Research Article

Syzygium Cumini : An Effective Cardioprotective via its Antiglycoxidation Potential

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ABSTRACT

Free radicals and glycation play an important role in the manifestation of diabetes and other cardiovascular diseases. Seeds of *Syzygium cumini* are traditionally used in Ayurveda and Unani medicine to fight against diabetes. In the present study, the antiglycoxidative potential of *S. cumini* was measured and the effect of *S. cumini* in glucose induced cardiac stress was observed. FTIR and HPLC studies were conducted for aqueous (ASE), ethanol (ESE) and methanol seed extracts (MSE) and a comparison of antiglycoxidative potential was studied. The antioxidative activities were evaluated by 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis 3- ethylbenzthiazoline-6-sulphonic acid (ABTS), nitric oxide (NO), hydrogen peroxide (H_2O_2), superoxide (O_2-) and lipid peroxidation assays. The safe dose of MSE and glucose was optimized on H9C2 cells by MTT assay and cell size was observed microscopically. Antiglycation potential of MSE was also estimated in glucose induced cardiac cells. MSE showed the maximum antiglycoxidative potential among ESE and ASE comparable to standard. The highest peak intensity in FTIR spectra of MSE for aliphatic and aromatic C-H stretching, aromatic C=C bonds and C-O single bonds. HPLC showed the gallic acid content (retention time at 2.33 ± 1.97 min) highest in MSE. MSE significantly suppresses the glucose induced stress on H9C2 cardiac cell lines by inhibiting glycation event. Our findings suggest that *S. cumini* MSE has maximum antiglycoxidative potential as compared to ESE and ASE, therefore proposed to play therapeutic role for diabetic complications associated with heart.

Keywords: Anti-AGEs potential, Antioxidant activity, Gallic acid, Polyphenol, Flavonoids.

INTRODUCTION

Reactive oxygen species (ROS) is an essential product of biochemical and physiological processes and normally they exist in balance with biochemical antioxidants.[1] Environmental stress increases the levels of free radicals drastically, thereby disturbing the equilibrium between free radical production and the antioxidant capability causing oxidative stress because of excess ROS, antioxidants depletion, or both.[2] However, when cellular production of ROS overwhelms its antioxidant capacity, damage to cellular macromolecules such as lipids, protein and DNA may ensue.[3] This damage has been associated with an increased risk of diseases such as diabetes, cardiovascular diseases, cancer, etc. Glycation and oxidative stress are closely linked and synergistic and are often referred as glycoxidation.[4] Free radicals have also been shown to participate in advanced glycation end products (AGEs) where nonenzymatic glycation of proteins alter their structure and functions, which further induce the cellular damage. Glycoxidation intensity increases in diabetes mellitus and its associated pathophysiologies as they can alter enzymic activity, modulate ligand binding to their receptor, modify protein functional ability and alter immunogenicity.[5] Hyperglycemia is considered as a clinical hallmark of diabetes, the phenomenon that results into the formation of AGEs as well as upregulation of reactive oxygen species. It is therefore essential to develop antiglycoxidative therapies against a global widespread incidence of diabetes and its complications in the recent time. Butylated hydroxyl toluene (BHT) and butylated hydroxyanisole (BHA) have been used as synthetic agents but they are reported to be toxic to human health shifting the focus towards herbal remedies.[6] Antioxidants can be extracted and purified from natural sources and used in medicine and as additives to neutraceuticals. Plant polyphenols have drawn increasing attention due to their potent antioxidant properties and their marked effects in the prevention of oxidative stress in diseases such as diabetes, diabetic associated cardiomyopathy, neuropathy and nephropathy.[7] The quantity and quality of the extracted antioxidant polyphenolics varies owing to the difference in the polarity of the solvent used for the extraction of phenolics from natural sources.[8,9] This makes it essential to analyze the fraction with maximum antioxidative potential in order to develop better and effective therapeutic strategies. Presently the drugs often prescribed for patients with diabetes have a limited ability to cure and can create more problems in the long run in different organs like eyes, kidneys, nerves and cardiovascular system.[10] In addition recent studies have shown that the drugs with combined antioxidative and antiglycative potential are more effective in treating diabetes mellitus.[11] We therefore selected *Syzygium cumini* (L.) Skeels, (Myrtaceae) having antidiabetic potential and aimed to evaluate antiglycoxidation potential of aqueous, ethanol and methanol extracts. Antihyperglycemic studies have been conducted extensively with aqueous extract of *S. cumini* fruit pulp, leaf and bark in the past *in vitro* and *in vivo*[12-14] however such antihyperglycemic effect of aqueous extract of *S. cumini* in patients with diabetes, could not be ruled out in

clinical trials conducted by Teixeira in 2004.[15] Due to such failure in results, we prepared various extracts of *S. cumini* seeds in different solvents and analyzed the pharmacologically most active *S. cumini* extract. In our previous study, we chose *S. cumini* seed, an unedible part and phytochemical screening of aqueous, ethanol, methanol, chloroform, n-hexane, benzene and di-ethyl ether seed extracts of *S. cumini* and methanol extract was found to be most enriched in phytocontents and reducing potential.[16] Also methanol extract of *S. cumini* had the maximum potential to suppress the intracellular ROS mediated stress induced in cardiac myocytes by various agents.[17,18] In the present study, we extended our research to compare the antiglycation and antioxidative potential of aqueous, ethanol and methanol *S. cumini* seed extracts and these effects were also validated *in vitro* using glucose stressed cardiac myocytes as hyperglycemia is reported to exert stress on heart leading to unignorable diabetic cardiomyopathies. Such a study would help in understanding the mechanism and the therapeutic application in preventing the diabetic complications associated with heart, that constitute a major problem with a life-threatening impact for diabetic patients worldwide.

