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Apoptotic Induction Mechanism of Artonin E in 3D Ovarian Cancer Cell Lines

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ABSTRACT

The three-dimensional culture model is gaining popularity because it is more physiologically relevant *in vivo* and predicts drug efficacy more accurately than conventional 2D culture. This study aimed to develop the 3D ovarian cells culture model and to investigate the cytotoxicity of artonin E in SKOV-3 cells in 2D and 3D cultures. The 3D cells culture was performed using BD™ Puramatrix™ Peptide Hydrogel. The Alamar blue assay and selectivity index analysis were conducted to examine the antiproliferative effects in both 2D and 3D cultures. The morphological apoptotic double staining and immunofluorescence studies were performed to investigate the possible apoptotic mechanism involved in treated 3D SKOV-3 spheroids. Result showed that the ovarian cell lines encapsulated in BD™ Puramatrix™ Peptide Hydrogel clearly demonstrated 3D-spheroids formation. The Alamar blue assay revealed that artonin E inhibited the growth of SKOV-3 cells in 2D and 3D culture, with IC_{50} values of 6.0 ± 0.8 µg/mL and 25.0 ± 0.8 µg/mL at 72 h treatment, respectively. This result indicated that the IC_{50} values of SKOV-3 cells treated with artonin E in 3D culture were four times higher than in 2D culture. Artonin E was found to be less toxic to normal human ovarian cell lines, T1074, with IC_{50} values at 72 h of 28.0 ± 0.8 µg/mL in 2D culture and 85.0 ± 0.5 µg/mL in 3D culture. The selectivity index analysis more than 2 suggested that artonin E was selective against cancer cells compared to normal cells. Artonin E treatment caused apoptotic morphological changes in 3D SKOV-3 spheroids. In a 3D immunofluorescence study, elevated levels of cleaved caspase-3, cleaved caspase-9, apoptotic proteins bax, and decreased levels of antiapoptotic proteins bcl-2, Hsp70, and survivin were observed. In conclusion, artonin E inhibits the growth of 3D SKOV-3 spheroids and induces cell death via a pro- and anti-apoptotic protein pathway. These findings demonstrate that 3D spheroids culture is an effective platform for testing artonin E therapeutic candidates in an *in vivo* mimic microenvironment.

Keywords: Apoptosis; ovarian cancer; artonin E; *Artocarpus elasticus*; 3D-culture

INTRODUCTION

Ovarian cancer is the most serious gynaecological malignancy (Torre *et al*., 2018). Approximately 300,000 ovarian cancer cases were registered in 2018, attributing for almost 4 percent of all new cases of cancer in women worldwide (Bray *et al*., 2018). This heritable cancer has been identified as a silent killer because its symptoms are nonspecific and usually diagnosed at late stage due to no effective screening test for the early detection (Jones, *et al*., 2017; Orr & Edwards, 2018). Surgery combined with a combination of chemotherapy drugs is the current treatment for ovarian cancer. Despite advances, ovarian cancer remains the most lethal female gynaecological cancer, with recurrence occurring frequently in this cancer population (Dochez *et al*., 2019; Stewart, *et al*., 2019).

Apoptosis is widely acknowledged as the main type of cell death, with a well-defined

molecular signaling pathway (Agrawal, 2019; Goldar, *et al*., 2015). It is a highly regulated process that plays a vital role in both development and maintaining tissue homeostasis (Hassan, Watari, AbuAlmaaty, Ohba, & Sakuragi, 2014). There are two major apoptotic pathways known to date: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (D'Arcy, 2019). A variety of intracellular and extracellular stimuli that initiate an intrinsic and extrinsic pathway can cause apoptosis (Boice & Bouchier-Hayes, 2020). The Bcl-2 protein family, which includes both pro-survival and pro-apoptotic protein, regulates intrinsic apoptosis pathway (Lee *et al*., 2019). Overexpression of antiapoptotic proteins and under-expression of proapoptotic proteins typically obstruct the apoptotic pathway in cancer (Pfeffer & Singh, 2018). Thus, modulating the apoptosis mechanism in the development of anticancer therapies has long been a mainstay and goal of clinical oncology.

Three-dimensional (3D) *in vitro* models have been gaining popularity in cancer research as they are closely mimicking *in vivo* condition (Langhans, 2021). These emerging techniques offer greater drug predictability, efficacy, and toxicity in preclinical drug identification (Breslin & Driscoll, 2016; Habanjar, *et al*., 2021). Among the numerous 3D culture models, spherical cancer is the most common 3D *in vitro* model (Weiswald, Bellet, & Dangles-Marie, 2015). Spherical cancer models embedded in various hydrogels were used to better mimic tissues *in vivo* and compatible for assessing drug targets in high-throughput screening (HTS) (Roth, *et al*., 2018).

