**Gracilaria changii** Derived Cholesterol Cause Necrotic Cell Death in an Ovarian Adenocarcinoma Cell Line, Caov-3 Through p38 MAPK and FasL Activation in Combination with Ethanol

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**Abstract:** A sterol compound found abundantly in Malaysia local red seaweed, *Gracilaria changii* diethyl ether fraction showed an unexpected in vitro cytotoxicity towards ovarian adenocarcinoma cell line, Caov-3, in combination with ethanol (GCM1-Et) without affecting the control cell line. Further assessment on p38 MAPK levels showed a transient expression of phosphorylated-p38 MAPK (p-p38) followed by increased expression of Fas and FasL upon treatment of GCM1-Et. In contrast to p-p38, FasL expressions were sustained until 24 hours. In the presence of p38 MAPK inhibitor (SB203580), both p-p38 MAPK and FasL signals were diminished, thus confirmed the correlation between p-p38 MAPK and increased level of FasL. Cell viability assay revealed the death of 37.8±6.87% Caov-3 cell population upon GCM1-Et treatment and was reduced to 10.5±8.03% in the presence of inhibitor. Morphology and DNA fragmentation analysis showed a dominant necrotic cell death which were characterized by high percentage of degraded chromatin without condensation (93.89%) and smearing of genomic DNA that took place upon GCM1-Et treatment. Thus, this *G. changii* cholesterol is believed to be responsible in inducing necrotic cell death of Caov-3 cells through increased level of p-p38 MAPK and FasL.

**Key words:** Necrosis ・ Cholesterol ・ *Gracilaria* ・ Ovarian ・ p38

**INTRODUCTION**

Seaweed has been known to contain substances able to maintain human health. This marine algae is a major producer of agar which has been used extensively in food industry. Other than commercial use, seaweed are traditionally used as human and animal source of nutrition because it contains significant amounts of essential proteins, vitamins and minerals. [1, 2]. Seaweed as a nutritional supplement has received much attention since many studies indicate low incidences of cancer in Asian communities especially estrogen-dependent diseases such as endometriosis, ovarian and endometrial cancer [3-5] apparently linked to seaweed phytosterol consumption [6, 7]. Seaweed has also been reported to have an antitumor properties [8] as well as antibacterial, antiviral, anticoagulant, antioxidant and antiinflammatory properties [9-12]. Several studies also showed an important role of seaweed in other medical condition such as goiter, dysentery and diarrhea [13].

In Malaysia, seaweed, especially *Gracilaria changii* is only consumed in certain areas along the east coast of Peninsular Malaysia, where it is eaten as a salad dish. *G. changii*, recorded from Malaysia and Thailand, is one of the most abundant agarophytic seaweeds found in Malaysia and is known for its good quality agar and adaptability to the harsh mangroves condition [14]. Recent studies have successfully regenerated *G.changii* protoplasts in order to produce a large mass of this seaweed [15] and many studies have been performed to manipulate its properties as a major agar supply, estimated at more than 60% of the world’s agar supply.
Despite a wealth of information on *G. changii* agar content, taxonomy, habitats, nutritional and biochemical composition [17], very few studies have been carried out to examine its medicinal value. *G. changii* extracts have been reported to show an antimicrobial [18], antioxidant [19] and antifungal [20] activities. Until now, however, there is little information on the cytotoxicity of *G. changii* extracts towards cancer cells.

Epithelial ovarian cancer is the most lethal [21] with worst prognoses with the highest fatality to case ratio of all gynecologic malignancies and is responsible for 140,200 deaths in 2008 worldwide [22]. In Malaysia, ovarian cancer remain in a top ten cancers with a high incidence in Malaysian women [23, 24]. The percentage of ovarian cancer occurrence was reported to increase from 4.1% in 2003 to 5.8% in 2006 [25, 26]. The early stage of cancer development is almost asymptomatic and most of the cases detected during carcinoma *in situ* or worst case, metastasis had already taken place [27, 28].

