RESEARCH ARTICLE

Antiproliferative Activity and Apoptotic Efficiency of Syzygium cumini Bark Methanolic Extract against EAC Cells In Vivo

Mst. Ayesha Siddika¹, Plabon K. Das¹, Saharia Y. Asha¹, Suraiya Akhtar¹, A.R.M. Tareq², Ayesha Siddika³, Md. Abdur Rakib¹, Farhadul Islam¹* and Jahan Ara Khanam¹*

¹Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh; ²Environmental and Organic Laboratory, Atomic Energy Centre, Dhaka, Bangladesh; ³Institute of Tissue Banking and Biomaterial Research, Atomic Energy Research Establishment, Ganakbari, Savar, Dhaka-1349, Bangladesh

Abstract: Background: Syzygium cumini is one of the evidence-based traditional medicinal plants used in the treatment of various ailments.

Objectives: Herein, the antioxidant property and anticancer property of Syzygium cumini against Ehrlich Ascites Carcinoma (EAC) were examined to find effective chemotherapeutics.

Methods: In vitro assays, and phytochemical and chromatographic analyses were used to determine antioxidant properties and chemical constituents of Syzygium cumini bark methanolic extract (SCBME). Functional assays were used to measure the anticancer activity of SCBME. Fluorescence microscopy and RT-PCR were used to examine morphological and molecular changes of EAC cells followed by SCBME treatment.

Results: Phytochemical and GC–MS analyses confirmed the presence of compounds with antioxidant and anti-cancer activities. Accordingly, we have noted a strong antioxidant activity of SCBME with an IC₅₀ value of ~10μg/mL. Importantly, SCBME exerted a dose-dependent anticancer activity with significant inhibition of EAC cell growth (71.08 ± 3.53%; p<0.001), reduction of tumor burden (69.50%; p<0.01) and increase of life span (73.13%; p<0.001) of EAC-bearing mice at 75mg/kg/day. Besides, SCBME restored the blood toxicity towards normal in EAC-bearing mice (p<0.05). SCBME treated EAC cells showed apoptotic features under a fluorescence microscope and fragment DNA in DNA laddering assay. Moreover, up-regulation of the tumor suppressor p53 and pro-apoptotic Bax and down-regulation of NF-κB and anti-apoptotic Bcl-2 genes implied induction of apoptosis followed by SCBME treatment.

Conclusion: The antiproliferative activity of SCBME against EAC cells is likely due to apoptosis, mediated by regulation of p53 and NF-κB signaling. Thus, SCBME can be considered as a useful resource in cancer chemotherapy.

Keywords: Syzygium cumini, bark, anticancer agents, EAC cells, apoptosis, p53.

1. INTRODUCTION

Cancer is posing an enormous global threat, a leading cause of death worldwide [1]. Despite the revolutionary advances in therapeutic approaches, the dilemma of emergent resistance upon long-term use and undesired side effects of conventional anti-cancer therapies lead researchers to discover cost-effective, suitable therapeutic options with higher efficacy and lower side effects [2, 3].

Nowadays, naturally occurring or plant-derived anticancer agents (alone or synergistically with synthetic agents) are getting more importance in the pharmacological evaluation of cancer therapy [4, 5]. Plants produce a wide range of active phytochemicals such as flavonoids, phenolics, and polyphenolics, which act as potent antioxidants and are capable of attenuating oxidative stress [6]. Accumulating data suggest that oxidative stress represses apoptosis by inducing NF-κB signaling, and this imbalanced condition could be ameliorated by antioxidants supplementation [7, 8]. In addition, results from cell cultures and animal studies reported that antioxidant-rich plant materials suppress cancer growth by promoting apoptosis [9-11]. Hence, screening of plant materials to figure out the antioxidant-rich plant and to evaluate its antiproliferative activity with apoptotic efficiency could reveal new potential anticancer drug candidates from natural resources.

Syzygium cumini (L.), commonly known as the Black plum in English is a member of the Myrtaceae family [12]. This evergreen tree is native to the Indian subcontinent and an important medicinal plant in various traditional medical applications in Bangladesh [13, 14]. In addition to the dietary use of its ripe fruit, every part of Syzygium cumini, especially the fruits and seeds are extensively used to treat different ailments such as diabetes, gastritis, constipation and diarrhea [12, 13]. Furthermore, fruits and seeds extracts have anticancer and chemo-preventive activity against breast, cervical and colon cancers [12, 14]. In addition, a number of studies have reported that stem bark of this plant possesses anti-dysenteric, antiviral, anti-rheumatic, and anti-diabetic properties [13]. It is also a rich source of bioactive compounds with antiproliferative properties and can induce apoptosis in cancer cells [14, 15]. However, to the best of our knowledge, the anticancer activities of Syzygium

*Address correspondence to these authors at the Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh; Tel: +61469857029; Fax +880-721-750064; E-mail: farhad_bio83@ru.ac.bd and Tel: +880-1914-254928; Fax: +880-721-750064; E-mail: jakbiochem@gmail.com

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cumin stem bark have never been studied elsewhere. Hence, we hypothesized that Syzygium cumini stem bark methanolic extract (SCBME) might inhibit proliferative activity as well as induce apoptosis of cancer cells. Therefore, in this study, we have investigated the anti-cancer property of SCBME and its mechanism of action against EAC cells in vivo.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

All the chemicals used in this study were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned. TIANamp Genomic DNA Kit (TIANGEN, Beijing, China), FvorPrep® blood/cultured cell total RNA mini kit (FAVORGEN, Taiwan), Go ScriptTM Reverse Transcription System (Promega, USA), Ethidium bromide (Genoxxon bioscience, Germany) and 100 bp DNA ladder (BIORON GmbH, Ludwigshafen, Germany) were of molecular grade.

