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Shikha Pandhi
Research Scholar,
Centre of Food Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi
Uttar Pradesh, India

Amrita Poonia
Assistant Professor,
Centre of Food Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi
Uttar Pradesh, India

Correspondence
Shikha Pandhi
Research Scholar,
Centre of Food Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi
Uttar Pradesh, India

Phytochemical screening of Jamun seeds using different extraction methods

Shikha Pandhi and Amrita Poonia

Abstract
Fruits contain a diverse assortment of bioactive compounds such as alkaloids, tannins, anthocyanins, flavonoids, phenolic acids, procyanidins that possess many functional properties such as antioxidant, anti-inflammatory, anti-diabetic, anti-carcinogenic properties and also protects against various degenerative and chronic diseases. These compounds are known to exhibit many health benefits and disease-preventive characteristics. The extraction of these phytochemicals from plant involves the use of various extraction techniques. *Syzygium cumini* (synonym *Eugenia jambolana*) or Jamun plant is known to possess diverse phytochemicals, most of which are observed to be of health benefits. The seeds are the most studied plant part. Polyphenols are present inherently in lots of fruits**,** which act as natural antioxidants and possess antioxygenic property with the advantage of low toxicity. The Antioxidant activity of phenolics in fruits is mainly because of their redox properties, which permit them to act as a reducing agent. The present study aims at screening various phytochemicals present in Jamun seeds extracted using different extraction methods both qualitatively and quantitatively.

Keywords: Phytochemicals, Jamun seeds, polyphenols, extraction

Introduction
Biochemical reactions occurring during biological processes such as respiration results in the generation of reactive oxygen species which when present in excess or are not eliminated properly leads to a condition called oxidative stress which initiates many diseases such as coronary heart diseases, stroke, diabetes, hypertension etc (Cai *et al*., 2004) [2]. Recently, there is an increasing interest in using natural antioxidants to protect human beings from the damage caused during the condition of oxidative stress (Scalbert *et al*., 2005) [14].
Many plant constituents possess the antioxidant property and oxygen scavenging activity as a metabolic response to endogenously generated free radicals during the various biochemical process (Grassmann *et al*., 2002) [4]. Among the diverse assortment of plant bioactive compounds, Phenolic compounds are known to possess good antioxidant property. The extraction of these phytochemicals from plant involves the use of various conventional extraction techniques such as Soxhlet extraction, maceration. The modern methods include microwave-assisted extraction (MAE), Ultrasonication assisted extraction (UAE), supercritical fluid extraction (SFE), solid phase microextraction (SPME), Soxhwave (Gupta *et al*., 2012) [5].
Syzygium cumini (synonym *Eugenia jambolana*) is a very large evergreen tropical tree belonging to the family Myrtaceae. India is the second largest producer of the jamun seeds in the world and contributes 15.4% in the world production of 13.5 million tonnes. Amongst the Indian states, Maharashtra is the largest jamun producer followed by Uttar Pradesh, Tamil Nadu, Gujarat, Assam, and others (Patil *et al*., 2012) [10]. Jamun plant is known to possess a diverse group of phytochemicals, most of which are observed to be of health benefits. The seedss are the most studied plant part and are reported to contain jambosine, gallic acid, ellagic acid, corilagin, 3,6-hexahydroxy diphenoylglucose, 4,6-hexahydroxydiphenoylglucose,1-galloylglucose, 3-galloylglucose, quercetin, β-sitosterol (Rastogi & Mehrotra 1990) [11]. The present study was undertaken to screen various bioactive compounds present in jamun seeds using three different methods of extraction namely soxhlet extraction, microwave-assisted extraction and ultrasonication assisted extraction. Phytochemical characterization of three extract variants was conducted both qualitatively and quantitatively for the presence of various bioactive compounds.

Materials and Methods

Materials

Dried Jamun (*Syzygium cumini*) seeds were procured from local market of Varanasi and were grounded into a coarse powder. High purity commercially available chemicals of AR grade were used in the investigation for different analyses.

Methods for preparation of Jamun seeds extract

Soxhlet extraction of Jamun seeds

The ethanolic extracts of *Syzygium cumini* seeds using Soxhlet apparatus were prepared as per the method described by Santhi (2016) [13] with slight modifications. 50 g of jamun seeds powder was mixed with the 250 ml of ethanol. The temperature was set at their boiling points and 10-12 cycles were run for concentrating the extracts. The rotary vacuum evaporator was used for further concentrating the extracts to a viscous mass which was then reconstituted at the concentration of 1 mg/ml.

