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Screening of *Syzygium cumini* (L.) Seed Ethanol and Hexane Extracts for Phytochemicals, Antioxidant, and Anti-Diabetic Efficacy: An *In-vitro* Study

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Many medicinal plant extracts have been known since ancient times to possess antioxidant activity to scavenge free radicals and anti-diabetic activity to control diabetes. In this study, seeds of *Syzygium cumini* were extracted in ethanol and hexane solvents. Primary and secondary metabolites were quantified. DPPH assay, nitric oxide scavenging (NOS) assay and ferric reducing antioxidant power (FRAP) assays were employed to study antioxidant activity. α-amylase inhibitory assay (AAI), yeast glucose uptake assay (YGU) and haemoglobin glycosylation inhibitory (HGI) assays were adapted to determine anti-diabetic properties. The results from the assays and the

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IC_{50} values (18.35 µg/ml in DPPH, 943.8 µg/ml in NOS, 871.3 µg/ml in FRAP, 886 µg/ml in AAI, 764 µg/ml in YGU, and 1495.1 µg/ml in HGI assay) indicate that *S. cumini* seed ethanol extract has higher antioxidant and anti-diabetic efficiency than the hexane extract. Our findings suggest that the rich phytochemical content of *S. cumini* seeds and its good antioxidant and anti-diabetic activity may be responsible for its popularity and wide traditional use and can be exploited to develop antioxidant and anti-diabetic drugs.

Keywords: Secondary metabolites; medicinal plant; amylase inhibition; DPPH; ferric reducing antioxidant power.

1. INTRODUCTION

Phytochemicals are biologically active compounds in plants that normally protect them from various stress conditions such as UV light, pollution, pathogen attack, and different drought conditions [1]. Most of these are called secondary metabolites, and they provide colour, aroma, flavor and taste to the plant parts. A few to mention are phenols, flavonoids, alkaloids, sterols, tannins, etc. Enriched with these chemicals, products from medicinal plants provide unlimited opportunities for the discovery of new drugs because of their great chemical diversity and unmatched availability [2]. There are several studies in the literature indicating a great variety of pharmacological activities of phytochemicals [3].

Free radicals generated from normal physiology causes oxidative stress that damages cell structure, including lipids and membranes, proteins and DNA and also cause several diseases such as heart diseases, neurodegenerative diseases, cancer, the ageing process, and diabetes [4]. Antioxidants are substances produced by our body to protect it from free radicals [5]. Medicinal plants are well-known for their protective role as antioxidants which can be a good exogenous source to counter-balance the insufficient endogenous antioxidants.

Diabetes mellitus is one of the most common chronic metabolic disorders that causes the insufficient production of insulin, and uncontrolled conditions lead to many complications, including peripheral vascular disease, nephropathy, neuropathy, and retinopathy [6]. It can be controlled by inhibiting the enzymes that reduce glucose absorption or increasing their uptake in muscle cells. Inhibiting α-amylase causes a delay in glucose absorption, thereby leading to a postprandial decrease in blood glucose [7]. Herbal products are rich in alkaloids, tannins, flavonoids, phenolic compounds, terpenoids, and other secondary metabolite constituents that help reduce blood glucose levels [8].

Syzygium cumini (L.) a member of Myrtaceae, is commonly known as black plum or Indian blackberry in English, and naaval in Tamil is traditionally claimed in the treatment of many disorders. Pharmacological potential includes anti-diabetic, anti-hyperlipidaemic, antioxidant, anti-ulcer, hepato-protective, anti-allergic, anti-arthritic, antimicrobial, anti-inflammatory, and anti-diarrhoeal activities [9].

In this study, we focused on the screening of phytochemicals in *S. cumini* seed ethanol and hexane extracts and evaluated their *in vitro* antioxidant and anti-diabetic property.

