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Induction of Apoptosis and ROS Production in Liver Cancer Cells by Saponin Fraction from *Alcea rosea* L. Seeds

N.M.T. Abutaha[1], R. Alghamdi[1], M. Al-Waddan[1]

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ABSTRACT

Background: Natural compounds are essential targets in anticancer research due to the toxic effects of current chemotherapy and drug resistance.

Methods: Saponin extraction from *Alcea rosea* L. was carried out using 20% methanol and liquid-liquid extraction coupled with the Solid phase extraction (SPE) approach. The saponin fraction was explored for its cytotoxic activity against Huh-7 and MDA-MB-231 cancer cell lines and a non–cancer cell line (HUVEC). The cytotoxic activity and oxidative stress were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 2,7 dichlorofluorescein diacetate dyes respectively. The apoptotic potential was investigated using DAPI and acridine orange-ethidium bromide dual staining.

Result: The saponin fraction exhibited cytotoxic potential against the cell lines investigated. The IC_{50} values of the saponin fraction were 49.02, 300 and 325 µg\mL against Huh-7, MDA-MB-231 and HUVEC cell lines, respectively. The cytotoxicity for the extract was dose-dependent. The saponin fraction calculated selectivity index (SI) value indicated high selectivity towards Huh-7 cells (SI = 6.62) compared with normal HUVEC cells. Huh-7 cells treated with saponin fraction showed cellular and nuclear morphological changes, such as apoptotic body formation and chromatin condensation, as observed using light and fluorescent microscopy. However, further investigation is required to assess the the saponin fraction as a potential therapeutic agent

Key words: *Alcea rosea*, Apoptosis, Cancer cells, Cytotoxicity, Selectivity index.

Abbreviations: Bak: Bcl-2 homologous antagonist/killer; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; Bcl-xL: B-cell lymphoma-extra large; Bim: Bcl-2-interacting mediator of cell death; Mcl-1: Myeloid cell leukemia 1.

INTRODUCTION

Hepatocellular carcinoma is the second-leading cause of cancer-related fatalities worldwide. Many risk factors can lead to this type of cancer. These factors include gender, race, chronic infections with hepatitis viruses, cirrhosis, non-alcoholic fatty liver disease, heavy alcohol and tobacco use, obesity, anabolic steroids, type 2 diabetes, aflatoxins and inherited metabolic diseases (Ananthakrishnan *et al*., 2006). The genetic diversity of this cancer type is likely why no effective targeted therapy has been discovered (Mansoori *et al*., 2017). Additionally, the application of current chemotherapeutic drugs is restricted due to the complex pathophysiological processes involved in cancer, significant side effects and unfavourable prognoses (Housman *et al*., 2014). Developing innovative, safe, natural treatments to target the primary dysregulated signalling mediators in cancer development is crucial while minimizing side effects (Talib *et al*., 2021).

Plants have been utilized in herbal medicine across various civilizations and regions worldwide. Plant species are considered valuable sources of compounds that can be employed in the development of active pharmaceutical or synthetic drugs. Plant-based medicines typically have fewer side effects, making them more appealing (Atanasov *et al*., 2021). Various reports suggest that compounds from different plant parts may help treat cancer. The anti-carcinogenic effects of botanical compounds, such as phenol, alkaloid, saponins and terpenoids are associated with their ability to suppress cell proliferation by inhibiting extracellular signal-regulated kinase, angiogenic factors and oncogenic factors signalling cascades, apoptosis and blocking cellular migration and metastasis (Ali Abdalla *et al*., 2022). Saponins, a group of secondary metabolites, are promising phytochemicals due to their well-documented anticancer activity. Saponins affect different signalling pathways and factors. They arrests the cell cycle mainly by downregulating different cyclins and increasing the recruitment of p27 and p15 to cyclin complexes. They also acts by upregulating pro-apoptotic Bim, Bax and Bak and downregulating anti-apoptotic Mcl-1, Bcl-xL and Bcl-2, leading to caspase-9 and caspase-3 activation. Additionally, saponins induce the internalization of caveolae, followed by an increase in Bim and Bax and a reduction of Bad. Saponins also act by increasing autophagy (Xu *et al*., 2016).

[1]Department Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia.