Ultrasonication assisted Extraction

25 g of jamun seeds powder was mixed with 100ml of ethanol and ultrasonicated for 40 sec at 20 KHz. It was then subjected to shaking for 3 hours and then filtered using Whatman paper no.1. It was then concentrated using vacuum rotary evaporator at 50 ^{0}C and stored at 4-5 ^{0}C for further analysis.

Microwave-assisted extraction

Microwave-assisted extract of jamun seeds was prepared by mixing 10g of jamun seeds powder was mixed with 100ml of ethanol. It was then incubated in a microwave oven at 110° C for 50 sec. After incubation, the mixture was kept for shaking for 3 hours and then filtered using Whatman filter paper no.1. It was then concentrated using vacuum rotary evaporator at 50℃ and kept at 4- 5 ^{0}C for further analysis.

Phytochemical Screening of Extract

Qualitative Analysis

The phytochemical examination was conducted for all the three extracts prepared using three different extraction methods as per the standard methods described by Brain and Turner (1975) [1].

Detection of alkaloids: Extracts were dissolved individually in dil. Hydrochloric acid and filtered. The filtered acidified extracts were then subjected to the following tests:

- **Mayer's Test:** Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colored precipitate indicates the presence of alkaloids.
- **Wagner's Test:** Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.
- **Hager's Test:** Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of a yellow colored precipitate.

Detection of carbohydrates: Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were then used to test for the presence of carbohydrates.

- **Benedict's test:** Filtrates were treated with Benedict's reagent and heated gently. Orange-red precipitate indicates the presence of reducing sugars.
- **Fehling's Test:** Filtrates were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

Detection of glycosides: (Coumarin glycosides) Alcoholic extract when made alkaline, shows blue or green fluorescence indicating the presence of glycosides.

Detection of saponins

- **Froth Test:** Extracts were diluted with distilled water to makeup 20ml of volume and was then shaken in a graduated cylinder for 15 minutes. Formation of the foam layer of 1cm indicates the presence of saponins.
- **Foam Test:** 0.5 g of the extract was shaken with 2 ml of water. Persistence of foam for ten minutes indicates the presence of saponins.

Detection of phytosterol

- **Salkowski's Test:** Extracts were treated with chloroform and filtered. The filtrates were then added with few drops of Conc. Sulphuric acid, shaken and allowed to stand for some time. The appearance of golden yellow color indicates the presence of triterpenes in the extract.
- **Liebermann Burchard test:** Extracts were treated with chloroform and filtered. The filtrates were then treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was then added. The appearance of the brown ring at the junction indicates the presence of phytosterols.

Detection of phenols

- **Ferric Chloride Test:** Extracts were treated with 3-4 drops of 10% ferric chloride solution. Development of a bluish black color shows the presence of phenols.

Detection of tannins

- **Gelatin Test:** To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white color precipitate shows the presence of tannins in the extract.

Detection of flavonoids

- **Alkaline Reagent Test:** Extracts were treated with few drops of sodium hydroxide solution. Development of deep yellow color, which becomes colorless on the addition of dilute acid, shows the presence of flavonoids.
- **Lead acetate Test:** Extracts were treated with few drops of lead acetate solution. Development of yellow color precipitate shows the presence of Flavonoids.

Detection of proteins and amino acids

- **Xanthoproteic Test:** The extracts were treated with few drops of Concentrated Nitric acid. Formation of yellow color indicates the presence of proteins.
- **Ninhydrin Test:** To the extract, 0.25% w/v Ninhydrin reagent was added and boiled for few minutes. Development of blue color demonstrates the presence of amino acid.

Quantitative Analysis

Total phenolic content

The total phenolic content (TPC) was determined as per the

method is given by Hinneburg *et al*. (2006) [6] with some modifications. 0.5ml of diluted sample was added to 2.5ml of 0.2N Folin-Ciocalteau reagent and kept aside for 5 minutes. 2ml of 75g/L of Na_2CO_3 was then added to the reaction solution. The above solution was then kept for incubation at room temperature for 2 hours. Absorbance was measured at 760nm using 1cm cuvette UV-1800 spectrometer (Shimadzu, Japan). Gallic acid (0-800mg/L) was used to produce a standard calibration curve. The total phenolic content was expressed in mg of Gallic acid equivalent (GAE)/100ml of extract.