Since ancient times, natural plant products have been the primary source of remedies for treating various diseases. Many medicinal plants used in traditional folk medicine have been scientifically identified as having bioactive compounds with the potential to be used in the development of new drugs (Nordin *et al*., 2014). Artonin E is one of the bioactive compounds isolated from a local medicinal plant, *Artocarpus elasticus.* Artonin E is a known prenylated flavonoid and can be found in several Artocarpus species such as *Artocarpus elasticus*, *Artocarpus dadah*, *Artocarpus rigida* (Nayak, *et al*., 2018; Ramli *et al*., 2016; Suhartati, *et al*., 2010). Artonin E (Figure 1) has shown vast biological activities such as antimicrobial, antiviral, tyrosinase inhibitor, anti-acne, and cytotoxicity activities (Kuete *et al*., 2011; Zajmi *et al*., 2015; Rahman *et al*., 2016). Previous research demonstrated artonin E's cytotoxicity properties in several cancer cell lines in 2D culture, which did not mimic the nature of cells *in vivo*. Thus, in this present study, we attempted to assess the antiproliferative effects of artonin E in 2D and 3D SKOV-3 cells, as well as the potential apoptotic mechanism involved in 3D culture.

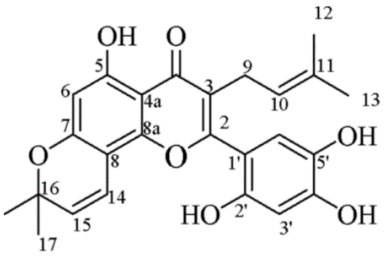

Figure 1. The chemical structure of artonin E

MATERIAL AND METHODS

Chemical and reagents

The chemicals were acquired from Sigma Chemical Co., St. Louis, MO, USA and Nacalai, Japan. All the reagents, solvents, and chemicals used are of analytical grade.

Artonin E and control drugs preparation

Artonin E was isolated from the stem bark of *Artocarpus elasticus* (Rahman *et al*., 2016). This compound was dissolved in dimethyl sulfoxide, with a maximum of 0.1 percent dimethyl sulfoxide dispensed to the cells. The tested cell lines were given a two-fold series dilution concentration (0.0, 1.0, 10, 50 and 100 µg/mL) of artonin E and the control drug carboplatin.

Cell culture

Metastatic ovarian adenocarcinoma SKOV-3 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). The normal immortalized human ovarian surface epithelial cell line T1074 was originally purchased from Applied Biological Materials (abm®) Canada.

PuraMatrix 3D cell culture model

BD™ Puramatrix™ Peptide Hydrogel (BD™ PuraMatrix™) was obtained from BD Bioscience, New Jersey, USA. The hydrogel scaffold was prepared in accordance with the manufacturer's protocol and previous published worked (Abu-Yousif, *et al*., 2010). SKOV-3 and T1074 healthy cells at concentration 1×10^3 cells per well were encapsulated in prepared BD™ Puramatrix™ Peptide Hydrogel. The encapsulated cells were allowed to form spheroids and were monitored in culture for up to 15 days. Every two days, the cells were supplemented with new fresh media.

Detection of 2D and 3D culture cell survival using Alamar blue assay

Alamar blue was used to detect the proliferation rate of artonin E in 2D and 3D cell culture (Bonnier *et al*., 2015). For 2D culture, the healthy SKOV-3 and T1074 cells were plated in 96-well and left overnight to attach before treatment. For 3D culture, the healthy SKOV-3 and T1074 cells were cultured in BD™ PuraMatrix™ peptide hydrogel for 7 days to form spheroids before being treated with Artonin E and control drug. The experiments were conducted for 24, 48 and 72 h. Following treatment, 10 µL of AlamarBlue® dye was dispensed into the designated wells and left in the dark for 4 h. The results were evaluated using a fluorescence microplate reader (Tecan Infinite M 200 PRO, Männedorf, Switzerland) at an excitement wavelength of 540 nm and an emission wavelength of 590 nm.

Selectivity index analysis

The selectivity index of a drug or compound is the most important and acceptable parameter to detect anticancer potential *in vitro* (López-Lázaro, 2015). It is a key metric for determining a drug's or compound's capability to kill cancer cells selectively while being less toxic to healthy cells. The immortalized normal human ovarian surface epithelial cell line T1074, which has a non-tumorigenic origin, was chosen as a reference control. The selectivity index (SI) was calculated according to the following equation:

$$Selectivity\ Index = \frac{IC_{50\ calculated\ for\ normal\ cells}}{IC_{50\ calculated\ for\ cancer\ cells}}$$