Corresponding Author: N.M.T Abutaha, Department Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia. Email: nabutaha@ksu.edu.sa

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Alcea rosea, or hollyhock, a member of Malvaceae, is native to Asia, particularly China, but has been widely cultivated and naturalized in various regions worldwide. Hollyhocks are popular ornamental plants, often grown in gardens and other landscape settings (Favretti and Favretti, 1997). Significant scientific investigations have revealed that *A. rosea* extracts exhibit a range of pharmacological properties, such as anti-inflammatory, antibacterial and analgesic properties, anticancer activity (Ahmed *et al*., 2023; Ahmed *et al*., 2016; Saha, 2019) and to treat several airway disorders like asthma and chronic bronchitis (Hanif *et al*., 2019).

Based on an extensive literature review, this report is the first to assess the cytotoxicity and apoptotic potentials of saponin derived from *A. rosea* seeds on human liver cancer cells. The effectiveness of these constituents was evaluated and compared in both cancerous and non-cancerous cell lines using an *in vitro* study.

MATERIALS AND METHODS

Study area

The research was conducted at King Saud University in the Kingdom of Saudi Arabia between October 2022 and October 2023.

Seed collection

Seeds of *A. rosea* were obtained from a herbal shop in Saudi Arabia. The seeds were preserved in the herbarium with voucher number KSU-RIY-90 at the Department of Botany at King Saud University, Saudi Arabia. The plant material was then ground into a coarse powder using a commercial mill (China) and kept in the refrigerator until used for extraction.

Extraction of crude saponins

To prepare the sample, 15 g of plant material was added to 20% methanol (150 mL) and kept at 55°C for 4 hours in a water bath. The extract was centrifuged at 3000 rpm and then the volume was reduced to 70 mL using a rotary evaporator. Next, 100 mL of diethyl ether was added to the extract (70 mL) in a separating funnel and the mixture was vigorously shaken. The diethyl ether was then separated and discarded. The aqueous phase underwent two successive extractions with 100 mL of n-butanol each time, followed by separation using a separatory funnel. The resulting n-butanol extract was washed with 50 mL of a 5% brine solution. The final volume was rotary evaporatored and yield was calculated (209 mg). The resulting stock solution was filtered and stored in the freezer until needed (Zeb *et al*., 2014).

A solid-phase extraction using C18 cartridge

Saponin separation from the extract was achieved through a solid-phase extraction method using a Hypersep C18 cartridge (Thermo Scientific ,USA). The cartridge was prepared by initially conditioning it with 3 mL of methanol followed by 6 mL of water.The crude saponin extract (209 mg) was eluted with methanol (20 mL). The extract was filtered using a 0.25 µm PVDF syringe filter (Millipore, England), rotary evaporatored and the yield was determined (44 mg).

Cell culture

Human liver cancer (Huh-7), human breast cancer (MDA-MB-231) and Human umbilical vein endothelial cells (HUVECs cell lines were obtained from DSMZ (Braunschweig, Germany). The cells were grown in Dulbecco's Modified Eagle Medium (Gibco, UK) with 10% fetal bovine serum (Gibco, UK), L-glutamine (BioBasic, Canada) and 5% CO_2 at 37°C.

Cytotoxicity assays

Following the established method, the cytotoxicity evaluation was performed using the MTT assay (Al-Zharani and Abutaha, 2023). Cells were seeded at 5×10^5 cells\well in 24-well plates and incubated overnight at 37°C in a 5% CO_2 environment. Subsequently, the extract at concentrations ranging from 0 to 500 µg/mL was introduced to the wells and incubated for 24 hours. Following the incubation, a 100 µL aliquot of a 5 mg/mL MTT solution was introduced into each well. After 2 h of incubation, the formazan crystals generated were solubilized by the addition of 1000 µL of dimethyl sulfoxide (DMSO). The absorbance was quantified (570_{nm}) using a microplate reader (ChroMate, England). DMSO (1%) was employed as the blank in the experiment. Cell viability was assessed by calculating the percentage of control based on the mean values of the data. The IC_{50} value, representing the concentration at which 50% inhibition occurred, was determined using OriginPro 8.5 Software. To determine the selectivity index (SI), the average IC_{50} value of the non-cancerous cell line (HUVECs) was divided by the IC_{50} values in the cancer cell lines (HuH-7 and MDA-MB-231).