2.2. Preparation of Syzygium cumini Barks Methanolic Extract

Mature Syzygium cumini stem barks were collected from the Rajshahi University campus in July 2018 and authenticated by the Department of Botany, University of Rajshahi, Bangladesh. The collected barks were first washed with distilled water, then chopped into small pieces and shed dried for 25 days. After complete drying, the entire portions were ground into coarse powder and stored in an airtight container at 4°C for further use. About 250 g of the powdered material was taken in a clean borosilicate glass reagent bottle and soaked in 750 ml of Methanol. The container was sealed and kept accompanying shaking and stirring with a magnetic stirrer. After 48 hours, the dissolved portion of the plant sample was separated. About 150 ml methanol was further added and the previous step was repeated. The resulting extract was filtered through Whatman No.1 filter paper and centrifuged at 6000 rpm for 10 minutes. Afterward, the solvent was evaporated under reduced pressure at 42°C using a rotary evaporator, and 29.68g (11.87% yields) SCBME obtained was kept at 4°C until further use.

2.3. Quantitative Phytochemical Analysis

Total phenolics and flavonoids contents were measured according to the protocol described in our previous study [16]. Total phenolics content of SCBME was expressed in term of gallic acid equivalent, GAE (standard curve equation: y = 0.0054x + 0.1112, R² = 0.9985), mg of GA/g of dry extract. Total flavonoids content was expressed in term of catechin equivalent, CAE (standard curve equation: y = 0.0021x + 0.037, R² = 0.9951), mg of CA/g of dry extract. Similarly, total flavonoids and proanthocyanidins in SCBME were estimated as described in our previous study [17]. Total content of flavonoids was expressed in term of quercetin equivalent, QUE (standard curve equation y = 0.002x+ 0.0187, R² = 0.9995), mg of QU/g of dry extract.

Total content of proanthocyanidins was expressed in terms of catechin equivalent, CAE (standard curve equation: y = 0.003x + 0.0185, R²= 0.9921), mg of CA/g of dry extract, β-carotene, lycopene and anthocyanin contents of SCBME were determined according to Vats et al., 2017 [18].

2.4. Antioxidant Activity of SCBME

Antioxidant activity of SCBME was determined following the assays including Antioxidant Capacity (TAC), Ferric Reducing Antioxidant Power (FRAP) assay as well as 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) and 2’-azino-bis(3-ethylbenothiazoline-6-sulfonic acid) (ABTS) free radical inhibition assays. TAC and FRAP of SCBME were determined by the methods as described in detail in a previous study of our research group [19]. Increased absorbance of the reaction mixture at 695 nm and 700 nm for TAC and FRAP, respectively indicated increased antioxidant activity.

DPPH and ABTS free radical scavenging activities of SCBME were determined according to the methods described previously by Das et al., 2019 [16]. Ascorbic acid was used as a reference compound. DPPH and ABTS absorbance at 517nm and 734nm, respectively were taken with a spectrophotometer, using methanol as a blank. Free radical scavenging activities for both DPPH and ABTS were measured using the following formula:

% of Inhibition = {(A₀ – A) / A₀} × 100

Where A₀ is the absorbance of the control (DPPH/ABTS radical solution without test sample) and A is the absorbance of the test sample.

2.5. Gas Chromatography-mass Spectrometry (GC-MS) Analysis

GC-MS analysis of active compounds from SCBME was carried out by Varian GC (Model Varian CP-3800, USA) with MS (Model: Varian Saturn-2200) spectrometer equipped with a flame ionization detector and capillary column with VF-5 ms (30m×0.25mm, 0.25μm). The instrument was operated in electron impact mode at the specified conditions (ionization voltage -70 eV, injector temperature - 250°C and detector temperature - 280°C). The carrier gas used was helium at a flow rate of 1 ml/minute and about 1 μl of the sample was injected. The temperature program for the column was from 40°C (1 minute) to 310°C at a gradually increased rate of 10°C/minute and then held finally 310°C for 10 minutes. The chemical compounds and measurements of peak areas were identified by GC-MS NIST LIBRARY (NIST 05).

2.6. Brine Shrimp Lethality Bioassay

Cytotoxicity of SCBME was screened against Artemia salina (brine shrimp) nauplii (hatched after 48 hours in saline water) in a 1-day in vivo bioassay according to a published protocol [17]. Concentrations of SCBME and gallic acids (standard) were 12.5, 25, 50, 100, and 200μg/ml with a final volume of 5ml in each vial in triplicates.

2.7. Experimental Animal, Cell Lines and Ethical Clearance

Healthy male Swiss albino mice, 6-7 weeks old (25 ± 2g body weight) were collected from the animal house of the Department of Pharmacy, University of Jahangirnagar, Dhaka, Bangladesh.

