99 ± 0.44	0.99

* Average of three determinations ± SD

Table 6 Loss on drying and moisture absorption of selected polysaccharide hydrogels

Hydrogel	Loss on drying (percentage)	Moisture absorption (percentage)
TSP	06.66	21.50
AG	17.33	6.40

Physical characterization of the hydrogels was carried out for bulk density and bulkiness, true density, total porosity, powder flow behavior (Table 7 and 8). The bulkiness values of the hydrogels were found to be 1.39 and 1.01, and the true densities of the hydrogels were found to be 1.015 and 1.103, respectively, for TSP and AG polysaccharides, which indicated that both the hydrogel powders were 'heavy' in nature.[27] This was supported by the low total porosity values (0.3 and 0.102% for Tamarind and almond hydrogels, respectively).

Certain powders contribute immensely to the porosity of the tablets. Porosity influences the rate of disintegration and dissolution. The higher the porosity, the faster the rate of dissolution.[27] The low total porosity values indicate that both the hydrogels might sustain the drug release from the dosage form (Table 7). The TSP hydrogel exhibited poor to passable flow. Hence, to improve the flow, it needs addition of glidants. The AG exhibited good flow (Table 8).

Table 7 Bulk density, bulkiness, true density and total porosity values of the selected polysaccharide hydrogels

Hydrogel	Bulk density ϱ_b (g/cc)	Bulkiness ($1/\varrho_b$)[a]	True density (ϱ, g/cc)	Total porosity (%ε)[a]
TSP	0.78	1.28	1.015	0.23
AG	0.83	1.20	1.103	0.25

[a] Derived property

Table 8 Powder flow properties and compressibility indices of selected polysaccharide hydrogels

Hydrogel	Angle of repose (θ, deg)* / Type of flow	Carr's index (%)* / Type of flow
TSP	39.79 / (Passable)	34.72 / (Poor)
AG	29.50 / (Good)	14.29 / (Good)

