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Phytochemical and *In Vitro* Antioxidant Activity of Various Bark Extracts of *Syzygium cumini (L.)*

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ABSTRACT

Syzygium cumini (L.) commonly known as *jamun* belongs to the Myrtaceae family. The aim of the present study includes phytochemical investigation and *in vitro* anti-oxidant capacity of various crude extracts from the bark of *syzygium cumini (L.)* by various anti-oxidant assays namely DPPH (2, 2-diphenyl-1-picrylhydrazyl), Nitric Oxide (NO) and Hydrogen peroxide (H_2O_2). Chloroform Ethyl acetate (EA) and Methanolic (MeOH) extracts of *S. cumini* gave positive results for steroids, alkaloids, tannins and flavonoids. The scavenging ability of chloroform, ethyl acetate and methanolic extracts along with standard Ascorbic acid were evaluated between the range of 20µg/ml to 300µg/ml using DPPH anti-oxidant assay and the IC_{50}values were found to 41µg/mL, 57 µg/mL, 53 µg/mL and 6.1 µg/mL respectively. To prove further its anti-oxidant activity, they were evaluated using NO and H_2O_2 antioxidant assays.

Keywords: Anti-oxidant Activity, DPPH, Phytochemical Screening and *Syzygium cumini (L.)*

INTRODUCTION

In the modern work culture, there are numerous reasons that lead to oxidative stress in body which is challenging to cope with and leads to many disorders like early aging and age related neuro degenerative disease. Imbalance between oxidants and antioxidants causes oxidative stress. Free radical is defined as any atoms (e.g. oxygen, nitrogen) which have at least one unpaired electron in the outermost shell, and is accomplished of independent subsistence. Oxygen is the most significant element for life which is the major resource of free radicals. Oxygen is used by cells for generating energy, which leads to free radical generation in the end of ATP (adenosine triphosphate) production by the mitochondria. The free radicals play a twin role, both as toxic and beneficial compounds**.** Formation of free radicals at a lower or moderate levels, contribute to good cellular responses and immune functions in human health and development. Free radicals occur not only in normal cellular process but also generated due to certain external factors like chemicals (polycyclic aromatic hydrocarbon, cadmium, lead, *etc.*), radiation, smoking and high fat diet. A balance

between formations of free radicals and its detoxification is essential for normal cellular function. When this balance gets disturbed due to any reason, it leads to cellular damage because of excess of free radical generated. The presence of excessive free radical is termed as oxidative stress. The free radicals can cause genetic instability by reacting with DNA which results in cancer[1], mutation, circulatory disturbance and early aging[2-5]. This makes researchers a way to work on this field and come out with entities which have good antioxidant activity and can be used to relieve the oxidative stress and help in maintaining good human health. Synthetic antioxidants have been reported to exhibit higher toxicity to humans, which makes it a need to look for natural source (herbs) for a good antioxidant candidate. Recent research work has confirmed this, as some of the medicinal plants are having good therapeutic anti-oxidant activity. Among them, a number of naturally occurring antioxidants; ascorbic acid, carotenoids and phenolic compounds are successful in scavenging reactive oxygen species (ROS) by inhibiting lipid peroxidation.

The *S. cumini* (L.) Skeels (*Syns. Syzygium jambolana* DC, *Eugenia cumini Eugenia jambolana* Lam.), commonly known as *Jamun*, belongs to the family Myrtaceae. The other common names are Indian blackberry, Java plum, Jambu, black plum and Jambul etc. The *jamun* fruit as well as different parts of the plants have high medicinal values, possess varied uses to mankind. The various extracts of different parts of *S. cumini* possess a range of pharmacological properties such as antibacterial[7-9], antimicrobial[10], antifungal[11], antiviral[12], antioxidant and free radical scavenging activity[13-17],cardioprotective[18],anti-inflammatory[19-20],neuropsychopharmacological[21], antiallergic[22], radioprotective[23],chemopreventive[24], larvicidal[25], and gastroprotective & antiulcerogenic[26] activities. However the reports pertaining to the antioxidant activity of extracts of *S. cumini* bark is very less. Hence, the present study was designed to screen for various phytochemicals present in the plant and to investigate the *in vitro* antioxidant potential of various extracts of *S. cumini* bark using DPPH, H_2O_2 and NO free radical scavenging assays.

