APOPTOSIS EFFECT OF ISOLATED PHENOLIC SECOIRIDOID GLUCOSIDE FROM AERIAL PARTS OF *GOMPHRENA GLOBOSA* IN A-431 CELL LINES

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ABSTRACT

The phenolic secoiridoid glucoside (Oleuropein) isolated from chloroform extract of aerial parts of *Gomphrena globosa* was evaluated for the apoptosis effect responsible for cytotoxic activity on A-431 cell lines. The isolated compound was elucidated by spectral characterization and was already reported. The presence of O-substituted dihydroxy phenolic group may be responsible for the activity. The possible mechanism of apoptosis was studied by LDH Leakage assay, DNA fragmentation and caspase -3 activity and was found out that the apoptosis may be through killing the target cell by cytotoxic T-lymphocyte and natural killer cells and caspase-3 dependent apoptosis.

KEYWORDS: *Gomphrena globosa*, cytotoxic activity, apoptosis, LDH leakage assay, caspase -3 activity, phenolic secoiridoid glucoside, A-431 cell lines.

INTRODUCTION

Apoptosis is a process of programmed cell death in multi cellular organisms that causes death in cells and lead to characteristic cell changes (morphology) and death. The cell changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Apoptosis is essential for survival of the body as a whole.
and this plays critical roles in many developmental processes and the immune system. It is active and genetically controlled processes that remove unwanted or damaged cells \[1, 2\]. It is fundamental to the initiation and progression of many human diseases. Diseases associated with increased cell survival or inhibition of apoptosis has been thoroughly reviewed \[3\]. It has been implicated in neurodegenerative disorders, AIDS \[4\], autoimmune disorders \[5\] and other viral disease \[6\], etc. Dysregulated apoptosis, results in excessive or insufficient cell death. Excessive apoptosis causes atrophy, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer.

Cancer is a dreadful disease in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. Among the types of cancer, skin cancer is one that causes major cancer death worldwide. In this non-melanoma skin cancer (NMSC) is one of the most commonly diagnosed malignancies in the world \[7, 8\]. Surgery and chemotherapy using 5% fluorouracil are the standard treatments for this. Even though there is a high rate of response to this therapy, most treated patients relapse with tumor \[9\]. Therefore, the treatments for skin cancer require novel strategies to target and eliminate skin cancer cells. The new therapies have been directed towards identifying agents that block proliferation and induce apoptosis of skin cancer cells, and thus, the researchers have been searching for new molecules from plants for skin cancer therapy.

A-431 cells are a model cell line (human skin carcinoma cell line) used more specifically in studies of the cell cycle and cancer-associated cell signalling pathways since they express abnormally high levels of the epidermal growth factor receptor (EGFR) and they contain no functional p53, a potent tumor suppressor gene, and so are highly sensitive to mitogenic stimuli (Horst Ibelgaufs’ Encyclopedia).

*Gomphrena globosa*, is commonly known as Globe Amaranth or Bachelor Button. It belongs to the family Amarantheceae, and is an annual plant that grows up to the height of 24 inches. The true species has magenta bracts, and the cultivars have colors such as purple, red, white, pink, and lilac. It is distributed in Brazil, Panama and Guatemala. The whole plant parts are used in the treatment of prostate problems \[10\] and gangrenous wounds. Previous study reports the presence of the phytochemicals such as saponins, alkaloids, reducing sugars and coumarins and pharmacological activities such as low cytotoxicity and antihypertensive
activity. Studies also reported the presence of twenty four phenolic compounds and eight betacyanins in three different extracts of *Gomphrena globosa* L. inflorescences which decreased nitric oxide content in LPS-stimulated RAW-264-7 cells \[11\]. Isolation of Triterpenoid saponin (gomphrenoside) and hopane derivative (hopan-7β-ol) from the aerial parts of the plant has been reported \[12\]. Petroleum ether extract of *G. martiana* was found to possess antitumor activity in the lipophilic flavanoid fraction. Similarly, *G. macrocephala* has been reported for anti-tumor activity. Literature reveals that different species of the same genus possess almost similar pharmacological activities \[13, 14\]. Based on the above factor, the present study aimed to investigate the cytotoxic activity of chloroform extract of *Gomphrena globosa* (GGC) and of its isolated compound, phenolic secoiridoid glucoside, and the possible mechanisms of apoptosis activity.