Analysis of cell and nuclear morphology

Morphological alterations for the control and the induced by saponin fraction in Huh-7 were observed and photographed using a phase-contrast microscope (Leica, Germany). For nuclear morphology, the treated and control cells (DMSO 1%) were fixed in ice-cold ethanol, washed with PBS, suspended in DAPI (1 µg/mL) staining solution and incubated in the dark for 10 min. Following that, the cells were examined for changes in nuclear morphology using a fluorescence microscope (EVOS, USA) (Alghamdi, 2024).

Acridine orange (AO)-ethidium bromide (EB) staining

Staining with AO-EB was carried out to assess the apoptotic potential. The permeability of AO (acridine orange) and EB (ethidium bromide) within cells varies depending on the membrane permeability differences observed among necrotic, apoptotic and living cells. Huh-7 were seeded in a 24-well culture plate and treated as reported earlier. Post

24 h, the cells were incubated with IC_{50} of saponin fraction. Following the staining with 1 µg/mL of AO-EB (1:1, v/v), the cells were imaged using a fluorescence microscope (EVOS, USA) (Abutaha *et al*., 2022).

Measurement of reactive oxygen species (ROS)

The dye 2,7 dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich) was used to monitor ROS production. Post-seeding cells in 24-well plates were treated with IC_{50} of saponin fraction or DMSO (1%). Subsequently, the cells were exposed to DCFH-DA (10 mM) for 30 minutes to induce the production of intracellular ROS. The ROS production was then examined by observing the fluorescence emission using a fluorescent microscope (Abutaha *et al*., 2022).

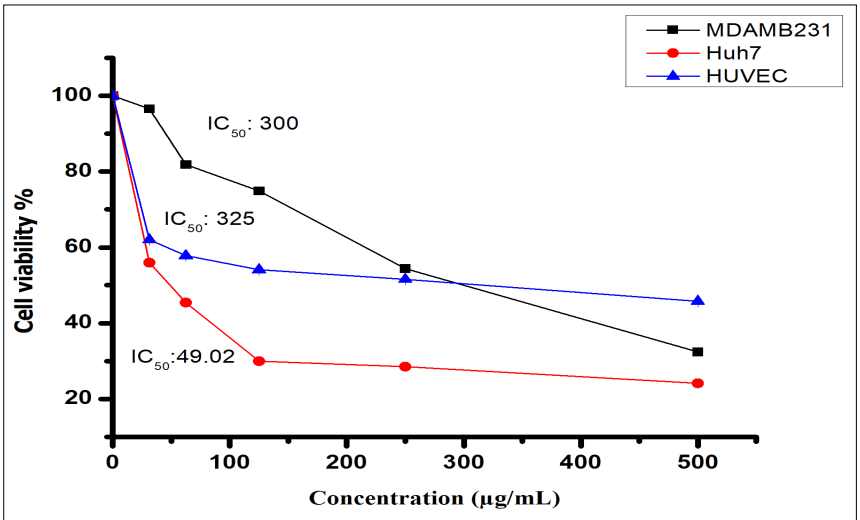
Statistical analysis

The results were analyzed using OriginPro 8.5 software and presented as the mean ± standard deviation from three independent replicates. Statistical analysis was performed using the Student's t-test. A P-value of 0.05 or less indicated a significant difference between the groups.

RESULTS AND DISCUSSION

The adverse effects effects observed with conventional chemotherapy and the emergence of drug resistance highlight the critical role of exploring natural compounds as potential therapeutic agents in combating cancer. Several publications show that natural substances exhibit strong *in vitro* and *in vivo* anticancer potentials (Hashem *et al*., 2022; Zhong *et al*., 2021; Agarwal *et al*., 2012). In this investigation, we found that the saponin extract of *A. rosea* seeds exhibited anticancer activity against cancer cell lines. As shown in Fig 1, the saponin fraction inhibited the growth of Huh-7 (IC_{50}: 49.02) and MDA-MB-231 (IC_{50}: 300) dose-dependently. However, it was less cytotoxic on non-cancerous HUVEC cell lines (IC_{50}: 325). Only a few reports are available on the *A. rosea* cytotoxic effect. Previous studies showed a cytotoxic effect of ethyl acetate extract of *A. rosea* seeds in HCT116 and SW480 cell lines. In a study conducted by Ahmed *et al*. (2016), it was observed that the ethyl acetate extract exhibited dose and time-dependent inhibition. Additionally, the extract induced apoptosis, as evidenced by the cleavage of PARP (poly ADP-ribose polymerase) and increased expression of Bax. A decrease in the levels of the BCL-xl protein accompanied this apoptotic effect.