The initial inoculums of EAC cells were obtained from the Indian Institute of Chemical Biology (IICB), Kolkata, India. The culture and aspiration of EAC cells were maintained in our laboratory as ascites tumor in Swiss albino mice by intraperitoneal inoculation bi-weekly. The use of mice as an animal model for cancer research was approved by the Institutional Animal, Medical Ethics, Bio-Safety and Bio-Security Committee (IAMEBBC) for Experiments on Animal, Human, Microbes, and Living Natural Sources (No. 125/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

2.8. Determination of Anticancer Properties of SCBME

2.8.1. Determination of Median Lethal Dose (LD₅₀)

LD₅₀ was determined according to the protocol described previously [20]. In short, SCBME was dissolved in 2% DMSO in PBS and injected intraperitoneally to six groups of mice (n=6) at various doses. Toxicity was evaluated by recording mortality after 24 hours.

2.8.2. Determination of EAC Cell Growth Inhibition (in vivo)

To determine the cell growth inhibition, 100μl EAC cells from 2 × 10⁶ cells/ml PBS were injected into the peritoneal cavity of the five groups of Swiss albino mice (n=6) at day ‘0’ [21]. Treatments were started after 24 hours of tumor inoculation and continued for
five consecutive days. Group I was used as control receiving solvent only, Group II – IV received SCBME at the doses of 25, 50 and 75 mg/kg per day and Group V was treated with bleomycin at the dose of 0.3 mg/kg. On the 6th day of treatment, cells were collected and viable cells were first identified with Trypan blue and then counted with a hemocytometer under an inverted microscope (XDS-1R, Optika, South Korea). Cell growth inhibition was calculated by the following formula:

\[ \% \text{Cell growth inhibition} = (1 - \frac{T_w}{C_w}) \times 100 \]

Where \( T_w \) = mean number of tumor cells of treated groups and \( C_w \) = mean number of tumor cells of control.

### 2.8.3. Determination of Average Tumor Weight and Survival Time

This experiment was conducted according to a method previously described by Islam et al., 2013 [22]. For therapeutic evaluation, 100 μl of EAC cells from adjusted 2 × 10⁶ cells/ml was inoculated per mouse to five groups of mice (n=6) on the 0th day. Treatments were started after 24 hours of tumor inoculation and continued for 10 days. Tumor growth was monitored by recording daily weight change up to 20 days. Host survival was recorded and expressed as mean survival time in days and percent (%) increase of life span was calculated by using the following formula:

\[ \text{Mean survival time (MST)} = \frac{\sum \text{Survival time (days) of each mouse in a group}}{\text{Total number of mice}} \]

\[ \text{Per cent increase of life span (ILS)} \% = \left( \frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \right) \times 100 \]

### 2.8.4. Estimation of Hematological Parameters

Hematological parameters (Hemoglobin, RBC and WBC) were measured as described by Ruksana et al., 2019 with slight modification [23]. A comparison was made among six groups (n=6) of mice on the 11th day after inoculation. All the groups were first injected with EAC cells (0.1 ml of 2.0 × 10⁶ cells/mouse) except the normal group. After 24 hours of inoculation, PBS (0.1 ml/kg/mouse/day) was administered to normal and EAC control, respectively, for 10 days. Then, SCBME at doses of 25, 50, 75 mg/kg/mouse and bleomycin (0.3 mg/kg), were administered. On the 11th day after EAC cell inoculation, hematological parameters were measured from freely flowing tail vein blood of each mouse of each group by using cell dilution fluid. Total WBC and RBC were counted by a hemocytometer, and the percentage of hemoglobin (Hb) was measured by Sahli’s haemometer.

### 2.9. Evaluation of Apoptotic Efficiency of SCBME

#### 2.9.1. Observation of the Morphological Appearances and Nuclear Damages of EAC Cells

The study of cellular apoptosis was carried out by observation of morphological changes of EAC cells from both control and treated mice using a fluorescence microscope (Olympus iX71, Japan) followed by staining the cells with 4′, 6-diamidino-2-phenylindole (DAPI) [24]. In brief, EAC cells were collected from both control and treated (75 mg/kg) mice after 5 days of treatment and the cells were washed 2-3 times with PBS. 0.15% of Triton-X-100 was added and incubated at 37°C for 10 minutes in dark to make the cell membrane permeable to DAPI. Subsequently, the cells were stained with 5 μl of 1 mg/ml of DAPI solution and incubated in dark at 37°C for 10 minutes. Finally, the cells were washed with PBS and resuspended in PBS for observation of morphological changes under a fluorescence microscope.

#### 2.9.2. DNA Fragmentation Assay

To observe the nucleotide cleavage, DNA fragmentation assay was carried out followed by SCBME treatment [22]. Total genomic DNA was extracted from control and treated (75 mg/kg) EAC cells using TIANamp Genomic DNA kit (TIangen, Beijing, China) according to the manufacturer’s protocol. DNA was run on 1% agarose gel containing 0.1% ethidium bromide and visualized under UV-light.

#### 2.9.3. Gene Expression Analysis by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA from both treated (75 mg/kg) and untreated (control) EAC cells were isolated using RNA extraction kit (Favorgen, Taiwan) following the manufacturer’s guidelines. Two μg total RNA was converted into cDNA by using 1 μl Random Primer, 2 μl 2.5 mM MgCl₂, 1 μl GoScript™ Reverse Transcriptase, 1 μl PCR Nucleotide Mix (final concentration 0.5 mM each dNTP), 4 μl GoScript™ 5X Reaction Buffer and required amount of Nuclease-free water to a final volume of 20 μl (Promega, Madison-Wisconsin, USA).

