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Cytotoxic effects of the *Crassula ovata* n-hexane fraction on human esophagus cancer KYSE-30 cells

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Abstract

The current study shows the cytotoxicity effect of the *Crassula ovata* n-hexane extract on esophagus cancer. *C. ovata* is a perennial succulent plant belonging to the Crassulaceae family. In Africa, the leaves were used medicinally to cure epilepsy and diarrhoea by boiling them in milk. The hexane fraction, which is obtained through the maceration method, demonstrates the presence of many compounds that have an anticancer effect, which are obtained by gas chromatography - mass spectroscopy. The phytosterol compound was isolated by a preparative thin layer chromatograph and was identified by liquid chromatography - mass spectroscopy. The hexane fraction was found to possess a strong anticancer effect against esophagus cancer. The obtained data from the human esophagus cancer KYSE-30 cell-line were analysed by one-way ANOVA, with a significance level of $p<0.05$.

KEYWORDS
b-sitosterol, *Crassula ovata*, cytotoxicity, esophagus cancer, phytosterol

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1. INTRODUCTION

Since ancient times, medicinal plants have been used to cure a wide range of diseases; in fact, in some places, the use of medicinal plants was thought to be witchcraft because people lacked the scientific concept at that time to explain and predict the plants' therapeutic effects [1]. About 70%-95% of the population depends on traditional medicines for primary care, and 70%-90% of the populations in developed countries use herbal medicines under the titles "competitive", "alternative", or "nonconventional" [2].

Crassula ovata is a plant belonging to the Crassulaceae family. The genus *Crassula* consists of nearly 200 species, most of which are found in southern Africa, which is considered their distribution center [3]. *Crassula ovata* is a perennial succulent plant with many branches and a bushy appearance, about 30-45 cm high, with thick, branching, succulent, and juicy stems. The leaves are

dark green, oval-shaped, and situated opposite one another on the stems. The white or light pink flowers are positioned at the apex of the stalks and only seldom bloom, especially in plants maintained indoors for ornamentation [4].

2. MATERIALS AND METHODS

Materials: This study used the human esophagus cancer KYSE-30 cell-line (Santa Cruz Biotechnology, USA). The sample consisted of whole plants (leaves, steam, and root) of *Crassula ovata* collected from the Babylon Nursery Plantation in March 2023. The plant was identified and authenticated by Dr Esraa Abdel-Al Razzaq Majeed (2023-1-29) at the Department of Biology, College of Sciences, University of Baghdad.

Extraction and isolation: About 350 gr of powdered plant were macerated for 48 h in n-hexane, and the plant material was then filtered. While the filtrate was evaporated and designated as the hexane extract, it was then submitted to gas chromatography - mass spectroscopy (GC-MS) as an identification step. The results indicated the presence of Y-sitosterol, and the isolation of phytosterol was performed by a preparative thin-layer chromatograph in acetone hexane (2.5 : 7.5) as the mobile solvent [5]. The sample was then sent for identification by liquid chromatography - mass spectroscopy (LC-MS).

Structural analysis: The structural analysis used for the n-hexane extract was GC-MS (7820A-5977E; Agilent Technologies, USA) and LC-MS (AB SCIEX 3200 QTRAP; Mashhad University of Medical Sciences).

MTT assay: The micro-culture tetrazolium assay (MTT assay) was used to evaluate the cytotoxicity of the n-hexane extract on the esophageal cell-line (KYSE-30 cells). The cells were grown inside the prepared media (10% fetal bovine serum, 100 g/mL streptomycin, and 100 units/mL penicillin), were transferred to 96-well plates (after trypsinization), and were incubated at 37°C. When a confluent monolayer was reached, the sample was applied. The sample was prepared by dissolving in dimethyl sulfoxide (DMSO; 11 mg/mL) as a stock solution, and by dilution, it was prepared into different concentrations (1,000, 500, 250, 125, 62.5, 31.25, 15.6, and 7.8 µg/mL) and it was applied on the plate wells. After 48 h, the MTT dye was applied, and after 4 h, the media was removed and the formazan violet crystal was dissolved by DMSO. The measurement was undertaken by a microplate reader at 570 nm.

Statistical analysis: The data of the n-hexane extract were analysed by one-way ANOVA, at a significance level of $p<0.05$. The data means were compared with the control mean (which represents only cancer cells), after testing the data with a normality test and ensuring a normal distribution. We used the Levene test to assess variance homogeneity, and *post hoc* analysis so as to explain the significant difference between the means.

3. RESULTS

The undertaken GC-MS of the n-hexane extract demonstrated several compounds with a similarity index above 95%, including: hexadecane,2,6,10,14-tetramethyl-, nonadecane, hexadecanoic acid, methyl ester, hexadecanoic acid (palmitic acid), vitamin E (α-tocopherol), α-tocopheryl acetate, and γ-sitosterol, while the undertaken LC-MS (Figure 1) confirmed the structure of the isolated phytosterol compound as Y-sitosterol, TMS (trimethylsilyl), with the IUPAC name of 17(4-ethyl,5-methyl-hexane)-10,13-dimethyl cyclopentaphenanthr-5-ene-3-trimethylsiloxyl.

The results demonstrate a very noteworthy cytotoxic activity against the esophageal cancer cells, and the ability of the n-hexane extract to significantly curb the growth of the esophageal cancer cell-line, in a concentration-dependent manner. The data were tested using the Shapiro-Wilk tests, since the number of cases were fewer than 2,000. The null hypothesis of normality cannot be rejected for most concentrations, since their p values surpass 0.05; therefore, the data are normally distributed. The variance homogeneity assessed by the Levene test with adjusted degrees of freedom and the deviations of the experimental groups (7.5, 15, 31, 62, 125, 250, 500, 1,000, and Control) show significant differences. The undertaken *post hoc* analysis using the Games-Howell test revealed a significant difference ($F=33.556$, $p<0.05$) among groups, in particular, between the 500 and the 1,000 µg/mL treatment groups.

