Jurnal Teknologi

Full Paper

A NOVEL ANTI-PROLIFERATIVE ACTIVITY (EC_{50}) OF PEGAGA (*Centella asiatica*) EXTRACT THROUGH *IN VITRO* 3-D CULTURE MICROENVIRONMENT

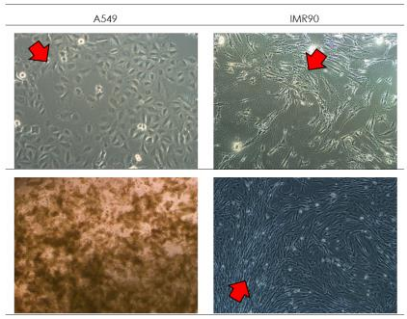
Syazwan Aizad[a], Nadzirah Mohd Khairiri[a], Badrul Hisham Yahaya[b], Saiful Irwan Zubairi[a,b*]

[a]School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
[b]Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia (USM) Bertam, 13200 Kepala Batas, Pulau Pinang, Malaysia

Article history
Received
11 May 2016
Received in revised form
5 November 2016
Accepted
10 December 2016

*Corresponding author
saiful-z@ukm.edu.my

Graphical abstract



Abstract

Centella asiatica or pegaga is one of the botanical plants that consists of many phytochemicals and is known for being able to offer various effects on wound healing, as well as functioning as an antioxidant and anticancer property. Therefore, this study was carried out to determine the efficacy of *Centella asiatica* water and alcohol-based extracts on the anti-proliferative activity of human lung cancer cells (A549) and normal fibroblast (IMR90) by mean of *in vitro* 3-D cell culture system. A porous 3-D scaffold was fabricated from poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) using solvent-casting particulate-leaching (SCPL) process. Antioxidant analysis (total phenolic content (TPC), DPPH and FRAP assays) was conducted prior to the 3-D cell culture study. The results showed that the extract contained 7.50 ± 1.10 mg/ml of asiaticoside and 0.74 ± 0.24 mg/ml of madecassoside. These bio-active compounds were believed to inhibit the proliferation of cancer cells (A549). The availability of phenolic compounds in the extract (TPC: 10133 ± 119.30 mg/100 g) had proven that the antioxidant properties existed. Moreover, the other values obtained from the antioxidant analysis revealed its capacity as a good source of antioxidant (DPPH: 87 ± 1.0%; FRAP: 127 ± 14.98 mg/100 g). Next, the lung cancer cells (A549) were cultured using a two-dimensional (2-D) system to generate the IC50 value of 5.75 ± 1.0 µg/ml. The A549 cell viability (MTS assay) after a 3-day incubation exhibited a good sign of mortality for the both treated models ranging from 55% to 70% as compared to control one (without treatment) ($p>0.05$). However, when the extract was exposed to a normal fibroblast IMR90, the cell growth of the both treated models exhibited an almost 2-fold greater cell numbers than that of the untreated models ($p<0.05$) indicating that the extract did not possess any possible threat to a normal and healthy cell. Therefore, the use of *Centella asiatica* extracts in terminating cancer cells has been proven to be able to inhibit cell growth (greater than 40%) in just 3 days of incubation.

Keywords: Biomimetics, anti-proliferation, *Centella asiatica*, A549, IMR90, PHBV, 3-D scaffold

Abstrak

Centella asiatica atau pegaga adalah salah satu tumbuhan botani yang terdiri daripada banyak fitokimia dan dikenali kerana dapat menawarkan pelbagai