A little opportunity for early stage detection and treatment, ovarian cancer remain fatal in contribution of increasing resistance against several line of cytotoxic drugs [29, 30]. However, pathway studies reveal a crucial involvement of stress kinases response to cytotoxic drug treatment which induce death signaling cascade [28] thus provide an alternative target for cancer treatment through phosphorylation of specific target.

Phosphorylation is the most common biochemical modification of cellular molecules regulating fundamental processes including proliferation [31] and mediated by protein kinases and phosphatases. Above all, mitogen activated protein kinases (MAPK) are well known to play a crucial role in regulating a variety of cellular events such as proliferation, differentiation and apoptosis [32]. Thus, the MAPK step of the transcription activation pathway may represent an important therapeutic target given its position as the final common pathway for cell proliferation and other activities relevant to cancer pathogenesis [33]. p38 MAPK is a member of MAPK family that is activated by a variety of environmental stress [34], inflammation process, cell regulation, migration and apoptosis [35]. p38 MAPK is built of threonine and tyrosine residues in its activation loop which require a phosphorylation by dual specificity MAPK (MKK3 and MKK6) to activate and consist of four isoforms (α, β, δ, γ) with 60% identical amino acid sequence encoded by a separate genes [36]. There is no argument that p38 also involved in carcinogenesis of some cancers, but its role in cell differentiation, growth inhibition and cell death is well established and has been reviewed in many studies [37]. Use of Cisplatin in combination with hepatocyte growth factor (HGF) in ovarian cancer cell line results in p38 activation and an enhancement of the cell sensitivity towards cytotoxic drugs [38, 39]. Previously, a chemoresistance in ovarian cancer cell line (2008C13) was reported to be associated with failure to up-regulate FasL and sustained activation of JNK/p38 pathway vanquished the state [40]. Specific antibody against CM1 (centrocyte/-blast marker 1) expressed mainly in Caov-3 cell exert an apoptotic machinery through modulation of Fas/FasL expression [41], thus, making these two components (p38 and FasL) as an interesting subject to explore.

**MATERIALS AND METHODS**

**Test Materials Preparation:** Methanol (MeGc), butanol (BuGc) and diethyl ether (E2Gc) extracts of *G. changii*, as well as GCM1 was retrieved from Dr. Habshah Mohamed from Malaysia University of Terengganu (UMT). The crude extract were diluted in DMSO making a 10 mg/mL of stock solution. The GCM1 compound was diluted in ethanol, chloroform and DMSO to make a stock solution with 10 mg/mL of concentration. Test materials were prepared by serial dilution in fresh medium (0.0078 mg/mL to 0.5 mg/mL). Specific inhibitor for p38 MAPK, SB203580 and paclitaxel® were purchased from Sigma, USA and prepared according to manufacturer’s recommendation. Briefly, SB203580 is reconstituted in DMSO to make 5 mg/mL stock solution and diluted in fresh medium to a final concentration of 10 µM. Paclitaxel® is reconstituted with ethanol to make 1 mg/mL stock solution and diluted in fresh medium to a final concentration of 1 µM. All stock solutions were kept at -20°C to maintain freshness [42].

**Cell Culture Preparation:** Caov-3 (HTB-75™) cell line was retrieved from American Type Culture Collection (ATCC), USA. As a comparison for cytotoxicity test, a mouse normal ovarian cell line, T-Ag-MOSE (JCRB0151) was retrieved from Japanese Collection of Research Bioresources (JCRB), Japan Health Science Foundation, Japan as there was no available normal human ovarian cell line at that time. Both cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C and 5% CO₂ insulated incubator as recommended by supplier.
Cell Synchronization: Prior to test, a total of 2 x 10^6 cells were cultured in 96-well plate for cytotoxicity test until cells reached 70% confluences. Synchronization was accomplished with starving medium which consist of 0.2% FBS supplemented DMEM for 24 hours and refreshed with fresh medium for another 24 hours. On the test day medium were replaced with test medium containing test material and controls. Tests were then carried out for 24 hours. For protein and cells assessment, the same method was applied in T-25 flask with a volume adjustment. Glass slide placed in a petri dish with 7 cm in diameter was used as a platform for nuclear assessment using DAPI.

