GC-MS ANALYSIS AND IN-VITRO CYTOTOXIC STUDIES OF BIXA ORELLANA SEED EXTRACT AGAINST CANCER CELL LINE

YOGESH KUMAR, LATHA PERIYASAMY*
Department of Biochemistry and Molecular Biology, Pondicherry University, Puducherry 605014, India
Email: yogeshkumarbiotech@gmail.com

Received: 24 Oct 2015 Revised and Accepted: 02 Dec 2015

ABSTRACTS

Objective: The present study was carried out to analyze the bioactive component of petroleum ether (PE) extract of Bixa orellana seed and its anticancer potential against B16F-10 melanoma cell line.

Methods: The presence of a bioactive component of PE extract was analyzed by GC-MS method. Cell viability was measured using MTT assay. Apoptotic inductive effect of the extract was evaluated by AO/EB staining; DAPI is staining and Annexin V/PI staining. DNA fragmentation analysis of the control and treated cell was carried out by agarose gel electrophoresis and comet assay. In vitro prevention of cell proliferation and migration was further estimated by colony forming assay and wound healing assay respectively.

Results: The cytotoxicity of the PE extract against B16F10 melanoma cell was dose and time dependent. The IC_{50} values for the B16F-10 melanoma cell were 145.98±7.49 µg/ml and 121.60±6.20µg/ml for 24h and 48h respectively. DAPI staining showed DNA fragmentation which was further confirmed by ladder-appearing pattern of DNA by gel electrophoresis and comet assay. PE extract has prevented the cell proliferation and migration of the treated cancer cell. GC-MS analysis shows the presence of three anticancer compound geranylgeraniol, squalene and beta-sitosterol.

Conclusion: The present study established that PE extract of Bixa orellana seed possesses potent anticancer and apoptotic inductive potential which can further be explored in-vivo model for possible cytotoxic activity.

Keywords: Bixa orellana, GC-MS, Geranylgeraniol, Comet assay, Annexin V/PI staining, AO/EB staining.

INTRODUCTION

Natural products as an anti-cancer agent have a long history. Many compounds isolated from plants are being employed in chemotherapy [1]. More than the 50% of the drugs sourced in clinical trials are from natural sources or related to it[2]. Many plant metabolites induce apoptosis in cancer cells but not in normal cells, thus it is a potential drug lead [3-5]. Cancer is a dreadful disease caused by abnormal and uncontrolled cell division and having the potential to invade other parts of the body. About 6 million new incidences of cancer are reported yearly worldwide [6] and more than 70 percent of all cancer deaths occurred in low-and-middle-income countries. Deaths from cancer are projected to continue rising worldwide with an estimated 12 million deaths by 2030 [7]. The number of melanoma cases is increasing worldwide faster than any other cancer and estimates suggested a doubling of melanoma incidence every 10-20 y [8, 9]. Bixa orellana is a plant native to Brazil and cultivated in other tropical countries. The seeds of this plant produce the dye most frequently used worldwide in the food, textile, paint, and cosmetic industries. The use of this dye has been authenticated by Professor N. Parthasarathy of Department of Ecology and Environmental Sciences, School of Life Sciences, Pondicherry University, Puducherry-605014, India.

MATERIALS AND METHODS

Chemicals and reagents

DMEM medium, Fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA, PBS (phosphate buffer saline), Acridine orange, Ethidium bromide, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) used in the work were purchased from HIMEDIA Pvt. Ltd, Bombay, India. Annexin V/PI kit was obtained from Sigma. The solvents used were of analytical grade.

Cell culture

B16F-10 melanoma cell was obtained from National Centre for cell sciences (NCCS), Pune. Cells were maintained as an adherent cell line in Dulbecco’s modified Eagle’s medium (DMEM medium, PH 7.4), supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37 °C in a humidified 5% CO_{2} atmosphere. Cells were passaged as needed using 0.5% trypsin-EDTA.

Collection and identification of plant material

The seeds of Bixa orellana used for the investigation were purchased from a local herbal shop in Pondicherry and later it was authenticated by Professor N. Parthasarathy of Department of Ecology and Environmental Sciences, School of Life Sciences, Pondicherry University, Puducherry-605014, India.