kesan ke atas penyembuhan luka, dan juga berfungsi sebagai antioksidan dan anti-kanser. Oleh yang demikian, kajian ini dijalankan untuk mengira keberkesanan ekstrak air dan alkohol *Centella asiatica* ke atas anti-proliferasi sel kanser paru-paru manusia (A549) dan sel fibroblas normal (IMR90) secara sistem pengkulturan 3-D *in vitro*. Struktur perancah berliang 3-D difabrikasi daripada *poly(hydroxybutyrate-co-hydroxyvalerate)* (PHBV) menggunakan kaedah larut-lesap zarah (SCPL). Analisis konstituen bioaktif ini telah dijalankan dengan menggunakan kromatografi cecair berprestasi tinggi (HPLC). Selain itu, analisis antioksidan (jumlah kandungan fenolik (TPC), DPPH dan FRAP) telah dijalankan menunjukkan bahawa ekstrak terkandung 7.50 ± 1.10 mg/ml asiaticoside dan 0.74 ± 0.24 mg/ml madecassoside. Sebatian bio-aktif dipercayai menghalang pembiakan sel-sel kanser (A549). Ketersediaan sebatian fenolik (TPC: 10133 ± 119.30 mg/100 g) telah membuktikan bahawa wujud sifat-sifat antioksidan. Selain itu, nilai-nilai yang lain yang diperoleh daripada analisis antioksidan mendedahkan kapasitinya sebagai sumber yang antioksidan yang baik (DPPH: 87 ± 1.0%; FRAP: 127 ± 14.98 mg/100 g). Seterusnya, sel-sel kanser paru-paru (A549) telah dikulturkan menggunakan sistem dua dimensi (2-D) untuk menjana nilai IC_{50} pada 5.75 ± 1.0 µg/ml. Sel A549 yang dieram selama 3 hari menunjukkan petanda kematian sel kanser yang baik untuk kedua-dua model yang dirawat. Walau bagaimanapun, apabila ekstrak telah didedahkan kepada IMR90 fibroblast normal, pertumbuhan sel bagi kedua-dua model yang dirawat menunjukkan pertambahan hampir 2 kali ganda bilangan sel daripada model-model yang tidak dirawat (p<0.05). Ini menunjukkan bahawa ekstrak itu tidak mempunyai apa-apa-apa ancaman kepada sel yang normal. Oleh yang demikian, penggunaan ekstrak *Centella asiatica* telah terbukti dapat menghalang pertumbuhan sel kanser (melebihi 40%) hanya dalam 3 hari pengeraman.

Kata kunci: Biomimetik, anti-proliferasi, *Centella asiatica*, A549, IMR90, PHBV, Struktur perancah 3-D

1.0 INTRODUCTION

Medicinal plants are valuable topics for the research on new biologically active compounds. Apart from the medicinal effects of traditional herbs, exploratory researches have also focused on and a wide variety of new biological activities involving traditional medicinal plants that have recently been reported, including anticancer activity [1]. *Centella asiatica* (Linn.) Urban sys. synonym *Hydrocotyle asiatica* Linn which is commonly known as Indian Pennywort, belongs to the family, *Apiaceae* (previously known as *Umbelliferae*). It is considered as one of the foremost herbs for treating skin problems, wound healing, revitalizing nerves and brain cells [2]. In fact, the use of *Centella* in food and beverages has increased over the years, basically due to its potential health benefits such as antioxidant, anti-inflammatory and a wound healing agent [3]. Meanwhile, the active components of *Centella asiatica* are mostly located in the fresh or dried aerial parts consisting of leaves and stem. They comprise triterpenic pentacyclic saponins that may vary from 1% to 8% (w/w). The principal saponins are asiaticoside (an ester of asiatic acid and a trisaccharide chain, constituted by rhamnose and two glucoses unit) and madecassoside (an ester of madecasic acid and a trisaccharide chain, constituted by rhamnose and two glucose unit) [3]. Asiaticoside is the most abundant triterpene glycoside in the water extract and it is transformed into asiatic acid *in vivo* by hydrolysis mechanism. This phytochemical has shown cytotoxic activity in fibroblast cells [4] and that it induces apoptosis in different types of cancer [5-7]. For instance, asiatic acid has been successively reported to possess a strong cell growth inhibition in hepatoma, breast cancer, melanoma, glioblastoma and gastrointestinal tumor cells [8, 9].