4. DISCUSSION

The observed cytotoxic effect is attributed to the presence of many compounds that have an anticancer effect, according to other studies focusing on compounds like vitamin E [6] or the n-hexadecanoic acid (palmitic acid) [7], which is indicated by our GC-MS and is known to act synergistically, especially with TMS sitosterol (which was isolated and confirmed by LC-MS). Phytosterols are plant sterol compounds synthesized by the isoprenoid biosynthesis pathway via squalene from acetyl coenzyme A, and are structurally similar to cholesterol except for an additional hydrocarbon chain at the C-24 position.

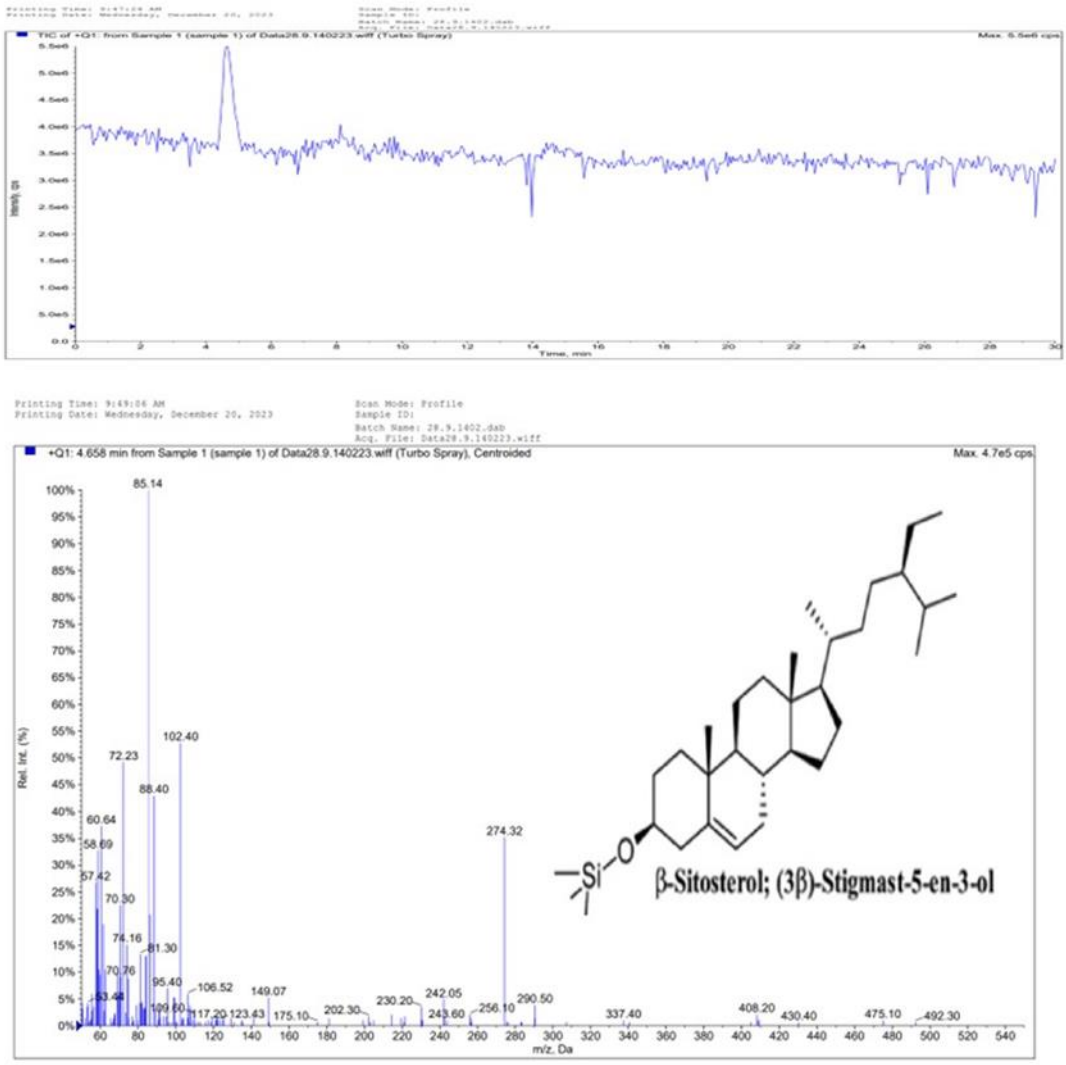

Figure 1. LC-MS chart of the isolated phytosterol compound (m/z of 486 $[M+H]^+$), where m/z=85 corresponds to the base peak result from the stable secondary carbocation fragment, m/z=274.32 corresponds to the steroid nucleus, m/z=72.23 corresponds to trimethylsilyl, and m/z=88.40 corresponds to trimethylsiloxyl.

Other studies have demonstrated that phytosterols could act through multiple mechanisms as anticancer agents and promote the inhibition of carcinogen production, cancer cell growth, angiogenesis, invasion, and metastasis, as well as cause the apoptosis of cancerous cells [8]. The apoptosis mechanism is known to be induced by the increased levels of the tumor suppressor protein p53 that interacts with kinase signalling pathways involving AMPK, PI3K/AKT/mTORR, RAS/RAF/MAPKK, and JAK/STAT [9], as well as the activation of procaspase-3 [10].

5. CONCLUSION

The n-hexane extract of *Crassula ovata* is found to have a strong anticancer effect against esophagus cancer cells, as it was observed from the application of different concentrations of the extract on a cell-line (KYSE-30).

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CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

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