Radical Scavenging Activity (DPPH inhibition)

Determination of antioxidant activity of the sample was done using DPPH inhibition method given by Nishino *et al*. (2000) [8] with slight modifications. 0.5 ml of ethanol extract of jamun seeds was taken and to it 2.5mL of DPPH solution (8mg/100mL ethanol) was added. A control was set up with 0.5ml distilled water as blank and left at room temperature for 30 min. The sample sets were centrifuged at 3000rpm for 15 min. In cuvette 0.5 ml of centrifuged solution was taken and to it, 1mL of ethanol was added. Absorbance was taken at 517 nm separately for blank and samples using ethanol as a reference.

% DPPH inhibition = (AB - AS/AB) × 100

Where,

AB = OD for blank AS = OD for sample

FTIR Analysis

When infrared radiation passes through a material, some intensity passes through without interacting with the molecules, while the remainder interacts with molecules and is absorbed. The proportion of absorbed intensity over the total intensity that enters the material is in direct relation to the concentration of absorbing molecules. This is the principle of Beer-Lambert's law. The method used was described by Sacithraa *et al*. (2013) [12]. The sample was analyzed using FT-IR spectroscopy. The infrared light source generates a wavelength from 4000 to 400 cm^{-1} 32 times per sample with a resolution of 4. The infrared spectrum was Fourier transformed and recorded in the absorption mode. The FT-IR Interferogram was obtained between wave number and absorption. IR solution software was used for getting the spectrum.

Results and Discussion

The present investigation aims at "Phytochemical Screening of Jamun seeds using different extraction methods". In the initial stages of the study, jamun seeds were extracted for bioactive compounds using ethanol as a solvent by adopting three different methods of extraction such as Soxhlet extraction, Microwave-assisted extraction, ultrasonication assisted extraction resulted in three concentrated extracts that were subjected to both qualitative and quantitative analysis for the characterization of bioactive compounds present in them. Phytochemical characterization of three extract variants was conducted both qualitatively and quantitatively for the presence of various bioactive compounds. The characterization of extracts was mainly done for phenols and flavonoids so as to assess the antioxidant property of the extracts as well. Comparative analysis of the three extracts for various parameters such as antioxidant activity, total phenol content, qualitative phytochemical profile, and % yield was conducted.

Yield of Extracts

Jamun seeds were subjected to three different methods of extraction yielding three different extracts. The three methods adopted possess varying extraction yields. The yield of extracts extracted using three different methods of extraction are indicated in Table 1:

Table 1: Extraction yields of Jamun seeds extract by different methods

Method Of Extraction	% Yield
Soxhlet Extraction (SJE)	9.83±0.35
Ultrasonication assisted extraction (UJE)	12.76±1.45
Microwave-assisted extraction (MJE)	11.65±1.06

Among the three methods adopted for extraction, the maximum yield of 12.76±1.45% was obtained from ultrasonication assisted extraction. The result shows that the % yield of different extracts was influenced by the method of extraction adopted. Ultrasonication assisted extraction gives the highest % yield as indicated in the results. The reason for this may be the use of sound waves during extraction to obtain quantitative analyte leaching from the solid matrix using a suitable solvent, which gives little or no matrix release, so that matrix effects can be kept to a minimum during the measurement steps and hence results in higher % yield. A similar study conducted by Sun *et al*. (2011) [16] reveals that Ultrasonication assisted extraction gives highest extraction yield of some flavonoids such as tectoridin, iristectorin B, iristectorin A, tectorigenin, iris-tectorigenin A, and total isoflavones, in lesser time in comparison to maceration and Soxhlet extraction.

Phytochemical screening of extracts

Qualitative Analysis of Extract

Extraction involves separation of biologically active constituents from inactive components using suitable selective solvents. During extraction solvent diffuse into solid material and solubilizes the compound having similar polarity. The variations involved in different methods of extraction will affect the quantity and composition of secondary metabolites extracted (Pandey *et al*., 2014) [9]. Phytochemical screening of the three extracts was performed based on various qualitative methods. The results are indicated below in Table 2 where '+' sign indicates the presence of that compound or '-' sign indicates the absence (Figure 1).