2. MATERIALS AND METHODS

2.1 Plant Collection and Authentication

The seeds of *S. cumini* were collected from Tirunelveli district, Tamil Nadu, India. The plant and seeds were authenticated at the Plant Anatomy Research Centre (PARC), Chennai, and a voucher specimen was deposited at the herbarium of PARC for further reference [PARC/ 2021/ 4520]. The washed, air-dried, and powdered seeds were used for further extraction.

2.2 Extraction

Extraction was done by maceration method [10]. 50 g of the powdered seeds were soaked in 200 ml hexane and ethanol solvents separately and was kept in constant shaking on a flask shaker for 72 h at room temperature. The filtered and concentrated extract was used for assays.

2.3 Qualitative Phytochemical Analysis

A preliminary qualitative phytochemical analysis was carried out to identify the primary and secondary metabolites in the extracts [11].

2.4 Estimation of Moisture Content

1 g of powdered *S. cumini* seeds was dried in an oven at 110 °C for 1 hour; and the moisture content was calculated (% w/w) = [(initial weight of sample – final weight of sample) / weight of sample] x 100.

2.5 Primary Metabolites Quantification

2.5.1 Estimation of carbohydrates

Carbohydrate content was estimated following standard procedures with little modification [12]. To 100 µl of extract, 900 µl of water, 1 ml of 5% phenol and 5 ml of H_2SO_4 were added; and the absorbance was read at 490 nm. The carbohydrate content was calculated from the glucose standard graph.

2.5.2 Estimation of proteins

Protein content was estimated by a standard procedure with minor modifications [13]. 20 µl of extract was added to 980 µl of water and 3 ml of Coomassie brilliant blue; incubated for 5 min, and the absorbance was read at 595 nm. The protein content was calculated from a standard graph of bovine serum albumin.

2.5.3 Estimation of lipids

The lipid content was estimated by a standard procedure with simple modifications [14]. To 20 µl powdered *S. cumini* seeds, 10 ml of chloroform and methanol (1:1) were added; filtered after 15 min, 0.1 ml of conc. H_2SO_4 was added to 1 ml of the filter; heated in water bath for 10 min, 2.4 ml vanillin reagent was added, and the absorbance was read at 490 nm. The lipid content was calculated from the cholesterol standard graph.

2.6 Secondary Metabolites Quantification

2.6.1 Determination of flavonoids

Flavonoid content was determined by the aluminium chloride method with a few modifications [15]. 10 µl of extract was mixed with 500 µl of distilled water and 300 µl of 5% sodium nitrate, incubated for 5 min at 25 ºC, added to 2 ml of 1M NaOH and 300 µl of 10% aluminium chloride, and the absorbance was read at 510 nm. The total flavonoid content was expressed as quercetin equivalent (mg QE / g) of extract.

2.6.2 Determination of glycosides

Glycoside content was determined by a standard protocol with a few modifications [16]. 100 µl of extract was mixed with 1 ml of Baljet reagent (9.5 ml of 1% picric acid and 0.5 ml of 10% NaOH), diluted with 2 ml of distilled water after an hour, and the absorbance was read at 495 nm. The total glycoside content was expressed as a digoxin equivalent (mg DQ / g) of extract.

2.6.3 Determination of terpenoids

Terpenoid content was determined by a standard protocol with minor modifications [17]. 20 µl of extract was dissolved in 200 ml of methanol and 1.5 ml of chloroform; vortexed for 3 min, mixed with 100 µl of conc. H_2SO_4, incubated at 30 $^{\circ}$C for 2 h, supernatant was removed, 1.5 ml of 95% methanol was added to the reddish brown precipitate and the absorbance was read at 538 nm. The total terpenoid content was expressed as linalool equivalent (mg LE / g) of extract.

2.6.4 Determination of saponins

Saponin content was determined by a standard protocol with small modifications [18] 1 g of *S. cumini* seeds was mixed with 2 ml of 2% acetic acid and 10 ml of ethanol, heated in a water bath at 50 °C for 24 h, filtered and conc. NH_4OH was added as drops, the precipitate was collected and weighed. The total saponin content was expressed as the disogenin equivalent (mg DE / g) of *S. cumini* seeds.