Preparation of extracts

A 100 g sample of dried seed powder of Bixa orellana was extracted in 400 ml of petroleum ether in soxhlet apparatus for 72 h. It was evaporated in rotavapor and kept in hot air oven at 40 °C overnight to remove traces of petroleum ether and obtained extract was kept in an airtight container at 4 °C for further use.

Gas chromatography-mass spectroscopic analysis

GC-MS analysis of the PE extract of Bixa orellana seed was performed using the equipment GC-MS-5975C (AGILENT). The equipment has a...
DB 5–MS Capillary Standard non-polar column with dimensions of 30 mm × 0.25 mm ID × 0.25 µm films. The carrier gas used is Helium with a flow of 1.51 ml/min. The injector was operated at 250 °C and the oven temperature was programmed as follows: 70 °C for 3 min, then gradually increased to 300 °C at 9 min. The identification of components was based on NIST libraries search.

Measurement of cytotoxicity by MTT assay

This assay was performed according to a slight modification of the procedure reported by Mosmann [12]. All cells were grown as a monolayer in 96 well plate seeded at a density of 5,000 cells/well/200 µl of complete medium. The cells were grown for 24 h before drug treatment. The different concentration of the extract was treated for another 24 and 48 h after that cytotoxicity was determined by MTT assay. 20 µl of MTT (5 mg/ml) were added to each well and kept in the dark for another 4 h at 37 °C. The formazan crystal formed was dissolved in 100 µl of DMSO and reading was taken at 570 nm in Elisa plate reader (Molecular Devices Inc, Sunnyvale, CA, USA). Doxorubicin was used as a positive control.

Acridine orange/ethidium bromide (AO/EB) staining

Cell morphological changes were assessed by differential staining using Acridine orange and ethidium bromide [13]. Approximately 1,00,000 cells were seeded into 24-well tissue culture plates, incubated for 24 h, treated with IC_50 drug concentration and incubated for 24 h. Untreated cells were used as negative control. After 24 h of treatments, cells were stained with AO/EB prior to microscopic examination. The stained cells were examined under a fluorescence microscope using a UV filter (450-490 nm).

DAPI staining

Detection of apoptosis by DAPI staining were performed by the method of Machana et al.[14]. 1,00,000 cells were seeded into 24 well culture plate for 24 h and treated with IC_50 drug concentration for another 24 h. Untreated cells were used as negative control. The cells were fixed in 4% paraformaldehyde for 20 min and then stained with 5µg/ml DAPI dye at 37 °C for 10 min. The image was taken with a Nikon Eclipse Ti fluorescence microscope (Nikon Instruments Inc., NY, USA) with ultraviolet illumination.

DNA fragmentation analysis by agarose electrophoresis

DNA fragmentation assay was performed as described by Sheng et al.[15]. The B16F-10 melanoma cells were plated in 60 mm culture dish at a density of 2 × 10^6 cells for 24 h and treated with PE extracts (121 µg/ml and 145 µg/ml) for 48 h. After that cells were collected and centrifuged to 1,500 rpm for 5 min at 4 °C to make pellet. DNA was prepared from the pelleted cells. Briefly, cells were lysed with 200 µl of lysis buffer containing 200 µg/ml RNaseA and 200 µg/ml proteinase K, after that it was incubated at 40 °C for 1h. 10 µl of the loading buffer (0.25% bromophenol blue, 30% glycerol) with equal quantities of DNA were electrophoresed in 0.8% agarose gel containing 0.2 µg/ml of ethidium bromide. After electrophoresis, the gel was photographed by syngeneic gel documentation system.

Comet assay

DNA fragmentation is considered as a hallmark of apoptosis. The alkaline version of the single cell gel electrophoresis assay (comet assay) was used to evaluate DNA damage as previously described by Lima et al.[16]. DNA damage was quantified using CASP (Comet Assay Software Project) software. The images were used to estimate the DNA content of individual nuclei and to evaluate the degree of DNA damage in the comet tail.

Colony forming assay

B16F10 melanoma cells were seeded at a density of 300 cells/ml in 6-well culture plates, treated with PE extract for 24 h, then washed with PBS and fresh medium was added. Cells were seeded in duplicate and allowed to grow for 10 d. Colonies were visualized by staining the cells with 0.5% crystal violet, and pictures were captured using a Nikon Coolpix L26 camera. The cell survival fraction was observed in treating cells to that of untreated control cells.