Expression of p53, Bax, Bcl-2 and NF-κB were studied by RT-PCR as described by Zahan et al., 2019 [25]. GAPDH, a housekeeping gene, was also studied as an internal control. The sequences of the primer used in the experiment are given in Table 1. Each 10 μl of PCR reaction mixture contained 5 μl of master mix (Promega, Madison-Wisconsin, USA), 1 μl each of forward and reverse primer, and template of 1 μl and 2 μl of nuclease-free water. Reaction conditions were initial PCR activation step of 2 minutes and 10 sec at 95°C, followed by 35 cycles of 95°C for 45 sec, 52°C for 45 sec and 72°C for a final extension of 72°C for 5 minutes. The annealing temperature for NF-κB was 48°C instead of 52°C. For analyzing the PCR reactions, PCR products were analyzed on 1% agarose gel staining with 0.1% ethidium bromide and visualized in UV-trans-illuminator (Cleaver bioscience, UK).

### 2.10. Statistical Analysis

All analyses were carried out in triplicates and data were expressed as mean ± SD (Standard deviation). Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Dunnett’s ‘t’ test for anticaner activity and Duncan’s multiple range test (DMRT) for antioxidant activity using Statistical Package for Social Science (SPSS) statistical software of v16 version. p<0.05 was considered to be statistically significant.

**Table 1.** The sequence of primers used for PCR amplification.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: (5’-GTGGAAGGACTCATGACCCAG-3’) Reverse: (5’-CTGCTACTAGTGTAAGCAGC-3’)</td>
<td>52°C</td>
</tr>
<tr>
<td>p53</td>
<td>Forward: (5’-CACAAAAACAGTTAAACCCAG-3’) Reverse: (5’-AGGATATGCAGGCAGGAC-3’)</td>
<td>52°C</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Forward: (5’-GTGGAAGAGTCTTTACGGGA-3’) Reverse: (5’-AGGCACCAAGGTTAGTCAAC-3’)</td>
<td>52°C</td>
</tr>
<tr>
<td>Bax</td>
<td>Forward: (5’-GCGGCAACAGTCTGAAGCAGA-3’) Reverse: (5’-GCCACGCTGGGCCCTTAAATG-5’)</td>
<td>52°C</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Forward: (5’-AAACAAAATGCCCCACCGTTA-3’) Reverse: (3’-GGGACGATGCAATGGACTGT-5’)</td>
<td>48°C</td>
</tr>
</tbody>
</table>
3. RESULTS

3.1. Phytochemical Constituents of SCBME

The qualitative phytochemical screening of extract showed the presence of phenols and flavonoids as major contents in the obtained extract (Table 2). The order of polyphenols content in SCBME was TPC>TFC>TPR>TFLC. Also, SCBME contains a considerable amount of β-carotene, lycopene, and anthocyanin of 2.54, 0.05, and 0.23mg/g, respectively.

3.2. Chemical Composition of SCBME

GC-MS analysis of SCBME enabled the identification of 23 compounds (Table 3) (Fig. 1) of different chemical families. The compounds were accounted for 95.33% of total plant extract.

3.3. SCBME Possesses Strong Antioxidant Activity in vitro

SCBME showed a dose-dependent antioxidant activity (p<0.05) (Fig. 2). In total antioxidant capacity (TAC) assay, the absorbance of SCBME (400μg/ml) and ascorbic acid was 0.54±0.010 and1.118±0.006, respectively (Fig. 2a). In Ferric Reducing Antioxidant Power (FRAP) assay, the absorbance of SCBME increased in a concentration-dependent manner, which was comparable to ascorbic acid (Fig. 2b). SCBME exhibited significant (p<0.05) concentration-dependent scavenging activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Fig. 2c) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Fig. 2d) free radical. The IC₅₀ values of SCBME and ascorbic acid against DPPH were 11.04 and 9.02µg/ml as well as against ABTS were 9.56 and 7.46µg/ml, respectively.

3.4. Toxicity of SCBME Against Brine Shrimp and Swiss Albino Mice

SCBME showed moderate cytotoxic activity with a median lethal concentration (LC₅₀) value of 98.07µg/ml in comparison with gallic acid, whose LC₅₀ value was 81.48µg/ml (Table 4).

LD₅₀ of SCBME was found to be 1050mg/kg body weight for intraperitoneal treatment in male Swiss albino mice.

3.5. SCBME Significantly Inhibits Cancer Cells Growth and Proliferation

The effect of SCBME on EAC cell growth is shown in Fig. 3a. The maximum cell growth inhibition with SCBME was noted at the dose of 75mg/kg/day (71.08%) compared with the control mice.

Table 2. Amounts of different Polyphenols and bioactive phytochemical constituents in SCBME.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Phenolics²</th>
<th>Flavonoids²</th>
<th>Flavono³</th>
<th>Proanthocyanidins²</th>
<th>Lycopene (mg/g)</th>
<th>β-carotene (mg/g)</th>
<th>Anthocyanin (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amounts in SCBME</td>
<td>136.64±0.62</td>
<td>117.42±1.04</td>
<td>82.60±1.26</td>
<td>54.67±0.76</td>
<td>0.05±0.01</td>
<td>2.56±0.04</td>
<td>0.23±0.00</td>
</tr>
</tbody>
</table>

Note: Results were expressed as mean ± SD (n=3). a, b and c expressed in terms of GAE, CAE and QUE, respectively (mg of GAE, CAE and QUE/g of dry extract, respectively).