2.6.5 Determination of phenols

Phenol content was determined by the Folin-Ciocalteau method [19]. 10 µl of extract was added to 1 ml of Folin-Ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate, shaken, and allowed to stand for 30 min. The absorbance was read at 725 nm. The total phenol content was expressed as gallic acid equivalent (mg GE / g) of extract.

2.7 *In-vitro* Antioxidant assays

2.7.1 DPPH radical scavenging assay

The DPPH assay was performed by the standard method with few modifications [20]. To 50 µl of extract (200, 400, 600, 800, and 1000 µg/ml), 50 µl of solvent (hexane or ethanol) was added; vortexed for 2 min, 2 ml of DPPH solution was added, incubated for 15 min, absorbance was

read at 515 nm, and the percentage inhibition of DPPH was calculated as: Inhibition (%) = [(OD of control – OD of sample) / OD of control] x 100. Ascorbic acid was used as a standard.

2.7.2 Nitric oxide radical scavenging (NOS) assay

Nitric oxide scavenging activity was performed by the standard method with few modifications [21]. 3 ml of 10 mM sodium nitroprusside in phosphate buffer was mixed with extract (200, 400, 600, 800, and 1000 µg/ml), incubated at 25 ºC for 2.5 h, 1.5 ml of Griess reagent was mixed with 1.5 ml of reaction mixture, made up to 100 ml with distilled water, absorbance was read at 546 nm and the scavenging effect of nitric oxide radical was calculated as: Inhibition (%) = [(OD of control – OD of sample) / OD of control] x 100. α-tocoferol was used as a standard.

2.7.3 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed by the standard method with few modifications [22]. Extract (200, 400, 600, 800, and 1000 µg/ml) was made up to 500 µl with sodium phosphate buffer, incubated in a water bath at 50 ºC for 20 min, 1 ml of 10% trichloro acetic acid was added, centrifuged at 3000 rpm for 10 min, 1 ml of the upper layer was mixed with 1 ml of distilled water and 500 µl of 1% ferric chloride, absorbance was read at 700 nm and the reducing effect was calculated as: Inhibition (%) = [(OD of control – OD of sample) / OD of control] x 100. Ascorbic acid was used as a standard.

2.8 *In-vitro* Anti-diabetic Assays

2.8.1 α-amylase inhibitory (AAI) assay

The α–amylase assay was performed by the 3, 5-dinitrosalicylic acid (DNSA) method [23]. To the extract (200, 400, 600, 800 and 1000 µg/ml) in 200 µl phosphate buffer, 50 µl of 1% α-amylase and 100 µl of 1% starch in 50 mM phosphate buffer were added, incubated at 37 ºC for 30 min, 400 µl DNSA reagent was added, placed in a water bath for 5 min, made up to 15 ml with distilled water, absorbance was read at 540 nm, and the percentage inhibition of α–amylase was calculated as: Inhibition (%) = [(OD of control – OD of sample) / OD of control] x 100. Acarbose was used as a standard.

2.8.2 Yeast glucose uptake (YGU) assay

The yeast glucose uptake assay was performed by the standard method with a few modifications [24]. 1 g of yeast was dissolved in 10 ml of distilled water; clear 1 ml suspension was added to extract (200, 400, 600, 800 and 1000 µg/ml), 200 ml of 50 mM glucose was added, incubated for 3 min, centrifuged at 3000 rpm for 5 min, to the 500 µl supernatant, 400 µl of dinitrosalicylic acid reagent was added; boiled in a water bath for 5 min, made up to 15 ml with distilled water, absorbance was read at 540 nm; and the percentage of glucose uptake was calculated as: Glucose uptake (%) = [(OD of control – OD of sample) / OD of control] x 100. Metronidazole was used as a standard.