**MATERIALS AND METHODS**

**General experimental methods**

IR spectra of isolated compound were recorded using a Fourier Transformed-Infra Red (FT-IR) spectrophotometer of Jasco make and model of 4100. NMR spectra of compound I were recorded using a Bruker Avance-111, 400MHz spectrometer 9.4 Tesla super-conducting magnet equipped with a BBO 400MHz, with Z-gradient nucleus probe, operating temperature range 360°. LC-ESI-MS and LC-ESI-MS/MS analysis was conducted on Agilent 6520 accurate mass Q-TOF LC/MS coupled with Agilent LC 1200 equipped with extend C18 column of 1.8 µm, 2.1x50 mm. Gradient elution was performed with methanol (solvent A, 70%) and 0.1% formic acid (solvent, 30%) at a constant flow rate of 0.3 ml/min. Column temperature was maintained at 30°C. The MS analysis was performed using ESI in the positive mode. The condition for mass spectrometry were as follows: drying gas (nitrogen) flow 5 L/min; nebulizer pressure 50 psig; drying gas temperature 325°C; capillary voltage +3000 V; fragmentor volt 250 V; Oct Rf Vpp 750 V.

**Plant material**

The plant, *Gomphrena globosa* are widely distributed in and around Tiruchengode of Namakkal district. The aerial parts of the plant were collected freshly in the month of June and authenticated (Specimen No. 1368) by Dr. G. V. S. Murthy, Joint Director, Botanical Survey of India, Coimbatore, Tamilnadu, India.
Extraction and isolation

Aerial parts of *Gomphrena globosa* were dried under shade and then powdered to coarse. Powdered material (500 g) was extracted with chloroform; ethanol and water by continuous hot extraction method for 48 h by using Soxhlet apparatus. The extracts were concentrated to a dry mass by vacuum distillation. All these extracts were subjected to *in-vitro* anticancer study by Trypan blue method to know the nature of the bioactive constituent. The results indicate that the bioactive compound nature is lipophilic with the lowest IC\textsubscript{50} values compare with other extracts. So the present was carried out to evaluate the cytotoxic activity and the possible mechanism for the activity of the isolated compound.

The GGC was subjected to column chromatography for the separation of phytoconstituents. A column of suitable size (1 m x 1.5 inch) was chosen and packed with silica gel 60-120 mesh by adding slurry of the adsorbent in hexane. GGC was dissolved in chloroform, and mixed with silica gel (60-120 mesh) and fed to the column through a funnel. Hexane was added to the column and kept aside without disturbance for overnight for the settlement of the extract. Maximum precautions were taken to remove the air bubbles. The column was eluted with different organic solvents in the order of increasing polarity (hexane, petroleum ether, chloroform, methanol and water). All the fractions were subjected to TLC studies. Fractions showing similar R\textsubscript{f} value, melting point and identification test, were pooled together and solvents evaporated to get residues. Two major fractions were obtained from analytical column, and denoted as fraction I and fraction II. Fraction I was collected from the elution of petroleum ether: chloroform (70: 30), (60: 40) and (50: 50) and fraction II from the elution of chloroform: methanol (80: 20), (70: 30) and (50: 50) yielded fraction II. Two compounds, compound I and compound II from fraction I and II respectively were isolated by preparative TLC method. The result of MTT assay indicate that the compound I posses cytotoxic activity. So the present study was focused to study the possible apoptosis mechanism through LDH Leakage assay, DNA fragmentation and Caspase -3 activity.

Apoptosis study

LDH leakage assay

The monolayer cell culture was trypsinized and the cell count was adjusted to \(1.0 \times 10^5\) cells/ml using medium containing 10\% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once
with medium and 100 µl of different compound I concentrations (1000, 500, 250, 125, 62.5, 31.25 and 15.625 µg/ml) were added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, microscopic examination was carried out and observations were recorded every 24 h. After 72 h, the supernatant was collected from individual wells, centrifuged at 2000rpm for 10 min. and collected the supernatant into clean vials. 100µl of each sample was transferred to a fresh clean 96 well plate, added 100µl of reaction mixture of the kit and incubated at room temperature for 30 min in dark. The absorbance was measured using microplate reader at a wavelength of 490 nm. The percentage activity was calculated over the untreated control samples \[15\].

