New Phytochemical Investigation of Atriplex halimus L. and Evaluation of its Cytotoxic and Anticancer Effect on Human Pancreas Cancer Cells

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DOI: 10.47750/pnr.2022.13.506.281

Abstract

Background: This study was to analyze for the first time the phytochemical composition of Atriplex halimus L. by advanced technique and evaluate its anticancer effect on pancreas cancer cells. Flavonoids are polyphenolic compounds synthesized in plants as bioactive secondary metabolites that have potent antioxidants.

Methods: High performance liquid chromatography techniques used to analysis bioactive compounds of Atriplex halimus and Total Flavonoid Content was determined. Evaluate the anticancer activity of Atriplex halimus extract on primary cell culture of human pancreas carcinoma done by MTT assay and compared it with a chemotherapy drug. Antioxidant activity for A. halimus was measured by 1,1 diphenyl2 picrylhydrazyl radical scavenging. Also, cell viability, catalase and superoxide dismutase enzyme activity were measured. Cancer cells were treated with a concentration of A. halimus, cisplatin, and a combination of them.

Results: The results showed two types of flavanols, quercetin and kaempferol. While, scavenging activity of A. halimus extract at 50 mg/ml concentration was 38.14696%. The MTT assay shows the highest cytotoxicity on cancer cells at concentrations of 100 and 200 µg/ml for all treatments. However, the catalase level decreased in treated cells compared to the untreated cells (P = 0.001) and no significant differences in SOD level in all these cells (P = 0.809).

Conclusions: Our research indicates that A. halimus has potent antioxidant and anticancer effects, which may be brought on by the presence of flavonoids in this plant.

Keywords: Atriplex halimus, Flavonoids, Pancreas Cancer cell, HPLC, catalase.

INTRODUCTION

Atriplex is a plant genus of about 250 species, known by the common names of saltbush and orache. It belongs to the subfamily Chenopodioideae of the family Chenopodiaceae. The genus is quite variable and widely distributed (OEP, 2017).

Atriplex halimus is a halophytic perennial shrub that thrives in arid and semi-arid environments. Its tolerance to high salinity and drought makes it an ideal species for landscaping in arid and salt-affected areas, where it provides valuable forage for livestock (Heuzé V., Tran G., Hassoun P., 2019).

A. halimus can grow from Europe to Northern Africa, western Asia, and W. Iraq and the NE, as well as the Arabian Peninsula (Ghazanfar, 2016). Depending on its morphological, anatomical and physiological adaptations to harsh environmental circumstances.

A. halimus contain a wide range of bioactive metabolites. In the aqueous extract, qualitative examination revealed the presence of phytocomponents such as tannins, flavonoids, saponins, and alkaloids (Chikhi I, Allali H, El Amine Dib M, Medjdoub H,
Flavonoids are polyphenolic compounds that give flowers their color and aroma, and they have antiviral, antibacterial, antioxidant, anti-allergic, and anti-inflammatory properties (Dirar et al., 2019). Flavonoids interfere with multiple signal transduction pathways during carcinogenesis, limiting proliferation, angiogenesis, and metastasis or increasing apoptosis (Agati et al., 2012).

Pancreatic cancer starts in the tissues of your pancreas, which is an organ in your abdomen located behind the lower part of your stomach. Pancreas secretes digestive enzymes and hormones that aid in blood sugar regulation (Tonini & Zanni, 2021). Pancreatic cancer is a type of gastrointestinal cancer with a high mortality rate (Siegel et al., 2021). Pancreatic cancer is expected to overtake breast, prostate, and colorectal cancers as the second highest cause of cancer-related deaths by 2030, according to the American Association for Cancer Research. (Rahib et al., 2014). Killing malignant cells and preventing the cancer from spreading are the two basic goals of pancreatic cancer treatment.

Chemotherapy and radiotherapy are among the current treatments. Chemotherapy can put patients under a lot of stress and damage their health. As a result, there is a focus on using alternative cancer treatments and therapies (de Martel et al., 2020). Many researchers have identified species of plants that have demonstrated anticancer properties that have been used in herbal medicine in developing countries (Abdulridha et al., 2020; Clemente et al., 2021; Luo et al., 2019). This is a novel study to phytochemical Analysis of A. halimus by HPLC and GC-Mass techniques to evaluate its cytotoxic effect on the primary cell culture of human pancreatic carcinoma and assess the anticancer activity of A. halimus extract.
2.4 Evaluation of antioxidant activity for A. halimus extracts by non-enzymatic methods

The electron donating ability of samples and standards Ascorbic acid (Vitamin C) were determined from bleaching of purple colored ethanol solution of DPPH. As a reagent in this spectrophotometric assay, the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is used. At a concentration of 0.002 percent, DPPH was prepared (Marinova & Batchvarov, 2011).