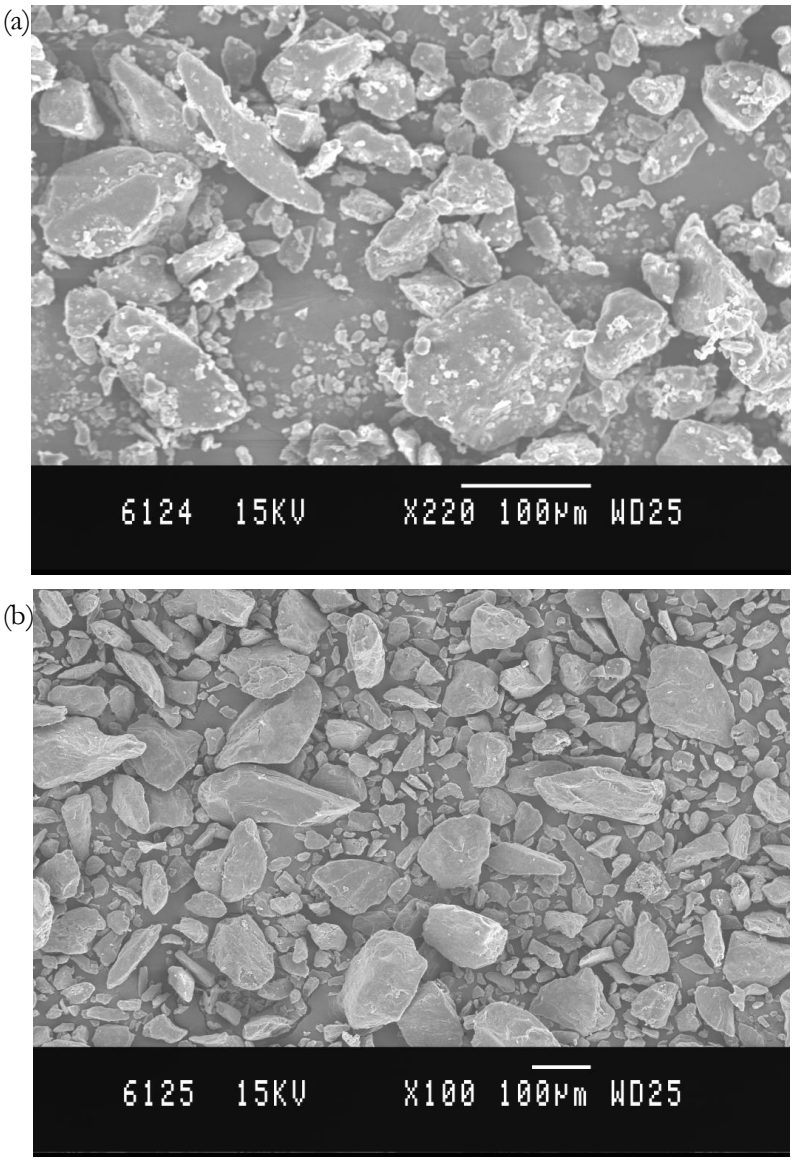
* Average of three determinations

To study the surface characteristics of hydrogels, SEM of the powder was taken. The SEM of TSP hydrogel exhibited rough surface with pores and crevices on it, whereas, the AG exhibited a comparatively smooth surface (Fig 1). Earlier, it has been reported that the drug release from the dosage form depends on surface characteristics of excipient.[31] If the surface is rough, drug release will be retarded because of the entrapment of drug particles in the pores and crevices. Hence, it can be stated that both the hydrogels can sustain the drug release because of their rough surface.

From the SEM, it was also evident that the particle size of both the powders was not uniform and the size distribution was not within a narrow range. The powder contains larger to ultra-fine particles. This might be the reason for the 'heavy' nature of the powders. The powders exhibit a 'closet' packing arrangement, in which, the smaller particles fill the voids between larger particles and reduce the bulkiness.[27] This packing arrangement is indicated by the low total porosity values as well. The close packing can also be responsible for poor flow properties of tamarind hydrogel, whereas the smooth surface of almond gum might be a reason for its good flow with close packing.

The FTIR spectra of both the hydrogels are given in Fig 2, which indicated that the hydrogels were carbohydrates in nature. These spectra can be used as standard spectra for quality control and determination of the purity of the hydrogels.

Fig 1 SEM of powder sample of TSP (a) and AG (b)

