**Isolation of Phytochemicals From Anticancer Active Extracts of Syzygium alternifolium Walp. Leaf**

B Komuraiah\(^1\), Srinivas Chinde\(^2\), A Niranjana Kumar\(^3\), K V N Satya Srinivas\(^1\), Ch Venu\(^1\), J Kotesh Kumar\(^1\)*, K P Sastry\(^1\) and Paramjit Grover\(^2\)

\(^1\)CSIR-Central institute of Medicinal and Aromatic Plants, Research Centre, Boduppal, Hyderabad-500 092, Andhra Pradesh, India
\(^2\)Toxicology Unit, Biology Division, CSIR-IICT, Hyderabad, Tarnaka, Hyderabad-500007, Andhra Pradesh, India

**ABSTRACT**

**Objective:** The aim of the present study was to isolate the phyto molecules from the leaf of endemic medicinal plant, *Syzygium alternifolium*. The phytochemical investigation of the leaf of the plant yielded a flavonoid Eucalyptin 1 and a triterpinoid Epibetulinic acid 2 in pure state. **Results:** The compound 1 is being reported for the first time from this plant. The anti-cancer activity showed leaf hexane extract (IC\(_{50}\) values 8.177 and 2.687 µg/ml) was significantly active, when compared to extracts and compounds, against human cancer cell lines MCF-7 and DU-145. Also, hexane extract potentially inhibited the growth of DU-145 cell lines when compared with the reference compound doxorubicin. Amongst the isolated compounds, 1 was better cytotoxic than 2. **Conclusions:** The hexane extract of leaves of *S. alternifolium* yielded compounds 1 and 2 and the structure elucidation, based on spectroscopy, revealed them as Eucalyptin and Epibetulinic acid respectively. The compound 1 is being reported for the first time from this plant. The anti-cancer activity showed leaf hexane extract (IC\(_{50}\) values 8.177 and 2.687 mg/mL) was significantly active, when compared to extracts and compounds, against human cancer cell lines MCF-7 and DU-145. Also, hexane extract potentially inhibited the growth of DU-145 cell lines when compared with the reference compound doxorubicin. Amongst the isolated compounds, 1 was better cytotoxic than 2.

**Keywords:** *Syzygium alternifolium*, Myrtaceae, Eucalyptin, Epibetulinic acid, anticancer activity.

**INTRODUCTION**

*Syzygium alternifolium* is an endangered tree species belongs to family Myrtaceae, which is very rare and its existence is confined to a few places in the Tirumala hills of Chittoor district in Andhra Pradesh. There are few reports available on the bio-activity and phytochemistry of the plant. The whole plant is an excellent source of secondary metabolites which have several medicinal values like Antimicrobial\(^{[1]}\), Anti-diabetic\(^{[2]}\), Anti-inflammatory\(^{[3]}\), Hypoglycemic and Anti-Hyperglycemic\(^{[4]}\).

**MATERIALS AND METHODS**

**Collection of plant material**

*S. alternifolium* leaves were collected from Thirumala Hills of Andhra Pradesh, and was identified by Taxonomist, Prof. V.S. Raju of Kakatiya University, Warangal, Andhra Pradesh. A voucher specimen was deposited at the CIMAP-Research Centre, Hyderabad, India under the accession number CP-SA-1/2012.

**Extraction and isolation**

Leaves of *S. alternifolium* were collected, shade dried and powdered. The powdered plant material (1 kg) was extracted successively with Hexane and Methanol using soxhlet apparatus at reflux temperatures. The extracts were filtered and evaporated at reduced pressures to obtain crude mass. The hexane extract (20 g) was purified by column chromatography by using silica gel 100-200 mesh size as stationary phase. The fractions collected in 20 % ethyl acetate in hexane resulted in the isolation of a yellow coloured compound (100 mg) which was further
refined with cold hexane washings to yield compound 1 (50 mg). Similarly, fractions collected in 30% ethyl acetate in hexane fractions gave a colourless compound 2 (200 mg). The structures of the compound 1 and 2 were elucidated on the basis of 1H/13C/2D-NMR, and Mass spectra. The methanol extract did not yield any pure compound.