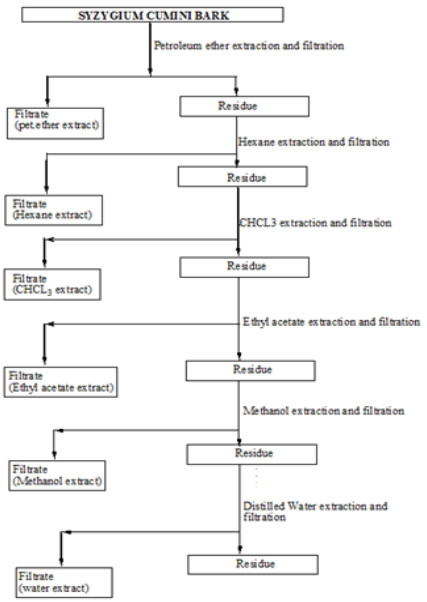
MATERIALS AND METHODS

Collection of Plant material

The barks of *S. cumini* were collected from the campus of Kalasalingam University, Krishnankoil, Virudhunagar District, Tamil Nadu. The taxonomical identification and authentication was done by Dr. Stephan. Professor, Department of Botany, The American College, Madurai. A Voucher specimen (AKCP/SCL/07/2016) has been deposited at the Department of Pharmacognosy, Arulmigu Kalasalingam College of Pharmacy, Srivilliputur for future reference.

Preparation of Plant extracts

The collected bark was dried under sun shade for 15 days at room temperature and then it was powdered by electrical grinder. The powdered bark of *S. cumini* has been percolated by continuous percolation method with Pet.-Ether, Hexane, $CHCl_3$, EA, MeOH and water respectively. The general scheme of the extraction protocol is depicted as scheme 1.



General Scheme of extraction protocol

Extracts were prepared as per the scheme, extraction of 250 g of bark powder in 1.5 L of solvent at room temperature for 7 days. The extracts have concentrated by rotary evaporator under reduced presser (SUPERFIT, INDIA) and then lyophilized, the resulting powder of extracts were stored at - 4°C and used for present study.

Chemicals and Reagents

Petroleum ether, Hexane, $CHCl_3$, EA, MeOH, Distilled water, Hydrogen peroxide, Griess reagent, DPPH (2, 2-diphenyl-1-picrylhydrazyl), Hydrochloric acid, Sulphuric acid, α-naphthol, Copper sulphate, Sodium hydroxide, Barfoed's solution, Benedict's solution, Potassium mercuric iodide, Potassium bismuth iodide, Iodine, Potassium iodide, Picric acid, con. HNO_3, NH_4OH, Millon's reagent, Ninhydrin, Biuret reagent, Ammonia, 95% Ethanol, Potassium hydroxide, Phenolphthalein, Lead acetate, Ferric chloride, *Agar-Agar*, Potassium dihydrogen phosphate and Calcium carbonate were obtained from Sigma-Aldrich Chemicals and other chemicals and reagents used were of analytical grade.

PHYTOCHEMICAL SCREENING

The powdered extracts were subjected to phytochemical investigation to detect for the presence of carbohydrate, protein, fat & oils, steroids, tannins, flavonoids, amino acids and volatile oils as described in literatures. The results were shown in table 1.

Determination of DPPH scavenging activity[27]

The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as:

$$\underset{\text{(Purple)}}{\text{(DPPH)}} + \text{(H-A)} \longrightarrow \underset{\text{(Yellow)}}{\text{DPPH-H}} + \text{(A)}$$

Antioxidants react with DPPH, which is a stable free radical and is reduced to the DPPH -H and as consequence the absorbance of DPPH-H decrease compared to DPPH radical. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. 1.0 mL of DPPH (0.1 mM) solution was added to 3.0 mL of various extracts in MeOH at different concentration (20-300 µg/ml) and allowed to react in dark room temperature for 30 minuters. The absorbance was measured at 517 nm. A blank solution was prepared without adding extract. Ascorbic acid at various concentrations (20 to 300 µg/ml).was used as standard. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH Scavenged (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

A_0 - Absorbance of control, A_1 - Absorbance in the presence of plant extract.

Determination of hydrogen peroxide scavenging activity[28]

A solution of H_2O_2 (2 mM) was prepared in phosphate buffer. Bark extracts at the concentration (20 - 300 µg/mL) were added to H_2O_2 solution (0.6 mL) and the total volume was made up to 3 mL. The absorbance of the reaction mixture was recorded at 230 nm in a spectrophotometer (Shimadzu, UV-1700). A blank solution containing phosphate buffer, without H_2O_2 was prepared.

The extent of H_2O_2 scavenging of the plant extracts was calculated as:

$$\text{\% Scavenging of } H_2O_2 = \frac{A_0 - A_1}{A_0} \times 100$$

A_0 - Absorbance of control, A_1 - Absorbance in the presence of plant extract.

