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Original Research Paper

Optimization of methodology for the extraction of polyphenolic compounds with antioxidant potential and α-glucosidase inhibitory activity from Jamun (*Syzygium cumini* L.) seeds

Arivalagan M.[1*], Priyanka D.R.[1] and Rekha A.[2]
[1]Division of Basic Sciences, [2]Division of Fruit Crops
ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru - 560 089, Karnataka, India.
*Corresponding author E. mail: arivalagan2100@gmail.com

ABSTRACT

Jamun (*Syzygium cumini* L.) seed is one of the rich sources of polyphenolic compounds with antioxidant potential and α-glucosidase inhibitory activity. A study was conducted to optimize the methodology for the extraction of polyphenolic compounds (total phenolic and flavonoid contents) with antioxidant potential and α-glucosidase inhibitory activity from jamun seed powder. The study showed that the nature of solvent and extraction conditions had a significant effect on total phenolic content (TPC), total flavonoid content (TFC), antioxidant potential, and α-glucosidase inhibitory activity. The TPC varied between 6.0 (mg/g jamun seed powder) for the acetone extract to 119.2 (mg/g) for 80% aqueous acetone extract, and TFC varied between 1.06 mg/g for the acetone to 10.81 mg/g for the 80% aqueous methanol. From the study, it was apparent that an aqueous form of acetone (acetone: water 80:20, v/v) is a better solvent system for extraction of polyphenolic compounds with high antioxidant potential and α-glucosidase inhibitory activity. Ultrasonication for 60 min increased the efficiency of phenolic extraction.

Keywords: á-glucosidase inhibitory activity, Antioxidant potential; Flavonoids, Jamun seed powder, Natural antioxidants and Polyphenols.

INTRODUCTION

Jamun (*Syzygium cumini* L.) is one of the most important indigenous fruits belongs to the family Myrtaceae. It is a treasure of wide range of secondary metabolites with numerous health benefits. The fruit pulp is sweet and the seeds are pungent and tangy. Both pulp and seeds are extensively used in traditional medicine against various ailments such as diabetes, diarrhea, and ringworm (Benherlal and Arumughan, 2007). While jamun fruits are a rich source of anthocyanins, their seeds are high in polyphenolic compounds. Oxalic acid, tannic acid, gallic acid, and some of the alkaloids are the major secondary metabolites responsible for the astringency taste of seed as well as pulp (Hameed *et al.,* 2020). Most of the secondary metabolites, especially polyphenolic compounds present in the jamun pulp and seeds are reported to possess free radical scavenging potential (Ayyanar and Subash-babu, 2012) as well as anti-inflammatory activities (De Bona *et al.,* 2016; Hossain *et al.,* 2016).

Earlier studies have suggested that some of the polyphenolic compounds present in jamun fruit pulp and seeds have the potential to inhibit the activities of the enzymes, alpha-amylase and intestinal alpha-glucosidase which are responsible for the digestion of dietary starch (Mahmood, 2016). These enzymes have been a target of many drugs developed for the treatment of type-II diabetes (Sim *et al.,* 2008). Since, diabetes is a major problem affecting the human population worldwide and synthetic drugs are risky due to associated side effects, plant-based secondary metabolites especially polyphenolic compounds are the ideal candidate to address the problems of hyperglycemia and diabetes in the human population. Investigation made earlier has reported that the presence of polyphenolic compounds in jamun seeds has appreciably good antioxidant potential and hypoglycemic effect (Aqil *et al.,* 2012). Thus, the extraction of polyphenolic compounds from jamun seeds and it's usage in the medication for regulation

of blood glucose level has attracted many researchers. Polyphenolic compounds possess diverse nature in terms of physical and chemical properties and some of them exist in the free as well as complex forms with carbohydrates and proteins. Due to this nature, the extent of the solubility of these polyphenolic compounds varies in different solvents (Khoddami *et al.,* 2013). Mostly water and other polar solvents such as ethanol, methanol, acetone, and diethyl ether have been extensively used for the extraction of polyphenolic compounds from plant sources (Arivalagan *et al.,* 2018). Since, the levels of active principle in the plant extract depends on the type of solvent used and the extraction methods being adopted (Sun and Ho, 2005; Turkmen *et al.,* 2006; Hayouni *et al.,* 2007), the present investigation was undertaken to optimize the suitable solvent system and extraction conditions for the extraction of polyphenolic compounds with antioxidant potential and alpha-glucosidase inhibitory activity from jamun seeds, as it is rich in polyphenolic compounds.