Tissue engineering and regenerative medicine (TERM) has recently being utilizing medicinal herbs as a newly approaches in dealing with the efficacy of killing cancer cells using biomaterial [9]. A popular approach is scaffold-based tissue engineering, which utilizes a biodegradable polymer scaffold for seeding cells [10]. A conventional method of two-dimensional (2-D) monolayer cell cultures did not truly represent it microenvironment and yet losses of physiological extracellular matrix (ECM) on artificial plastic surfaces and high serum concentrations are mostly considered as its big flaw. Consequently, cells started to lose relevant properties such as differentiation, polarization, cell-cell communication and extracellular matrix contacts [11]. For that reason, this study was carried out to determine the relationship between the total phenolic content and antioxidant activity of an aqueous extract of *Centella asiatica* as regard to the anti-proliferative activity via 3-D cell culture system. A porous 3-D scaffold of poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) was fabricated by means of solvent-casting particulate-leaching (SCPL)

to support the efficacy of the extract that has the anti-proliferative activity in a faithful 3-D microenvironment.

2.0 METHODOLOGY

2.1 Chemicals and Materials

Centella asiatica fresh leaves were obtained from the local night market located in Bangi, Malaysia. Chemicals such as 80% (v/v) methanol, 70% (v/v) ethanol, 0.1% (v/v) acetic acid, acetonitrile, 1% streptomycin, 100 µg/ml penicillin, 4 mM L-glutamine, hydrochloric acid, phosphate buffered saline (PBS), trypan blue, Presto Blue® MTS, Folin-Ciocalteau, sodium carbonate, sodium acetate buffer, pentylenetetrazol (PTZ), anhydrous ferrous chloride, 2 2-diphenyl-1-picrylhydrazyl (DPPH) methanolic solution, poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV), sodium chloride and chloroform 99.9% in purity were purchased from Sigma-Adrich™ (Malaysia). Two cell lines were purchased from American Type Culture Collection (ATCC) and cyro-preserved in liquid nitrogen gas prior to cell culture. The details of the selected cell lines are shown in Table 1.

2.2 Water and Alcohol-based Extraction

The extraction of ground *Centella asiatica* were carried out and modified based on the previous study done by Babykutty *et al.* (2009) [12]. Samples were placed in a freeze dryer for 24 hours for them to dry. Later, 1 g of finely dried powdered material was mixed uniformly with methanol and water based on the ratio of 8:2 (10 ml) and stirred with a hot plate stirrer for 6 hours at an ambient temperature in a conical flask. Then, the extract was filtered using Whatman No. 1 filter papers and was rotary-evaporated until methanol vaporized. For the aqueous extract, 10 ml of distilled water was used as a solvent and was vaporized under a fume hood overnight.

2.3 Bio-active Constituents Analysis

The concentration of triterpenoid compounds in the *Centella asiatica* extract was determined using the Waters HPLC system consisted of a high pressure constant flow pump (Model 510), auto injector (WISP 712), a PDA spectrophotometric detector (490 multi-wavelength detectors) and a data station (840 with a Digital 350 computer). Chromatographic separation was carried out by using a column of µBondapak, C_{18}, with stationary phase particle size of 10 µm (internal diameter 3.9 mm x length 120 mm) and with a water-acetonitrile mobile phase (70:30), as well as PDA detection at 220 nm and attenuation. A 20 µl volume of sample was injected into the column.

2.4 Total Phenolic Contents (TPC) Determination

The TPC of the extracts was determined by using Folin–Ciocalteau method described by Wong *et al.* (2006) [13]. An aliquot (100 µl) of *Centella asiatica* extract was mixed with 2.5 ml of Folin–Ciocalteu phenol reagent (10x dilutions). After 5 minutes, 2.5 ml of saturated Na_2CO_3 solution was added and allowed to stand for 1 hour prior to absorbance reading at 740 nm. All samples were analyzed in triplicates (n = 3) and the results were averaged. The total phenolic contents (TPC) of the *Centella asiatica* extract was calculated using a gallic acid calibration curve (five different concentrations in the range of 1.7 to 3 mM, R^2 = 0.98) and expressed as mg gallic acid equivalent per gram of plant on dry basis (g db).