**Anticancer Activity**

**Cell culture**

Human breast cancer cell line (MCF-7) and human prostate cancer cell lines (DU-145) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) the cell lines were grown in DMEM medium supplemented with 10% FBS, 0.3% sodium bicarbonate, 10 mL/L antibiotic antifungal solution (10,000 U/ml penicillin, 10 mg/L streptomycin and 25µg/mL amphotericin B), 1 mL/L of 4mM L-glutamine and 1 mL/L of 100 mM sodium pyruvate culture was maintained in CO2 incubator at 37°C with a 90% humidified atmosphere and 5% CO2.

**Preparation of samples for MTT assay**

Test compounds, extracts and isolated compounds, were taken in 10 mg/ml of DMSO and various dilutions were made with sterile PBS (1X) to get desired concentrations. All formulations were filtered with 0.22 µm sterile filter and 20 minutes of UV eradication before adding to the 96 well plates containing cells.

**Cytotoxicity evaluation (MTT assay)**

Cytotoxicity of formulations was assessed using MTT assay to determine the cell viability according to a reported method. The assay is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells into purple formazan crystals which gets dissolved in DMSO by the mitochondrial dehydrogenase of viable cells into purple formazan crystals which gets dissolved in DMSO. Briefly, 1x104 exponentially growing cells were seeded into each 96 well plate (counted by Trypan blue exclusion dye method) allowed to grow till 60-70% confluence then compounds (name of the compounds if applicable) were added to the culture medium with the final concentrations ranging from of 10, 25, 50 and 100 µg/ml and along with controls (negative/without compound) and positive (Doxorubicin) incubated for 24 hours CO2 incubator at 37°C with a 90% humidified atmosphere and 5% CO2. Then the media of the wells were replaced with 90 µl of fresh serum free media and 10 µl of MTT (5mg/ml of PBS), plates were incubated at 37°C for 2h, there after the above media was discarded allow to dry for 30 minutes. Add 100µl of DMSO in each well at 37°C for 5min. The purple formazan crystals were dissolved and immediately read absorbance at 570nm was measured using Spectra Max plus 384 UV-Visible plate reader (Molecular Devices, Sunnyvale, CA, USA). IC50 values were determined by probit analysis software package of MS-excel. % Cell viability (from control) versus concentration.

**RESULTS AND DISCUSSION**

**Chemistry**

Structure elucidation of compounds 1 and 2 were done using IR, 1D/2D NMR and Mass spectral data. Basing on the spectroscopy and also comparison from the literature compound 1 was identified as Eucalyptin (Figure 1). Compound 1 was obtained as a yellow powder (Eucalyptin); TLC Rf: 0.41 in n Hexane/ethyl acetate (1:2); 1H-NMR (300 MHz, CDCl3, δ ppm) 6.560 (s), 6.300 (s), 6.306 (s), 2.156 (s), 2.016 (s), 2.336 (s), 2.781 (d), 6.976 (d), 3.84 (s); 13C-NMR: (75 MHz, CDCl3, δ ppm): 163.07, 103.27, 182.45, 152.20, 113.30, 7.47, 59.70, 161.80, 104.41, 7.76, 157.30, 107.98, 123.10, 127.19, 113.77, 156.51, 54.73. ESI-MS of 1 gave molecular ion peak at m/z 327 (M+1) corresponding to the molecular formula C18H36O5.