MATERIALS AND METHODS

Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2- carboxylic acid), TPTZ (2,4,6-tris-2,4,6-tripyridyl-2-triazine), ABTS (2,2-azino-di-(3-ethylbenzothialozine-6-sulphonic acid) diammonium salt), potassium persulfate, and neocuproine (2,9-dimethyl-1,10-phenanthroline) were purchased from Sigma-Aldrich Co St. Louis, MO, United States of America. Analytical grade Ethanol, methanol, acetone, acetic acid (glacial), sodium acetate, hydrochloric acid (conc.), ferric chloride, ammonium acetate, copper (II) chloride, Folin–Ciocalteu's phenol reagent, aluminium chloride, sodium nitrite, and sodium carbonate were purchased from Merck KGaA, Darmstadt, Germany.

Sample preparation

The experiment was condcuted at ICAR- Indian Institute of Horticultural Research (IIHR), Bengaluru, Karnataka, India during 2020. Matured and fully ripened fruit samples were randomly collected from jamun germplasm (IC-0621954, 8 years old tree with semi-spreading type with broad leaves) and brought to the laboratory in the icebox. After removal of pulp, the seeds were dried in a mechanical tray dryer (Make: M/s Servewell Instruments Pvt. Ltd, Karnataka, India) at 50°C until the constant dry weight was achieved. The seed coat was then removed and the kernels were powdered finely using a ball mill (Mixer Mill MMOL400, RETSCH GmbH, Germany) to get uniform particle size and stored in a vacuum desiccator for further analysis.

Solvent system and extraction procedure

About 100 mg jamun seed powder was mixed with 5 mL of selected solvent in 15 mL centrifuge tube and extracted. For extraction seven solvent systems *viz*. water, methanol, aqueous methanol (methanol: water 80:20 v/v), ethanol, aqueous ethanol (ethanol: water 80:20 v/v), acetone, and aqueous acetone (acetone: water 80:20 v/v) were used. The extraction procedure was carried out at two different conditions (room temperature [25±2°C] and ultra-sonic bath set at 50±2°C), each with 30 min and 60 min separately. Extracts were centrifuged (at 5°C for 10 min) and the supernatant was collected in amber reagent bottles. The residue was re-extracted twice using the same procedure as mentioned above. Filtrates collected from all three successive extractions were pooled and dried under a nitrogen atmosphere. The dried samples were dissolved in 5 mL of water, centrifuged, and then the clear supernatant collected was used for the analysis.

Determination of total phenolic and flavonoid content

Determination of total phenolic content (TPC) in the extract was done by Folin-Ciocalteu (FC) assay as described by Singleton *et al.* (1999) using gallic acid as a standard, and TPC was expressed as mg gallic acid equivalent (GAE), per g dry weight of jamun seed powder. The total flavonoid content (TFC) was determined according to Zhishen *et al.* (1999). Quercetin was used as a standard and the results were expressed as mg of quercetin equivalent (QE) per g jamun seed powder.

Determination of antioxidant potential

The DPPH and ABTS radical scavenging activities (IC_{50}) of the different solvent extracts of jamun seed powder were determined by the methods of Brand-Williams *et al.* (1995) and Arnao *et al.* (2001), respectively. IC_{50} value signifies the concentration of test samples to scavenge 50% of the DPPH and $ABTS^{\bullet+}$ radical and expressed as mM TE/g jamun

seed powder. The FRAP (Ferric Reducing Antioxidant Power) assay was done according to Benzie & Strain (1996) and CUPRAC (Cupric ion reducing antioxidant capacity) of the extract was determined according to the method of Apak *et al.,* (2004). In both methods, Trolox served as a positive control and results were expressed as mM TE/g dry jamun seed powder.

Determination of alpha-glucosidase inhibitory activity

Individual sample extracts of varying concentrations were made up to 1 mL with phosphate buffer (0.1M, pH 6.9) and 100 µL of alpha-glucosidase enzyme (25 µg of enzyme) was added. The contents were incubated at 25°C for 5 min. After pre-incubation, about 100 µL of p-nitrophenyl-α-D-glucopyranoside (250 µg/mL) added, contents mixed well and the reaction mixture incubated for 20 min at 25°C. The reaction was terminated by the addition of 1.0 mL of 1 N Na_2CO_3. After 10 min, the yellow color developed was measured at 405 nm and the α-glucosidase inhibition % (IC %) was calculated using the following equation:

IC% = [(Acontrol–Asample)/Acontrol]×100;

Where, Acontrol is the absorbance of the blank control (containing both enzyme and substrate except jamun seed extract): Asample is the absorbance of the test sample.