Morphological assessment of spheroid apoptotic cells

The morphological apoptotic were ascertained using double stained acridine orange (AO) and propidium iodide (PI). The cells were plated at 1×10^3 cells per well in a 96 well plate for 7 days to form spheroids. The spheroids were then exposed to the IC_{50} values of artonin E (28 µg/mL) for 24, 48, and 72 h. After incubation, the media were gently removed from the culture well. The spheroids were fixed in 4% paraformaldehyde for 20 minutes. Following fixation, the spheroids were washed 3× with cold PBS. Inside the well, the fresh spheroids were stained with an equal volume of AOPI solution and directly observed under an inverted fluorescence microscope (Nikon, ECLIPSE TI-S). The morphological criteria used for the classification of healthy, apoptotic, and necrotic spheroids were followed as published (Rahman *et al*., 2016).

Fluorescence immunostaining

The apoptotic mechanism involved in 3D SKOV-3 spheroid treated with artonin E was investigated using immunofluorescence staining. Bax, bcl-2, survivin, Hsp70, cleaved caspase-3, cleaved caspase-8, and cleaved caspase-9 were the proteins selected in this study. The immunofluorescence staining was conducted with some modifications to the method of Karimian *et. al.* (2014). The cells were plated in 96 well plate at 1×10^3 cells per well and cultured for 7 days to form spheroid. The spheroids were then exposed to artonin E (28 µg/mL) for 48 hours. The spheroids were fixed in 4% paraformaldehyde for 30 minutes, washed with PBS and blocked in a blocking solution Then, the primary antibody was added in a blocking solution and left overnight at 4°C before adding a secondary antibody. The secondary antibody was added and incubated for four hours. The imaging analysis of samples was performed directly in the cell culture wells using Inverted fluorescence microscope (Nikon, ECLIPSE TI-S).

Statistical analysis

All the data were denoted as mean ± SD and assessed employing one-way ANOVA and post hoc Tukey HSD multiple comparison tests. Statistical analysis was performed using SPSS-16.0 package and GraphPad prism 5.0, with $p<0.05$ was considered significant.

RESULT AND DISCUSSION

Cells grown in a 3D culture system are more physiologically similar to their *in vivo* natural microenvironment, which has been shown to be more clinically relevant than 2D conventional culture. (Breslin & Driscoll, 2016). In this study, we attempt to create the 3D ovarian spheroids using BD™ Puramatrix™ peptide hydrogel. The formation of ovarian spheroids was characterized and then subjected to cytotoxicity evaluation, morphological apoptotic induction, and protein analysis. The antiproliferative results in 3D culture were then compared to those in 2D culture.

Optimization of 3D SKOV-3 spheroid formation

Brightfield microscopy was used to observe the spheroids formation, size, shape, and diameter in every two days for 15 days.