Basing on the various spectroscopy data and comparison from the literature compound 2 was identified as epibetulinic acid (Figure 1). White amorphous solid (m. p. 279–280°C); TLC Rf: 0.41 in n Hexane/ethyl acetate (1:3); IR In KBr (nue max cm⁻¹): 3448 (OH), 2934 (C-H), 1687 (C=O), 1641(C=C), 1450. 1H-NMR (300 MHz, CDCl3, δ ppm) (ppm): 4.53 (2H, d, J = 39 Hz), 3.17 (m), 3.00 (m), 2.29 (q), 2.23 (d), 2.21 (s), 0.75-1.61 (m, rest of protons); 13C-NMR: (75 MHz, CDCl3, δ ppm): 88.77, 77.44, 78.05, 38.90, 55.41, 18.33, 34.38, 40.76,50.58, 37.26, 20.90, 25.56, 38.45, 42.49, 30.61, 32.20,57.23, 46.93, 49.34, 150.41, 29.74, 37.06, 28.02, 15.36, 16.06, 16.15, 14.73, 179.40, 109.71, 19.41; ESI-MS m/z 455 [M-H] corresponding to the molecular formula C30H48O5.

Basing on the various spectroscopy data and comparison from the literature compound 2 was identified as epibetulinic acid (Figure 1). White amorphous solid (m. p. 279–280°C); TLC Rf: 0.41 in n Hexane/ethyl acetate (1:2); 1H-NMR (300 MHz, CDCl3, δ ppm) (ppm): 4.53 (2H, d, J = 39 Hz), 3.17 (m), 3.00 (m), 2.29 (q), 2.23 (d), 2.21 (s), 0.75-1.61 (m, rest of protons); 13C-NMR: (75 MHz, CDCl3, δ ppm): 88.77, 77.44, 78.05, 38.90, 55.41, 18.33, 34.38, 40.76,50.58, 37.26, 20.90, 25.56, 38.45, 42.49, 30.61, 32.20,57.23, 46.93, 49.34, 150.41, 29.74, 37.06, 28.02, 15.36, 16.06, 16.15, 14.73, 179.40, 109.71, 19.41; ESI-MS m/z 455 [M-H] corresponding to the molecular formula C30H48O5.

![Figure 1: Structures of the isolated compounds](image-url)
**Anticancer activity**

Anticancer activity, in *in vitro* mode, was performed on leaf hexane and methanol extracts and on the isolated compound 1 and 2 respectively (Table 1). The data from table 1 infers that leaf hexane extract (IC$_{50}$ values 8.177 and 2.687 µg/mL) was significantly active when compared with others against human cancer cell lines MCF-7 and DU-145. Hexane extract potentially inhibited the growth of DU-145 cell lines when compared with the reference compound doxorubicin. Amongst the isolated compounds, 1 was better cytotoxic on the above cell lines than 2.

**Table 1: Anticancer activity of leaf extracts and isolated compounds**

<table>
<thead>
<tr>
<th>Extract and Compound</th>
<th>IC$_{50}$ in µg/mL</th>
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<tbody>
<tr>
<td></td>
<td>MCF-7</td>
<td>DU-145</td>
<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>8.177±0.035</td>
<td>2.687±0.009</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>36.238±0.011</td>
<td>55.746±0.003</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>51.466±0.023</td>
<td>31.081±0.035</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>Doxorubicine</td>
<td>1.856±0.003</td>
<td>13.707±0.02</td>
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</tr>
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</table>

**CONCLUSION**

The Hexane extract of leaves of *S. alternifolium* yielded compounds 1 and 2 and the structure elucidation, based on spectroscopy, revealed them as Eucalyptin and Epibetulinic acid respectively. The compound 1 is being reported for the first time from this plant. The anti-cancer activity showed leaf Hexane extract (IC$_{50}$ values 8.177 and 2.687 mg/mL) was significantly active, when compared to extracts and compounds, against human cancer cell lines MCF-7 and DU-145. Also, Hexane extract potentially inhibited the growth of DU-145 cell lines when compared with the reference compound doxorubicin. Amongst the isolated compounds, 1 was better cytotoxic on the above cell lines than 2.

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**REFERENCES**