A graph was plotted with concentration along x axis and IC% along y axis, and IC_{50} values were calculated. IC_{50} value signifies the concentration of tested samples to inhibit 50% of the α-glucosidase enzyme.

Statistical analysis

All the experiments were conducted on triplicate samples. The effect of different extraction solvents, time, and conditions on TPC, TFC, antioxidant potential, and alpha-glucosidase inhibitory activity was evaluated by ANOVA using SAS (SAS, 2011). Pearson's linear correlation was performed to measure the correlation and strength of the relationship between TPC, TFC, antioxidant potential, and alpha-glucosidase inhibitory activity of different solvent extracts.

RESULTS AND DISCUSSION

Effect of solvent type with varying polarity, condition, and extraction time on the extraction of total phenolics and total flavonoids from jamun seeds

Total phenolics (TPC), flavonoids (TFC), antioxidant potential, and alpha-glucosidase inhibitory activity of different solvent extracts of jamun seeds are given in Table 1.

Total phenolic content (TPC)

The TPC varied between 6.0 (mg/g) jamun seed powder for acetone to 119.2 (mg/g) for 80% aqueous acetone. Significant variation was observed for the yield of total phenolic content with respect to solvent type, extraction condition, and time. The phenolic extraction ability was found significantly higher for aqueous solvents compared to their absolute forms. Absolute forms of methanol, ethanol, and acetone extracted less amount of TPC compared to their aqueous counterparts irrespective of extraction time and conditions. Among the solvents with absolute form, extractability of phenolics was found more in methanol (69.8 mg/g) followed by ethanol (42.3 mg), while acetone extracted significantly less TPC (19.3 mg). Water alone extracted a significantly high amount of TPC (78.1 mg) compared to solvents with absolute from. The addition of water to the solvents significantly increased the extraction ability of the solvents. Among the aqueous forms of solvents, aqueous acetone extracted higher amount of TPC (111.9 mg) followed by aqueous methanol (89.8 mg) and aqueous ethanol (83.6 mg). About 5 to 10-fold increase in TPC content was observed for aqueous acetone with different conditions and extraction time compared to its absolute form. From the results, it is clear that the solvents with varying polarity had a significant effect on extractability, and aqueous acetone was superior among the solvent systems studied.

Earlier studies also reported similar findings with high extractability of phenolics by aqueous acetone with varying proportions starting from 50 %, 70%, and 80% (Zhao *et al.,* 2006; Sulaiman *et al.,* 2011, Wijekoon *et al.,* 2011). In the present study, two extraction conditions were employed, one at room temperature [25±2°C] and the other using ultrasonication maintained at 50±2°C. Ultra sonication extracted significantly high amount of TPC compared to room temperature irrespective of solvent type. Similarly, in most cases, 60 min extraction time significantly increased the extraction ability of solvents compared to 30 min extraction time. From the results, it is clear that the aqueous form of acetone with ultrasonication for 60 min is the ideal condition for the extraction of more TPC from jamun seed powder.

Table 1: Total phenolics, flavonoids, antioxidant potential and alpha-glucosidase inhibitory activity of different solvent extracts of jamun seeds

