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Antidiabetic assays' based fractionation and characterization of Jamun (*Syzygium cumini* Skeels) fruits

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Abstract
Jamun (*Syzygium cumini* Skeels) fruits are well known for their anti-diabetic properties. The present study aimed at characterization of promising accessions for anti-diabetic and anti-oxidant metabolites in seeds and pulp. Among 7 accessions characterized, fruit pulp of CISH J-36 and CISH J-37 recorded high DDPH scavenging activity (anti-oxidant potential), phenol and flavonoid content. Fractionation of the methanol extracts by liquid partitioning yielded 3 fractions (ethyl acetate, butanol and water) which were evaluated based on bioactivity assays. α-Amylase inhibition was maximum with seed (methanol extracts) of all accessions, successive water extracts having anti-diabetic potential as demonstrated by the inhibition assay. Five fractions collected by liquid-liquid partition using ethyl acetate (EA), butanol and water showed varying levels of bioactivity, EA fraction with high DPPH scavenging activity and butanol fraction with high α-Amylase inhibition activity. Sixth fraction (Sc/EA/A6) had best bioactivity (aldose reductase assay: IC_{50} 4.20µg/ml). The anti-oxidant and anti-diabetic potential based on DPPH and α-Amylase inhibition assays are pioneering attempts to evaluate Jamun germplasm and its fruit parts for recovery of bioactive principles for development of novel phytomedicines.

Keywords: jamun, DPPH scavenging activity, α-amylase inhibition, fractionation, methanol extract, anti-diabetic activities

Introduction
Java plum or Jamun (*Syzygium cuminii* Skeels) is an emerging fruit crop of the twenty-first century yielding nutritious table fruit of high therapeutic value. It could be equated to *Kalpvriksha* in Sanskrit (meaning each part of tree has importance) as it has nutraceutical and medicinal properties. In the present era, there has been an increased interest in natural antioxidants that include various phytochemicals, alkaloids, flavonoids, terpenoids, tannins, and vitamins (Ayyanar and Pandurangan, 2012) [2] because of their promising medicinal and therapeutic properties. The medicinal significance of Jamun is well documented in ancient Indian literature and utilized in homoepathic, ayurvedic and siddha medicines. Being native to India, there is huge diversity of germplasm resources with enormous potentials for extracting pharmaceutically important metabolites.

Among lifestyle diseases, *Diabetes mellitus* is fast gaining the status of a potential epidemic in India with more than 62 million diabetic individuals diagnosed with the disease (Kaveeshwar and Cornwall, 2014) [6]. Persistent and chronic hyperglycemia is known to result in high oxidative stress, which depletes the activity of antioxidative defense systems and thus promotes *de novo* free radical generation. Hence there is immense potential in search for novel antioxidants from natural sources (Kade *et al.*, 2013) [4]. There is renewed interest in plant-based medicines and functional foods modulating physiological effects in the prevention and cure of diabetes and obesity. Several indigenous medicinal plants have a high potential in inhibiting α-amylase enzyme activity including *Syzygium cuminii* (Karthic *et al.*, 2008) [5]. It is established fact that jamun fruits possess hypoglycaemic and antioxidant properties that have been proven on animal models (Ravi *et al*., 2004 and 2004a) [11]. At present, large numbers of accessions from different parts of the country have been collected and maintained at field gene bank at Central Institute Subtropical Horticulture, Lucknow. The antioxidant activity and fractionation of the fruit extract for evaluating the anti-diabetic potential using various assay is crucial for promoting Jamun as potential anti-diabetic functional fruit and formulating utilization strategies. The present investigation was carried out to characterize the germplasm resources for their anti-oxidant and anti-diabetic properties and also to further characterize the bioactive fractions that are responsible for the anti-diabetic activity.

Material and Methods

Collection of plant samples and preparation of extracts for antioxidant assays

Fruits of seven Jamun accessions (CISH J-23, CISH J-26, CISH J-34, CISH J-36, CISH J-37, CISH J-43, CISH J-49) (Fig.1) were utilized for evaluating the anti-diabetic potential based on antioxidant, flavonoid and phenol contents. Different parts of ripe fruits such as skin, pulp and seeds were separated and lyophilized. 1–2 g of lyophilized fruit samples were homogenized in mortar and pestle with 25 ml of ethanol/water (80:20 v/v) and kept in shaking water bath at 60 °C for 30 min. The samples were filtered through Whatmann No.1 filter paper by applying vacuum. The crude extracts were then concentrated by flash evaporation to 10 ml and used for antioxidant assays. Unripe whole jamun was used as a control. All the assays were carried out in triplicates.