In separate test tubes, different concentrations of samples (10, 20, 30, 40, 50 mg/ml) were taken and volumes of ethanol were made up to 2 mL. Then, in each test tube, 2 mL of DPPH solution (2.0 to 0.001 mg/mL) was added and the solutions were kept in the dark for thirty minutes. All the samples were tested in triplicate. The optical density was recorded with spectrophotometer UV at 517 nm. Ethanol with DPPH was used as control. The formula used for calculation is:

\[
\text{\% Inhibition of DPPH activity} = \left( \frac{A - B}{A} \right) \times 100
\]

Where A as Optical density of control while B as Optical density of sample

2.5 High Performance Liquid Chromatography

A. halimus extract at a concentration of 2 mg/mL in methanol, then (20 μL) of methanol extract of A. halimus was injected to column C18 (25 cm X 4.6mm) particles of size 5 μm for separation and compared to various standards of flavonoid that were carried out by HPLC (Table 1) (Ding et al., 2022). The separation parameters were determined by the gradient of mobile A (1% acetic acid in HPLC grade water) and mobile B (acetonitrile). However, peak wavelengths were monitored at 272, 280, and 310 nm (Seal, 2016).

Table 1. System components of HPLC

<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th>Model or version</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Binary high pressure gradient pump</td>
<td>P6.1L</td>
</tr>
<tr>
<td>2</td>
<td>Diode array detector</td>
<td>DAD 2.1L</td>
</tr>
<tr>
<td>3</td>
<td>Sample loop (20 μl) and injector</td>
<td>D1357</td>
</tr>
<tr>
<td>4</td>
<td>Analyses and system control software</td>
<td>Claritychrom, V 7.4.2.107</td>
</tr>
</tbody>
</table>

2.6 Primary cell culture of Human Pancreatic Carcinoma

2.6.1 Specimen collection

Patient sample: a true-cut biopsy was obtained from a male, 64 years old, has carcinoma in the pancreatic head. The diagnosis was made by ultrasound imaging and confirmed on tissue samples by a pathologist at the Cancer and Cellular Biological Unit (CCBU) in AL-Najaf Specialized Laboratory in AL-Najaf province, Iraq.

2.6.2 Ethical Approval

A valid consent was obtained from each patient before their inclusion in the study according to the Helsinki Declaration-Ethical Principles for Medical Research on Human Subjects. Ethical approval of the sample procedure was conducted by the Cancer and Cellular Biological Unit in AL-Najaf Specialized Laboratory.

2.6.3 Tissue dissociation

Tissues were dissected and incubated in 100μl collagenase (0.25%) for 20 minutes at 37°C. It was vigorously shaken and mechanically digested with a syringe plunger to facilitate cell shedding.
2.6.4 Tissue inhibition

Using an inhibitor solution (900 µl of RPMI 1640 and 10% fetal bovine serum FBS1), the cells were mechanically digested and washed with 300µl of RPMI 1640 for 5 minutes at 37°C and centrifuged at 1500 RPM for 5 minutes. The supernatant was discarded, and 200µl of RPMI 1640 containing 10% FBS and 1µl Penicillin/Streptomycin solution was added before resuspending and incubating at 37°C for 15 minutes (Hayon, 2015; Hayon et al., 2003).

2.6.5 Tissue growth

Cells were cultured in 24 non-TC treated plates. Cells were cultured for 7 days and replaced daily. Cells were monitored and photographed using an inverted microscope (Hayon, 2015).

2.7 Preparation of A. halimus extract, Cisplatin And Combination

Cisplatin (Cis) and A. halimus extract (AHE) was dissolved separately in RPMI 1640 medium and variable concentrations (200, 100, 50, 25, 12.5 µg/ml) were prepared for each. However, combination (AHC) is a 50% dose of A. halimus extract and cisplatin in the same concentrations.

2.8 Cell cytotoxicity determination using MTT assay on human Pancreatic carcinoma

Cells were seeded at 1× 105 cells/mL in 96 well micro titer plates in RPMI medium. For 24 hours, the cells were incubated. AHE, Cis and AHC at various concentrations (200, 100, 50, 25, 12.5 µg/ml) were dissolved in media and control cells received only serum free media, added in triplicate, and incubated for 24 hours. Following that, the cells were treated for 1 hour with 50µl MTT at a concentration of 2µg /ml (Arslan et al., 2021).