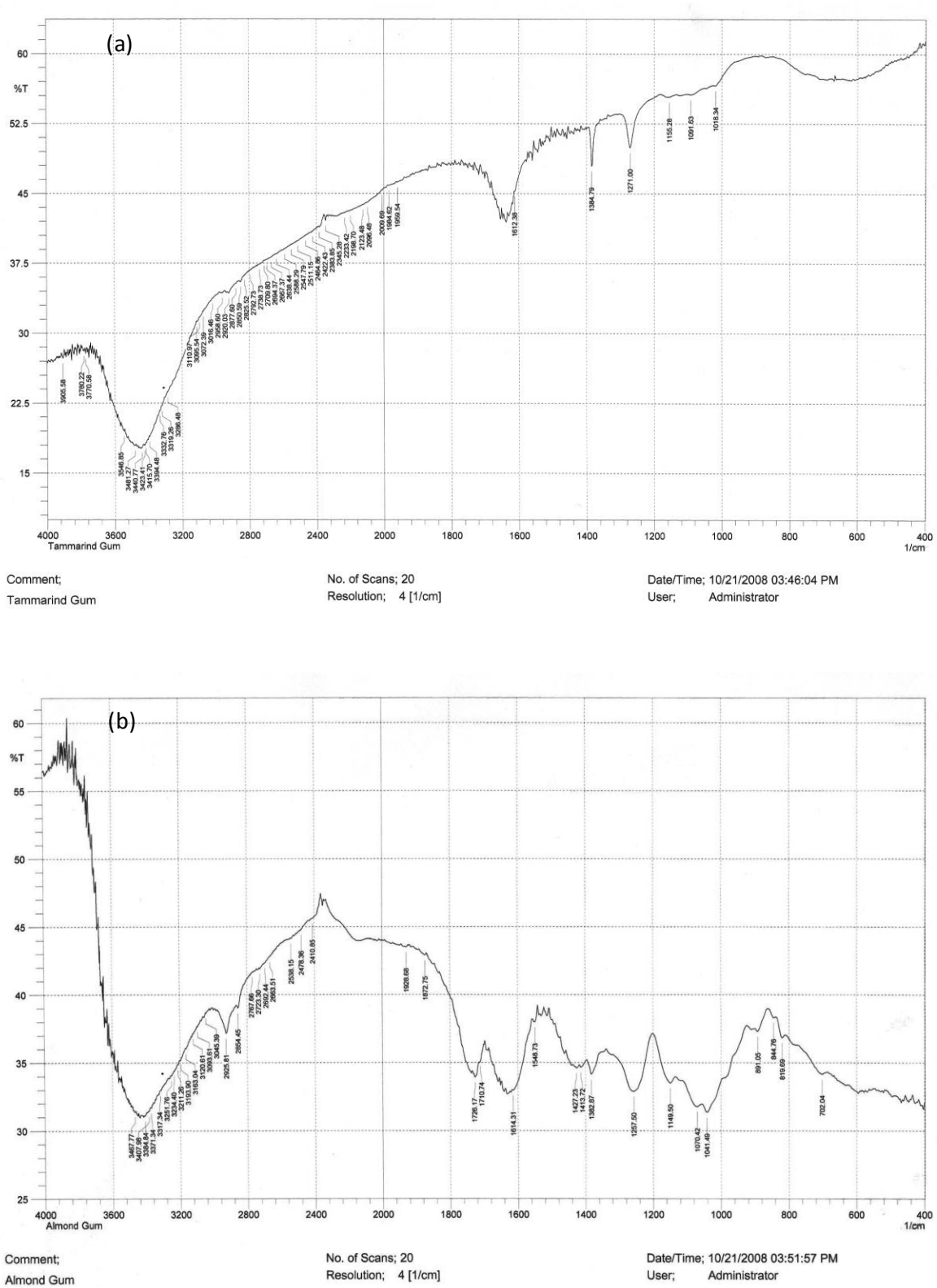


The total microbial load of the selected polysaccharide hydrogels is given in Table 9. The total microbial load is an important parameter which decides the suitability of a substance for use as excipient in pharmaceutical dosage forms. According to many Pharmacopoeias, for synthetic and semi-synthetic substances, the total aerobic count should not be more than 100 colony forming units (cfu) per gram, and the total fungal count (including yeasts and molds) should not exceed 50 cfu/g. In case of excipients from natural origin, the total aerobic count should not be more than 1000 cfu/g and total fungal count should not exceed 100 cfu/g. In the present study, both the hydrogels exhibited bacterial and fungal counts less than the specified limits and the pathogenic organisms were absent in both the polysaccharides after purification.

Table 9 Microbial load of the selected hydrogels

Hydrogel	Total bacterial count (cfu/g)	Total fungal count (cfu/g)
TSP	93	78
AG	92	81

Fig 2 FTIR spectra of tamarind seed polysaccharide (a) and almond gum (b)



CONCLUSION

From the results of the above studies, it can be concluded that the selected hydrogels from *Tamarindus indica* and *Prunus amygdalus* have physicochemical properties suitable for multifunctional excipients. They may be used as binders, mucoadhesive agents and also for sustaining the drug release. These predictions can be further proved by utilizing these hydrogels in different formulations.

DECLARATION OF INTEREST

It is hereby declared that this paper does not have any conflict of interest.

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