Table 3. Chemical composition of SCBME analyzed by GC/MS.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compounds</th>
<th>Retention Time (Minutes)</th>
<th>Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-butoxy-Ethanol</td>
<td>5.07</td>
<td>49.35</td>
</tr>
<tr>
<td>2</td>
<td>Cyclohexanone</td>
<td>6.31</td>
<td>0.72</td>
</tr>
<tr>
<td>3</td>
<td>1,2,3,5-tetramethyl-Benzeno</td>
<td>10.57</td>
<td>1.41</td>
</tr>
<tr>
<td>4</td>
<td>Cyclohexalectoxane, 1-(methyl)amino-4-[1-(4-methylphenyl)amino]-</td>
<td>13.57</td>
<td>0.63</td>
</tr>
<tr>
<td>5</td>
<td>2-Butenoic acid, 2-methyl-, 1,1a,b,4,4a,5,7a,b,8,9-decachydro-4a,7b-di-hydroxy-3-(hydroxy)methyl-1,1,6,8-tetramethyl-1,5-oxo-9aR-tetraprop[3,4][benz]c]-azulen-9a-diy ester, [1aR-[1a, alpha., 1b, beta., 4a, beta., 7a, alpha., 7b, alpha., 8, alpha., 9 beta. (E, 9a, alpha, E)]-[</td>
<td>48.31</td>
<td>0.16</td>
</tr>
<tr>
<td>6</td>
<td>2,4-Imidazoledinedione, 5-[3,4-bis([trimethylsilyloxy][phenyl]-3-methyl-5-phenyl-1-(trimethylsilyl)-</td>
<td>50.09</td>
<td>7.13</td>
</tr>
<tr>
<td>7</td>
<td>.psi., psi- Carotene, 3,3',4,4'-tetradehydro-1,1',2,2',-tetrahydro-1-hydroxy-1'-methoxy-</td>
<td>50.58</td>
<td>1.42</td>
</tr>
<tr>
<td>8</td>
<td>9,10-Anthracenedione, 1-(methylamino)-4-[1-(4-methylphenyl)amino]-</td>
<td>50.81</td>
<td>1.59</td>
</tr>
<tr>
<td>9</td>
<td>Acetic acid, 1,1',4'-triacetoxy-5,5'-disopropyl-6,6',7'-tetramethoxy-3,3'-dimethyl-[2,2']binaphthalenyl-4-yl ester</td>
<td>51.19</td>
<td>2.16</td>
</tr>
<tr>
<td>10</td>
<td>3,9 beta., 14,15-Diepoxyregn-16-en-20-one, 3,11 beta., 18-triacetoxy-</td>
<td>51.33</td>
<td>3.77</td>
</tr>
<tr>
<td>11</td>
<td>Canthaxanthin</td>
<td>51.49</td>
<td>3.59</td>
</tr>
<tr>
<td>12</td>
<td>Cephalotaxine, 11-(acetoxy)-, acetate (ester), (11, alpha.)-</td>
<td>51.57</td>
<td>3.74</td>
</tr>
<tr>
<td>13</td>
<td>1H-Cyclopent[c]oxazolone, 1-[2,3,5,6-bis-O-(1-methylhydroxyle) alpha., d-mannofuranosyl]hexahydro-4,5,6, tri-(phenylmethoxy), [3aR-[3a, alpha., 4, alpha., 5, beta., 9a, alpha, E)]-[</td>
<td>51.87</td>
<td>5.37</td>
</tr>
<tr>
<td>14</td>
<td>9,15-Desoxy-9-x-acetoxy-3,8,12-tri-O-acetylingol</td>
<td>52.12</td>
<td>3.57</td>
</tr>
<tr>
<td>15</td>
<td>Spiro[9,9][difluorene, 2', 2, (2,5,8,11-tetraoxadodecan)-1,12-diy]-</td>
<td>52.44</td>
<td>3.81</td>
</tr>
<tr>
<td>16</td>
<td>3,8,12-Tri-O-acetylingol 7-phenylacetate</td>
<td>55.95</td>
<td>1.47</td>
</tr>
<tr>
<td>17</td>
<td>2H-1,4-Benzodiazipin-2-one, 7-chloro-1,3-di-hydroxy-1-methyl-5-[4-[trimethylsilyloxy][phenyl]-</td>
<td>56.01</td>
<td>0.51</td>
</tr>
<tr>
<td>18</td>
<td>alpha., d-lumicolchicine</td>
<td>56.31</td>
<td>1.55</td>
</tr>
<tr>
<td>19</td>
<td>Pregn-16-en-20-one, 11,18-bis(acetyloyl)-3,9-epoxy-3-methoxy-, (3, alpha., 5, beta., 11, alpha.)-</td>
<td>57.68</td>
<td>0.51</td>
</tr>
<tr>
<td>20</td>
<td>3-Hydroxybromazepan, bis(trimethylsilyl)- deriv</td>
<td>57.84</td>
<td>0.62</td>
</tr>
<tr>
<td>21</td>
<td>6,6'-Diactetyl-7,7'-dihydroxy-2,2',4,4',5,5'-hexamethoxy-1,1'-binaphthalene</td>
<td>58.73</td>
<td>1.31</td>
</tr>
<tr>
<td>22</td>
<td>Pregnan-11,20-dione, 3,17,21-tris(trimethylsilyloxy)-, 20-(O-phenylmethyl)oxime, (3, alpha., 5, alpha.)-</td>
<td>59.23</td>
<td>0.62</td>
</tr>
<tr>
<td>23</td>
<td>Silane, ([(3, beta., 5, alpha., 11, beta., 20S)-pregn-3,31,17,20,21-pentienyl pentakis(oxyl)]pentakis(oxyl)trimethyl-</td>
<td>60.27</td>
<td>0.22</td>
</tr>
</tbody>
</table>
Anticancer Activity of Syzygium cumini