Maximum cytotoxicity against cancer cells and minimal harm to normal cells are the best choices in chemotherapy. The selectivity index (SI) quantifies the contrasting cytotoxic effects of a substance on non-cancerous and cancer cells. An SI value surpassing 3 signifies selective cytotoxicity, indicating that the substance exhibits higher toxicity towards cancer cells than non-cancerous cells (Bézivin *et al*., 2003). The extract displayed higher cytotoxicity against Huh-7 than HUVEC, with SI value of 6.62, indicating a selective cytotoxic effect of saponins fraction against Huh-7 cells. Similarly, in a study conducted by (Abdel-Salam *et al*., 2018), the extract of *A. rosea* flowers was evaluated for its anticancer properties against HepG2 cells. The extract demonstrated an IC_{50} value of 3.8 µg/mL, indicating its effectiveness in inhibiting the growth of HepG2 cells. Furthermore, the calculated selectivity index (SI) values showed a high level of selectivity towards HepG2 cells, with an SI value of 15.2. To examine the effect of the saponin fraction on the nuclear morphology of Huh-7 cells, a DNA-binding fluorescent dye



The cells were treated with various concentrations of the saponin fraction for 48 hours. The results were obtained from three replicates, presented as mean ± SD.

Fig 1: A dose-response curve was constructed to assess the effects of the saponin fraction derived from *A. rosea* seeds on cell lines: Huh-7, MDA-MB-231 and HUVEC (a non-cancerous cell line).

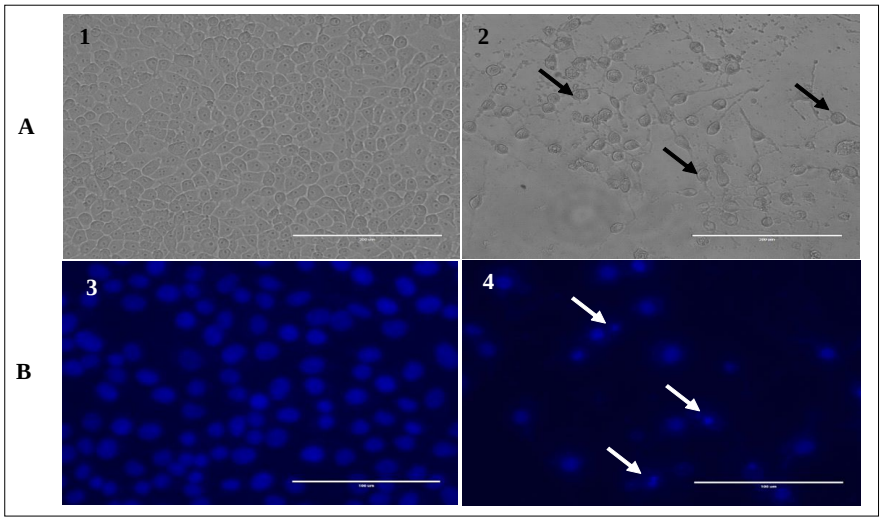
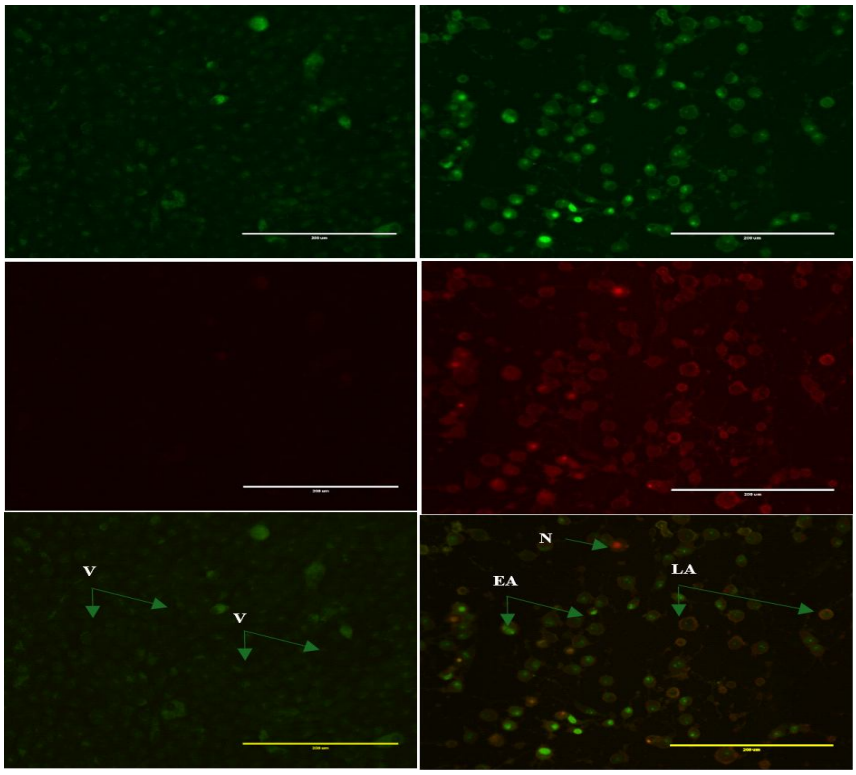