**DNA fragmentation study**

Cells (3 x 10⁶ /ml) were seeded into 6 well plates and incubated at 37°C with 5% CO₂ atmosphere for 24 h. The cells were washed with medium and were treated with different doses (50 and 100 µg/ml) of the compound I, standard drug (camptothecin, 3µg/ml) and incubated at 37°C, 5% CO₂ for 24 hrs. At the incubation time ended, the chromosomal DNA of cancer cells was prepared with apoptotic DNA ladder kit (Apoptic DNA ladder kit was purchased from Roche, Germany). Briefly, cells were harvested and lysed with lysis buffer for 10 min. Then the samples were mixed with isopropanol before passing through the filter and washed. The DNA was eluted from the filter and treated with RNAse at 37°C for 30 min before loading on to 2% agarose gel electrophoresis and run 50 V/cm for 3 hrs. The gel was visualized under UV transilluminator and photographed.

**Effect on Caspase-3 activity**

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10⁵ cells/ml using medium containing 10% FBS. To each well of the 6 well plates, 2ml of the diluted cell suspension was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and different compound I concentrations (25 and 50 µg/ml) prepared in DMEM were added to the cells. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, microscopic examination was carried out and observations were recorded every 24 h. After 72 h, cells were scrapped and centrifuged at 2000rpm for 10 min to separate the pellet. The pellet was resuspended in 50 µl of chilled cell lysis buffer and incubated cells on ice for 10min. Centrifuged at 15000rpm for 1 min and supernatant was transferred to a fresh tube. The vial was maintained in ice and protein concentrations of the samples were measured by Bradford
assay. The samples were diluted to get 4 mg per ml. 50µl of samples was mixed with 50µl of 10mM DTT, followed by 5µl of 4 mM DEVD-p-nitroanilide and incubated at 37°C for 120 min. The absorbance was measured using microplate reader at a wavelength of 405 nm.

RESULTS AND DISCUSSION

The IC₅₀ value of the cytotoxic activity of GGC was (189.5±1.44µg/ml) and that of isolated Oleuropein (Fig 1), was (90±2.1µg/ml) in A-431. The activity may be due to the phenolic groups which are produced as secondary metabolites in plants via the shikimic acid pathway. Phenylalanine ammonia lyase (PAL) is the key enzyme catalyzing the biosynthesis of phenolics from the aromatic amino acid phenylalanine [16]. These secondary metabolites are reported to possess various biological activities like antioxidant, antimutagenic, anticarcinogenic and also modify the gene expression [17].

The inhibition of cell cycle progression and / or induction of apoptosis are the promising strategies for the control of the proliferation of cancer cells [18], the mechanism of apoptosis which produced cytotoxicity was further investigated by LDH leakage assay [15]. DNA fragmentation and Caspase-3 dependent apoptosis.

Lactate dehydrogenase (LDH) cytotoxicity assay measures the activity of a cytosolic LDH enzyme. When the substance having cytotoxic effect is applied on the cells, there is an increased LDH activity which correlates with increased cell wall damage and LDH leakage to extracellular space, thus acting as an indicator of cell membrane integrity. Nuclear DNA degrades into nucleosomal units is one of the important factor of apoptotic cell death, which identified that, this process is specific to DNase (CAD, caspase-activated DNase). When cells are induced to undergo apoptosis, caspases-in particular caspase 3-cleave ICAD (Inhibitor of CAD) to dissociate the CAD: ICAD complex, allowing CAD to cleave chromosomal DNA. Cells that lack ICAD or cells that express caspase-resistant mutant ICAD thus do not show DNA fragmentation during apoptosis, although they do exhibit some other features of apoptosis and die.