Fig. (1). GC-MS chromatogram of SCBME. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

Fig. (2). Antioxidant activity of SCBME. (a) Total antioxidant capacity of SCBME and ascorbic acid; (b) Ferric reducing antioxidant power assay of SCBME and Ascorbic acid; (c) DPPH free radical inhibition activity of SCBME and Ascorbic acid; (d) ABTS free radical inhibition activity of SCBME and Ascorbic acid. (A higher resolution / colour version of this figure is available in the electronic copy of the article).
Table 4. Concentration dependent cytotoxic potential of SCBME and standard gallic acid against Brine shrimp nauplii.

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>LC₅₀ (µg/ml)</th>
<th>95% Confidence Limits (µg/ml)</th>
<th>Regression Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>81.48 ± 0.47</td>
<td>45.113 to 147.178</td>
<td>y = 2.239 + 1.44x</td>
</tr>
<tr>
<td>SCBME</td>
<td>98.07 ± 0.39</td>
<td>49.99 to 192.41</td>
<td>y = 2.054 + 1.47x</td>
</tr>
</tbody>
</table>

Fig. (3). Anticancer activity of SCBME. (a) Effects of SCBME on EAC cell growth inhibition; (b) Tumor weight of EAC bearing mice treated with SCBME; (c) Effects of SCBME on survival time of tumour-bearing mice. Results are shown as mean ± SD (n=6), where significant values are *p<0.05, **p<0.01 and ***p<0.001 when treated (EAC+SCBME) mice compared with EAC bearing control mice (EAC only). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

where bleomycin (a clinically used anticancer drug) treated mice showed cell growth inhibition of 80.15% (0.3mg/kg/day).

It was noted that the treatment of SCBME on EAC-cells bearing mice resulted in dose-dependent inhibition of tumor growth, which is represented in Fig. 3b. In the case of the control group, tumor weight was increased by 16.92g after 20 days compared to the normal. However, treatment with SCBME at doses 25, 50 and75mg/kg (i.p.) increased tumor weight by 11.04, 6.28, and 5.16g, respectively. Therefore, treatment with SCBME at the doses 25, 50, and 75mg/kg decreased tumor burden of EAC cell bearing mice by 34.75%, 62.88%, and 69.50% respectively, compared to the control. Mean survival time and life span of tumor-bearing mice were increased dose-dependently after SCBME treatment (Fig. 3C). Mean survival time for control mice was 22.33 ± 0.58 days, whereas treatment with SCBME at the highest dose increased the mean survival time by 34.00 ± 0.58 days (Fig. 3e). Treatment with SCBME at doses 25, 50, and 75mg/kg resulted in increased life span significantly, which were 25.37%, 52.33%, and 73.13% respectively, when compared to control mice.

3.6. SCBME Restored the Depleted Hematological Parameters Towards Normal

Blood parameters in EAC-bearing mice were found to be significantly (p<0.01) deteriorated as compared to normal mice due to
the toxicity of tumourigenesis. Interestingly, these deteriorated parameters reverted towards the normal level when SCBME supplementation was given (Table 5).

### 3.7. SCBME Induced Apoptosis of EAC Cells

It was noted that nuclei of EAC cells were round, regular, and homogeneously stained with DAPI in control, whereas chromosome condensation, membrane blebbing, apoptotic body, and fragmented DNA were observed in SCBME treated EAC cells (Fig. 4). In addition, we found a non-fragmented distinct band on agarose gel for control EAC cell’s genomic DNA, whereas a fragmented band in the case of SCBME treated cell’s genomic DNA (Fig. 4e).

These results indicated that SCBME treatment could induce apoptosis in EAC cells.

### 3.8. SCBME Induced Alteration in the Expression of Apoptosis-related Gene

The expression of internal control GAPDH was consistent between the control and treated groups (Fig. 5a), whereas p53 was found to be overexpressed in SCBME treated EAC cells compared to control (Fig. 5b). Similarly, the expression of pro-apoptotic gene Bax was increased in SCBME treated EAC cells compared to control EAC cells (Fig. 5c). On the other hand, the expression of Bcl-2, an anti-apoptotic gene, was lower compared to control (Fig. 5d).