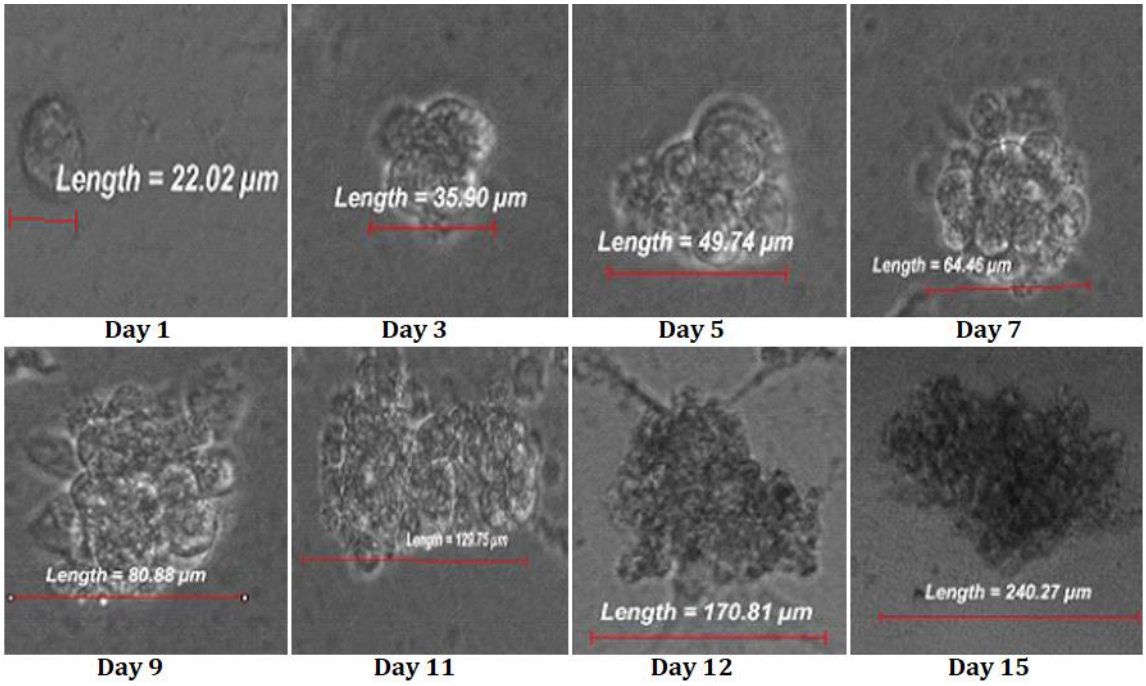

Figure 2. Phase-contrast images of SKOV-3 spheroids formation (magnification 20×). Spheroid size and morphology were observed for 15 days. Spheroids images and diameter (µm) were taken and measured at day 1, 3, 5, 7, 9, 11, 13 and 15.

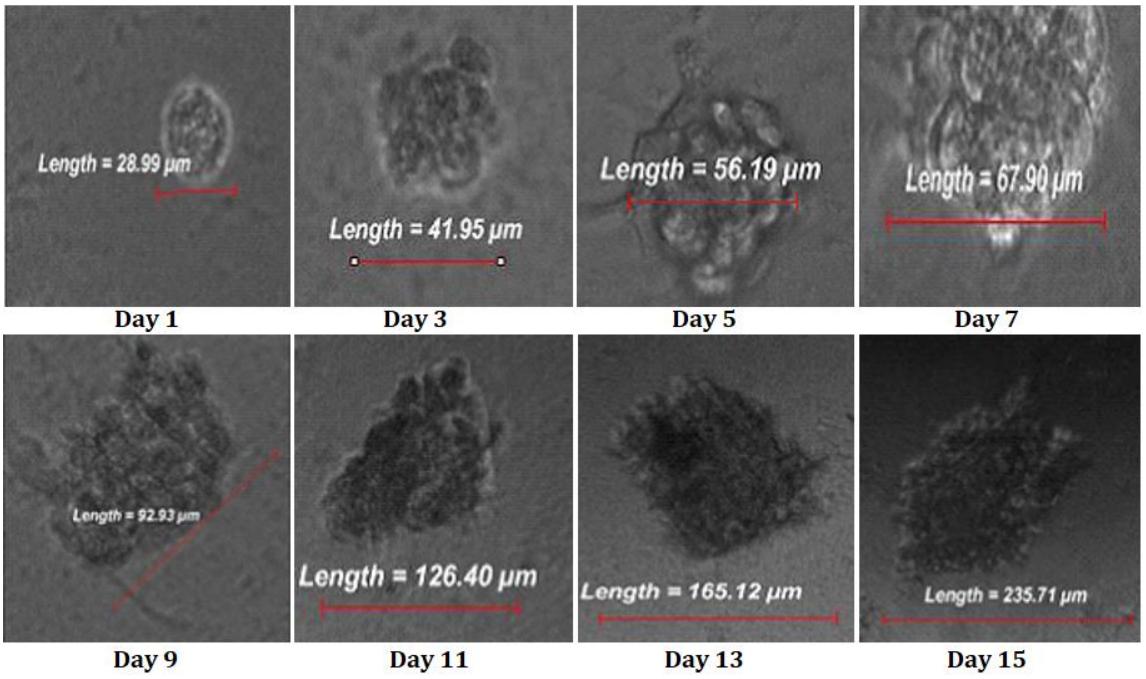

Figure 3. Phase-contrast images of T1074 spheroids formation (magnification 20×). Spheroid size and morphology were observed for 15 days. Spheroids images and diameter (µm) were taken and measured at day 1, 3, 5, 7, 9, 11, 13 and 15.

The diameter of spheroids was measured as soon as 24 h after plating. SKOV-3 cells encapsulated in BD™ Puramatrix™ Peptide Hydrogel demonstrated the formation of 3D-spheroids with the diameter of 22.02 µm after 24 h post plating (Figure 2), and the diameter of spheroids gradually increased over 15 days of culture from 22.02 µm to 240.27 µm. The healthy compact cellular aggregate spheroids appeared at day 3 until day 9 of culture. Similar pattern of 3D-spheroid formation was observed in T1074 cell lines (Figure 3).

After 7 days in culture, the spheroids had grown to a diameter of >50 µm with healthy compact aggregates. Based on this finding, spheroids cultured for 7 days reached a size range of >50 µm, which can be considered an appropriate size in 3D *in vitro* ovarian tumour models without the risk of necrosis (Abu-Yousif *et al*., 2010; Hadi, *et al*., 2018). This result was also consistent with previous research, which suggested that epithelial ovarian cancer cell spheroids with growth rates ranging from 50 to 750 µm exhibited a more physiological 3D microenvironment mimicking peritoneal ascites (Loessner *et al*., 2010). As a result, optimised 3D ovarian spheroids with diameters greater than 50 µm were used throughout the study.