Wound healing assay

B16F10 cells were allowed to grow confluent in 6-well plates and after it had reached confluent wound was made scraping with 200 µl pipette tips and washed with PBS. Cells were then incubated with fresh complete medium containing 100 µg/ml of PE extract. Photographs were taken at the wound at 0 and 24h of time interval.

Annexin V/PI apoptosis detection

An Annexin V/PI kit (Sigma, USA) was used according to the manufacturer’s instructions. Briefly, 500 µl of binding buffer was added to the PE extract treated and control B16F10 cells followed by staining with an Annexin-V-FITC and PI solution for 30 min at room temperature in the dark. The images were taken in Nikon Eclipse Ti fluorescence microscope. Annexin V (Green fluorescence) uptake was observed in early apoptotic cells and red fluorescence was observed in necrotic cells or late apoptotic cells which uptake the PI dye.

Statistical analysis

All analysis was performed in triplicate, and the data were reported as means±SD. IC_{50} values were calculated by linear regression analysis using Microsoft excel 2010. Comparisons of the results were made using analysis of variance (ANOVA) by SPSS 16. Significant differences (* p<0.05) between the means of control and treated cells were analyzed by Tukey test.

Results

GC-MS analysis

The identified principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (peak area %) are presented in table S1 and fig. 1 which shows the presence of 43 compounds in the PE extract of Bixa orellana. Out of the 43 compound identified, Trans-Geranylgeraniol (19.69%) [17, 18] Squalene (3.14%) [19] and β-sitosterol (3.56%) [20, 21] have been reported for anticancer activity on the other cell lines.

Fig. 1: GC-MS chromatogram for PE extract of Bixa Orellana L.
Acridine orange/ethidium bromide (AO/EB) staining

The results obtained with acridine orange & ethidium bromide double staining of control and treated B16F-10 cells are presented in the fig. 3. The untreated control cells fluoresced uniformly green and had normal features whereas cells treated with PE extract fluoresced orange-red that indicated the apoptotic features such as cell shrinkage, chromatin condensation, nuclear fragmentation and apoptotic body formation.

DAPI staining

Programmed cell death plays an essential role for an effective cancer therapy [22]. The morphological changes of the nuclei DNA after PE extract treatment are shown in fig. 4. The result indicated that the PE extract induces nuclear morphological changes compared to control cells, and this may be indicative of apoptosis. Morphological changes observed in the treated cells included broken nuclei into discrete fragments.

DNA fragmentation and comet assay

The comet assay is a rapid and sensitive technique for measuring DNA damage at the level of a single cell. The control cells showed little or negligible DNA damage, but the treatments with PE extract
produced a high percentage of damaged cells. The treated cancer cell shows the 21.17 % and 37.35 % of DNA damage in tail after treatment of 121 µg/ml and 145 µg/ml PE extract for 24h respectively (fig. 5a&b). For cell undergoing apoptosis fragmentation of Genomic DNA into the characteristic ladder on agarose is an important feature. Treatment of B16F10 melanoma cell with 121 and 145 µg/ml PE extract produces a DNA ladder with a smear (fig. 6). The smear could be due to the some post-apoptotic cell necrosis. In comparison, of treated cells, control cells did not produce any ladder or smear.

Colony forming and wound healing assay

The use of a colony formation assay further demonstrated that the treatment of PE extract resulted in decreased cell proliferation of B16F10 melanoma cell (Fig.7). As shown in Fig.8, treatment of B16F10 melanoma cell with 100 µg/ml of PE extract for 24h, reduced the migration of cell at the wound site. But in the control, major part of the wound site was covered by the cell. This shows the in vitro prevention of cell proliferation by PE extract.

Annexin V/PI apoptosis detection

Annexin V-FITC is a fluorescent probe, which binds to Phosphatidylserine in the presence of calcium. At the onset of apoptosis phosphatidylserine which is normally found on the external part of the plasma membrane, becomes translocated to the internal part of the membrane, so it becomes available to Annexin V whereas PI detects necrotic cells and late apoptotic cell with permeabilized plasma membrane. Untreated cell did not show any green fluorescence but treated cell shows green fluorescence in early apoptotic cell and green with red or only red in late apoptotic and necrotic cells (fig. 9).