---

**Table 5.** Effect of SCBME on blood parameters of tumour bearing and normal mice on day 11 of tumour inoculation.

<table>
<thead>
<tr>
<th>Group</th>
<th>% of Hb (gm/dl)</th>
<th>RBC (Cells/ml)</th>
<th>WBC (Cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td>13.76 ± 0.50</td>
<td>(6.78 ± 0.19) × 10⁹</td>
<td>(11.60 ± 0.36) × 10⁶</td>
</tr>
<tr>
<td>Control (EAC cell bearing mice)</td>
<td>6.17 ± 0.61*</td>
<td>(3.1 ± 0.34) × 10⁹</td>
<td>(26.790 ± 0.47) × 10⁶</td>
</tr>
<tr>
<td>EAC + SCBME (25mg/kg)</td>
<td>8.87 ± 0.31*</td>
<td>(4.56 ± 0.22) × 10⁹</td>
<td>(20.24 ± 0.75) × 10⁶</td>
</tr>
<tr>
<td>EAC + SCBME (50mg/kg)</td>
<td>11.57 ± 0.25*</td>
<td>(5.65 ± 0.13) × 10⁹</td>
<td>(15.78 ± 0.25) × 10⁶</td>
</tr>
<tr>
<td>EAC + SCBME (75mg/kg)</td>
<td>13.13 ± 0.59*</td>
<td>(6.1 ± 0.09) × 10⁹</td>
<td>(11.07 ± 0.48) × 10⁶</td>
</tr>
<tr>
<td>EAC + Bleomycin (0.3 mg/kg)</td>
<td>13.72 ± 0.70*</td>
<td>(6.72 ± 1.6) × 10⁹</td>
<td>(11.16 ± 0.63) × 10⁶</td>
</tr>
</tbody>
</table>

Results are shown as mean ± SD (standard deviation) (n = 6), where control mice (EAC only) were compared with normal mice (no EAC) and treated mice (EAC + SCBME) were compared with control mice. The significant values are *p < 0.05, **p < 0.01 and ***p < 0.001.
Interestingly, a decreased level of \( \text{NF-\( \kappa \)} \text{B} \) expression in SCBME treated EAC cells was found (Fig. 5e). The results of gene amplification study reported that the treated mice showed up-regulation of p53 and up-regulation of pro-apoptotic gene Bax mRNA levels as well as down-regulation of \( \text{NF-\( \kappa \)} \text{B} \) and anti-apoptotic gene Bcl-2 mRNA levels (when compared with their respective control), which implied that SCBME inhibited EAC cells growth and proliferation by inducing apoptosis.

4. DISCUSSION

A growing number of experimental studies have suggested that the anticancer activity of plant materials is attributed to the presence of bioactive compounds with antioxidant potentialities [26-28]. Phytochemical analysis revealed that SCBME possessed a high amount of polyphenols such as phenolics, flavonoids, flavonols, and proanthocyanidins, which reflected the antioxidant and anti-cancer properties observed in the present study. SCBME also contains a considerable amount of anthocyanin, \( \beta \)-carotene, and lycopene, which are well-known antioxidants and have been thought of as potent anticancer agents [14]. This study also revealed that SCBME is a strong free radical scavenger activity and has potent antioxidant activity with a very strong potential to eradicate free radicals. SCBME also showed prominent dose-dependent reducing capacity in Ferric Reducing Antioxidant Power assay, which is supported by a previous study [29]. The IC\(_{50}\) value of 11.04 µg/ml in the DPPH assay is significantly lower than a previous finding (600 µg/ml) from Tamil Nadu, India [29]. It is suggested that variations in antioxidant content may result from different geographical positions.

It has been suggested that the compound having an LC\(_{50}\) value less than 250µg/ml against brine shrimp lethality bioassay is a significantly active and useful candidate for further research [11]. Considering this, having an LC\(_{50}\) value of 98.07µg/ml, SCBME may be a potent source of biologically active compounds. GC-MS analysis suggested significant bioactive compounds in SCBME. Many of the identified compounds are known to possess anticancer activities [30-37]. For example, 2,4-Inimidazolidinedione, one of the bioactive compounds found in SCBME, reported inhibiting the proliferation of human erythroleukemic K562 cells and human prostate cancer PC-3 cells through induction of apoptosis [30].

![Fig. (5). Agarose gel electrophoresis of extracted DNA and PCR product from untreated and SCBME (75mg/kg) treated EAC cells. Lane (M), (C), (T) represent molecular marker, control, and SCBME treated, respectively. (a) Equal expression of GAPDH in case of both control and treated EAC cells; (b) Upregulation of p53 genes in treated cells in comparisons to control; (c) Upregulation of Bax gene in treated cells in comparison to control; (d) Downregulation of Bcl-2 gene in case of treatment; (e) Downregulation of NF-\( \kappa \)B gene in case of treatment compared to control. (A higher resolution / colour version of this figure is available in the electronic copy of the article).](image-url)
Canthaxanthin, another prominent bioactive compound of SCBME, appeared to prevent cancer initiation. It was suggested that dietary supplementation of Canthaxanthin resulted in a 65% reduction of mammary cancer in rats induced by dimethylbenzanthracene [31]. Carotene, another compound found in SCBME, has shown in vitro anti proliferative activity against HepG2 cells [32]. Cephalotaxine (CET), a natural alkaloid present in SCBME, was derived from the Chinese coniferous tree *Cephalotaxus hupehensis* and found to have tumor cell growth inhibitory effect [33]. One of the Cephalotaxine ester called omacetaxine methyl succinate is FDA approved drug for both chronic- and blast-phase and in clinical trials [34]. Another compound present in SCBME was 9, 10-Anthracenedione which showed strong inhibition against fatty acid synthase expression in human breast cancer MDA-MB-231 cells [35]. Cyclohex- anone was also found in SCBME, which had been reported to exert anticancer potential against various human carcinoma cell lines by inducing apoptosis [36, 37]. The presence of these bioactive compounds in SCBME indicated that the noted significant anticancer activity could be the synergistic activity of these compounds against EAC cells.