Table 2: Phytochemical Screening of the JSE

Test For Detection	Soxhlet Extract	Microwave Extract	Ultrasonicated Extract
Saponins:			
a) Foam test-	+	-	-
b) Froth test-	+	-	-
Alkaloids:			
a) Mayer's Test-	-	+	+
b) Hager's Test-	-	-	-
c) Wagner's Test-	+	+	+
Carbohydrates:			
a) Fehling's Test-	-	-	-
Phenols:			
a) $FeCl_3$ test-	+	+	+
Tannins:			
a) Gelatin Test-	+	+	+
Flavonoids:			
a) Alkaline agent Test-	+	+	+
b) Lead acetate Test-	+	+	+
Glycosides:			
a) Coumarins-	-	-	-
Phytosterols:			
a) Salkowski's Test-	-	-	-
b) Liebermann Burchard Test	-	-	-
Proteins:			
a) Xanthoproteic Test-	-	-	-

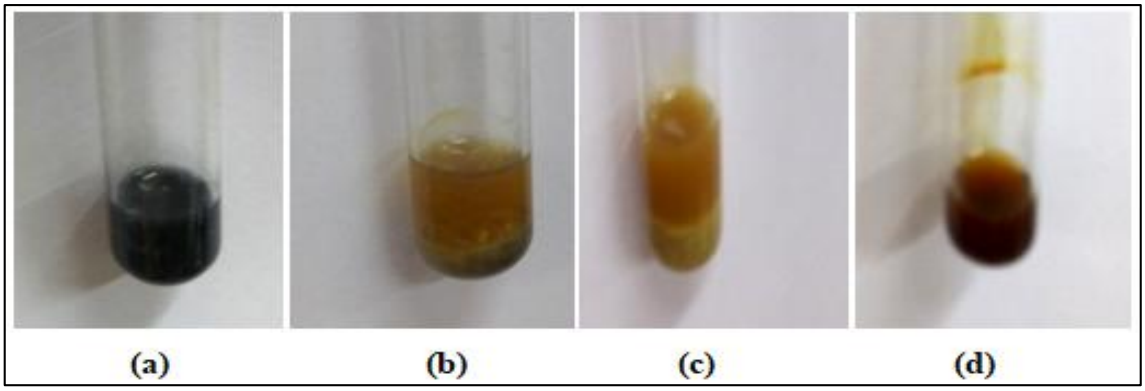

Fig 1: Showing "+" results of (a) Presence of Phenols (b) Presence of Tannins (Gelatin test) (c) Presence of Flavonoids (Lead acetate test) (d) Presence of Alkaloids (Wagner's Test).

Basic parameters influencing the quality of an extract are the part of the plant used as starting material, the solvent used for extraction and extraction procedure. Effect of extracted plant phytochemicals depends on:

- The nature of the plant material
- It's origin
- Degree of processing
- Moisture content
- Particle size

The possible cause of variation in different extraction methods that will affect the quantity and composition of secondary metabolite of an extract depends upon:

- Type of extraction
- Time of extraction
- Temperature
- Nature of solvent
- Solvent concentration
- Polarity

On comparing the results obtained from a phytochemical screening of three variants of extract we can conclude that there is the absence of saponins, Phytosterols, carbohydrates, glycosides in both microwave assisted and ultrasonication assisted extract but saponins were detected in the Soxhlet extract. All the three extracts show positive results for Phenols, Tannins, and Flavonoids.

Quantitative analysis of extracts

Total Phenolic Content

Using the standard curve, the total phenolic content of the three extracts was determined. The Total phenolic content of the three extracts was found to be 376.28±6.11, 399.39±2.75, 425.90±15.2 mg of gallic acid equivalent/100mL of extract for soxhlet extract, microwave extract & ultrasonicated extract respectively (Table 3). The results indicate that the ultrasonicated extract contains higher total phenols as compare to the other two extracts. A study was conducted by Cho *et al*. (2006) [3] indicated that UAE (Ultrasonicated assisted extract) of resveratrol from grapes was considered to be more effective than other conventional extraction methods as degradation of resveratrol from grapes may be negligible within a certain extraction time period with the use of UAE. This reveals high efficiency and efficacy of ultrasonication assisted extraction in the extraction of various bioactive constituents from the inactive solid matrix without any detrimental effects.