Necrotic spheroids can be seen after day 11 and up to day 15 of culture as shown in Figure 2 and Figure 3. In nature, spheroids have a layered-like cellular structure, with an outer proliferating layer, an inner quiescent zone, and possibly a necrotic core at their centre, similar to the structure of a real tumour (Brüningk, *et al*., 2020). The current finding was also consistent with previous research, which suggested that two weeks was an ideal time to grow spheroids with less risk of necrotic lesion (Godugu *et al*., 2013).

Detection of 2D and 3D culture cell survival using Alamar blue assay

Alamar blue assay was used to examine the antiproliferative effect of artonin E and carboplatin in 2D and 3D culture. The IC_{50} values obtained from 2D, and 3D culture were compared. The antiproliferative activity using the Alamar blue assay showed that the IC_{50} values of SKOV-3 and T1074 spheroids culture treated with artonin E and carboplatin were higher than their 2D counterparts. IC_{50} values of SKOV-3 cells treated with artonin E and carboplatin for 24, 48 and 72 h in 2D culture were found to be in the range of 6.0 ± 0.8 µg/ml to 12.0 ± 0.4 µg/ml and 24.0 ± 0.8 µg/ml to 35.0 ± 0.4 µg/ml, respectively (Table I). SKOV-3 cells treated with artonin E and carboplatin in 3D culture exhibited the IC_{50} values range of 25.0 ± 0.8 µg/ml to 34.0 ± 1.0 µg/ml and 61.0 ± 1.4 µg/ml to 72.0 ± 0.8 µg/ml, respectively (Table 1). These results showed that the IC_{50} values of SKOV-3 spheroids treated with artonin E and control drug were 1.8 to 4.1-fold higher than their 2D counterparts (Table III). A similar pattern of results was discovered in the treated T1074 spheroids culture (Table II). The IC_{50} values were found to be higher in 3D-spheroid culture (Table II), ranging from 1.2 to 6-fold higher than in 2D culture (Table III).

Several studies have found that cells grown in 3D culture have a higher survival rate and are more resistant to the anticancer drug (Borghese, *et al*., 2020; Nunes, *et al*., 2019). Increased IC_{50} values in cell spheroids treated with artonin E and anti-cancer drug could be due to a number of mechanisms, including decreased anti-cancer drug penetrance, increased pro-survival signalling, and/or upregulation of drug resistance genes (Godugu *et al*., 2013; Nunes *et al*., 2019). Previous research has also discovered that tumour cells grown in 3D are more resistant to cytotoxicity drugs due to phenotypic changes that do not occur in 2D culture (Jensen & Teng, 2020).

Selectivity index analysis

The most difficult aspect of drug development is therapeutic selectivity. Many drug candidates fail because there is insufficient therapeutic effect at non-toxic concentrations (Lehar, *et al*., 2009). Therefore, the determination of the SI value for any drug candidate is critical in deciding whether additional work can be carried out (Indrayanto, *et al.,* 2020).

Artonin E displayed SI values ranging from 2.8 to 4.6 in both 2D, and 3D culture (Table IV). This compound had higher SI values than the control drug, carboplatin. Carboplatin had an SI value ranging from 0.3 to 0.9, indicating that the drug is unable to distinguish between normal and cancer cells. A drug or compound with a higher SI ration would theoretically be safer and more effective in *in vivo*, potentially providing a safer therapy (Calderón-Montaño *et al*., 2019; Segun *et al*., 2019). The high selectivity of artonin E towards ovarian cancer cell lines deserves further extensive studies.

Table I. The IC_{50} values of artonin E and carboplatin on SKOV-3 cells at different time-point in 2D and 3D culture condition.

Compounds/ Treatment period (h)	**IC_{50} values at different time points (µg/mL)**					
	2D Culture			**3D Culture**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Artonin E	12.0 ± 1.4	8.0 ± 1.2	6.0 ± 0.8	34.0 ± 1.0	28.0 ± 1.4	25.0 ± 0.8
Carboplatin	26.0 ± 1.4	18.0 ± 0.8	12.0 ± 1.0	48.0 ± 1.8	38.0 ± 1.2	32.0 ± 1.6

Table II. The IC_{50} values of artonin E and carboplatin on T1074 cells at different time-point in 2D and 3D culture condition.

Compounds/ Treatment period (h)	**IC_{50} values at different time points (µg/mL)**					
	2D Culture			**3D Culture**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Artonin E	38.0 ± 1.8	35.0 ± 0.5	28.0 ± 0.8	98.0 ± 0.8	90.0 ± 0.5	85.0 ± 0.5
Carboplatin	22.0 ± 0.4	16.0 ± 0.6	8.0 ± 0.8	68.0 ± 0.8	55.0 ± 1.2	48.0 ± 1.5

Table III. The comparison of the fold differences in IC_{50} values between 2D and 3D cultures at various time points.