MTT Cytotoxicity Test: After 24 hours incubation with test materials, 10 µL of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg/mL) was added in each well and plate was further incubated at 37°C, 5% CO₂ for 4 hours. Medium with MTT were discarded and DMSO was added to each well to dissolve the formazan crystals, a product generated by the activity of succinate dehydrogenase in viable cells. Adsorbance value at 540 nm and 660 nm were taken and percentages of living cells were calculated using a formula below;

\[
\%TC = \frac{(GT – CT)}{GC – CB} \times 100
\]

where; TC, total cells; GT, cells grown in test material; CT, empty medium with test material; GC, cells grown in medium without test material; CB, empty medium.

The amount of formazan detected by optical density measurement at 540 nm is proportional to viable cells in test cultures. LC₅₀ were determined from probit analysis which involves graphs generated from percentage of TC against log concentration of test materials.

Western Blot: Caov-3 cells were incubated in the presence and absence of SB203580 prior to GCM1-Et treatment for 24 hours. Cells were washed twice, scraped and lysed in ice-cold buffer (Tris, SDS, sodium orthovanadate, PMSF, pepstatin A, aprotinin, leupeptin, DTT, EDTA) for 30 minutes. Lysates were centrifuged at 12,000 g for 20 minutes at 4°C and supernatant were used for protein amount determination using BCA reagent. Equal amount of protein (20 µg) were mixed with 2X SDS gel buffer and boiled at 100°C for 5 minutes. Samples were separated in 12% SDS-PAGE and transferred on to nitrocellulose membrane. Membrane was blocked with 0.5% skim milk and probed with different primary antibodies. Anti-p38 MAPK, anti-phosphorylated p38 MAPK (p-p38), anti-Fas, anti-FasL and anti-Actin were purchased from Santa Cruz Biotechnology. Anti-rabbit and anti-mouse IgG conjugated peroxidase act as a secondary antibodies and were detected using PIERCE detection reagents on X-ray film.

Trypan Blue Dye Exclusion Assay: Cell counting is carried out based on standard method [43]. Basically, a 100 µL of 0.4% trypan blue solution was added to 0.1 mL cell suspension following a five minutes incubation at room temperature (~25°C). The cell counting was done using haemocytometer where percentages of live cells were determined.

DAPI Staining: A total of 2 x 10^6 Caov-3 cells were cultured in a petri dish containing sterile glass slide and allow to attach overnight. Cells were then incubated in medium containing 10 µg/mL GCM1-Et with paclitaxel® as a positive control. After 24 hours, slides were washed in PBS and fixed in a serial diluted methanol (70%, 85%, 100%) for 5 minutes followed by immersion in 3.7% paraformaldehyde for 10 minutes before air-dried. Slides were stained with 1 µg/mL DAPI in dark for 15 minutes and washed off with methanol. Slides were mounted with glycerol-PBS (10:1) and covered with a clean coverslip. Cells were observed under fluorescence microscope with 100x oil immersion objective. A percentage of condensed and degraded chromatin were then determined in 300 cells for each samples [44, 45].

DNA Fragmentation Assay: Caov-3 cells were cultured in the absence or presence of GCM1 (10 µg/mL) and paclitaxel (1 µM) for 24 hours. Suspensions of treated and non-treated Caov-3 cells were washed two times in PBS prior to DNA extraction. DNA fragmentation assay were done according to rapid protocol developed previously [46] to minimise mechanical and chemical damage on DNA materials. Basically, a freshly prepared lysis buffer (10 mM Tris-Cl, pH 7.4; 100 mM NaCl; 25 mM EDTA; 1% sarsosyl) and 10 µg/µL proteinase K were added to cells pellet followed by 1 hour incubation at 45°C. Another 2 µL of RNAsa (10 µg/µL) was added to lysates and further incubation was carried out at room temperature (~25°C) for another hour. Samples were then mixed with 4 µL of 6x loading buffer and separated on 2% agarose gel. Visualisation was carried out by ethidium bromide staining.
Table 1: Summary of LC_{50} values of each tested materials against Caov-3 and T-Ag-MOSE cell lines