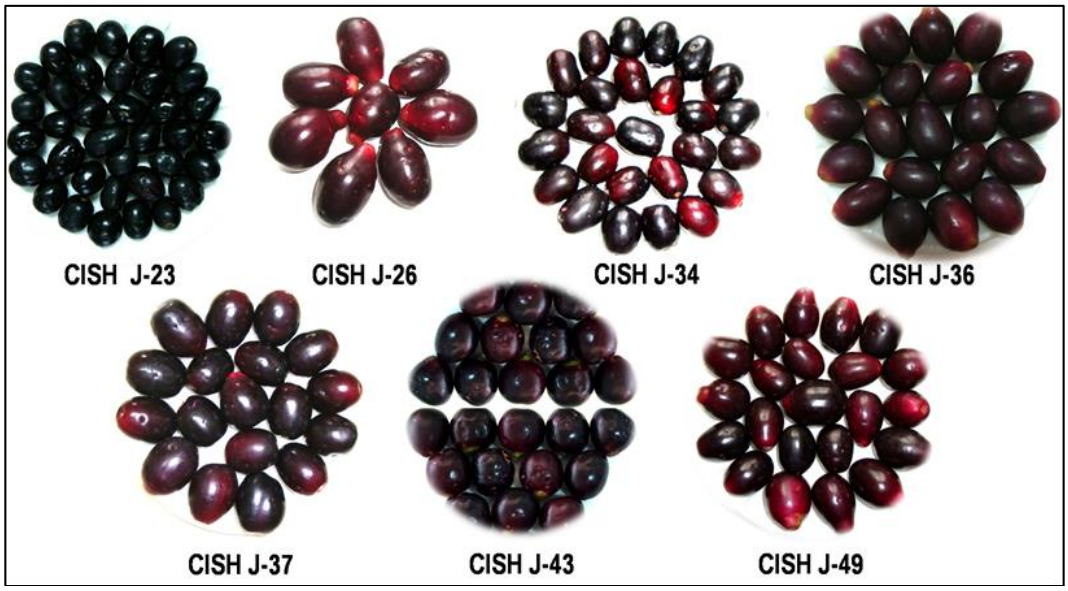

Fig 1: Fruits of germplasm accessions of Java plum (Jamun) used in the present study

Preparation of methanolic extracts

A known quantity of the powdered plant material was taken in a round bottom flask and extracted with methanol under reflux, on a water bath (70ºC) for 1.5 hrs. The extraction was repeated two times with fresh solvent each time. Each extract was filtered through a filter paper. The combined methanol extract was concentrated under vacuum (400mm of Hg) at 60ºC, to obtain a dry extract which was subsequently refluxed with de-mineralized water in the same manner as methanol extraction (2 extraction, 1.5 hrs each). All the three water washings were combined, concentrated and dried under vacuum (600mm of Hg) at 75ºC to obtain a successive water extract.

DPPH Free Radical Scavenging Activity Assay

Free radical scavenging activity of jamun pulp and seed extracts against stable DPPH was determined spectrophotometrically (Brand-Williams *et al.*, 1995) [3]. After 30 min of incubation at room temperature the reduction of the DPPH free radical was measured at 517 nm. Inhibition percent was calculated from the following equation:

% Inhibition = [Absorbance of control - Absorbance of sample/absorbance of control] x100

Where, Ascorbic acid was included as a positive control

Bioactivity guided fractionation of methanolic extracts (Sc/MeOH)

The methanolic extract was subjected to liquid-liquid partition with ethyl acetate, butanol and water. The ethyl acetate layer (Sc/EA) was separated and dried. The aqueous layer was again subjected to liquid-liquid partition with butanol. The butanol layer (Sc/BU) and aqueous layer (Sc/Aq), were further concentrated, and all fractions were evaluated in *in vitro* anti-diabetic bioassays. The selected ethyl acetate extract was subjected to column chromatography using different solvent systems with silica gel used as the stationary phase saturated with lowest polar 100% petroleum ether as eluting solvent. The ethyl acetate fraction (SC/EA) was loaded onto the bed of the column and was chromatographic run was started by gradient elution technique (increasing the polarity of eluting solvent) using petroleum ether and ethyl acetate as mobile phase. With the view to identify and characterize the bioactive compounds present in the sample. Fractionation was done and the resulting fractions with bioactivity were further used for subsequent studies.