2.8.3 Haemoglobin glycosylation inhibitory (HGI) assay

The haemoglobin glycosylation inhibitory assay was performed by the standard method with minor modifications [25]. 2% glucose, 0.06 % haemoglobin, and 0.02% azithromycin were mixed in 10 ml of 0.01 M phosphate buffer (pH - 7). To this 1 ml of buffer and extract (200, 400, 600, 800, and 1000 µg/ml) were added, made up to 1 ml with distilled water, incubated for 72 h, absorbance was read at 720 nm, and the percentage inhibition of haemoglobin glycosylation was calculated as: Inhibition (%) = [(OD of control – OD of sample) / OD of control] x 100. Aspirin was used as a standard.

3. RESULTS

3.1 Phytochemicals in *S. cumini*

Table 1 lists the phytochemicals in the hexane and ethanol extracts of *S. cumini* seeds as examined by qualitative analysis. The ethanol extract reveals a high content of flavonoids, tannins, phenols, terpenoids, sapnonins, glycosides, anthraquinones, carbohydrates, and a moderate level of proteins. Alkaloids and steroids are absent from the extract. The hexane extract is rich in alkaloids, flavonoids, phenols, saponins, and proteins but poor in terpenoids, glycosides, and carbohydrates, and completely lacks tannins, steroids, and anthraquinones.

Table 1. Phytochemicals in *Syzygium cumini* seed

Phytoconstituents	Ethanol	Hexane
Alkaloids	-	+++
Flavonoids	+++	+++
Tannins	+++	-
Phenol	+++	+++
Steroids	-	-
Terpenoids	+++	++
Saponin	+++	+++
Coumarin	-	-
Glycosides	+++	++
Anthroquinones	+++	-
Carbohydrate	+++	++
Protein	++	+++

(+: mild, ++: moderate, +++: high, - : Absence)

Table 2. Quantified primary and secondary metabolites in *S. cumini* seeds

Constituents	Ethanol extract	Hexane extract
Carbohydrate	58.43 mg/g	16.93 mg/g
Protein	2.53 mg/g	3.06 mg/g
Lipid	111.61 mg/g*	
Phenol	296 mg GE/g	135.6 mg GE/g
Flavonoid	54.12 mg QE/g	41.31 mg QE/g
Terpenoid	16.64 mg LE/g	0.42 mg LE/g
Glycoside	229 mg DQ/g	201 mg DQ/g
Saponin	186.2 mg DE/g*	

*(GE: gallic acid equivalent, QE: quercetin equivalent, LE: linalool equivalent, DQ: digoxin equivalent, DE: disogenin equivalent, *: in raw seeds*

Table 3. Calculated IC_{50} of *S. cumini* seed extracts and reference compounds in antioxidant assays

Assay	Sample	IC_{50} (µg/ml)
DPPH	*S. cumini* seed ethanol extract	18.35
	S. cumini seed hexane extract	145.47
	Ascorbic acid	21.97
NOS	*S. cumini* seed ethanol extract	943.8
	S. cumini seed hexane extract	1154
	α-tocopherol	42.5
FRAP	*S. cumini* seed ethanol extract	871.3
	S. cumini seed hexane extract	2020
	Ascorbic acid	44.7

The moisture content was estimated to be 10% in *S. cumini* seeds. The phyto-compounds in *S. cumini* seeds were quantified and are represented in Table 2. Total carbohydrate content was 58.43 mg/g in ethanol extract and 16.93 mg/g in hexane extract. Total protein content was 2.53 mg/g in ethanol extract and 3.06 mg/g in hexane extract. Total lipid content was 111.61 mg/g in *S. cumini* seeds. Secondary metabolites in the ethanol and hexane extracts of *Syzygium cumini* seed were quantified and expressed as milligram equivalents of the standard reference compound in one gram of the extract. The phyto-constituent content was estimated as: 54.12 and 41.31 mg QE/g flavonoid in ethanol and hexane extract respectively; 16.64 and 0.42 mg LE/g terpenoid in ethanol and hexane extract, respectively; 229 and 201 mg DQ/g glycoside in ethanol and hexane extract, respectively; 296 and 135.6 mg GE/g phenol in ethanol and hexane extract, respectively; and 186.2 mg DE/g saponin in *S. cumini* seed.