<table>
<thead>
<tr>
<th>Test materials</th>
<th>Caov-3 LC_{50}</th>
<th>T-Ag-MOSE LC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeGc</td>
<td>39.07±1.53</td>
<td>&gt;500</td>
</tr>
<tr>
<td>BuGc</td>
<td>24.19±1.26</td>
<td>&gt;500</td>
</tr>
<tr>
<td>E2Gc</td>
<td>18.58±0.77</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>79.43±0.00</td>
<td>8.54±1.03</td>
</tr>
<tr>
<td>GCM1-DMSO</td>
<td>177.46±7.82</td>
<td>&gt;500</td>
</tr>
<tr>
<td>GCM1-Etanol</td>
<td>7.19±1.07</td>
<td>224.9±4.78</td>
</tr>
<tr>
<td>DMSO</td>
<td>&gt;1%</td>
<td>&gt;1%</td>
</tr>
<tr>
<td>Ethanol</td>
<td>&gt;10%</td>
<td>&gt;10%</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.078±4.84%</td>
<td>0.1±2.99%</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Cytotoxicity against Caov-3 cell line is Exerted by Ethanol Diluted GCM1 (GCM1-Et): Cytotoxicity test of all extract showed only diethyl ether extract of G. changii (E2Gc) gave a significant cytotoxic activity against Caov-3 cell line by exerting the lowest LC_{50} value (18.58±0.77 µg/mL) (Table 1). Methanol (MeGc) and butanol (BuGc) extract did show some activity towards the same cell line, however, the high LC_{50} value of more than 20 µg/mL indicate an insignificant cytotoxic activity (Table 1). None of the three extracts showed any relevant activity against normal mouse ovarian cell line, T-Ag-MOSE. A high cytotoxic activity of E2Gc against Caov-3 cells suggests a potential cytotoxic bioactive fraction presence. Diethyl ether is a non-polar solvent with a capability to extract a plant sterol compound [47]. Previous study showed that diethyl ether extract of G. changii possesses antimicrobial activities against wide range of microbes, including bacteria, yeast and fungi [48]. Further purification of E2Gc by other group resulted in an abundance of crystal white substances with similar chemical structure to animal cholesterol. Sterols, a primary metabolite, are organic molecules which function is critical in cellular processes (eg. hormone precursor) and maintaining cell integrity (eg. cholesterol). Being a common metabolite, sterols somehow contribute to some biological activity in the form of extracts [49].

This substance, named as GCM1, was reported to have a weak interaction with commonly used amphipatic solvent, DMSO. Other option, such as ethanol and chloroform were taken into account as these solvents are known to have a better interaction with sterol based compound. A cytotoxicity test carried out using both solvents on Caov-3 and T-Ag-MOSE cell lines showed that only DMSO and ethanol appear to be safe towards both cell lines. Chloroform on the other hand, was found to be toxic towards both cell lines and unsuitable to be used in this study as been shown by a very low LC_{50} value recorded from both cell lines. A cytotoxicity test was carried out on ethanol and DMSO diluted GCM1 against both cell lines reveal a specific cytotoxic activity executed by ethanol diluted GCM1 (GCM1-Et) towards Caov-3 cell line. As been shown in Figure 1a, a high percentage of Caov-3 cell death was recorded from GCM1-Et as compared to DMSO diluted GCM1 (GCM1-Dm) in figure 1b with a low LC_{50} value (7.19±1.07 µg/mL). Neither combination have a significant cytotoxic effect towards control cell line, T-Ag-MOSE as been shown by low percentage of cell death with high LC_{50} values. Further investigations were carried out on p38 MAPK protein expression profiles which demonstrate a significant involvement in ovarian cancer response to several anti-cancer drug treatments.