Results and Discussion

Antioxidant potential of fruit and seed extracts

Antioxidant potentials of fruit pulp and seed extracts of 7 important jamun accessions were assessed using DPPH assay, flavonoid and phenol estimations. The results referring to the estimated antioxidant activity of 7 important jamun accessions are shown in Fig. 2. Anti-oxidants present in fruit extract reduce DPPH to 2, 2-diphenyl-1-picryl hydrazine, a colorless compound, the degree of discoloration indicating the scavenging potential of antioxidants or plant extracts. DPPH is a stable, nitrogen-centered free radical producing violet purple color in ethanol solution and fades to shades of yellow color in the presence of antioxidants (Koleva *et al*., 2002) [7]. The purple color of DPPH solution was reduced to a yellow colored product, diphenylpicryl hydrazine on the addition of fruit extract in a concentration-dependent manner. While seed extracts of all accessions gave good DPPH inhibition, the fruit pulp of CISH J-36 and 37 demonstrated highest antioxidant potential, based on DPPH assay and metabolite content. Shrikanta *et al.* (2013) [13] also showed jamun seed extracts to exhibit the highest polyphenol content (55.54 mg gallic acid equivalent g dry weight) and highest antioxidant property.

Aquil *et al.* (2012) [1] demonstrated antioxidant property of fruit and seed extract by various biological assays and HPLC based identification, where in it was stated that seed has high polyphenols and tannins over pulp, which was also evident in our results.