Compounds/	**SKOV-3 cells**			**T1074 cells**		
Treatment period (h)	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Artonin E	2.8	3.5	4.1	2.1	2.14	2.4
Carboplatin	2.0	2.3	2.5	3.0	3.4	6.0

Table IV. The selectivity index analysis of artonin E and carboplatin on SKOV-3 cells at different time points in 2D and 3D culture.

Compounds/	**2D culture**			**3D culture**		
Treatment period (h)	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Artonin E	3.1	4.3	4.6	2.8	3.2	3.4
Carboplatin	0.6	0.5	0.3	0.9	0.8	0.7

Evaluation of apoptotic spheroid morphology

3D ovarian spheroids treated with Artonin E induced apoptosis specific morphological changes as exhibited in AOPI analysis. Control SKOV-3 spheroids had a bright green color with an intact structure, indicating healthy viable spheroids, whereas artonin E-treated spheroids had a bright yellow stain, indicating the presence of apoptotic cells (Figure 4). As the incubation time with artonin E increased, the relative number of green spheroids decreased while the number of yellow spheroids increased, indicating the transition of viable spheroids to apoptotic spheroids. After 48 and 72 hours of treatment, there was a statistically significant *($p<0.05$)* increase in the induction of apoptosis in SKOV-3 spheroids exposed to artonin E. This study suggested that 3D ovarian spheroids treated with Artonin E induced apoptosis specific morphological changes. Increase in the percentage of early apoptosis spheroids over time suggesting that the integrity and size may have compromised and penetrated by artonin E even in 3D microenvironment. This study was similar to the previous published works showed that there were markedly increased in the apoptosis morphological changes in breast cancer spheroids staining with acridine orange (AO)/ ethidium bromide (EtBr) (Rolver, *et al*., 2019; Salehi, *et al*., 2017).

Fluorescence immunostaining

Immunofluorescence staining was used to gain a better understanding of the role of artonin E in inducing apoptosis at the protein level. Artonin E significantly *($p<0.05$)* induced the expression of apoptotic protein bax and decreased the expression level of antiapoptotic bcl-2 (Figure 5).