Solvents	Condition	Time (min)	TPC	TFC	FRAP	CUPRAC	DPPH	ABTS	α-glucosidase inhibitory activity*
Water	RT	30	53.0	4.00	0.51	2.06	0.62	0.58	285.2
		60	53.6	3.93	0.52	1.92	0.62	0.92	206.0
	Sonication	30	67.7	5.65	0.61	2.54	0.86	0.86	222.2
		60	78.1	5.47	0.83	2.74	0.89	1.06	197.0
Methanol	RT	30	50.2	6.68	0.55	2.00	0.62	0.55	120.9
		60	57.1	6.82	0.54	2.09	0.61	0.77	57.7
	Sonication	30	68.8	8.41	0.65	2.24	0.76	0.93	194.2
		60	69.8	8.13	0.75	2.64	0.77	0.96	111.2
Aqueous methanol (80% v/v)	RT	30	74.7	9.20	0.89	3.09	0.90	0.98	58.4
		60	76.7	8.69	0.80	2.90	0.92	0.98	90.1
	Sonication	30	89.8	10.81	0.72	3.56	0.99	1.17	107.5
		60	72.4	9.09	0.62	2.38	0.70	1.19	86.6
Ethanol	RT	30	16.5	2.59	0.18	0.62	0.21	0.12	313.7
		60	17.8	3.20	0.16	0.50	0.23	0.22	206.8
	Sonication	30	31.2	3.86	0.34	1.13	0.35	0.34	215.7
		60	42.3	5.31	0.44	1.50	0.52	0.58	173.8
Aqueous ethanol (80% v/v)	RT	30	63.8	7.13	0.64	2.59	0.80	1.02	93.1
		60	65.9	6.61	0.66	2.53	0.83	0.90	83.8
	Sonication	30	79.0	8.48	0.78	3.05	0.94	1.06	120.6
		60	83.6	8.33	0.94	3.16	0.94	1.08	78.8
Acetone	RT	30	7.0	1.50	0.10	0.30	0.06	0.06	972.7
		60	6.0	1.06	0.07	0.16	0.10	0.28	771.0
	Sonication	30	14.0	1.94	0.16	0.53	0.12	0.28	536.9
		60	19.3	3.98	0.21	0.58	0.20	0.35	268.7
Aqueous acetone (80% v/v)	RT	30	83.7	8.60	0.93	3.54	1.08	1.24	81.6
		60	94.4	7.44	0.95	3.70	1.11	1.12	58.0
	Sonication	30	97.2	8.98	0.88	3.85	1.08	1.35	54.0
		60	111.19	9.01	1.08	4.23	1.17	1.42	47.0
Interaction effect with CD									
Solvent		2.29	0.48	0.03	0.09	0.05	0.08	51.7	
Condition		1.23	0.26	0.02	0.05	0.03	0.04	27.7	
Time		1.23	NS	0.02	NS	NS	0.04	27.7	
Solvent * Condition		3.24	NS	0.05	0.13	0.07	0.11	73.2	
Solvent * Time		1.73	0.36	0.02	0.07	0.04	0.06	NS	
Condition * Time		NS	NS	0.05	0.13	NS	NS	NS	
Solvent * Condition * Time		4.58	0.96	0.07	0.18	0.10	NS	103.5	

Three independent experiments were performed and data are presented as mean per gram of dried jamun seed powder;

RT- Room temperature [25±2°C]; TPC- Total phenolic content; TPC was expressed as GAE - Gallic acids equivalent (GAE); TFC- Total flavonoid content; TFC was expressed as QE - Quercetin equivalent.

DPPH and ABTS values are expressed as mM trolox equivalent/ g dry jamun seed powder, obtained from a trolox solution having a free radical scavenging activity (IC50) equivalent to that of sample.

FRAP and CUPRAC values are expressed as mM trolox equivalent / g dry jamun seed powder, obtained from a trolox solution having reducing power equivalent to that of sample. CD- Critical Difference at 5% level of significance;

*α-glucosidase inhibitory activity was expressed as the amount of sample (in µg) required to inhibit 50% of the enzyme alpha-glucosidase (25 µg) activity (IC50) in the presence of substrate p-nitro phenyl glucopyranoside (25 µg)

Total flavonoid content (TFC)

The TFC varied between 1.06 mg/g for acetone to 10.81 mg/g for 80% aqueous methanol. Significant variation was observed for the yield of TFC in terms of solvent type and extraction condition. Among the absolute solvents used, methanol was found to be a better solvent for TFC extraction (6.68-6.82 mg at room temperature and 8.13-8.41mg under ultra-sonication) followed by ethanol. Absolute acetone was found to be poor solvent that could extract only 1.06 to 1.50 mg of TFC at room temperature and 1.94-3.98 mg TFC under ultra-sonication. The addition of water to the absolute solvents significantly increased the TFC extraction ability. Among the aqueous forms, 80% methanol extracted the maximum amount of flavonoids (10.81 mg) followed by 80% acetone (9.01 mg). The increase in extraction ability was found high for acetone followed by ethanol and methanol. The extraction condition significantly increased the TFC, and ultra-sonication extracted a high amount of TFC compared to room temperature, and the effect was high for acetone. Extraction time did not vary significantly among the condition and solvent system used for extraction of flavonoids, except for acetone and methanol. In case of acetone, 60 min extraction time significantly increased the TFC content under ultra-sonication (1.94 mg to 3.98 mg), but in case of methanol, 60 min extraction time significantly reduced the TFC content from 9.20 to 8.69 mg and 10.8 to 9.09 mg for absolute methanol and 80% methanol, respectively). From the study, it was found the aqueous form of methanol with ultrasonication for 30 min is the ideal condition for extraction of more TFC from jamun seed powder.