3.2 Antioxidant Activity of *S. cumini*

The antioxidant potential of *S. cumini* was tested with the three widely accepted methodologies, and the results are represented as a graph in Fig. 1. In all the assays, one could clearly witness that *S. cumini* seed ethanol and hexane extracts showed a concentration-dependent increase in the percentage inhibition of the reactive species. To specify, in the DPPH test at 1000 µg/ml, the highest concentration used in the study, the ethanol extract inhibited 87.74% of the radicals while the hexane extract inhibited 77.25% of the free radicals. A similar curve pattern was also exhibited by the NOS assay, where the ethanol extract at 1000 µg/ml scavenged 54.39% of the nitrite radicals and the hexane extract scavenged 44.05% of the nitrite radicals at the same concentration. Likewise, in the FRAP assay, another significant assay to deduce the antioxidant power, at 1000 µg/ml, 64.96% reducing power was visible in the ethanol extract, while only 21.4% reducing power was seen in the hexane extract. With regard to the reference standard antioxidants, ascorbic acid and α-tocopherol, used in the assays, they demonstrated a higher efficiency in their antioxidant power at lower concentrations than predicted (Fig. 2). IC_{50} values were calculated using a dose-response linear / logarithmic fitting curve and are tabulated in Table 3. All the results together suggest the higher antioxidant ability of the ethanol extract over the hexane extract of *S. cumini* seeds.

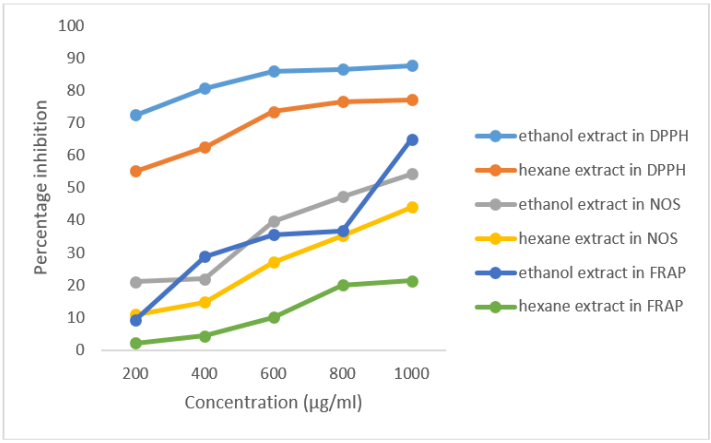

Fig. 1. Graph showing percentage inhibition of *Syzygium cumini* seed ethanol and hexane extracts in DPPH radical scavenging, nitric oxide radical scavenging and ferric reducing antioxidant power assays

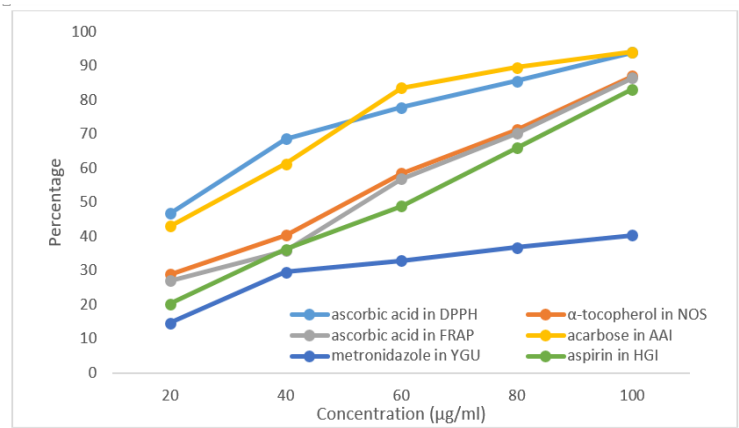

Fig. 2. Graph showing values of reference compounds in *in vitro* antioxidant and anti-diabetic assays