Table. 1: Phytochemical Screening of various extracts of *Syzygium cumini (L.)*

S.No	Phyto-constituents	Pet.-ether	Hexane	$CHCl_3$	EA	Methanol	Water
1	Carbohydrate	-	-	-	-	-	+
2	Protein	-	+	-	-	-	+
3	Amino acid	-	-	-	-	+	-
4	Steroid	+	-	+	-	-	-
5	Glycoside	-	+	-	-	-	-
6	Alkaloid	-	+	+	+	-	+
7	Tannin	-	-	+	+	+	-
8	Fat & Oils	+	-	-	-	-	-
9	Flavonoids	-	-	+	-	+	+
10	Volatile Oils	-	-	-	-	-	-

Determination of nitric oxide scavenging activity[29]

Sodium nitroprusside in Phosphate buffered saline (PBS), at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that are estimated spectrophotometrically at 546 nm.

Sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentrations (20-300 µg/mL) of methanol extract of each plant were dissolved in methanol and incubated at 30°C for 2 hours. The same reaction mixture without the extract but the equivalent amount of ethanol served as the control. After the incubation period, 0.5 mL of Griess reagent (1% sulfanilamide, 2 % H_3P0_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophores that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 550 nm. Inhibition of nitrite formation by the plant extracts and the standard antioxidant ascorbic acid were calculated relative to the control. Inhibition data (% inhibition) were linearized against the concentrations of each extract as standard antioxidant.

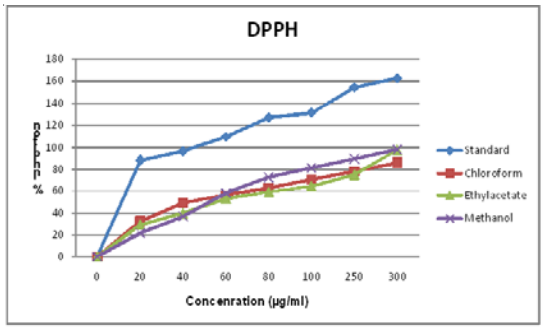


Fig. 1. DPPH Radical scavenging activity of various extracts ($CHCl_3$, Ethyl acetate and Methanol) against standard (Ascorbic acid)

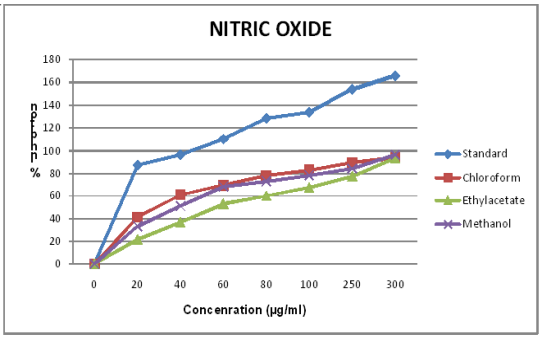


Fig. 3. NO Scavenging activity of various extracts ($CHCl_3$, Ethyl acetate and Methanol) against standard (Ascorbic acid)

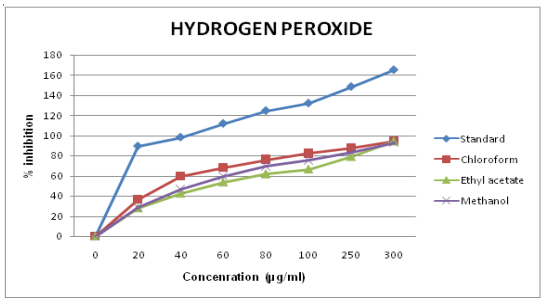


Fig. 2. H_2O_2 Scavenging activity of various extracts ($CHCl_3$, Ethyl acetate and Methanol) against standard (Ascorbic acid)

$$\% \text{ Scavenging of NO} = \frac{A_0 - A_1}{A_0} \times 100$$

A_0 - Absorbance of control, A_1 - Absorbance in the presence of plant extract.

Statistical Analysis

All the experiments were performed at least in triplicate using fresh bark sample in each time and all the data points were expressed as Mean ± SEM. Linear regression analysis was used to calculate IC_{50} for each bark extracts.