Cell growth inhibition, reduction of tumor weight, increase in survival time and restoration of hematological parameter towards normal levels are considered as the reliable criteria for evaluating an anticancer drug [21, 22]. We found that SCBME fulfills all these criteria when compared with data obtained by running a parallel experiment with a clinically-used anticancer drug (bleomycin at the dose 0.3mg/kg) (Fig. 3 & Table 5). EAC-cell bearing mice were found to reduce hemoglobin concentrations and RBC counts gradually [21]. AEC was in EAC cell bearing mice reversed all the altered hematological parameters more or less to normal level suggesting the protective action of SCBME on the haemopoietic system. These findings indicated the compatibility of SCBME as a natural anticancer agent.

Escaping of apoptosis is the early incidence in tumor growth, which allows the cell to proliferate abnormally and leading to the progression of cancer [38]. Therefore, the induction of apoptosis of cancer cells without damaging surrounding normal cells is one of the mechanisms of anticancer agents [39]. Hence, to study the underlying mechanism of the anticancer potential of SCBME, we evaluated its apoptotic efficiency. It was found that among the three doses, 75mg/kg was more effective against EAC cells. Therefore, it was chosen to evaluate the apoptotic efficiency. Fluorescence and optical microscopy of SCBME treated EAC cells showed significant morphological changes, including cell membrane blebbing, the formation of apoptotic bodies, chromosomal condensation, and nuclear fragmentation. On the contrary, normal, round size cells and regular nucleus were observed in control mice (Fig. 4). These morphological alterations indicated the apoptosis of EAC cells. Further evaluation of EAC cells apoptosis was conducted by performing a DNA ladder test, which revealed fragmented bands in gel electrophoresis for SCBME treated mice’s DNA. However, the DNA extracted from control mice showed a relatively unique and distinct band. The activation of the endogenous Ca²⁺/Mg²⁺ dependent endonucleases mediated the cleavage of inter-nucleosomes and generated oligonucleotide fragments, which is the most distinctive biochemical hallmark of apoptosis [25]. Thus, the appearance of fragmented bands in agarose gel implies the induction of apoptosis in EAC cells by SCBME treatment.

To investigate the mechanisms by which SCBME inhibited cell growth and promoted apoptosis in EAC cells, we analyzed the expression of some genes related to cell proliferation and apoptosis. Cancer cells can use a number of diverse strategies to evade apoptosis [39]. One of the common strategies is a disruption in the balance of pro-apoptotic and anti-apoptotic proteins due to an under-expression or over-expression of the pro-apoptotic proteins and anti-apoptotic proteins, respectively, or a combination of both [38, 39]. We focused on this strategy because several compounds present in SCBME as well as some antioxidants rich plant extracts are reported to induce apoptosis by activating intrinsic pathways of apoptosis [8, 21, 28, 30, 35]. Interestingly, we found that SCBME up-regulated pro-apoptotic gene Bax, tumor suppressor gene p53 and down-regulated anti-apoptotic gene Bcl-2. p53 is a nuclear transcription factor, which trans-activates numerous target genes involved in the induction of cell cycle arrest and/or apoptosis [40]. In addition, p53 induces apoptosis through up-regulation of p53-responsive pro-apoptotic gene Bax. Furthermore, we observed the downregulation of NF-kB in SCBME treated EAC cells. In cancer cells, NF-kB is always constitutively activated and contributes to enhancing the proliferation and evasion of apoptosis [41, 42]. Thus, NF-kB is an important inhibitor of apoptosis and can protect cancer cells from death and inhibition of NF-kB and its signaling pathway could be a target of chemotherapy. Once activated and released into the nucleus, NF-kB trans-activates approximately 300 target genes encoding cytokines, growth factors, chemokines, and most importantly anti-apoptotic factors such as Bcl-2 [42]. Additionally, p53 and NF-kB can cause cell cycle arrest by regulating the expression of different cytokines and growth factors such as cyclin D and cyclin E, thereby inhibiting cell proliferation [43, 44]. Thus, a decreased level of NF-kB expression causes decreased expression of Bcl-2. The present study revealed that SCBME up-regulated p53 and Bax as well as down-regulated NF-kB and Bcl-2, which induced apoptosis of EAC cells.

CONCLUSION

In light of the above observations, it can be concluded that *Syzygium cumini* bark is a promising source of different bioactive phytochemicals, having significant anti proliferative, antioxidant and cytotoxic activities. The anticancer effects of SCBME could be due to the synergistic effects of various bioactive natural compounds, which appear to be mediated by multiple mechanisms, including induction of apoptosis through the regulation of target genes, p53, Bax, Bcl-2 and NF-kB. Therefore, SCBME may be considered as a promising resource in cancer chemotherapy with a better host safety profile. In order to ascertain this as a potential source of anticancer drugs, it is necessary to carry out further experiments against human cancer cell lines both in vivo and in vitro.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The use of mice as an animal model for cancer research was approved by the Institutional Animal, Medical Ethics, Bio-Safety and Bio-Security Committee (IAMEBBC) for Experiments on Animal, Human, Microbes, and Living Natural Sources (No. 125/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. All research procedures involving were in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf).

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIALS

Data incorporated in this manuscript are generated from primary research.
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The authors declare no conflict of interest, financial or otherwise.

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Anticancer Activity of Syzygium cumini


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