Effect of solvent type with varying polarity, conditions and time on antioxidant potential and alpha-glucosidase inhibitory activity.

Antioxidant potential

Jamun seeds contain a wide array of polyphenolic compounds with different characteristics, thus employing a single method to evaluate the total antioxidant potential may not be appropriate to accurately measure the antioxidant potential. Thus, in the present study four complementary methods *viz*. DPPH and ABTS radical scavenging activity, FRAP, and CUPRAC reducing power – based on the single electron transfer mechanism were tested to evaluate the antioxidant activity due to their simplicity, stability, and accuracy. The results obtained in the study were expressed as mM TE per g jamun seed powder (Table1). Significant differences were observed between various solvent systems and extraction conditions for antioxidant potential measured by various methods. The antioxidant potential measured by FRAP and ABTS method showed significant variation with respect to extraction time. Among the antioxidant methods employed, CUPRAC method measured higher antioxidant potential, followed by ABTS and DPPH. Correlation studies clearly showed that the total phenolics and flavonoids contents have a significant positive correlation with antioxidant potential [0.987**, 0.991**, 0.987**, 0.967** for TPC with FRAP, CUPRAC, DPPH and ABTS; and 0.884**, 0.891**, 0.881**, 0.882**for TFC with FRAP, CUPRAC, DPPH and ABTS, respectively, at the 0.01 level ($P<0.01$)] (Fig. 1, 2). Since the antioxidant potential of the plant samples is directly related to the total polyphenolic content, the polarity of the solvents had a similar response to the total antioxidant potential. Among the studied solvents with different polarities, absolute methanol extract showed higher antioxidant potential measured by all methods followed by ethanol. Absolute acetone extract showed lesser antioxidant potential. Antioxidant potential due addition of water to the solvents had a similar effect observed for TPC. Aqueous solvent extracts had significantly higher amount of antioxidant potential compared to their counterpart with absolute forms.

Samples extracted using ultra-sonication had significantly higher amount of antioxidant potential compared to the samples extracted under room temperature. The time of extraction did not vary significantly for antioxidant potential measured by CUPRAC and DPPH, while FRAP and ABTS significantly varied with the time duration of extraction. In most cases, radical scavenging values obtained for ABTS radical scavenging