3.3 *In-vitro* Anti-diabetic Activity

The anti-diabetic efficacy of *S. cumini* was evaluated *In vitro* with three common protocols, and the effects are depicted as a graph in Fig. 3. In all the assays, *S. cumini* seed ethanol and hexane extracts showed a well-defined concentration-dependent increase in either inhibiting α-amylase, or up take of glucose by yeast, or inhibiting haemoglobin glycosylation. This is evidence to confirm the anti-diabetic properties of the extracts. To specify, in α-amylase inhibitory assay at 1000 µg/ml, the highest concentration used in the study, the ethanol extract inhibited 56.34% of the enzyme while the hexane extract inhibited 42.16% of the enzyme. A comparable curve was also seen in the yeast glucose uptake assay, where the presence of ethanol extract at 1000 µg/ml increased glucose uptake up to 62.38%, and in the presence of hexane extract, glucose uptake was 52.43% at the same concentration. Similarly in the haemoglobin glycosylation assay, another significant assay to detect the anti-diabetic potency, at 1000 µg/ml 46.57% inhibition of haemoglobin glycosylation was observed in the ethanol extract and around 36.2% inhibition of haemoglobin glycosylation was seen in the hexane extract. With regard to the reference compounds, acarbose, metronidazole, and aspirin, used in the assays, they established a higher anti-diabetic effect when compared to the plant extracts at lower concentrations, as expected (Fig. 2). Also, the IC_{50} values were calculated using a dose-response linear / logarithmic fitting curve and are tabulated in Table 4. The results substantiate that the ethanol extract of *S. cumini* seeds is more anti-diabetic than the hexane extract.

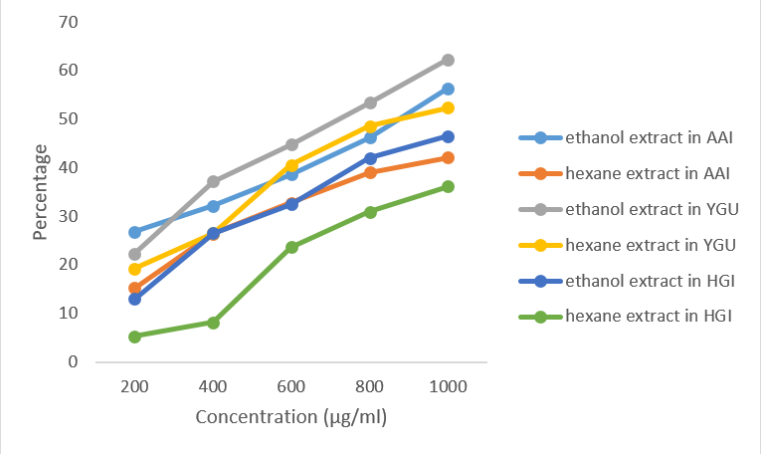

Fig. 3. Graph showing percentage values of *Syzygium cumini* seed ethanol and hexane extracts in inhibiting α-amylase, up taking of glucose by yeast and inhibiting haemoglobin glycosylation

Table 4. Calculated IC_{50} of *S. cumini* seed extracts and reference compounds in anti-diabetic assays

Assay	Sample	IC_{50} (µg/ml)
AAI	*S. cumini* seed ethanol extract	886
	S. cumini seed hexane extract	1116.9
	Acarbose	25.02
YGU	*S. cumini* seed ethanol extract	764
	S. cumini seed hexane extract	886
	Metronidazole	179.4
HGI	*S. cumini* seed ethanol extract	1495.1
	S. cumini seed hexane extract	2038
	Aspirin	58.8

4. DISCUSSION

Medicinal plants such as *Syzygium cumini* and many others have been used worldwide since ancient times for the treatment of various diseases, including asthma, abdominal disorders, skin diseases, respiratory and urinary complications, and liver and cardiovascular disease [26]. The chemicals in the plant established their protective role.