Table S1: Identified compound by GC-MS analysis of seed extract of Bixa orellana

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>RT</th>
<th>Name of the compound</th>
<th>Molecular formula</th>
<th>Molecular mass</th>
<th>Peak area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.956</td>
<td>Naphthalene</td>
<td>C10H8</td>
<td>128.17</td>
<td>1.31</td>
</tr>
<tr>
<td>2</td>
<td>13.047</td>
<td>Alpha-Muuroleone</td>
<td>C20H30</td>
<td>284.35</td>
<td>0.21</td>
</tr>
<tr>
<td>3</td>
<td>14.930</td>
<td>Ledene oxide-(E)</td>
<td>C10H20</td>
<td>204.35</td>
<td>0.19</td>
</tr>
<tr>
<td>4</td>
<td>17.895</td>
<td>(E,E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene</td>
<td>C28H42</td>
<td>272.46</td>
<td>1.83</td>
</tr>
<tr>
<td>5</td>
<td>17.927</td>
<td>1,5,9-decatrione, 2,3,5,8-tetramethyl-</td>
<td>C12H24</td>
<td>192.34</td>
<td>0.28</td>
</tr>
<tr>
<td>6</td>
<td>18.219</td>
<td>(E,E)-3,7,11,15-Tetramethylhexadeca-1,3,6,10,14-pentaene</td>
<td>C26H32</td>
<td>272.46</td>
<td>0.15</td>
</tr>
<tr>
<td>7</td>
<td>18.385</td>
<td>Alpha-Farnesene</td>
<td>C12H24</td>
<td>204.35</td>
<td>0.47</td>
</tr>
<tr>
<td>8</td>
<td>18.448</td>
<td>1,5,9-cycloctadecatriene, 1,5,9-trimethyl-12-(1-methylethenyl)-</td>
<td>C22H34</td>
<td>272.46</td>
<td>0.17</td>
</tr>
<tr>
<td>9</td>
<td>19.714</td>
<td>Androsta-3,5-dien-7-one</td>
<td>C22H34</td>
<td>270.40</td>
<td>0.89</td>
</tr>
<tr>
<td>10</td>
<td>20.529</td>
<td>Trans-Geranylgeraniol</td>
<td>C22H32</td>
<td>290.48</td>
<td>19.69</td>
</tr>
<tr>
<td>11</td>
<td>20.713</td>
<td>Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-</td>
<td>C24H32</td>
<td>290.48</td>
<td>0.33</td>
</tr>
<tr>
<td>12</td>
<td>21.375</td>
<td>1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-(E,E)-</td>
<td>C26H34</td>
<td>290.48</td>
<td>0.46</td>
</tr>
<tr>
<td>13</td>
<td>21.432</td>
<td>4-Cyclopropylmethylbenzonoitrile</td>
<td>C13H14N</td>
<td>157.21</td>
<td>0.34</td>
</tr>
<tr>
<td>14</td>
<td>22.876</td>
<td>Butyl 9,12-octadecadienoate</td>
<td>C22H34O2</td>
<td>336.55</td>
<td>0.56</td>
</tr>
<tr>
<td>15</td>
<td>22.921</td>
<td>Myrcenylacetat</td>
<td>C15H20O2</td>
<td>196.28</td>
<td>0.18</td>
</tr>
<tr>
<td>16</td>
<td>24.626</td>
<td>Tetracosane</td>
<td>C24H32</td>
<td>338.65</td>
<td>0.25</td>
</tr>
<tr>
<td>17</td>
<td>25.351</td>
<td>Octacosane</td>
<td>C20H32</td>
<td>394.76</td>
<td>0.27</td>
</tr>
<tr>
<td>18</td>
<td>25.453</td>
<td>Squalene</td>
<td>C24H30</td>
<td>410.71</td>
<td>3.14</td>
</tr>
<tr>
<td>19</td>
<td>26.038</td>
<td>Octadecane</td>
<td>C18H36</td>
<td>254.49</td>
<td>0.37</td>
</tr>
<tr>
<td>20</td>
<td>26.713</td>
<td>Heneicosane</td>
<td>C20H36</td>
<td>296.57</td>
<td>0.32</td>
</tr>
<tr>
<td>21</td>
<td>26.763</td>
<td>7-Amino-4-methyl-1,8-naphthyridin-2-ol</td>
<td>C22H32N2O</td>
<td>175</td>
<td>0.11</td>
</tr>
<tr>
<td>22</td>
<td>27.029</td>
<td>3,5,9-Undecatrien-2-one, 6, 10-dimethyl-(E,Z)-</td>
<td>C20H30O</td>
<td>192.29</td>
<td>25.77</td>
</tr>
<tr>
<td>23</td>
<td>27.381</td>
<td>Tricosane</td>
<td>C22H40</td>
<td>324.62</td>
<td>0.37</td>
</tr>
<tr>
<td>24</td>
<td>27.940</td>
<td>Benzenepropanonitrile, 3,4-dimethoxy-</td>
<td>C16H13NO2</td>
<td>191.23</td>
<td>3.60</td>
</tr>
<tr>
<td>25</td>
<td>28.119</td>
<td>Biosane</td>
<td>C20H32</td>
<td>282.54</td>
<td>0.40</td>
</tr>
</tbody>
</table>
DISCUSSION
Plants are directly or indirectly associated with human life and are an essential source of human well-being. This is a valuable source for medicine including cancer. They have contributed to fighting against numerous diseases. Many of the modern medicines are produced from the different pharmacological active ingredient of medicinal plants. The analysis and process of extraction of plant material play a crucial role in the development, modernization and quality control of herbal formulations. Studying about medicinal plants conjointly facilitates to understand plant toxicity and also helps to safeguard human and animals from natural toxins. Several anticancer studies have focused on the ability of pure compounds; however, there have been reported that the total contents of a whole herb show a significantly better synergistic effect than a single isolated active ingredient [23, 24]. The cytotoxicity of PE extract against B16-F10 melanoma cell was dose and time dependent manner. As the concentration of PE extract increased the number of cell death was more this was evident by MTT assay and morphological observations. In our study, the control cells showed nuclei with homogenous chromatin distribution, whereas the chromatin amount was less in PE treated cancerous cell [25] and morphological modifications were clearly visible during apoptosis. Fluorescence microscopy analysis revealed that the PE extract induced apoptosis and caused morphological changes in cells. Some of the changes observed include chromatin condensation; plasma membrane blabbing and DNA fragmentation [26]. The characteristic of apoptosis was also further confirmed by DNA ladder which is a result of DNA fragmentation.