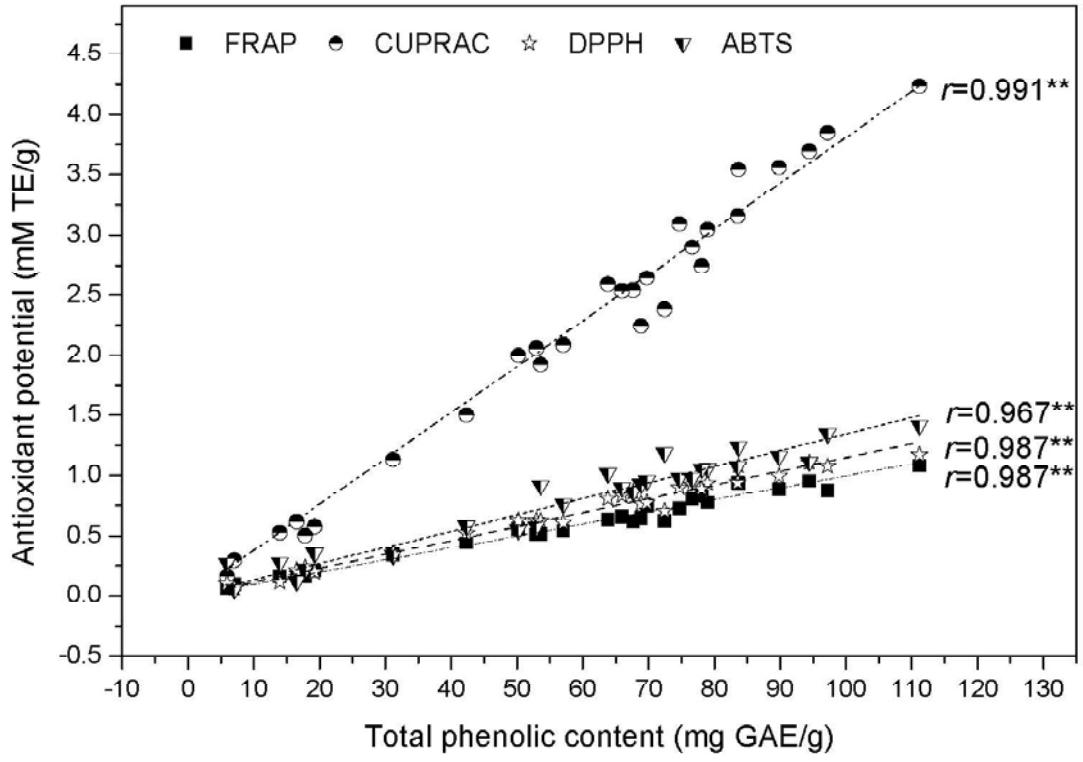

Fig. 1: Relationship between TPC and antioxidant potential.
**Correlation is significant at the 0.01 level (P<0.01).

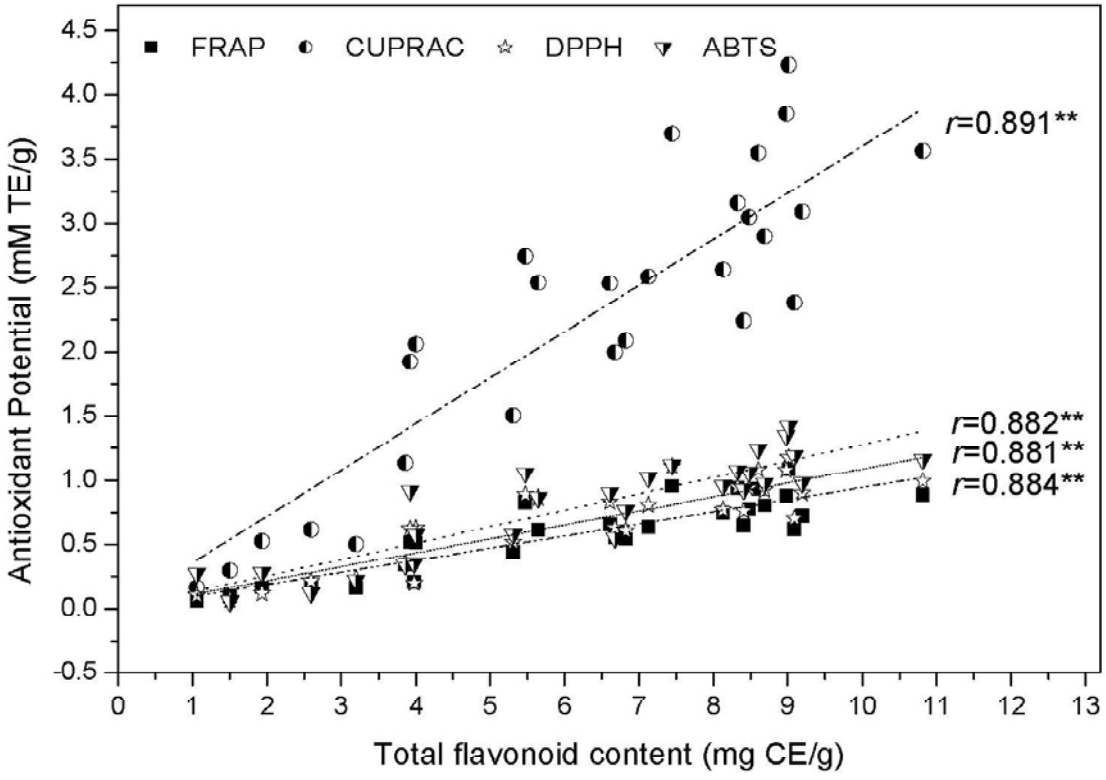

Fig. 2: Relationship between TFC and antioxidant potential.
**Correlation is significant at the 0.01 level (P<0.01).

activity were significantly higher than DPPH radical scavenging activity. This could be because of the ABTS radical's solubility in hydrophilic and lipophilic solvents, and its activity in a wide range of pH (Cano *et al.,* 1998). In case of reducing power, CUPRAC method resulted in higher reducing power compared to FRAP, and it is mainly due to the pH of the reaction medium. In FRAP method, the reaction is carried out in acidic pH (3.6) whereas CUPRAC assay is carried out in neutral pH (7.0). Acidic pH may cause a reduction in reducing power due to protonation of antioxidant compounds, while in neutral pH and basic conditions, antioxidant potential increases due to proton dissociation (Apak *et al.,* 2004).