Fig 2: A: Morphology of human liver cancer cells Huh-7 in the absence or presence of the saponin fraction of *A. rosea* seeds. (1): Untreated Huh-7cells; (2): Huh-7 cells incubated with IC_{50} dose of the saponin fraction (48 h); B: Nuclear staining of cells using DAPI on Huh-7 cells after treatment with the saponin fraction of *A. rosea* seeds; (3): Untreated Huh-7cells; (4): Huh-7 cells incubated with IC_{50} dose of the saponin fraction; (48 h): Arrow indicates several characteristic features associated with apoptotic cell death, including cell shrinkage, floating of cells, nucleus margination and nuclear fragmentation-magnification 200x.



(a): Untreated Huh-7 cells; (b): Huh-7 cells were incubated with an IC_{50} dose of the saponin fraction (48 h); Viable cells (V): Uniformly green; early apoptotic cells; (EA): Appear green, with “dots” of condensed chromatin; late apoptotic cells; (LA): Bright green-orange and apoptotic nonviable cells; (N): Stained bright orange-magnification 200x.

Fig 3: Cellular staining of liver cancer cells Huh-7 using acridine orange/ethidium bromide staining in the absence or presence of the saponin fraction of *A. rosea* seeds.

(DAPI stain) was used. Fig 2 illustrates the cellular changes observed after treating the cells with the saponin fraction for 24 hours. These changes include chromatin fragmentation, condensation and clustering around the nuclear periphery. These alterations correlate with a higher fluorescence intensity, as depicted in Fig 2. In contrast, control cells showed uniform staining of the nuclei. These findings indicate that the saponin fraction affected the nuclear morphology of Huh-7 cells, altering the chromatin structure. To investigate the mechanism by which saponin fraction caused cell death, we carried out dual staining to assess apoptosis. This staining is commonly used in research to determine the viability of cells and is particularly useful in distinguishing between live, apoptotic and necrotic cells. The morphological analysis of Huh-7 cell using the AO-EB double-staining technique elucidated the apoptotic potential of the saponin fraction (Fig 3). In this representation, viable cells are characterized by a green color, whereas apoptotic cells with chromatin condensation (suggestive of early apoptosis) or fragmentation are denoted by orange-stained nuclei (indicative of late apoptosis).

Apoptosis is a crucial process for maintaining tissue homeostasis and the induction of apoptosis in cancer cells represents a significant therapeutic strategy for cancer treatment (Suh *et al*., 2009). The cell and nuclear morphology have been used to evaluate the apoptotic features and DNA damage of synthetic and natural product compounds (Al-Zharani and Abutaha, 2023). In the present investigation, the apoptotic potential was seen by the saponin fraction in the Huh-7 cell line. The fragmentation of nucleic acids represents a key aspect of natural cell death, which can occur during the early stages (Bortner *et al*., 1995). The level of ROS generation in Huh-7 cells was compared using DCFH-DA fluorescent dye, both in the absence and presence of the saponin fraction of *A. rosea*. Fluorescent microscopy images revealed a rise in ROS production within the treated cells (Fig 4B) in contrast to the control group (Fig 4A). Although ROS plays a vital role in normal cell growth and division, excessive amounts can lead to severe DNA and protein damage, consequently triggering apoptosis. (Simon *et al*., 2000; Wang *et al*., 2011). Several studies suggest that the generation of ROS by saponin initiates cascades of cell death signals in human cancer cell lines (Ji *et al*., 2012; Zhang *et al*., 2020; Zou *et al*., 2009). Elevated levels of ROS have been documented to induce DNA damage, ultimately resulting in the apoptosis of cancer cells (Erkisa *et al*., 2020).