Antioxidant Activity (% DPPH inhibition)

The total antioxidant activity of ethanolic extracts was determined using DPPH radical scavenging assay. Based on DPPH assay the antioxidant capacity of the extracts was found to be 84.73±1.4, 94.03±1.6, 95.83±0.50% for Soxhlet, Microwave and ultrasonicated extract respectively. Ultrasonicated extract exhibit the highest % DPPH inhibition indicating high free radical scavenging property. Margaret *et al*. (2015) [7] evaluated the antioxidant activity of different parts of *Syzygium cumini (Linn.)* and reported that antioxidant activities of the plant extracts can be often explained by their total phenolics and flavonoid contents as higher phenolic content generally relate to high antioxidant activity. From Figure 2 it can be concluded that Total phenolic content has a positive relationship with antioxidant activity. It can be concluded from the results that UJE exhibit highest Total phenolic content as well possess the highest % DPPH inhibition among the three extracts. SJE contains less total phenols among the other variants and hence possess least antioxidant activity among others. % DPPH inhibition is directly related to the total phenolic content of the extract as these phenolic compounds were known to possess antioxidant property (Table 3).

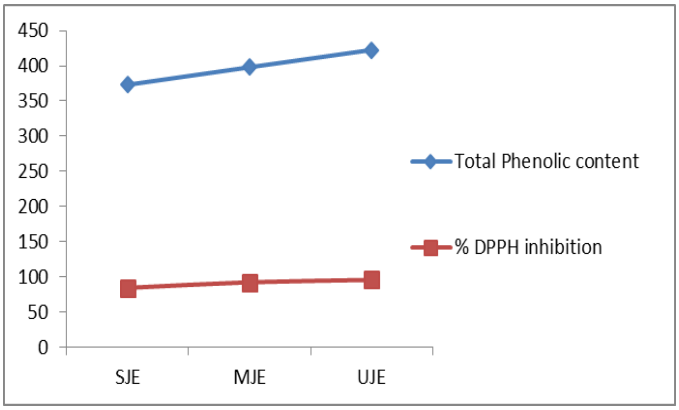

Fig 2: Relationship between Total Phenolic Content and% DPPH inhibition.

Table 3: Total Phenolic content and % DPPH inhibition of Three Different Extracts

JSE	Total Phenolic content (mg of gallic acid equivalent /100mL of extract	% DPPH inhibition
SJE	376.28±6.11	84.73± 1.4
MJE	399.39±2.75	94.03±1.6
UJE	425.90±15.2	95.83± 0.50

FTIR- Analysis of Extract

The resulting spectrum represents the molecular absorption creating a molecular fingerprint of the sample. Like a fingerprint, no two unique molecular structures produce the same infrared spectrum. The mid-infrared spectrum of range 4000–400 cm^{-1} can be approximately divided into four regions and the nature of a group frequency may generally be determined by the region in which it is located. The regions are generalized as follows:

- The X–H stretching region (4000–2500 cm^{-1})
- The triple-bond region (2500–2000 cm^{-1})
- The double-bond region (2000–1500 cm^{-1})
- The fingerprint region (1500–600 cm^{-1})

The fundamental vibrations in the 4000–2500 cm^{-1} region are generally due to O–H, C–H and N–H stretching. O–H stretching produces a broad band that occurs in the range 3700–3600 cm^{-1}. By comparison with standard values N–H stretching is usually observed between 3400 and 3300 cm^{-1}. Principle bands observed in the range 700-600cm^{-1} are due to out of plane =C–H bending. Carbonyl stretching is one of the easiest absorptions to recognize in an infrared spectrum. It is usually the most intense band in the spectrum and depending on the type of C=O bond, occurs in the 1830– 1650 cm^{-1} region. Functional groups were identified in Ultrasonicated jamun seeds extract by comparing corresponding functional group peak absorption value at a particular wave number with FT-IR Standards (Figure 3). Functional groups identified were listed in Table 4 along with standard FT-IR wavenumber range (Stuart B, 2004) [15].