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory action of the different solvent extract of jamun seed powder was assessed and the results are expressed as the amount of sample (in µg) required to inhibit 50% of the enzyme alpha-glucosidase (25 µg) activity (IC50) in the presence of substrate p-nitrophenyl glucopyranoside (25 µg) (Table 1). Lesser the amount required to inhibit the α-glucosidase enzyme indicated the better inhibitory activity. As stated earlier, the α-glucosidase inhibitory activity is mainly due to the presence of polyphenolic compounds, the solvent system which extracted higher polyphenolic compounds showed better α-glucosidase inhibitory activity. Total phenolic content and flavonoid content negatively correlated [r=-0.754** and -0.784**, at the 0.01 level (P<0.01), respectively] with the amount of jamun seed powder required to inhibit the α-glucosidase enzyme (Fig. 3, 4). Solvent types, extraction condition, and time duration for extraction significantly affected the α-glucosidase inhibitory activity of the extract. Among the absolute solvents, the amount of jamun seed powder extract required to inhibit the enzyme is lesser for methanol followed by ethanol irrespective of extraction condition and time duration of extraction. Significantly very high amount of jamun seed powder is required to inhibit the enzyme when extracted with absolute acetone. Among the aqueous solvents, 80 % acetone showed better inhibitory activity compared to 80% methanol and 80% ethanol. Solvent with extraction condition and extraction condition with time duration did not vary significantly for α-glucosidase inhibitory activity, but compared to 30 min, 60 min extraction duration had better α-glucosidase inhibitory activity especially for 80 % acetone extract. From this assay, it was found the aqueous form of acetone is a better solvent for extraction of more α-glucosidase inhibitory compounds under ultra-sonication for 60 min. Presence of significant amount of antioxidant compounds such as phenolic acids, and flavonoids attributed to jamun seed's medicinal values such as anti-diabetic properties (Ayya *et al.,* 2015). Laboratory studies revealed that the antidiabetic effect of jamun seeds are mainly due to their inhibitory activity on the major enzymes *viz* alpha-amylase and alpha-glucosidase which involves in hydrolysis of carbohydrate (Omar *et al.,* 2012). Kim *et al.,* (2016) stated that the phenolics can impact carbohydrate metabolism and help maintain blood glucose homeostasis through multiple pathways. Results from the present study corroborated the earlier reports that the increased amount of phenolics compounds with antioxidant potential can inhibit more amount of α-glucosidase, which helps in maintaining blood glucose homeostasis.

Polyphenolic compounds derived from plant sources gaining more interest among researchers, food manufacturers, and consumers due to their numerous health-benefits. Jamun seed powder is rich in polyphenolic compounds with both antioxidant and α-glucosidase inhibitory activity. The present study showed that the nature of solvent and extraction condition had a significant effect on TPC, TFC, antioxidant, and α-glucosidase inhibitory activity. The aqueous form of acetone (acetone: water 80:20, v/v) is the better solvent system for extraction of polyphenolic compounds with antioxidant and α-glucosidase inhibitory activity. Ultrasonication for 60 min facilitated higher extraction of phenolics.