GCM1-Et Cytotoxicity Increased the Level of Phosphorylated-p38 MAPK in Caov-3: A western blot of Caov-3 cell lysate with the absence and presence of GCM1-Et were carried out to determine the phosphorylated form of p38 MAPK (p-p38) expression profiles at different time interval. Within 70 minutes, p-p38 expressions were observed at as early as 5 minutes after exposure to GCM1-Et and remain visible until 60 minutes of exposure (Fig. 2). On the contrary, a completely different result obtained in the absence of GCM1-Et where p-p38 was not observed at any duration. A densitometry analysis of blot showed a peak in signal intensities achieved at 30 and 40 minutes of treatment before the signal declined to its original state (Fig. 2). Phosphorylation of p38 MAPK has a significant implication in ovarian cancer response towards anti-cancer drug treatment which are shown by presence or elevation of p-p38 signal intensities. However, phosphorylated form of p38 MAPK does not affect the toxic outcome of these drugs directly, instead, a constitution of cell death signaling pathway components are required to finish up the task. A cell death component such as Fas ligand (FasL) is proven to be vital in p38-mediated apoptosis or necrosis in several types of cells. To investigate further, Caov-3 cells were exposed to GCM1 in the absence and presence of SB203580. Fas and FasL expression profiles, as well as p38 and p-p38 within 24 hours time frame were observed and compared.

Increased Level of p-p38 MAPK Affect Level of FasL in GCM1-Et treated Caov-3: A western blot analysis of Caov-3 cells treated with GCM1-Et in the absence and
Fig. 1: Ethanol diluted gcm1 is more toxic to caov-3 cell line as compared to dmsos diluted gcm1. A. Gcm1-et resulted in more caov-3 cells death compared to t-ag-mose cells. B. Gcm1-dm only shows a significant percentage of cells death at a higher concentration than gcm1-et.

Fig. 2: Gcm1-et affects p-p38 expression profiles within 70 minutes of treatment. A densitometry analysis of blot showed a p-p38 expression profiles was increased after 5 minutes of gcm1-et treatment and declined after 60 minutes.
Fig. 3: Western blot analysis of gcm1-et treated caov-3 cell line in the absence or presence of inhibitor sb203580 within 24 hours. A. p38 expression profiles were not significantly affected by gcm1-et and inhibitor treatment. Only p-p38 was observed at 0.5 hour, consistent with earlier finding. B. Fas expression was elevated significantly upon gcm1-et treatment and signal reduced in the presence of inhibitor. mFasL (c) and sFasL (d) expression were affected by gcm1-et treatment at 0.5 hour. mFasL and sFasL signals were restrained in the presence of inhibitor, except at 1.5 hours where sFasL can still be detected at low intensity.

Both FasL isoform (mFasL and sFasL) were reported to have a different function in response to inflammation [50, 51]. mFasL is associated with proinflammation and proapoptotic effect, while sFasL prevent the inflammation effect [52, 53]. In Figure 3c, mFasL was rarely detected in the baseline sample, C, but emerged significantly after 0.5 hour of GCM1-Et treatment and continue to express up to 24 hours at the same time p-p38 signal was observed earlier. Presence of inhibitor reversed the effect where no signal was observed at any duration. In Figure 3d, sFasL exhibited the same profile as mFasL but at a lower signal intensities. However, in the presence of inhibitor, sFasL signal can still be observed at 1.5 hours, indicating an incomplete inhibition of sFasL. Based on the result, the appearance of both mFasL and sFasL occur at the same time of p-p38 expression. It may suggest a relation between pathways involving these proteins or may also indicate a distinct pathway being activated at the same
GCM1-Et Treatment Affect Caov-3 cells Viability: Cells counting using trypan blue dye exclusion assay were carried out where viable cells with clear morphology were differentiated from dead cells with blue morphology. Prior to test, synchronized cells were counted before seeded into T-25 flask for incubation. After left overnight to allow attachment, Caov-3 cells were incubated with medium containing 10 µg/mL GCM1-Et in a presence and absence of SB 203580 for 24 hours. Caov-3 cells incubated with 1 µM paclitaxel® is used as a positive control. After 24 hours, cells were trypsinized, washed and incubate with trypan blue solution at room temperature. Cell counting was carried out using haemocytometer under light microscope. The result showed GCM1-Et treatment cause 37.8±6.87% cells dead as compared to baseline control cells (Fig. 4). In the presence of SB203580, the percentage of dead cells is reduced to 10.5±8.03%. Paclitaxel, as expected, cause a massive cells death after only 24 hours of treatment with each samples control condition.