Following the incubation period, the entire contents of the well were aspirated. After incubation at 37°C, 5% of CO2 and, 50µl of DMSO was added to each well, and absorbance was measured at 492 nm using a microplate reader (Hayon et al., 2003).

To visualize the shape of cells under an inverted microscope, 200µL of cell suspensions were seeded in 96-well micro-titration plates at a density of 1 x 104 cells mL-1 and incubated for 48 hours at 37°C. The medium was then removed, and 200µL of each AHE, Cis, and AHC, concentration were added. After 24 hours, the plates were stained with 50 µL of Crystal Violet and incubated at 37°C for 10-15 minutes before being gently washed with tap water until the dye was removed. The cell was observed under an inverted microscope and calculated the cell growth inhibition rate then photographed with a digital camera (Alexandrov & Dergalev, 2019).

2.9 Assessment Catalase Activity

Primary cells of human carcinoma after 24 hours of treatment were collected at concentrations (100 µg/ml) from plates cultured at 13,000 rpm (4°C) for 10 min, and the supernatant was isolated. The method is based on the reduction of vanadium(V) to vanadium (III) by H2O2 and involves the reaction of ammonium metavanadate with H2O2 under acidic conditions. Although H2O2 is a strong oxidant, under certain redox conditions it can act as a reductant. As a result, vanadium (V) reduction lead to the formation of a red-orange peroxovanadium complex with a maximum absorbance at 452 nm (Hadwan & Abed, 2016). The absorption of the red-orange peroxovanadium complex at 452 nm was used to determine catalase enzyme activity. The procedure for enzyme activity was elucidated in (Table 2) (Hadwan & Ali, 2018).

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Test</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>1000 µl</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>2000 µl</td>
<td>2000 µl</td>
<td>----</td>
</tr>
</tbody>
</table>

Mix with vortex and incubate at 37 °C for 2 min, after that, add:

| Vanadium reagent  | 2000 µl  | 2000 µl  | 2000 µl |

Table 2. Procedure for assessments of catalase activity
The tubes then kept at 25 °C for 10 minutes. Changes in absorbance were measured at 452 nm in comparison to the reagent blank.

### 2.10 Superoxide Dismutase (SOD) Activity

Primary cells of human carcinoma after 24 hours of treatment were collected at concentrations (100 µg/ml) from plates cultured at 13,000 rpm (4°C) for 10 min, and the supernatant was isolated. (Cu-Zn) SOD activity was evaluated using a simple and quick method based on the enzyme's ability to inhibit pyrogallol autoxidation. In the presence of EDTA at pH 8.2, the autoxidation of pyrogallol is 50%. The principle of this approach is based on the competition between pyrogallol autoxidation by O2• and radical dismutation by SOD. (Cu-Zn) SOD activities are measured in units per millilitre. Prepared control and sample test tubes then pipetted into test tubes (Table 3). One unit of (Cu-Zn) SOD activity is defined as the amount of enzyme required to inhibit pyrogallol autoxidation by 50%. At zero time and after 1 minute of pyrogallol addition, absorption was measured at 420 nm against Tris-EDTA buffer (Magnani et al., 2000; Marklund & Marklund, 1974).

**Table 3. Amount of reagents added in experiment**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Test (µl)</th>
<th>Control (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>DW</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Tris buffer</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

### 2.11 Statistical analysis

Data was collected and analyzed in triplicate for the MTT assay using a graph pad prism. An automated python IC50 calculator was used to calculate (IC50) and for enzyme assay, a Paired Samples Statistics was used. Graphs and data were represented using Microsoft Excel 2010.

### 3. Results and Dissection

Flavonoids have been extracted from the whole plant of A. halimus by fractionation of methanol crude extract through the use of different solvents for re-extraction of free (hexane fraction) and bound (ethyl acetate fraction) flavonoids (Truong et al., 2019).

The results showed a total flavonoid yield of 9% w/w. However, the total flavonoid content (TFC) of A. halimus was quantified from the calibration curves (Y = 0.073x, R² = 0.9955) in (Figure 1). The results showed that the TFC value was 2.04 mg QE/g of dry material in the methanol extract, but in the aqueous extract, it was 1.48 mg QE/g of dry material. The highest value of flavonoids found in methanol extract compared with aqueous extract and this is in agreement with a previous study (Kheira, Slama, Mahieddine Boumendjel, Faiza Taibi, 2018). The highest concentration of flavonoids was in methanol extract compared with aqueous extract. The variation of TFC in the extracts depends on the influence of the difference in the polarity of the organic solvent on the extracted phytochemical compound.
The DPPH assay is a common test for determining the antioxidant activity of plant extracts. A. halimus extract exhibited good antioxidant activity relative to that of ascorbic acid as a standard antioxidant (Figure 2). From the result it was noted percentage scavenging activity at 50 mg/ml concentration of A. halimus extract was 38.14696% (Table 4). Addition to DPPH scavenging activity increased with an increase in concentration for ascorbic acid and A. halimus extract.