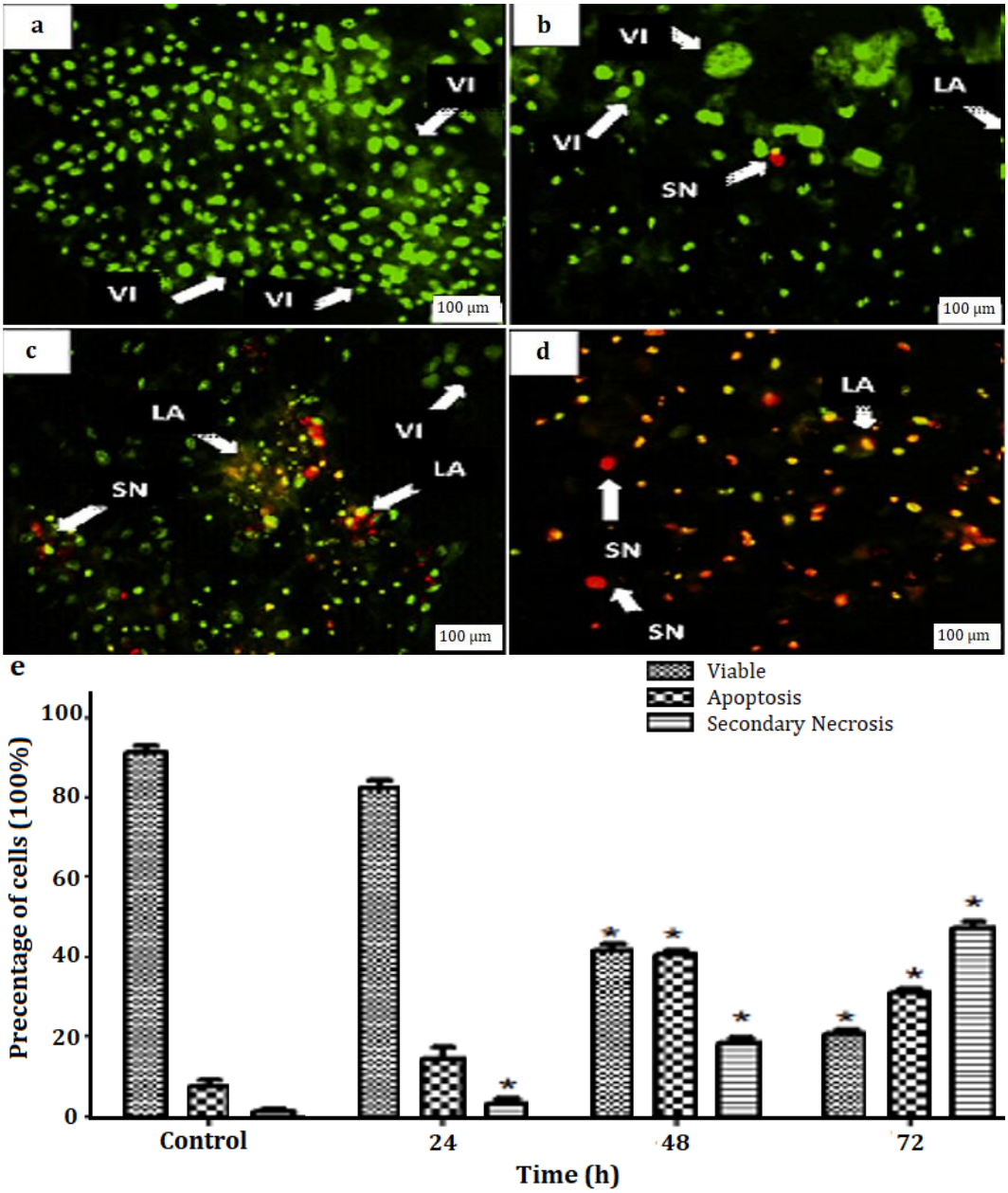

Figure 4. Untreated and treated 3D spheroids were stained with fluorescence dyes AO and PI (magnification 20×). SKOV-3 spheroid treated with artonin E (28 µg/mL) at various time points. (A) Untreated SKOV-3 spheroid (B) SKOV-3 spheroid at 24 h treatment (C) SKOV-3 spheroid at 48 h treatment (D) SKOV-3 spheroid at 72 h treatment. (E) Quantitative analysis of an artonin E-treated SKOV-3 spheroid over time (EA: early apoptosis; LA: late apoptosis; and SN: secondary necrosis). The results are given as the mean SD of three replicates. * Each phase has a significant difference from the control (p<0.05).

These findings are consistent with previous research, which discovered that arsenic-disulfide increased bax expression in 2D and 3D MCF-7 cells (Zhao *et al*., 2018). In addition, a combination of drugs targeting Bcl-2 family proteins has recently been shown to reduce cell viability in melanoma and cervical 3D spheroids (Lee *et al*., 2019; Rahman *et al*., 2020).