MATERIALS AND METHODS

Methanol, gallic acid, sodium carbonate, vanillin, aluminium chloride, potassium acetate, ferric thiocyanate (FTC), ammonium thiocyanate, linoleic acid, potassium ferricyanide (K3[Fe(CN)6]), trichloroacetic acid (TCA), butylated hydroxy toluene (BHT) and ferrous chloride (Fecl3) were purchased from Qualigens. 2-Thiobarbituric acid (TBA), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2,-2´azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), potassium persulphate, Griess reagent, sodium nitroprusside, hydrogen peroxide, glacial acetic acid, acetonitrile, nitroblue tetrazolium bromide (NBT), -NADH, phenazine methosulfate, glucose, bovine serum albumin, gallic acid, potassium bromide (KBr) etc. were purchased from Sigma Aldrich.

Seed collection

Seeds were collected in the month of June - July and were identified. The authentication was done by Dr. Anshu Rani, Department of Botany, Govt. P.G. College, Abu Road, Rajasthan, India. They were washed and dried and ground. The powder was collected for the preparation of aqueous (ASE), ethanol (ESE) and methanol seed extracts (MSE).

Preparation of aqueous and organic solvent extracts of *S. cumini* seeds

Powdered seeds (5 g) were mixed in 100 ml of boiled water and kept for 2-4 h at 60°C for the preparation of aqueous extract. The extract was filtered and centrifuged at 5000 rpm at room temperature and the supernatant was dried for quantified at the concentration of 1 mg/ml. Ethanol and methanol extracts of *S. cumini* seeds were prepared by using Soxhlet solvent extraction method.

Seed powder (20 g) was mixed with the 200 ml of ethanol and methanol respectively. The temperature was set at their boiling points and 12-14 cycles were run for complete. The rotary vacuum concentrator was used for drying the extracts and the dried mass of ethanol and methanol extracted polyphenolics were weighed and reconstituted at the concentration of 1 mg/ml.

Antiglycation assay

Antiglycation assay was performed by the method of McPherson.[19] The reaction mixture contained 10 mg/ml BSA, 50 mg/ml glucose, and the extract at the conc. of 0.2-1 mg/ml in the proportion of 20 µl each. Glycated control contained 20 µl of, 10 mg/ml BSA, 50 mg/ml glucose and sodium phosphate buffer while blank contained the same except glucose. Reaction mixture was incubated for 7 days at 37°C. After incubation, TCA was added and centrifuged at 13000 rpm for 10 min. Supernatant was discarded and pellet containing AGEs-BSA was dissolved in 60 µl PBS. Florescence spectrum based on AGEs formation was monitored by using spectroflourometer (Shimadzu). Percent inhibition was calculated by following formula: % inhibition = 1-[(Fluorescence of sample)/Fluorescence of glycated control)] X 100

Fourier transform infrared analysis

FTIR analysis was performed for the aqueous (ASE), ethanol (ESE) and methanol extracts (MSE) of *S. cumini* seeds in order to identify the bonds and the functional groups present in the extracts. The extracted fractions of *S. cumini* seeds were dried overnight to remove humidity and encapsulated in dried potassium bromide (KBr) discs. Transmission mode of these discs was scanned using a Fourier transform infrared spectrometer (Shimadzu) within a wavelength range of 400 to 4000 cm^{-1}.

Estimation of total flavonoids

Aqueous, ethanol and methanol extracts (1 ml each) were mixed individually with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water and kept at room temperature for 30 min. The absorbance of the resulting mixture was taken at 420 nm with UV visible spectrophotometer (Shimadzu). The flavonoid content was determined by gallic acid standard curve.

High performance liquid chromatography

HPLC was performed by taking gallic acid as the reference standard. Aqueous, ethanol and methanol extracts were prepared and clarified by low speed centrifugation followed by filteration by syringe filters before loading on HPLC system (Waters). The final concentration was set as 10 µg/ml as stock. HPLC analyses was carried by using a HPLC system and equipped with high pressure binary pump and photodiode array detector. The column specification and solvent systems are as follows: Column, C-18 Preparative, 4.6 x 250 mm size, particle size 15 µ. Mobile phase: Solvent - methanol:acetonitrile (90:10),

total flow rate - 1mL/min, wavelength range 200 - 450. The polyphenols were detected with that of standards by comparing retention times and UV-VIS spectra (Shimadzu).

Antioxidant activities of ASE, ESE and MSE of S. cumini seeds

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH free radical scavenging activity of *S. cumini* ASE, ESE and MSE was estimated by the method of Brand-Williams.[20] Equal volumes of extract at various concentrations were mixed with 0.135 mM DPPH prepared in methanol. The reaction mixture was incubated in dark for 30 min. The absorbance was measured spectrophotometrically at 517 nm against the blank. The scavenging ability of all the three extracts was calculated using the following equation:

$$\frac{OD_{control} - OD_{sample}}{OD_{control}} *100$$

where $OD_{control}$ is the absorbance of DPPH + methanol; OD_{sample} is the absorbance of DPPH radical + sample (i.e. extract or standard). The data was represented as the mean of triplicates. The scavenging activity of seed extracts was compared to the ascorbic acid standard.

2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) scavenging activity

The ABTS scavenging activity of *S. cumini* aqueous, ethanol and methanol extracts was determined by the method of Re.[21] 7 mM ABTS solution and 2.4 mM potassium persulphate was added in equal volume and incubated for 12-16 h at room temperature in the dark. Freshly prepared ABTS·+ solution (1 ml) was added in the resulting mixture. The samples of extract and standard were mixed individually with the resulting mixture in 1:1 ratio. After 10 minutes of incubation the absorbance was measured at 734 nm. The quenching or inhibition capacity of the extracts for ABTS·+ and standard butylated hydroxyltoluene (BHT) was calculated. The percentage of scavenging capacity of the extracts was calculated by the equation as mentioned above.