RESULTS AND DISCUSSION

The preliminary phytochemical analysis of *S. cumini (L.)* bark extracts showed the presence of various phytochemical constituents such as steroids, alkaloids, tannins and flavonoids in which tannins were present in all three extracts. Glycosides, volatile oil and fat & oil were absent in most of the bark extracts. The herbal research studies revealed that tannins and flavonoids are responsible for antioxidant activity in order to treat neuro-degenerative disorders, CVS diseases, diabetes and treatment of cancer. In DPPH radical scavenging assay, the compound's ability to reduce the DPPH (a stable free radical). DPPH is nitrogen-centered stable free radical having a maximum absorbance at 517 nm in methanolic solution. It becomes a stable diamagnetic molecule on accepting an electron or hydrogen atom. In the presence of an extract capable of donating a

hydrogen atom, the free radical nature of DPPH is lost and the purple colour change to yellow (diphenyl picrylhydrazine). The scavenging ability results were showed that absorbance value with 1.342 ± 0.0016, 1.359 ± 0.0017 and 1.265 ± 0.0015 (Mean ± SEM) for $CHCl_3$, EA and MeOH extracts respectively at minimum concentration of 20 µg/mL and maximum concentration of 300 µg/mL about 0.453 ± 0.0034, 0.542 ± 0.0035 and 0.582 ± 0.0036 (Mean ± SEM) respectively. The IC_{50} value of $CHCl_3$, EA and MeOH extracts were found to be 41, 57 and 53 µg/mL respectively. The scavenging ability as % inhibition of the *S. cumini (L)* bark extracts of $CHCl_3$, EA and MeOH by DPPH method has clearly showed a dose-dependent antioxidant activity.

To further prove the antioxidant activity, the antioxidant scavenging activity of the selected extracts of *S. cumini(L.)* was carried out by hydroxyl radical scavenging assay. The Fenton reaction generates $OH^{\cdot}$ radicals which degrade DNA using Fe^{2+} salts as an important catalytic component and may cause to DNA fragmentation and DNA strand breakage. The results of antioxidant scavenging assay showed absorbance value of 1.490 ± 0.0018, 1.371 ± 0.0016 and 1.37 ± 0.0017 (Mean ± SEM) for $CHCl_3$, EA and MeOH extracts at minimum concentration of 20 µg/mL respectively and 0.623 ± 0.0036, 0.683 ± 0.0033 and 0.612 ± 0.0036 (Mean ± SEM) for $CHCl_3$, EA and MeOH extracts at maximum concentration of 300 µg/mL respectively. Moreover, the IC_{50} value of $CHCl_3$, EA and MeOH extracts of *S. cumini (L.)* bark was found to be 31, 46 and 44 µg/mL respectively.

Nitric oxide (NO) is one of the strong pleiotropic mediators in physiological processes as well as pathological conditions. Formation of peroxynitrite ($ONOO^-$) as a strong oxidant is responsible for oxidative damage of proteins in living systems. Hence, we also evaluated the extracts antioxidant activity using NO scavenging assay. The results of NO scavenging activity showed an absorbance value of 1.160 ± 0.0015, 1.192 ± 0.0014 and 1.120 ± 0.0012 (Mean ± SEM) for $CHCl_3$, EA and MeOH extracts at minimum concentration of 20 µg/mL respectively and 0.613 ± 0.0036, 0.624 ± 0.0031 and 0.654 ± 0.0034 (Mean ± SEM) respectively at a maximum

Table. 2: DPPH Radical scavenging activity of various extracts ($CHCl_3$, Ethyl acetate and Methanol) against standard (Ascorbic acid).

S.No	Conc.(µg/mL)	Standard		Chloroform		Ethyl acetate		Methanol	
		Absorbance (Mean±SEM)	% Inhibition	Absorbance (Mean±SEM)	% inhibition	Absorbance (Mean±SEM)	% inhibition	Absorbance (Mean±SEM)	% inhibition
1	20	1.059 ±0.0017	88.28	1.342±0.0016	32.15	1.359±0.0017	29.15	1.265 ±0.0015	21.58
2	40	0.809 ±0.0018	96.52	1.166 ±0.0019	48.71	1.199±0.0020	39.36	1.106 ±0.0017	36.62
3	60	0.685 ±0.0024	109.25	0.957 ±0.0021	56.16	0.997±0.0023	52.78	0.974±0.0021	58.13
4	80	0.469 ±0.0027	126.72	0.803 ±0.0024	63.15	0.846 ±0.0026	59.12	0.823 ±0.0025	72.52
5	100	0.273 ±0.0029	131.05	0.643 ±0.0028	70.65	0.683 ±0.0029	64.69	0.730 ±0.0028	81.34
6	250	0.243 ±0.0031	154.05	0.592 ±0.0030	78.32	0.637±0.0031	75.06	0.656±0.0032	89.75
7	300	0.239 ±0.0033	162.34	0.453 ±0.0034	86.19	0.542±0.0035	97.58	0.582±0.0036	97.86
IC_{50}		IC_{50} = 6.1 µg/mL		IC_{50} = 41 µg/mL		IC_{50} = 57 µg/mL		IC_{50} = 53 µg/mL	