Quantification of plasma membrane damage of isolated compound was measured by lactate dehydrogenase (LDH) release assay. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme in every cell and releases its enzyme activity when the cell death occurs. The enzyme activity detected in the culture supernatant correlates with the proportion of lysed cells. Detection of LDH in the supernatant of target cells can be used to measure cell-mediated
Cytotoxicity\textsuperscript{[19]}. Lytic and apoptotic cell death have been suggested as mechanisms by which cytotoxic T lymphocyte (CTL) and natural killer (NK) cells may kill target cells\textsuperscript{[20, 21]}. The LDH leakage assay (Fig 2), of isolated compound; phenolic secoiridoid glucoside, produced high LDH activity (75 ± 0.58\%) at high concentration (1000 µg/ml), starting from 62.5 µg/ml with 21.33 ± 0.88\% activity in a dose dependent manner.

![Figure 1 Isolated compound (Oleuropein) from Gomphrena globosa](image)

DNA gel electrophoresis was performed in order to verify DNA fragmentation\textsuperscript{[22]}. The criteria for assessment of apoptosis generally include morphologic changes examined by light or electron microscopy and DNA fragmentation detected by colorimetric assay or visualization of fragmented DNA ladder patterns by agarose gel electrophoresis\textsuperscript{[23]}. The visualized photography of DNA fragmentation (Fig 3) indicates induced apoptosis of the compound and the effect was in a dose dependent manner. The apoptosis activity of the compound was found to be less than that of the standard camptothecin. The efficacy of cancer therapy might be determined by the ability to induce caspase-dependent apoptosis.

Caspase–3, apoptosis-related cysteine peptidase encoded by CASP3 gene, plays a central role in the execution-phase of cell apoptosis. It has been found to the peptide sequence DEVDG (Asp-Glu-Val-Asp-Gly) with the cleavage occurring on the carboxy side of the second aspartic acid residue (between D and G) with the isolated compound, phenolic secoiridoid glucoside, in a dose dependent manner. The results of the study indicate that the isolated compound, phenolic secoiridoid glucoside, induced apoptosis through LDH leakage assay, DNA fragmentation and Caspase-3 activity.

Table 1 indicated that treatment with isolated compound, phenolic secoiridoid glucoside, increased the Caspase-3 activity (1.49 ± 0.015, 1.79 ± 0.015 and 1.84 ± 0.029) in a dose dependant manner (25, 50 and 100 µg/ml respectively, and p<0.001 with respect to standard).
Standard drug, Camptothecin increased the Caspase-3 activity significantly (2.14 ± 0.026 for 5 µg/ml) compared to tumor control (0.57 ± 0.012). These observations, confirm that the extract induced apoptosis in A-431 cells involves the activation of caspase-3.

**Table 1 Apoptosis activity by Caspase-3 activity**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Drug</th>
<th>Concentration (µg/ml)</th>
<th>Caspase-3 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CPD-1 (Isolated)</td>
<td>100</td>
<td>1.81 ± 0.035&lt;sup&gt;ns,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>1.80 ± 0.015&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>1.45 ± 0.015&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Camptothecin (Standard)</td>
<td>5</td>
<td>2.15 ± 0.026&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>--</td>
<td>0.57 ± 0.012</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, where n = 3.

<sup>a</sup> P<0.001 compared with control,  <sup>b</sup> P<0.001 compared with standard,  <sup>c</sup> P<0.001 compared among doses, ns – non-significant.

**Figure 1 Isolated compound (Oleuropein) from Gomphrena globosa**

**Fig 2 Apoptosis activity by LDH leakage assay**
Fig 3 Apoptosis indicated by DNA fragmentation

Lane 1: A-431 Cells (Normal)
Lane 2: A-431 Cells treated with CPD 1 (50µg/ml)
Lane 3: A-431 cells treated with CPD 1 (100µg/ml)
Lane 4: A-431 cells treated with CPD 1 (200µg/ml)
Lane 5: A-431 cells treated with Camptothecin (3µg/ml)
Lane 6: DNA ladder

CONCLUSION
It can be concluded that the isolated compound, phenolic secoiridoid glucoside, may be beneficial for treatment of skin cancer. Further work in derivatization of the isolated compound may open up avenue for new molecules with potent and safe anticancer property for skin cancer.

REFERENCES