Free radical scavenging activities

Nitric oxide scavenging activity

The nitric oxide radical scavenging activity of all the three extracts was determined by the method of Garratt.[22] 2 ml of 10 mM sodium nitroprusside was mixed with 0.5 ml of ASE/ESE/MSE at various concentrations.

The mixture was incubated at 25°C for 2.5 h.

The resulting solution was further mixed with Griess reagent in equal proportion and incubated at room temperature for 30 minutes.

The absorbance was measured at 540 nm. The amount of nitric oxide radical inhibition was calculated by following equation:

$$\text{\% Inhibition} = \frac{A_0 - A_1}{A_1} *100$$

Where A_0 is the absorbance before reaction (without sample) and A_1 is the absorbance after reaction has taken place.

Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by *S. cumini* aqueous, ethanol and methanol extracts was determined by the method of Ruch.[23] ASE/ESE/MSE at various concentrations were mixed with 4 mM H_2O_2 solution (prepared in 0.1 M phosphate buffer, pH 7.4) in equal proportion and incubated for 10 minutes at room temperature. The absorbance of the solution was measured at 230 nm. The percent of scavenging capacity of H_2O_2 by all the three extracts was calculated using the % inhibition equation mentioned above.

Scavenging activity of superoxide anion

The scavenging activity of superoxide anion was determined by the method of Nishimiki.[24] The reaction mixture contained 1 ml of 156 µM Nitro blue tetrazolium (NBT) prepared in 100 mM phosphate buffer, 0.1 ml of extract at various concentrations and 1 ml of 468 µM NADH. 100 µl of 60 µM Phenazine methosulfate (PMS) was added with the mixture and incubated at 25°C for 5 min. The absorbance was measured at 560 nm. The percent of superoxide scavenging capacity of the extracts was calculated individually using the above mentioned equation.

Lipid peroxidation assays

The antioxidant activity of the extracts was determined using ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods. The FTC method was used to evaluate the peroxides at the initiation of lipid peroxidation and TBA method was used to analyze free radicals after the oxidation of peroxides. The inhibition of lipid peroxidation was estimated by the % inhibition formula.

Ferric thiocyanate (FTC) method

The inhibition of linoleic acid peroxides was evaluated using FTC method as described by Kikuzaki.[25] The reaction mixture contains 4 mg of the ASE, ESE, MSE samples and standard BHT at various concentrations, 4 ml of 99.5% ethanol, 4.1 ml of 2.5% linoleic acid in 99.5% ethanol, 8.0 ml of 0.02 M phosphate buffer and 3.9 ml of distilled water.

The mixture was incubated in dark for 30 min. To measure the extent of antioxidant activity, 9.7 ml of 75% (v/v) ethanol was added in the reaction mixture, followed by 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloric acid.

After 5 min ferrous chloride was added and the absorbance was measured at 500 nm.

Thiobarbituric acid (TBA) method

The method of Ottolenghi[26] was used for the determination of free radicals present in all the three extracts. The reaction mixture composition used in FTC assay was used for TBA also. Equal volume of 20% trichloroacetic acid and 0.67% of thiobarbituric acid were added to the mixture. The mixture was incubated in water bath at 100 °C for 10 min and centrifuged after cooling at 3000 rpm for 20 min. The absorbance activity of the supernatant was measured at 552 nm.

Cell culture

Heart-derived H9C2 cardio myoblast cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. All the cells were cultured with Dulbecco's modified Eagle's Medium (DMEM) supplemented with penicillin, streptomycin, glucose, L-glutamine, sodium bi-carbonate and 10 % fetal bovine serum (FBS) in humidified CO2 incubator (New Brunswick) with 5 % CO_2 at $37^{\circ}C$.[27]

In vitro cytotoxicity assay for glucose and MSE on H9C2 cells

Cell viability was measured by 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) which was converted to purple formazan by the action of cellular enzymes present in the cytosol of living cells.[28] For MTT Assay, 8000 cells were seeded in 96-well plates. After treatment with glucose and MSE at different concentrations on H9C2 cells, 10 µl MTT solution (5 mg/ml) was added and incubated at 25 $^{\circ}C$ for 3 h. Formazan salt crystals were further dissolved in 100 µl dimethylsulfoxide (DMSO) and samples were analyzed at 570 nm. Cell viability is defined relative to untreated control cells as follows: cell viability = absorbance of treated sample/absorbance of control. Optimized doses were taken for the treatment of cells and cultured in serum-free DMEM supplemented with ITS.

Treatment of H9C2 cells with glucose and MSE

Optimal concentrations of Glucose and MSE were analyzed by MTT assay as mentioned above. H9C2 cells were cultured in serum-free DMEM supplemented with ITS (insulin 50 mg/ml, transferrin 27.5 mg/ml and selenium 0.025 mg/ml) and treated with optimal concentration of glucose.

To observe the effect of MSE, an optimal concentration was taken under similar culture conditions simultaneously along with Glucose in the culture media.

These treatments were carried out for 48 h along with an untreated control.

Morphological analysis

Cells were observed under inverted microscope for morphological changes at 40X magnification. H9C2 cells were treated in four experimental sets - untreated control, glucose induced (GI), MSE treated glucose induced (MSE +GI) and treated with MSE alone (MSE).

Statistical analysis

Experimental results were expressed as Mean ± SD. Statistical analysis was done by SPSS 16 software. A one-way ANOVA with the Tukey's test was used to evaluate the significance of the results obtained. IC50 values were also calculated for MSE. All the results were significant at P<0.05 level. Each experiment was conducted in triplicates.