Table. 3: H_2O_2 scavenging activity of various extracts ($CHCl_3$, Ethyl acetate and Methanol) against standard (Ascorbic acid)

S.No	Conc.(µg/mL)	Standard		Chloroform		Ethyl acetate		Methanol	
		Absorbance Mean±SEM	% Inhibition	Absorbance Mean±SEM	% inhibition	Absorbance Mean±SEM	% inhibition	Absorbance Mean±SEM	% Inhibition
1	20	1.059±0.0017	89.58	1.490±0.0018	36.58	1.371±0.0016	27.95	1.377 ±0.0017	28.54
2	40	0.809±0.0018	98.35	1.283±0.0020	59.78	1.201±0.0019	42.56	1.214 ±0.0020	46.82
3	60	0.785±0.0021	112.01	1.087±0.0023	68.05	1.056±0.0021	53.69	1.071±0.0023	59.51
4	80	0.682±0.0024	124.73	0.901±0.0027	76.25	0.892±0.0025	61.78	0.925 ±0.0026	69.42
5	100	0.563±0.0027	132.24	0.842±0.0030	82.32	0.806±0.0029	66.56	0.830 ±0.0029	75.36
6	250	0.421±0.0030	148.62	0.765±0.0032	88.09	0.756±0.0031	78.92	0.732±0.0032	83.78
7	300	0.395±0.0034	165.36	0.623±0.0036	94.61	0.683±0.0033	94.36	0.612±0.0036	92.75
IC_{50}		IC_{50}=5.5 µg/ml			IC_{50}=31 µg/ml	IC_{50}=46 µg/ml		$_0$=44 µg/ml	

Table. 4: NO scavenging activity of various extracts ($CHCl_3$, Ethyl acetate and Methanol) against standard (Ascorbic acid)

S.No	Conc.(µg/mL)	Standard		Chloroform		Ethyl acetate		Methanol	
		Absorbance Mean±SEM	% Inhibition	Absorbance Mean±SEM	% inhibition	Absorbance Mean±SEM	% inhibition	Absorbance Mean±SEM	% Inhibition
1	20	1.063 ±0.0017	87.26	1.160±0.0015	41.32	1.192±0.0014	21.52	1.120±0.0012	32.96
2	40	0.819 ±0.0018	96.37	1.118±0.0021	61.01	1.143±0.0017	36.56	1.014 ±0.0015	50.51
3	60	0.735±0.0021	110.01	1.005±0.0024	69.74	1.017±0.0018	52.74	0.913±0.0018	68.52
4	80	0.698±0.0024	128.23	0.921±0.0027	78.05	0.939±0.0020	60.04	0.839±0.0021	73.02
5	100	0.525 ±0.0027	133.54	0.855±0.0029	83.12	0.878±0.0024	67.58	0.755 ±0.0024	78.51
6	250	0.465±0.0030	153.73	0.739 ±0.0032	89.45	0.754±0.0028	77.45	0.721±0.0029	84.23
7	300	0.336±0.0034	165.78	0.613±0.0036	93.78	0.624±0.0031	93.71	0.654±0.0034	96.27
IC_{50}		IC_{50}=6.8 µg/ml		IC_{50}=30 µg/ml		IC_{50}=58 µg/ml		IC_{50}=39 µg/ml	

concentration of 300 µg/mL. The IC_{50} value of $CHCl_3$, EA and MeOH extracts of *S. cumini (L)* bark was found to be 30, 58 and 39 µg/mL respectively. The extracts showed similar IC_{50} value irrespective of the solvents used for extraction. Since, tannins were present in all the extracts and it showed similar IC_{50} values irrespective of the solvents used, it can be concluded that tannins present in *S. cumini (L.)* may be the reason for its antioxidant property which can be further studied.

CONCLUSION

Results from current research revealed that $CHCl_3$, EA and MeOH extracts of *S. cumini* possessed good antioxidant activity using DPPH, H_2O_2 and NO scavenging assay. The preliminary phytochemical screening of that indicated the presence of tannins in all the three extracts. Based on the antioxidant assay data, it could be concluded that tannins present in *S. cumini* may be the reason for its antioxidant activity which has to be studied further. Also extracts of *S. cumini* can be further characterized and studied in detail for its mechanism of action in order to develop harmless, cost-effective and targeted antioxidant agent for the benefit of mankind.

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