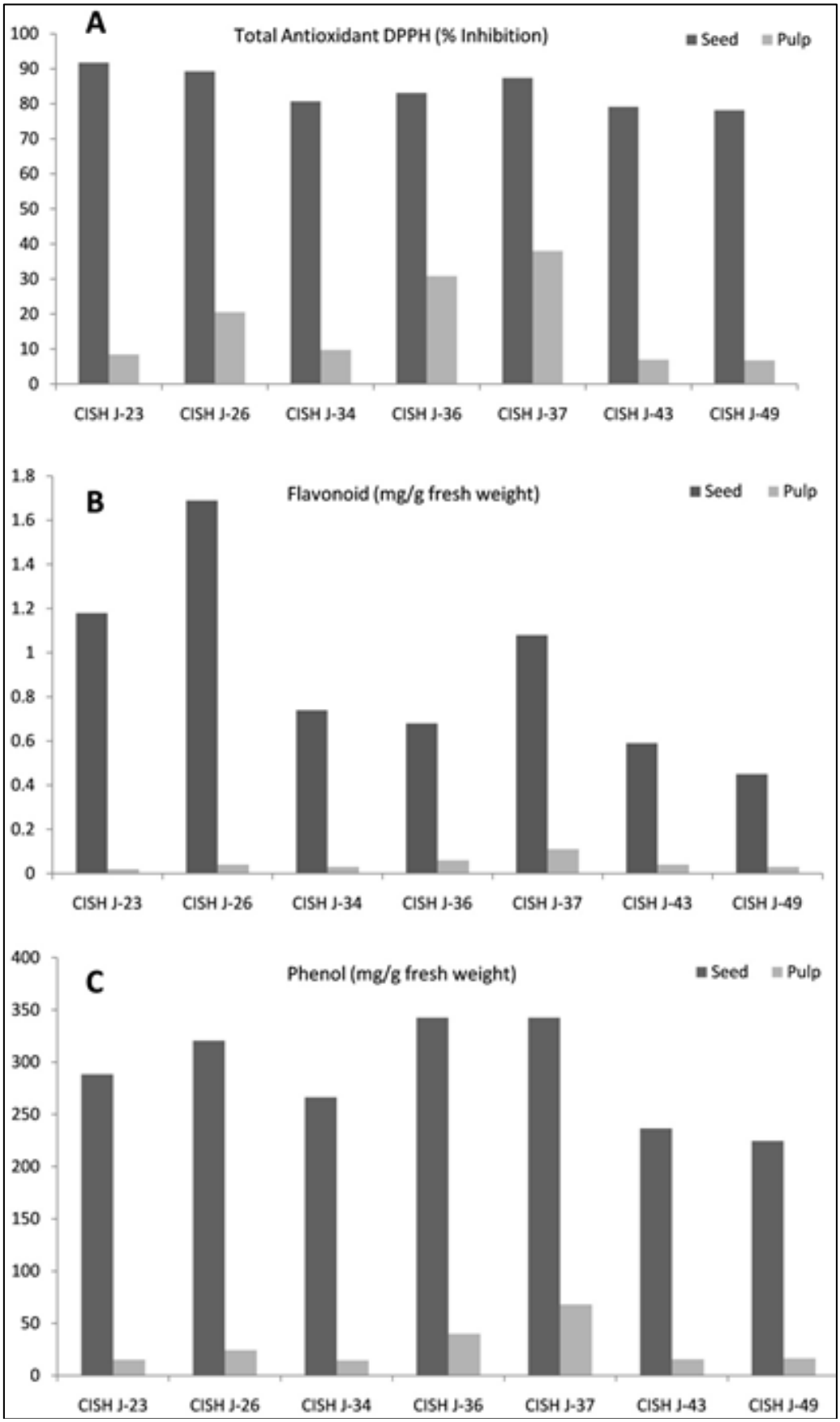

Fig 2: Profiling of antioxidant activities in seed and pulp extracts from mature fruits of superior java plum or jamun accessions. A) Total antioxidants based on DPPH assay (expressed in % inhibition, B) Flavonoid content (expressed in terms of mg/g of fresh weight of seed or pulp), C) Phenol content (expressed in terms of mg/g of fresh weight of seed or pulp)

α-Amylase inhibition assay

α-amylase inhibition is an important assay for screening anti-diabetic plant-derived phyto medicines for managing diabetes, as they are known to display fewer or no side effects. Some well-known and critical *Diabetes mellitus* targets include, α-glucosidase, DPP-IV, aldose reductase, PPAR-γ, AMP kinase and GLUT4 (Sawant *et al.,* 2015) [12]. Accordingly, the shade dried fruit extracts from different varieties of jamun were screened in α-amylase inhibition assay at a concentration of 100 µg/ml. Inhibition of the enzyme activity by plant extract decreased the absorbance at 405 nm which could be directly correlated to the glucose levels as α-amylase is the key enzyme involved in the production of glucose by break down of complex polysaccharides. The activity of the jamun methanolic extracts from different accessions in α-amylase inhibition assay revealed inhibition by both types of extracts, from pulp and seed (Table 1), CISH J-49 seeds displayed highest inhibition in methanol extracts. Fully ripe jamun pulp (MeOH extract) showed lesser inhibition, similar to the results obtained in pear and kiwifruit extracts (Pech *et al*., 1973; Matsui *et al*., 1989) [9, 8] corroborating our findings. However, successive water extract of the fruit and seeds displayed higher inhibition of α-amylase. CISH J-37 having good potential for table purpose, also demonstrated anti glycemic properties in seed and pulp in ripe stages.

Table 1: Profiling of various accessions of Jamun pulp and seed extracts displaying α-amylase inhibition property

Inhibition of α-amylase (% Inhibition at 100 µg/ml)														
	CISH J-23		CISH J-26		CISH J-34		CISH J-36		CISH J- 37		CISH J-40		CISH J-49	
	Met.	Wat.	Met.	Wat.	Met.	Wat.	Met.	Wat.	Met.	Wat.	Met.	Wat.	Met.	Wat.
Fruit extract	19.7	23.1	2.8	35.5	17.2	5.7	2.8	12.0	19.7	23.1	28.0	29.2	90.0	25.0
Seed extract	64.9	39.5	71.5	53.5	34.0	16.0	76.1	54.7	64.9	39.5	56.9	47.3	90.0	41.8