RESULTS AND DISCUSSION

Advanced glycation end products (AGEs) are proteins or lipids that become glycated after exposure to sugars during hyperglycemic conditions that contribute to a variety of complications during diabetes.[29] For the identification of a natural, effective and less toxic glycation inhibitor, aqueous, ethanol and methanol seed extracts were tested for their potential in preventing advanced glycation end product formation. ASE, ESE and MSE showed 66.64% ± 2.08, 72.54% ± 1.56 and 78.61% ± 1.51 anti-glycation activities as compared to gallic acid 91.97% ± 1.06 at the concentration of 1 mg/ml (Fig. 1). Upon comparison we found least antiglycation potential in ASE, moderate potential in ESE and highest potential in MSE. To investigate the differential AGE potential of selected extracts, FTIR analysis was performed with ASE, ESE and MSE of *S. cumini* seeds. The analysis resulted in a spectrum of peaks by which the presence of various bonds and functional groups in the extracts can be determined. Though all the three extracts showed the presence of polyphenolic compounds, but the highest absorbance peaks found in MSE confirms it to be the richest source of polyphenolic compounds. The absorbance peak at 2547-2973 cm^{-1} corresponds to aliphatic C-H stretching and aromatic C-H out of plane deformation bands occurs below 700 cm^{-1}. The absorbance peak observed at 1505 cm^{-1} represents aromatic C=C bonds. The absorbance peak around 1000-1220 cm^{-1} indicates C-O single bonds and peak at 1610 cm^{-1} indicates the presence of carbonyl groups [C=O]. MSE shows better results with high intense peak of C-H stretching vibrations at 2547-2973 cm^{-1}. The FT-IR spectra of methanol extract shows more intense peaks at 2890, 1216, 1040 and 697 cm^{-1} as compared with aqueous and ethanol extracts (Fig. 2A). The C-O single bonds and carbonyl groups may be present due to polyphenols such as flavonoids, gallic acid, kaempferol, epigallocatechin gallate etc.[30,31] We further estimated the total flavonoid content in all three extracts against gallic acid. Amongst the three extracts, MSE showed highest amount of flavonoid content (0.642 ± 1.98 mg/ml of gallic acid) followed by ESE (0.435 ± 0.98 mg/ml of gallic acid), while aqueous extract showed least amount of flavonoid content (0.298 ± 2.08 mg/ml of gallic acid) (Fig. 2B). The seeds of *S. cumini* have been reported to contain gallic acid polyphenols as a major constituent. We therefore performed HPLC analysis of aqueous, ethanol and

methanol *S. cumini* seed extract to measure gallic acid content. The chromatogram of all three extracts showed that the fraction was enriched with gallic acid and compared with pure gallic acid (Fig. 3A & 3B).

The retention time for ASE, ESE and MSE was found to be 2.33 ± 1.97 min, which was found to be equivalent to gallic acid.

The gallic acid peak intensity and area was found to be highest in MSE as compared to ESE and ASE.

HPLC analysis further validated that MSE is nutritionally and therapeutically most enriched extract among all the three extracts.

We further compared the antioxidative potential of ASE, ESE and MSE as there is accumulating evidences that advanced glycation end products (AGEs), mediate adverse effects via generating oxidative stress in the cells.[32]

DPPH and ABTS assays showed that methanol extract (MSE) has maximum free radical scavenging activity while ASE has least scavenging activity. MSE, ESE and ASE showed DPPH inhibition 60.87%±0.06, 54.91%±0.12, 50.24%±1.17 correspondingly and ABTS inhibition 84.86%±2.18, 69.09%±2.15, 60.04%±2.11 respectively comparable to relevant standards at 1 mg/ml concentration (Fig. 4A & 4B).

Concentrations providing 50% inhibition (IC50) was also calculated for most enriched *S. cumini* MSE extract, which was found to be higher in case of DPPH than that of ABTS. MSE showed 50% inhibition at a concentration of 0.56 and 0.38 mg/ml for DPPH and ABTS respectively as compared to the standard 0.20 mg/ml and 0.17 mg/ml.

Free radical such as nitric oxide, hydrogen peroxide and superoxide play important physiological roles and is involved in AGE induced oxidative stress during hyperglycemia, which is the cause of long-term diabetic complications.[33]