The presence of various bioactive compounds were detected using GC-MS analysis of the PE extract justifies the use of Bixa orellana seed for various elements by traditional practitioners. Oboh et al. have reported that non-polar fraction of Bixa orellana seed has a more protective of oxidative stress than the polar fraction [27]. Lopes et al. have reported gerangeralin from Bixa orellana seed as a death inducer in Leishmania amazonensis [28], trans-Geranylgeraniol [17], Squalene [19] and β-Sitosterol [21] have a report for anticancer activity on another cell line. This is the first time evaluation of PE extract of Bixa orellana seed on B16F-10 melanoma cell line. Medicinal plant forms the backbone of traditional medicine. In the last few decades, plants have been the subject for very intense pharmacological studies. This has been brought about by therapeutic value and as sources of lead compounds in drug development.

CONCLUSION
Considering the results of our in vitro studies, it clearly shows a cytotoxicity and apoptosis inductive effect indicative of an anti-cancer activity. The presence of trans-Geranylgeraniol, Squalene and β-Sitosterol seems to be responsible for cytotoxicity and apoptotic-like characteristics. Further studies are being carried out on the expression of marker enzymes and protein expressed in apoptosis in order to provide more comprehensive data on the anticancer activity of the extract.

ACKNOWLEDGMENT
The author thanks the Bureau Veritas Consumer Products Services (I) Pvt. Ltd., Guindy, Chennai for carrying out GC-MS analysis of the sample. The author thanks the DIT IAPS, Pondicherry University for providing the infrastructural facility.

CONFLICT OF INTERESTS
Declared None

REFERENCES