Wat: water, Met: Methanol

Bioactivity guided fractionation

On the basis of anti-diabetic potential of extracts, methanolic extract of *Syzygium cumini* seeds (CISH J 37) was chosen for further analysis guided by bioactivity of fractions. The ethyl acetate fraction (Sc/EA) was found to be active in DPPH assay (IC50: 42.63 µg/mL), while for α-Amylase inhibition assay (IC50: 22.64 µg/mL) butanol fraction was more active in the bioassay (Table 3). Five different fractions viz. Sc/EA/A1 (14% EA + 86% PE), Sc/EA/A2 (28% EA + 72% PE), Sc/EA/A4 (62% EA + 38% PE), Sc/EA/A5 (79% EA + 21% PE), Sc/EA/A6 (100% EA), were collected from the column, dried in a rotary evaporator and further evaluated in aldose reductase inhibition assay (as per Sawant *et al*., 2015) [12].

Among the resulting ethyl acetate fractions, Sc/EA/A6 (100% EA fraction) was found to be most active. It showed inhibition of aldose reductase activity with an IC_{50} of 10.85 and 4.20 µg/mL, respectively (Table 2). Further fractionation of Sc/EA/A6 was done for subsequent analysis. The fraction (Sc/EA/A4) which was eluted with 62% EA and 38% PE aldose reductase activity with an IC_{50} of 11.42 and 20.25 µg/mL, respectively. Overall, A4 and A6 fractions were found to be the bioactive fractions and taken up for further fractionation. Further separation of metabolites responsible for anti-diabetic bioactivity can be done using column chromatography and characterized using HPLC and NMR spectroscopy. Recent study (Poongunran *et al.,* 2017) [10] revealed strong α-amylase and α-glucosidase inhibitory effects of ursolic acid and oleanolic acid constituents isolated from jamun leaves for the first time validating its use in anti-diabetic therapy. This study confirms bioactivity of fruit and seed extracts and it is useful for identification of phyto metabolites in Jamun towards augmenting anti-diabetic plant based supplements. As described in Ayurveda and demonstrated by studies in jamun and other berries, these Jamun derivatives provide anti-oxidant benefits in addition to anti diabetic characteristics.

Table 2: Bioactivity assays of ethyl acetate, butanol and water fractions of methanolic extracts (Sc/MeOH) and determination of IC 50

Ethyl acetate fraction (Sc/EA), Butanol fraction (Sc/BU) fraction and Water fraction (Sc/Aq) of methanolic extract (Sc/MeOH)					
Inhibition assay	**Conc. tested**	**(Sc/EA), % Inhibition/ IC 50 (µg/mL)**		**(Sc/BU) % Inhibition/ IC 50**	**(Sc/Aq) % Inhibition/ IC 50**
α-Amylase	100 µg/mL	46.50/87.01		71.21/22.64	19.99/--
DPPH	50 µg/mL	69.67/42.63		55.63/51.33	29.95/--
IC_{50} inhibition assay (µg/mL) (Fractions Sc/EA/A1 to A6)					
	A1	A2	A4	A5	A6
Aldose reductase	69.77	38.44	20.25	21.5	4.20

Conclusion

Jamun seeds have proved to display anti-diabetic principles, yet there is no systematic characterization and identification of the active compound with anti-diabetic property. In this direction, CISH J-36 and CISH J-37 demonstrated highest antioxidant potential, based on DPPH assay and metabolite content. Both water and methanol extract displayed anti-diabetic potential of fruit extracts based on α-amylase inhibition assay. Further (Sc/EA) fractions were enabled wherein, Sc/EA/A6 (100% EA fraction) was found to be most active. It showed inhibition of aldose reductase activity with an IC_{50} of 10.85 and 4.20 µg/mL, respectively. These fractions needs to be further characterized through HPLC, GC-MS or LC-MS profiling which would enable identification of active compounds governing anti-diabetic property exhibiting therapeutic significance. Furthermore, fractionation based bioactivity of the extracts confirm anti-diabetic and antioxidant potential of choicest variety CISH J-36 and CISH J-37 which can be promoted for crop diversification. Advanced tools of genomics and computational biology could help in designing novel drugs enabling developing new formulations of next generation drugs for ailment of long standing and chronic diseases like diabetes.

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Declaration of interest statement
Authors declare no conflict of interest

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