Nitric oxide is a reactive species under the aerobic condition. NO generates peroxynitrite and free radicals that was estimated by Griess reagent.
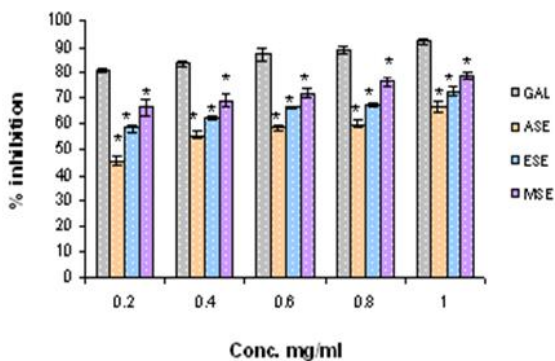
Figure 1: Antiglycation assay for *S. cumini* aqueous (ASE) ethanol (ESE) and methanol seed extracts (MSE). Antiglycation assay for *S. cumini* ASE, ESE and MSE as compared to standard gallic acid.
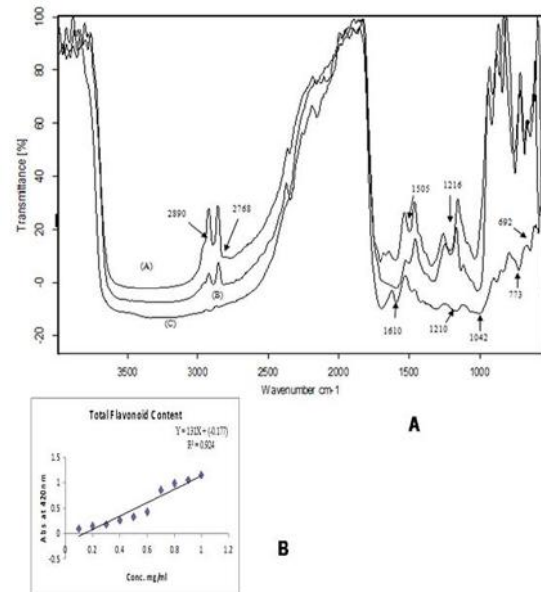
Figure 2: (A) FTIR analysis for *S. cumini* aqueous (ASE) ethanol (ESE) and methanol seed extracts (MSE). **(A)** FTIR spectra for *S. cumini* (A) MSE, (B) ESE and (C) ASE **(B)** Gallic acid standard curve for the estimation of flavonoid content of *S. cumini* ASE, ESE and MSE
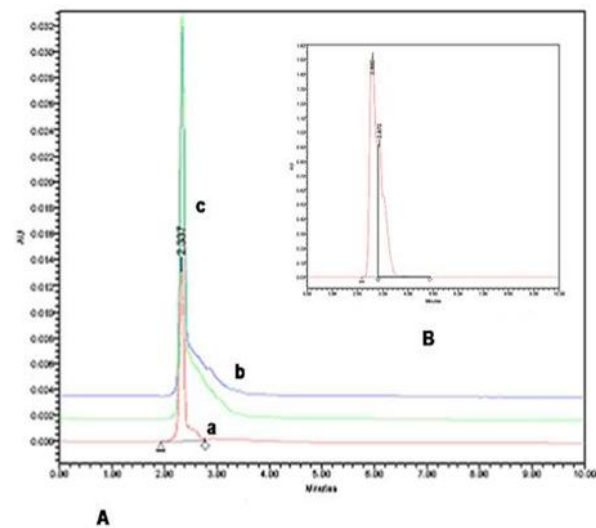
Figure 3: (A) HPLC analysis for *S. cumini* aqueous (ASE) ethanol (ESE) and methanol seed extracts (MSE). HPLC analysis for *S. cumini* (a) aqueous (ASE) (b) ethanol (ESE) and (c) methanol seed extracts (MSE) as compared to the **(B)** standard gallic acid

S. cumini MSE was found to most powerful scavenger of the NO radicals.

The NO Scavenging activity was found to be increased in a dose dependent manner. MSE, ESE and ASE showed 79.65% ± 1.18, 75.76% ± 1.05, 69.87% ± 1.19 NO scavenging activity (Fig. 4C).

The IC50 values were found to be 0.38 and 0.20 mg/ml for most enriched MSE and standard BHT respectively.

Results showed that MSE most effectively inhibit the NO free radicals.

Hydrogen peroxide is an important reactive oxygen species because of its ability to penetrate biological membranes and damage biomolecules in living cells.

It gives more toxic effects when converted to hydroxyl radicals in the cell.

Among the three extracts, the scavenging activity of MSE of *S. cumini* was also increased in a concentration dependent manner.

MSE had a highest level of free radical scavenging activity 79.98%±1.45 followed by ESE 65.91% ± 1.79 and ASE 59.47% ± 1.19 scavenging activity comparable to the standard BHT 87.81% ± 0.98 at the concentration of 1 mg/ml (Fig. 4D).

The IC_{50} value was 0.37 mg/ml for MSE and 0.16 mg/ml for BHT.

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated and are also precursor to more damaging reactive oxygen species.

MSE showed highest inhibition of superoxide radicals at the concentration of 1 mg/ml. The scavenging activity of MSE showed a maximum activity of (64.95% ± 2.04) inhibition than that of ESE (54.92% ± 1.60) and ASE (50.87% ± 2.04) respectively which was comparable to standard BHT (79.98% ± 1.00) (Fig. 4E). The IC50 value of MSE was 0.60 mg/ml as compared to the BHT (0.22 mg/ml). Collectively our results demonstrated that MSE had maximum capacity to scavenge free radicals possibly due to the electron donating ability, attributed to its phenolics. Polyphenolics are the secondary metabolites which are the outcome of secondary metabolism in plants, and retain antioxidative potential due to redox properties, which are essential for chelating transitional metals as well as free radicals scavenging and showed that the polar phenolics play a central role in free radical scavenging.[34] Increasing evidence in both experimental and clinical studies suggests that free radicals formed disproportionately during diseases lead to increased lipid peroxidation. We therefore investigated the effect of *S. cumini* ASE, ESE and MSE on non enzymatic peroxidation of lipids by measuring the levels of malondialdehyde (MDA), which is produced based on the acid-catalyzed decomposition of lipid peroxides. The FTC method indicates the amount of peroxide in the initial stages of lipid peroxidation[35] whereas the thiobarbituric acid method shows the amount of peroxide in the secondary stage of lipid peroxidation.[36] This analysis confirmed that MSE significantly inhibit the peroxides with increasing concentrations followed by ESE and ASE respectively. The maximum value of percentage inhibition of lipid peroxide at the initial stage of oxidation was found to be 67.27% ± 1.46 for MSE, 63.25% ± 0.63 and 52.96% ±1.46 for ESE and ASE correspondingly than that of the standard BHT which was found to be 71.98% ± 2.12 at a concentration

of 1 mg/ml. The IC50 value for MSE based on FTC assay was found to be 0.51 mg/ml as compared to 0.37 mg/ml for BHT (Fig. 5A). All the three extracts could also reduce thiobarbituric acid reactive substances (TBARS). TBA assay showed the highest value of percentage inhibition of malondialdehyde at a concentration of 1 mg/ml which was found to be 74.98% ± 1.00 for MSE, 54.32% ± 0.50 for ESE and 43.90% ± 1.00 for ASE compared to 81.89%±0.58 of the standard BHT (Fig. 5B). The IC_{50} value for BHT and MSE was found to be 0.20 mg/ml and 0.56 mg/ml respectively.