Fig. 4: Gcm1-et treatment affects caov-3 cells viability. Gcm1-et cause 37.8% caov-3 cells death. In the presence of inhibitor sb 203580, percentage of cells death was reduced to 10.5% suggesting a prominent role of p-p38 as a mediator of cells death upon treatment of gcm1-et. *p<0.05 statistically significant when compared to each samples control condition.

The results clearly showed that FasL expression is dependent to the presence of p-p38. Previous studies reported the importance of sustaining the activation of p38 MAPK to execute the death pathway. Various durations of p38 activation had been reported, but of all reason, a resistant cancer cell lines resulted in transient activation of p38 in response to drug treatment [40, 55]. However, in this study, p-p38 expression in Caov-3 without any treatment was hardly detected and almost null. GCM1-Et treatment induces the expression of p-p38 as early as 5 minutes exposure and transiently expressed up to 40 minutes before declining to its original state. The p-p38 temporary presences however escalate the expression level of Fas and Fas ligand up to 24 hours. This relation is then confirmed by using the specific inhibitor of p38 MAPK. But the question remain to be clarify is whether this transient effect of GCM1-Et through p-p38 along with increased expression of FasL is enough to cause a Caov-3 cells death. To resolve this issue, a few analyses were carried out to determine cells viability as well as nucleuses profiles upon treatment of GCM1-Et.

Cytotoxicity of GCM1-Et is Mediated by Necrosis: Cell morphological changes are one criteria which can be used to characterized modes of cell death. Cell death is distinguished into two large categories, apoptosis and necrosis. Apoptosis is an active form of cell death with a supervised programmed by intrinsic factors in regulation of cell population [56]. A common feature of apoptosis are nuclear and cytoplasm condensation [57], genomic fragmentation [58] and membrane blebbing or vesiculation [59]. In contrast, necrosis is a passive form of cell death mostly occurred in pathological circumstances characterized by cell swelling and loss of membrane integrity [60] as well as membrane spike formation [61].
Fig. 5: Morphology analysis of caov-3 cells reveal mixed features of apoptosis and necrosis characteristics upon gcm1-et treatment. A. Caov-3 cells without treatment. B. Caov-3 treated with ethanol as a vehicle control. C. Gcm1-et treated caov-3 cells showed a mixed features of cytoplasm condensation (arrow 1), loss of membrane integrity (arrow 2) and membrane spike formation (arrow 3). D. Paclitaxel treated caov-3 revealed mixed features of apoptotic bodies (arrow 1), formation of filopodia (arrow 2) and membrane blebbing and vacuolation (arrow 3).

Morphology analysis of Caov-3 cells in the presence of GCM1-Et under phase contrast microscope showed a mixed feature of cytoplasm condensation which suit the apoptosis criteria (Fig. 5c1), loss of membrane integrity (Fig. 5c2) and membrane spike formation (Fig. 5c3) which suit the necrosis criteria. Morphology analysis of paclitaxel treated Caov-3 cells also showed a mixed feature of both apoptosis and necrosis which were characterized by apoptotic bodies (Fig. 5d1), formation of filopodia (Fig. 5d2) and membrane swelling with vacuolation (Fig. 5d3) which persistent with secondary necrosis. Non-treated Caov-3 (Fig. 5a) and vehicle control cultures (Fig. 5b) did not show any cellular depreciation.