In our study, many compounds were detected in *Syzygium cumini* seed ethanol and hexane extracts. This is similar to the reports on the phytochemicals in *S. cumini*'s various plant parts recorded by many authors, especially in aqueous and methanol leaf extracts [27], ethanol, methanol, choloroform, petroleum ether, and water leaf extracts [28], hydro-alcoholic leaf extract [29], and methanol seed, leaf, and bark extracts [30]. There are also GC-MS studies that have revealed new compounds from the hexane and ethanol extracts of *S. cumini* seed, such as: oleic acid, n-hexadecanoic acid, cyclooctasiloxane, hexadecamethyl, octadecanal, and 2-bromo [31,32].

Although several researchers have performed *in vitro* antioxidant activities of various parts of *S. cumini* using different methodologies such as DPPH, FRAP, ABTS, hydroxyl radical scavenging assay and nitric oxide assay [33-35], in our study with DPPH, FRAP and NOS assays for ethanol and hexane seed extracts of *S. cumini*, we could confirm from the above research reports that the percentage of inhibitions had gradually increased from lower to higher concentrations in a concentration-dependent manner that is congruent to our results. In our study, our IC_{50} value suggests that the ethanol extract of *S. cumini* seed is more potent in inhibiting the radicals. By IC_{50} value definition, the lower the IC_{50} greater is its potential to scavenge. The highest activity in ethanol extract can be correlated with the phytochemicals in the extract. Being a polar solvent, ethanol extract is found to test positive for flavonoids, tannins, phenols, terpenoids, saponins, quinones and glycoside compounds. On the other hand, hexane, being a non-polar solvent, has only few compounds in it, which led to a lowering of its antioxidant activity when compared to the ethanol extract [29].

This is the first record of alpha-amylase inhibition, yeast glucose uptake, and haemoglobin glycosylation inhibition in *S. cumini.* α-amylase inhibitors inhibit the enzyme α-amylase to reduce hyper-postprandial blood glucose. In yeast glucose uptake assay, the excess of glucose left in the medium after a particular time is considered an indicator of glucose uptake by yeast cells, which mimics higher animal cells. Good anti-diabetic drugs inhibit the formation of haemoglobin glycosylation. Our study confirms both the hexane and ethanol extracts of *S. cumini* seed to be effective, and it is likely that the ethanol extract of *S. cumini* exhibits a greater potency to inhibit the pathogenesis of diabetes than the hexane extract when tested using *in vitro* models. Studies on several other plants claimed to be anti-diabetic have substantiated this, and the inhibitory activity can certainly be attributed to the phytoconstituents in the plants [36]. In many reports, ethanol extract is found to be more efficient than hexane extract [37], and ethanol extract is known to exhibit better pharmacological activity than most other tested extracts [38]. Our study validates previous research [39].

5. CONCLUSION

In this study, we have shown that both the hexane and ethanol extracts of *Syzygium cumini* seeds have concentration-dependent inhibitory activity towards radicals and anti-diabetic properties. However, the effects are more potent in ethanol extract than in hexane extract, and it seems that compounds of high polarity are most potent. We have also screened the phenol, flavonoid, terpenoid, saponin, and glycoside content of the extract. Our results indicate that the hexane and ethanol extracts of *Syzygium cumini* seeds are enriched with antioxidants and thus probably prevent oxidative stress-related disorders. It also appears that several different compounds mediate even the anti-diabetic efficiency of the extract, as witnessed by the amylase inhibition, haemoglobin glycosylation inhibition, and yeast glucose uptake. Although the exact *in vivo* mechanism of action remains unexplored, subsequent studies might open up new facts about its inherent properties.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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