The time dependent studies were also evaluated for the FTC and TBA assay. The % inhibition using FTC and TBA method was observed for 0, 2nd, 4th, 6th and 8th day of incubation. The % inhibition by ASE, ESE and MSE for FTC assay on the 6th day was found to be 49.98% ± 1.00, 56.95% ± 3.00 and 66.47% ± 2.3 respectively for the concentration of 1 mg/ml and on the 8th day it reduced significantly (Fig. 5C & 5D). The inhibition of TBARS species were found to be 30.04% ± 0.52, 40.43% ± 2.12, 70.12% ± 1.10 for ASE, ESE and MSE correspondingly. The percentage inhibition of the ASE, ESE and MSE decreased after 6th day of incubation at every concentration. Higher amount of peroxides were inhibited at the later stages of peroxidation which was observed using the TBA assay.

Thus, our results suggest that MSE has the maximum ability to limit the lipid peroxide generation while ESE has the minimum potential to inhibit lipid peroxide formation.

The percentage inhibition of scavenging activities and the IC_{50} values of the methanol extract of the Jamun seed for DPPH, ABTS, Nitric oxide, Hydrogen peroxide, superoxide and lipid peroxidation were summarized in Table 1.

Further cell line based experiments were conducted to validate the antiglycation potential of MSE in H9C2 cardiac cells under high glucose, a well known *in vitro* model for diabetic cardiomyopathy. For that, hyperglycemic stress is generated on cardiac cell by previously optimized MTT doses of glucose (25mM), and cardioprotective effect of safe MSE dose (9µg/ml) was tested by observing the changes in the morphology as terminally differentiated cells reported to undergo hypertrophy in stress conditions where cells enlarge in size. Morphological analysis revealed the excessive stress in glucose induced H9C2 cells and MSE treatment on glucose induced cells reverses the stress comparable to control (Fig. 6). MSE alone did not show any change in the cellular integrity confirming nontoxic nature of MSE. AGEs contribute to diabetic complications through a series of pathological changes such as increased atherogenicity of LDL, increased basement membrane permeability and decreased insulin binding to its receptors thereby causing cardiac pathologies we therefore tested the formation of glycation products in our H9C2 cell line based experimental set

Table 1: Free radical scavenging activities of ASE, ESE and MSE of *S. cumini* at different concentration (Standard values in parentheses)

| Conc. (mg/ml)	DPPH[a] (%Inhibition)	ABTS[a] (%Inhibition)	NO[a] (%Inhibition)	H_2O_2[a] (%Inhibition)	Superoxide[a] (%Inhibition)	FTC[a] (%Inhibition)	TBA[a] (%Inhibition)
0.2(MSE)	30.87±0.90	40.32±1.13	45.32±2.06	49.32±1.09	40.62±0.62	43.36±1.36	38.98±0.55
(ESE)	24.89±0.16	25.65±1.66	28.08±2.01	28.62±1.07	24.91±1.00	42.13±3.24	22.77±1.82
(ASE)	18.57±1.73	20.04±2.62	26.05±2.06	20.85±1.14	22.87±0.62	34.65±2.27	20.87±1.82
Standard	(49.01±1.00)	(58.05±1.13)	(48.01±1.42)	(59.33±1.52)	(44.35±0.55)	(48.17±1.70)	(46.98±1.57)
0.4(MSE)	42.78±1.49	54.98±2.01	58.21±2.04	54.98±0.99	46.95±2.04	49.67±1.13	44.98±1.52
(ESE)	38.28±0.58	39.04±1.08	33.65±2.0	39.87±1.09	39.61±1.51	44.62±0.55	37.88±0.58
(ASE)	26.35±0.39	33.54±2.01	32.76±2.02	35.64±0.98	28.64±2.04	39.86±1.13	26.98±1.52
Standard	(54.31±2.30)	(64.01±1.51)	(64.69±1.11)	(63.65±0.95)	(54.62±1.6)	(52.95±1.89)	(52.87±2.05)
0.6(MSE)	52.91±0.08	68.32±2.05	65.25±2.90	59.22±0.43	50.47±2.09	58.97±1.89	47.56±0.55
(ESE)	44.04±0.54	58.98±0.96	54.98±2.86	52.55±0.50	44.01±0.14	55.82±0.27	42.18±1.10
(ASE)	35.68±1.05	45.98±2.05	44.50±2.90	42.59±0.38	36.89±2.09	41.61±1.89	34.89±0.53
Standard	(56.25±3.06)	(79.04±1.71)	(69.21±1.12)	(75.87±1.01)	(64.98±3.46)	(61.86±0.10)	(60.98±0.58)
0.8(MSE)	56.72±0.55	72.83±2.06	69.32±1.97	72.43±1.03	59.65±2.56	61.77±0.90	57.54±0.53
(ESE)	48.89±1.01	59.86±0.56	60.89±1.90	60.87±0.501	47.62±0.54	60.81±0.20	52.98±2.30
(ASE)	43.83±1.16	48.77±2.05	49.54±1.17	54.89±1.09	40.85±2.56	43.39±0.90	38.77±0.55
Standard	(60.34±0.51)	(83.33±1.10)	(74.09±1.44)	(79.88±0.86)	(70.98±2.16)	(68.08±2.98)	(68.89±2.99)
1 (MSE)	60.87±0.06	84.86±2.18	79.65±1.18	79.98±1.45	64.95±2.04	67.27±1.46	74.98±1.00
(ESE)	54.91±0.12	69.09±2.15	75.76±1.05	65.91±1.79	54.92±1.60	63.25±0.63	54.32±0.50
(ASE)	50.24±1.17	60.04±2.11	69.87±1.19	59.47±1.39	50.87±2.04	52.96±1.46	43.90±1.00
Standard	(68.30±2.06)	(89.76±0.11)	(77.76±1.05)	(87.81±0.98)	((79.98±1.00)	(71.98±2.12)	(81.89±0.58)
	DPPH IC_{50} mg/ml	ABTS IC_{50} mg/ml	NO IC_{50} mg/ml	H_2O_2 IC_{50} mg/ml	Superoxide IC_{50} mg/ml	FTC IC_{50} mg/ml	TBA IC_{50} mg/ml
S. cumini MSE	0.56	0.38	0.38	0.37	0.60	0.51	0.56
Standard	0.20	0.17	0.20	0.16	0.22	0.37	0.20