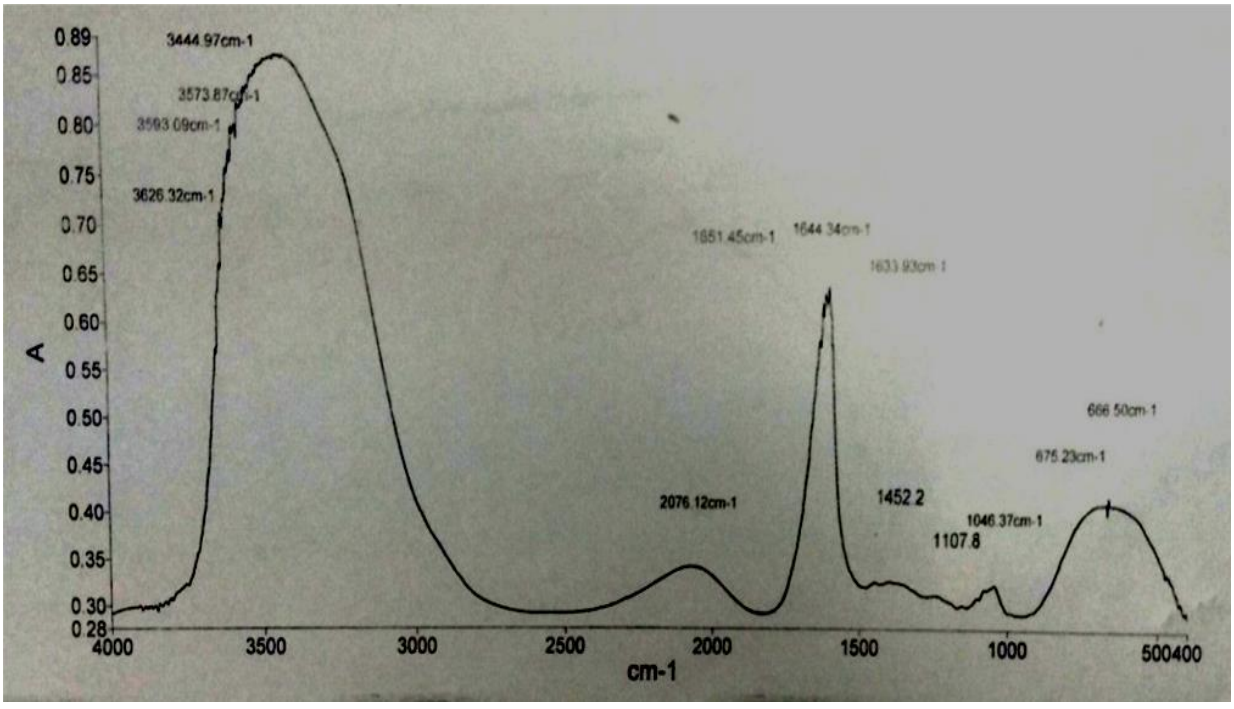

Fig 3: FT-IR Spectrum for Ultrasonicated *JSE*

Table 4: The functional group identified in Ultrasonicated *JSE* by FTIR Analysis

Wave Number (cm^{-1})	Wave number Range(cm^{-1})	Functional groups Identified
666.50	700–600	Alkynes =C–H bending (out of plane)
675.23	700–600	Alkynes =C–H bending (out of plane)
1046.37	1275–1000	In-plane C–H bending (Aromatic)
1107.8	1100	C–O stretching band (Ether)
1452.2	1485–1445	Methylene CH- bend
1633.93	1680–1600	C=C stretching
1644.34	1680–1600	C=C stretching(Alkene)
1651.45	1830–1650	C=O bond(Carbonyl)
2076.12	2300 -2050	C≡C(Alkyne)
3444.97	3500–3200	N–H stretching band (Heterocyclic)
3573.87	3620–3540	Tertiary alcohol, OH stretch
3593.09	3640–3530	Phenols, OH stretch
3626.32	3635–3620	O–H stretching band Secondary alcohol (Alcohol or Phenol)

Conclusion

The *Syzygium cumini* also known as the jamun plant contains a diverse assortment of secondary metabolites i.e. alkaloids, flavonoids, terpenoids, steroids, tannins, saponins and reducing sugars that play a vital role in preventing various diseases. The antidiabetic, anti-inflammatory, antiviral, anti-bacterial, antianalgesia, anti-oxidant, anti-abortifacient of the various parts of plants is due to the presence of diverse secondary metabolites. The phytochemical analysis of the plants is also important and pharmaceuticals companies for the novel drugs for the treatment of various diseases. The present study compares different methods for phytochemicals extraction. It also reveals various medicinally important bioactive compounds present in jamun seeds and justifies their use as a conventional medication for treatment of different diseases. Further purification, identification, and characterization of the bioactive chemical constituent's compounds would be our priority in future studies. Efforts should be geared up to exploit the biomedical applications of these screened fruit seeds due to the presence of a certain class of phyto compounds for their full usage.

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