Table 4. Concentration of A. halimus during the Time

<table>
<thead>
<tr>
<th>Time (15Min)</th>
<th>Concentration mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>7.644</td>
</tr>
</tbody>
</table>

Ascorbic acid (vitamin C) is a potent scavenger of free radicals and used as pure concentrate as standard in DPPH assay. therefore, was given more potent effect than a plant extract. Antioxidant activity of the plant extract on total antioxidant activity is due to the oxidisation of the antioxidant molecules and the test activity on DPPH may be attributed to a direct role in trapping free radicals by donating hydrogen atom to DPPH radical to become stable and in the reducing power assay, it was supposed to be due to the presence of reductants in the A. halimus extract by donating an electron. Results confirm that AHE is effective in stabilising these free radicals due to the flavonoids contents (Tailor, 2014). This influence is traced back to the flavonoids present in this extract.
This is the first study used to identify flavonoids in this species of A. halimus by HPLC that uses retention time and UV/vis spectrum matching with its respected standard material to quantify flavonoids compounds. The HPLC chromatogram showed the presence of quercetin and kaempferol (Figure 3), and the concentration of quercetin (Figure 4), and kaempferol (Figure 5), was (8.5387941 and 7.6326572 µg/ml) (Table 5).

Table 5. Identified qualitative and quantity of flavnoids in A. halimus Extract

<table>
<thead>
<tr>
<th>Sample</th>
<th>peak area</th>
<th>µg/ml</th>
<th>total µg</th>
<th>plant weight µg/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>340.191</td>
<td>8.5387941</td>
<td>25.6163823</td>
<td>0.256163823</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>360.031</td>
<td>7.6326572</td>
<td>22.8979716</td>
<td>0.228979716</td>
</tr>
</tbody>
</table>

Quercetin and kaempferol consider natural antioxidants and could help us to understand the action of plant behaviors in biological functions and to discover new targets (Ye et al., 2019). And that is agrees with a previous study used LC-MS to identify quercetin of A. halimus extract (Maria Clauser, Stefano Dall’Acqua & Innocenti, 2013).

Figure 3. HPLC Chromatogram of A. halimus extract by retention Time and UV spectrum.
The anticancer activity of AHE was tested on human pancreas carcinoma cells and compared with the chemotherapy drug like Cisplatin (Cis) as well as the combination (AHC) to produce a new treatment and assess the synergistic or antagonistic effects on cells. It was also compared with A. halimus and Cisplatin. The results from the MTT assay of AHE, Cis and AHC show the highest cytotoxicity on carcinoma cell growth inhibition at concentrations (100 and 200 µg /ml) and were (43.14 and 25.41%) (Figure 6), (46.14 and 28.41%) (Figure 7) and (49.16 and 27.41%) (Figure 8), respectively.

The IC50 value of this assay for AHE, Cis, and AHC was (117, 104.6, and 110 µg/ml), respectively. The results from IC50 values indicate Cis was the most effective concentration compared to AHE and AHC, whereas the combined shows the highest cytotoxicity effect than AHE.

Cisplatin is used as first-line chemotherapy treatment for patients diagnosed with various types of malignancies such as leukemia, lymphomas, breast, testicular, ovarian and sarcomas. Once cisplatin enters the cell it exerts its cytotoxic effect by losing one chloride ligand, binding to DNA to form intra-strand DNA adducts, and inhibiting DNA synthesis and cell growth. The DNA lesions formed from cisplatin-induced DNA damage activate DNA repair response via NER (nuclear excision repair system) by halting cisplatin-induced cell death by activation of ATM (ataxia telangiectasia mutated) pathway (Brown et al., 2019), Therefore cisplatin appeared high effected compared with another treatment.

At the same time, the cytotoxic effect of AHE induced cancer cell death may be due to induction of intracellular ROS, leading to altered mitochondrial function and caspase 3 activity as shown in this study (Al-senosy & Ahmad, 2018). The cytotoxic effect of AHE on cancer cells can increase by an increased dose of AHE due to no side effects of this plant (Benarba, 2015), in contrast to most chemotherapy drugs like cisplatin that may be causes nephrotoxicity, hepatotoxicity, cardiotoxicity, and neurotoxicity (Dasari et al., 2022).