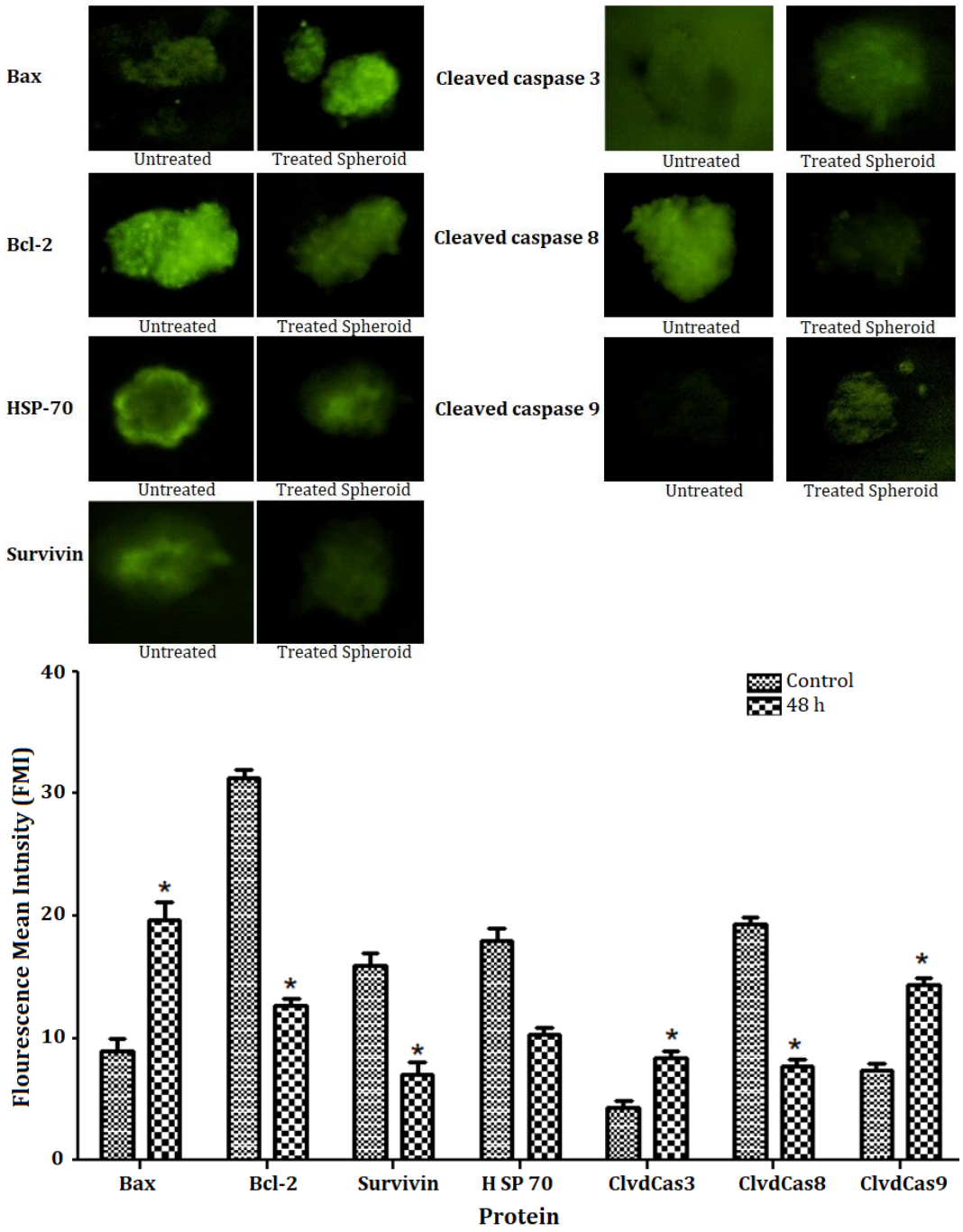

Figure 5. (A) The 48-hour effect of artonin E (28 µg/mL) on apoptosis protein expression (magnification 20×). (B) Artonin E-treated SKOV-3 cells were quantified. All data was expressed as mean ± SD. *p<0.05 denoted statistical significance.

Decrease in the expression of the anti-apoptotic proteins survivin and Hsp70 (Figure 5). Survivin and Hsp70 belong to the family of apoptosis inhibitors. Both molecules have been found in a variety of cancers, and their overexpression have been linked to aggressiveness and resistance to chemotherapy (Elmallah *et al*., 2020; Hennigs *et al*., 2020). Previous work reported YM155, a small molecule survivin promoter suppressant, downregulated survivin in

3D MCF-7 spheroids, making it a potential target for cancer biomarker (Xin, *et al*., 2019). A recent study also reported that microwave treatment inhibits HPV oncoprotein expression and downregulates Hsp70 in 3D *in vitro* cervical tumour tissues, and this decreased level of Hsp70 was correlated with increased expression of caspase-3 (Badea *et al*., 2019).

Figure 5 depicted elevated levels of cleaved caspase-9, cleaved caspase-3, and downregulated levels of cleaved caspase-8. These findings suggest that intrinsic apoptosis pathways are involved in artonin E-treated SKOV-3 spheroids, with the bcl-2/bax protein family involved. The intrinsic pathway is activated by the release of cytochrome *c* from the mitochondria, which recruits caspase-9, while the extrinsic pathway recruit's caspase-8 (Li-Weber, 2013).

Several studies have been published that show the effects of natural compounds on the deterioration of malignancies in 3D culture. Resveratrol, for instance, has been shown in 3D culture to induce luminal apoptosis but not in 2D culture (Tsunoda *et al*., 2014). Thymol, a natural compound derived from the essential oil of *Oliveria* induced apoptosis in MDA-MB-231 spheroids in 2D and 3D culture by activating both intrinsic and extrinsic apoptosis pathways (Jamali, *et al*., 2020; Jamali, *et al*., 2018). Therefore, such studies supported that natural compound such as artonin E have a good potential in inducing apoptosis in 3D ovarian spheroids culture model.

CONCLUSION

Taking the advantage of 3D culture, the current study investigated the cytotoxicity and apoptotic induction mechanism of artonin E in 3D BD™ Puramatrix™ peptide hydrogel. This study showed that normal and human ovarian cancer cell lines grown in 3D puramatric hydrogel resulted in the formation of multicellular spheroids. The IC_{50} values in 3D culture were increased when compared to the 2D culture model suggested that SKOV-3 spheroids showed higher resistance to artonin E and chemotherapeutic drug with high selectivity index towards 3D normal ovarian spheroids. The current study into the mechanism of action suggests that artonin E induced apoptosis in 3D SKOV-3 ovarian spheroids via apoptotic morphological changes as shown in AOPI analysis, involvement the intrinsic apoptosis pathway and altered the expression levels of bcl-2/bax, Hsp70, and survivin.

CONFLICT OF INTEREST

All authors declared to have no competing of interest relevant to this article.

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