Further analysis on nucleus morphology showed a mixed character of apoptosis and necrosis in both GCM1-Et and paclitaxel treated Caov-3 cells. As seen in figure 6a1, a nucleus of Caov-3 cell resembles a chromatin material in non-treated condition. The same feature was observed in ethanol treated Caov-3 cells (Fig. 6b). In the presence of GCM1-Et, a condensed chromatin (Fig. 6c1), a dispersed nucleus content as a result of ruptured cell membrane (Fig. 6c2) and a degraded chromatin without condensation (Fig. 6c3) were observed. The latest two features match a necrosis characteristic. A degraded chromatin without condensation is related to necrotic cell death which is marked by homogenous blue DAPI stain as a result of random cut on chromatin material [62, 63]. Paclitaxel also display a mixture of apoptotic and necrotic cell death which were characterized by apoptotic bodies (Fig. 6d1) as well as exploded cell with scattered genomic material (Fig. 6d2) as a result of ruptured cell membrane. DNA fragmentation analysis also supports the previous finding. Figure 7 showed a DNA fragmentation analysis of GCM1-Et treated Caov-3 cells resulted in a consistent feature of necrotic cell death as been proved by smearing of DNA without any fragment (ladder) observed. DNA content of paclitaxel treated ovarian carcinoma cells were mostly available after more than 24 hours until 96 hours of treatment [64-66].
Fig. 6: Nucleus morphology of treated and non-treated caov-3 cells with DAPI staining. A. Non-treated and (b) ethanol treated caov-3 showed a chromatin structure unaffected (arrow 1). C. Gcm1-et treated caov-3 cells displayed mixed features of condensed chromatin (arrow 1), dispersed nucleus content as a result of membrane rupture (arrow 2) and degraded chromatin without condensation (arrow 3). D. Paclitaxel treated caov-3 also revealed a distinct features of apoptotic bodies (arrow 1), as well as scattered nucleus content (arrow 2) as a result of cell lysis which is common in necrotic cell.

Fig. 7: Gel electrophoresis analysis of genomic DNA of treated and non-treated caov-3 cells. DNA profiles of caov-3 cells after 12 hours (lane 1 and 4) and after 24 hours (lane 2 and 3). DNA smear of paclitaxel treated caov-3 cells after 12 hours (lane 5) and 24 hours (lane 6). DNA smear of gcm1-et treated caov-3 cells after 12 hours (lane 8) and 24 hours (lane 7).

CONCLUSION

Red algae are well known for its cytotoxic secondary metabolite [67]. Three G. changii crude extracts had been tested for their cytotoxic properties and only diethyl ether fraction gave a promising output, resulting in GCM1 purification. GCM1 is a cholesterol compound found abundantly in red algae and was not suspected to expel toxic effect. However, cytotoxic test carried out on two different GCM1 solutions producing an unconventional result. Ethanol diluted GCM1 exert a significant cytotoxic activity against Caov-3 cells without affecting a control normal ovarian cell line, T-Ag-MOSE. A phosphorylated form of stress p38 MAPK (p-p38) was analyzed upon GCM1 treatment where elevated level of Fas ligand was detected in conjunction with increased level of p-p38 MAPK within 24 hours of treatment. Alteration of p-p38 MAPK and Fas/FasL level of expression upon GCM1-Et treatment was found to be correlated through specific inhibitor implementation. Thus, it is clear that GCM1-Et is capable of affecting the viability of Caov-3 cells through
modulation of p38 MAPK phosphorylation and Fas/FasL activation. Even the end result is in contrary to commonly expected outcome, necrosis in actual has been proposed as the ultimate consequence of cell death mechanism. However, the mechanical of interaction between GCM1-Et and Caov-3 cell remain an interesting subject to be further elucidated. It is presumed that structure compatibility between GCM1 and ethanol enhances the toxic effect of GCM1 either through increment of membrane permeability or disrupted membrane liquid-raft formation. Either way could lead to cell distress and begin signaling cascade in response to cell survival as had been proved before.

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