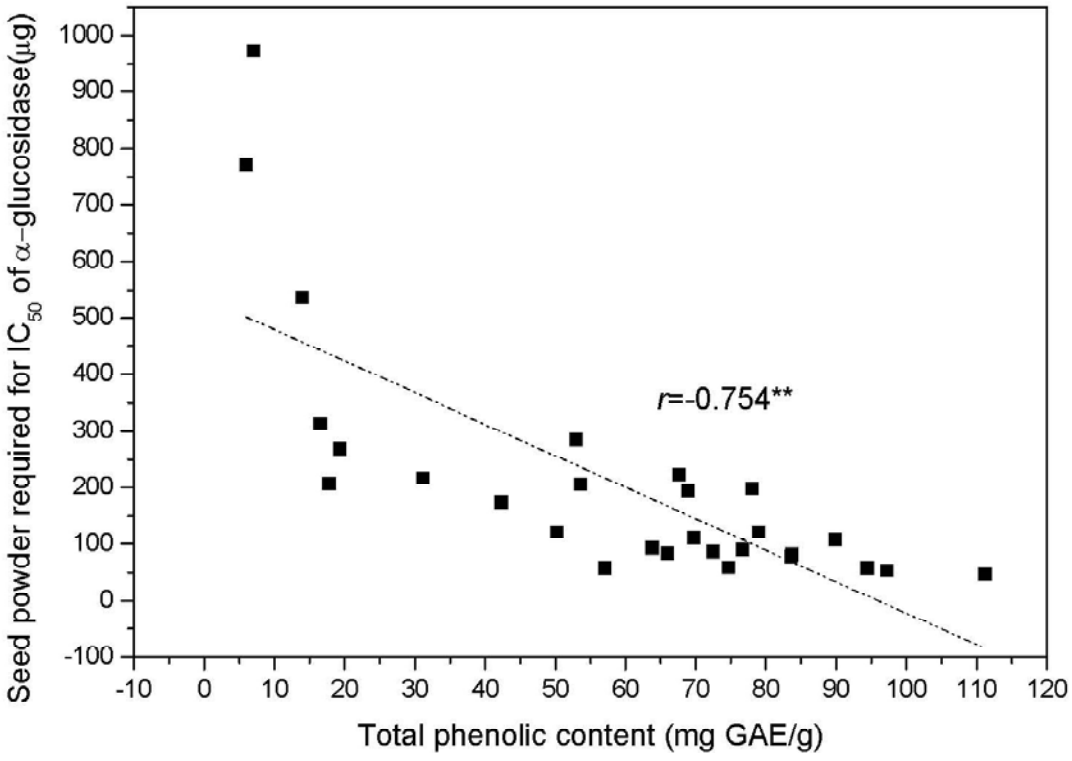

Fig. 3: Relationship between total phenolic content and α-glucosidase inhibitory activity.
**Correlation is significant at the 0.01 level (P<0.01).

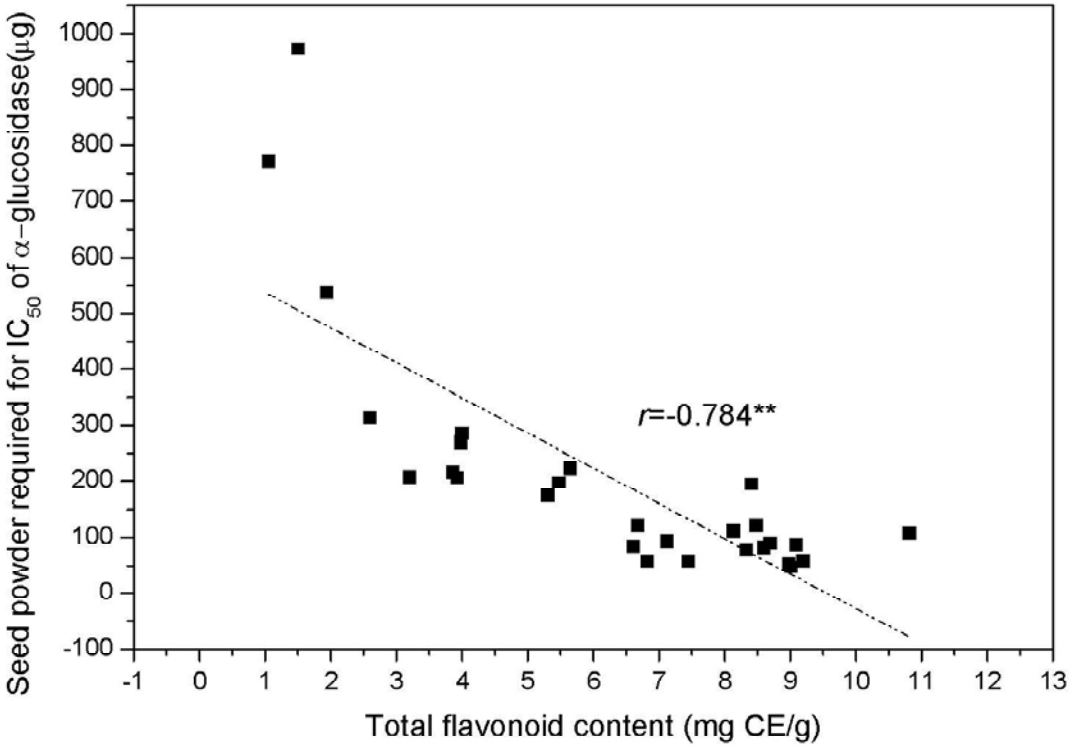

Fig. 4: Relationship between total flavonoid content and α-glucosidase inhibitory activity.
**Correlation is significant at the 0.01 level (P<0.01). 4. Conclusion

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