[a]Values represent mean ± SD
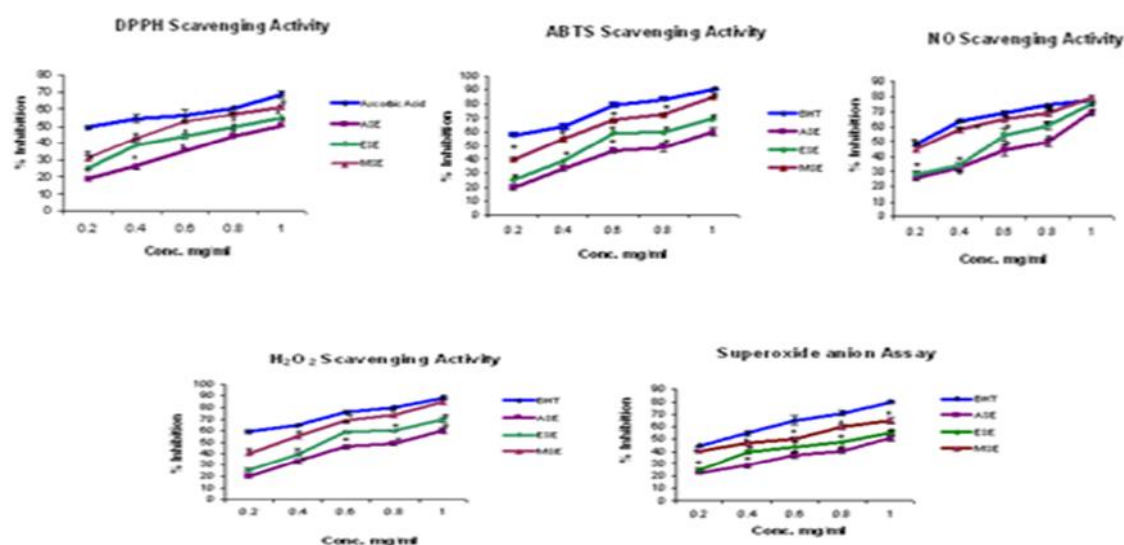
Figure 4: Antioxidative assays for *S. cumini* aqueous (ASE) ethanol (ESE) and methanol seed extracts (MSE). **(A):** DPPH Scavenging activity **(B):** ABTS Scavenging activity **(C):** NO Scavenging activity **(D):** H_2O_2 Scavenging activity **(E):** Superoxide Anion Scavenging activity. All assays were compared to their respective standards. Values indicated as % inhibition of peroxides on 2, 4, 6 and 8 days. '*' showed the level of significance (P≤0.05).
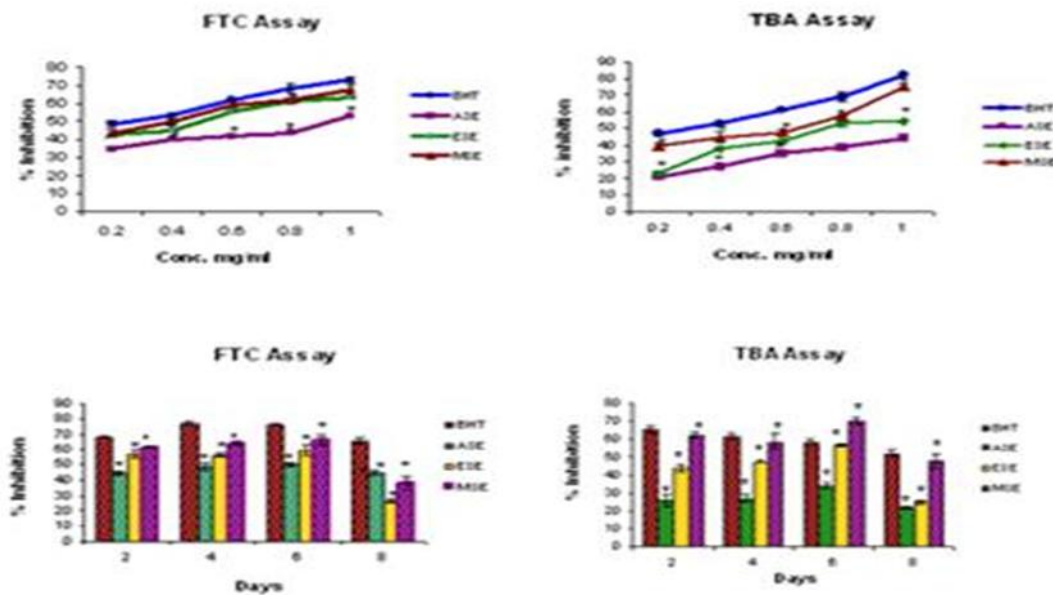
Figure 5: Lipid peroxidation assay for *S. cumini* aqueous (ASE) ethanol (ESE) and methanol seed extracts (MSE). **(A):** FTC assay and **(B):** TBA assay of *S. cumini* aqueous, ethanol and methanol seed extracts. All assays were compared to their respective standards. The time dependent studies were also done for **(C):** FTC Assay and **(D):** TBA Assay. Values indicated as % inhibition of peroxides on 2, 4, 6 and 8 days. '*' showed the level of significance (P≤0.5).
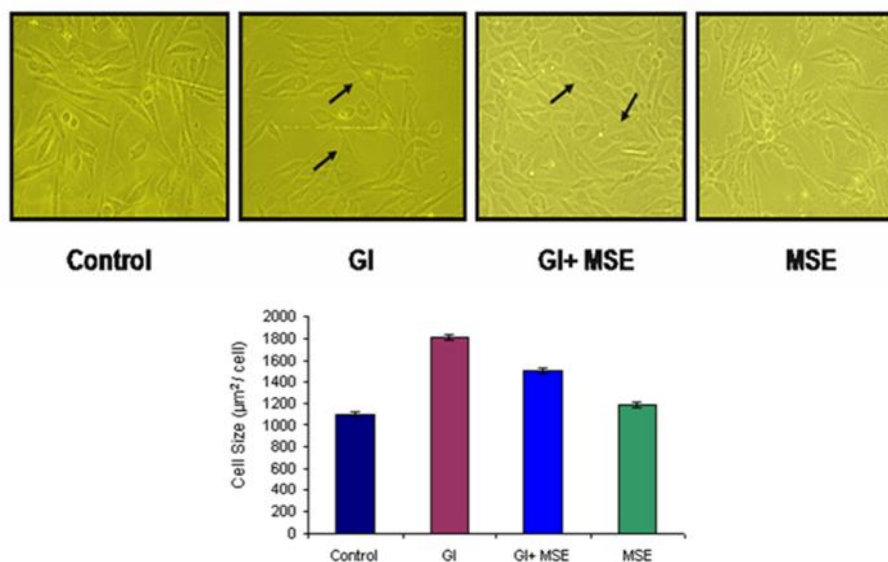
Figure 6: Morphological analysis in H9C2 cells. H9C2 cells were cultured for 48 h with/without glucose in following experimental sets: (1) untreated control, (2) glucose induced (GI): induced with 25 mM glucose, (3) GI + MSE: treated with glucose (25 mM) and MSE (9 μg/ml) and (4) treated with MSE (9 μg/ml) alone. Glucose-induced cells showed stress (increase in cell size) and MSE treatment reversed the stress nearby control as indicated by arrows. Images were captured at 40X magnification. Quantitation was done by image J Software.
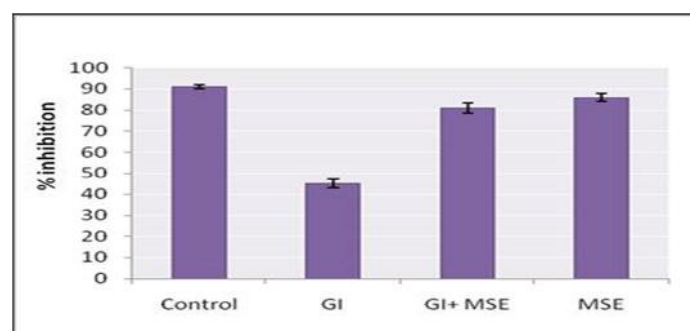
Figure 7: Antiglycation assay in H9C2 cells. AGEs production was found to be increased in glucose induced cells whereas MSE treatment reduced it by showing ~ twofold increase in scavenging of AGEs. Results are comparable to control (P≤0.05).