However, AHC shows better efficacy than AHE, this combination gives a synergistic effect. At the same time, AHC can be useful in reducing the side effects of cisplatin because it contains powerful antioxidants and maintains the efficiency of
chemotherapy, as shown in this previous study, which clearly indicates that combination therapy with cisplatin and natural products is effective in reducing resistance to cisplatin therapy (Dasari et al., 2022).

Figure 6. Graphical representation of the cytotoxic effect of AHE against cancer cells.

Figure 7. Graphical representation of the cytotoxic effect of Cis against cancer cells.
The level of Catalase enzyme was measured on primary cells culture of human pancreatic carcinoma. cancer cells were exposed to A. halimus extract, cisplatin drug and combination AHC at a concentration of (100 ug/ml) for 24 hours and untreated cells were used as a control.

Human pancreatic carcinoma cells were exposed to A. halimus, Cisplatin and Combination the result showed significant differences by an decrease in catalase level in pancreatic cancer cells that were exposed to these treatments compared to the control cell (P=0.006), as shown in (Figure 9).

Human pancreatic carcinoma cells were treated with (200 µg/ml) AHE, Cis, and AHC for 24 hours.

Catalase is one of the crucial antioxidant enzymes which plays a significant role in hydrogen peroxide metabolism as a key regulator. The result showed that catalase enzyme levels are high in the control cancer cells hence acquiring resistance against oxidative stress (Glorieux et al., 2011). While, after exposure of these cancer cells to various treatments, there was a decrease in the levels of catalase enzyme, and significant differences between treated and untreated cells As shown in the previous study (Fonseca et al., 2019). This decrease in the levels of catalase enzyme in the treated cells leads to an increase in oxidative stress, which in turn leads to cell death by apoptosis. So obviously A. halimus extract has potent antioxidants and gives the same effect on cancer cells when compared with chemotherapeutic drugs and combinations.
SOD activities are low in many cancers, implying reduced protection against ROS (Xu et al., 1999). Human pancreas carcinoma cells were exposed to AHE, Cis, and AHC for 24 hours at a concentration of 100 µg/ml, while untreated cells were used as a control. The results revealed increased in the SOD level obviously as compared to control cells and the same time no significant difference (P = 0.809) (Figure 10). The absence of significant differences is attributed to the short period cells exposed to treatments. This proves that low levels of the SOD enzyme in cancer cells could be supplemented by exogenous antioxidants delivered by exposure to A. halimus extract, which has potent antioxidants. The same effect was given on cancer cells when compared with chemotherapeutic drugs and was better than combined.

![Figure 10. Effect of AHE, Cis and AHC on SOD activity in cancer cells (p value<0.05)](image)

Results show the reduced proliferation of human pancreas carcinoma cells in all treated compared with non-treated cells. Cis showed a higher effect on cancer cells, while the effect of the AHE was lower than the AHC (Figure 11). Cisplatin showed a higher effect on cancer cells because the antitumor activity of cisplatin is believed to be due to its interaction with chromosomal DNA. Only a small fraction of cisplatin actually interacts with DNA and the inhibition of DNA replication cannot solely account for its biological activity (Alakananda and Soumya, 2010). In addition, the efficacy of chemotherapeutic drugs depends not only on their ability to induce DNA damage but also on the cell's ability to detect and respond to DNA damage (Lomonaco et al., 2009).

While the effect of the AHC was higher effect than A. halimus due to make a synergistic effect on cancer cells compared with AHE alone, this A. halimus content potent antioxidant of flavonoids like quercetin and kaempferol and this is useful by inducing apoptosis in cancer cells and reversal of multidrug resistance by working as inducers which induce more ROS production that can reach the toxic threshold to activate apoptosis. Major players in maintaining balanced ROS in cells include Mrf2, GSH, both of which can be inhibited by certain flavonoids to exert their re-sensitizing effects (Cui et al., 2018). Additionally, a previous study indicates that flavonoid can be used in chemotherapy not only because of its antitumor ability but also due to the enhanced cytotoxic effects of chemotherapeutic agents (Qian et al., 2014). As a result, this will reduce the dose of chemotherapy and have the same effect on the growth and proliferation of cancer cells.
Conclusion

A. halimus has specific flavonoids quercetin and kaempferol therefore appeared to potent an antioxidant activity on human carcinoma so can protect cells from damage induced by free radicals. A. halimus extract showed high cytotoxic activity against primary cell cultures of pancreatic carcinoma with no side effects compared with cisplatin, at the same time appeared remarkable anti-cancer effects while combining them and synergism effects on cancer cells during triggered morphological, biochemical changes and enzymes assessments. Consequently can use this plant in pharmaceutical product.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

I would like to appreciate the professors supervising this research because it was part of the doctoral requirements for the researcher.