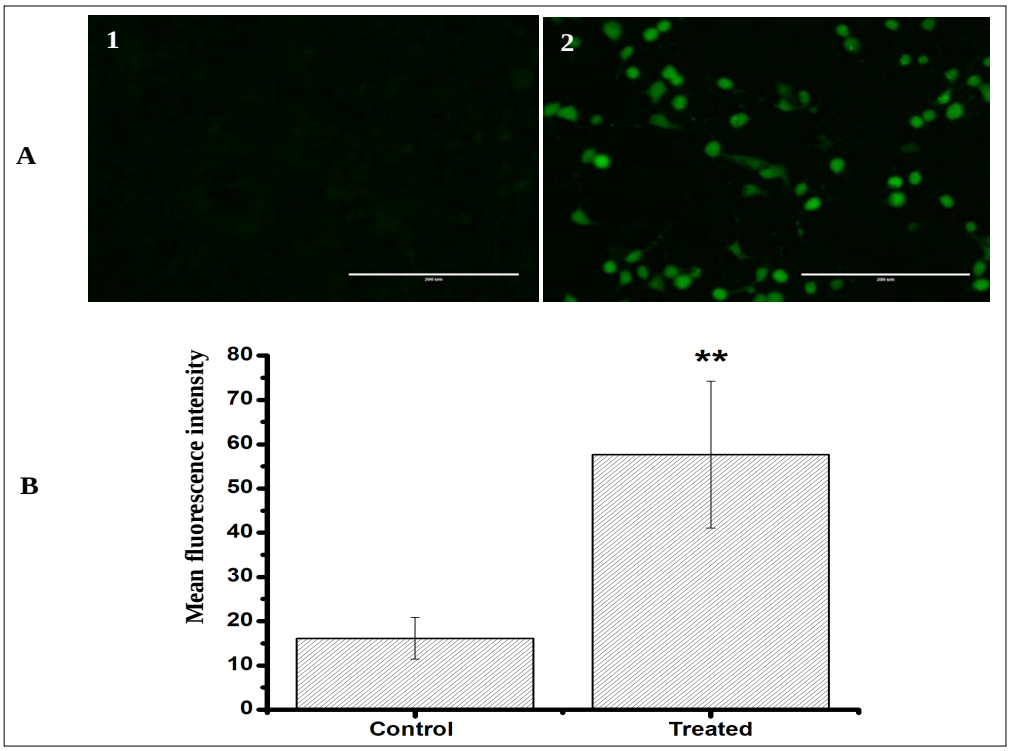


Fig 4: A: DCFDA staining was employed to assess the generation of reactive oxygen species (ROS) in Huh-7 liver cancer cells, both in the absence and presence of the saponin fraction derived from *A. rosea* seeds. (A): Untreated Huh-7 cells; (B): Huh-7 cells were incubated with an IC_{50} dose of the saponin fraction (48 h); B: ImageJ software was used to quantify the fluorescence intensity of reactive oxygen species (ROS) in cells treated with the saponin fraction. The measured fluorescence values were presented as mean ± SD from three replicates. Statistical significance was denoted by asterisks (**p-value).

It was observed that DNA-damaging substances can induce selective cytotoxicity towards cancer cells. These cells commonly harbour mutations in DNA repair genes, rendering them more susceptible to the cytotoxic effects of specific DNA-damaging substances. In contrast, normal cells maintain an intact DNA damage response mechanism, allowing them to repair DNA damage and survive the treatment with these DNA-damaging substances (Helleday *et al*., 2008; Lord and Ashworth, 2012). Several triterpenoid saponins are selectively cytotoxic against cancer cells *in vivo* and *in vitro* (Du *et al*., 2014).

CONCLUSION

In conclusion, our study demonstrated the potent apoptotic-inducing effects of the saponin fraction derived from the *A. rosea* seed extract in Huh-7 cells. Notably, the extract exhibited lower toxicity towards non-cancerous human cells than the liver cancer cells tested. These findings suggest that the *A. rosea* saponin fraction holds promise as a potential alternative anticancer treatment. Furthermore, its potential combination with conventional chemotherapeutic regimens could enhance treatment safety and efficacy.

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Authors' contribution

Abutaha designed the study, Alghamdi and Abutaha conducted experiments. Abutaha and Wadaan helped write the manuscript and performed data analyses.

Data availability statement

All the data is available within the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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