The glucose induced H9C2 cells showed 50% reduction in antiglycation potential as compared to control and the MSE treated glucose induced cells showed significant increase (~30%) in AGEs scavenging capacity as compared to cells treated with glucose alone (Fig. 7). MSE alone had effects similar to control. Collectively our cell based assays confirmed that MSE had antiglycation potential in glucose induced cardiac cells and also it is nontoxic and is safe for consumption.

Clinical trial conducted by Teixeira failed to prove the antihyperglycemic potential of tea and aqueous extracts prepared from leaves of *Syzygium cumini*. Therefore such comparative study was the need of hour because of successful and effective implementation of phytomolecules at the onset of drug discovery. We have performed experiments which showed that all extracts prepared from seeds of *Syzygium cumini* are not equally pharmacologically active. The result of the present study show that aqueous extract of *S. cumini* is least enriched in its phytocontents, antioxidative and anti-AGE potential. Methanol extract of *S. cumini* contain the highest amount of flavonoids and exhibit the greatest antioxidative and antiglycation activity through scavenging the free radicals and reducing the AGEs which participate in various pathophysiology particularly in diabetes associated long-term complications in terms of morbidity and mortality for individual diabetic patient. We propose that future clinical studies should not solely rely on the putative antihyperglycemic effect of crude plant aqueous preparations but select for a formulation that is pharmacologically most active. We suggest utilizing the methanol extracted *S. cumini* seed fraction for trials in order to show potent pharmacological activity of this plant. Also in spite of having a pharmcological potential, certain barriers like poor bioavailability sometimes might limit its potential. Thus future studies can be designed to understand the mechanisms of action of the seed fractions and to observe its effect during clinical trials. We conclude that *S. cumini* methanol seed extract could be used as a potential source of natural antioxidant as well as anti-AGE agent. However, the study conducted has plenty of scopes further thorough investigation for better understanding of its exact pharmacological activities, mechanism of action as well as the purification of active compound(s) responsible for these actions.

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Source of Support: Nil, **Conflict of Interest:** None.