2.5 DPPH Free Radical Scavenging Assay

DPPH free radical scavenging activity was measured using the method described by Brand-Williams *et al.* (1995) [14]. DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable free radical red color solution. If the free radicals have been scavenged, DPPH will generated a new colour formation which is from red to yellow. The Ultraspec 1601 UV-visible spectrophotometer (Shimadzu) was used with the absorbance wavelength of 515 nm. An aliquot (40 µl) of *Centella asiatica* extract was added into 3 ml of methanolic DPPH solution (0.1 mM) and incubated for 30 minutes prior to the measurement. The absorbance changes was measured every 30 minutes interval until the reaction curve reached plateau. The antioxidant activity was expressed as Trolox equivalent per gram of plant material on a dry basis. All samples were analyzed in triplicates of an average ± SD (n = 3).

Table 1 General description for both A549 and IMR90 cells that were used for the proliferation study

Types of cells used	A549	IMR90
Description	Human lung carcinoma cells	Human Caucasian fetal lung fibroblast
Biological source	Lung from human	Lung, foetal from human
Growth mode	Adherent	Adherent
Morphology	Epithelial	Fibroblast
Medium used for culture	Dulbecco's Modified Eagle's Medium (DMEM) + 10% (v/v) fetal bovine serum (FBS) + 1% (v/v) antibiotics-antimycotic	Roswell Park Memorial Institute (RPMI) 1640 + 10% (v/v) fetal bovine serum (FBS) + 1% (v/v) antibiotics-antimycotic

2.6 Culturing Cells

The vials of the frozen cells were removed from the liquid nitrogen tank (-196 °C) and immediately thawed in a water bath (37 °C). Then the cells were transferred into tissue culture flasks (75 cm^2) (Greiner, Germany) which includes of a media containing 10% (v/v) of Fetal Bovine Serum (FBS) and supplemented with 1% (v/v) of antibiotics-antimycotic. As for the IMR90, RPMI growth medium was utilized with the same ratio as above. All cells were incubated in a humidified incubator under 5% CO_2 at 37 °C. The media were examined for any colour changes and changed on a daily basis (if necessary).

2.7 IC_{50} of *Centella asiatica* Extract

Approximately 1.0 x 10^4 cells of A549 (cell seeding) were transferred into 96-well plate and incubated for 24 hours (37 °C, 5% CO_2). After an overnight incubation, a serial dilution of extract was put into a media containing A549. Then, the incubation period was continued for 48 hours to observe the effect of the extract on cell growth. After 48 hours of incubation, 10 µl of PrestoBlue ® solution was added into the well plates, and incubated 37 °C for 3 hours. The well-plates containing coloured media were analysed using a micro-ELISA automatic reader (wavelength: 570 nm). IC_{50} values were determined based on the log concentrations against normalized cell growth absorbance (%).

2.8 Fabrication of PHBV Porous 3-D Scaffold

The fabrication of PHBV porous 3-D scaffold was carried out based on the previous studies [15-17]. About 2.4 g of PHBV (12% of polyhydroxyvalerate (PHV)) was dissolved in 60 ml of boiled reflux chloroform (99.9% purity) at 60 °C. The polymer solutions were cooled down for 10 minutes prior to the co-mixing with sodium chloride (NaCl), which acts as a leachable porogen. Once cooled, the polymer solutions were then poured evenly over a coated aluminium foil glass Petri-dish (internal diameter, 8.8 cm × height, 1.8 cm). Sodium chloride crystals with a salt weight fraction of 99% (w/w) were then poured into and stirred evenly in the polymer solutions with a perpetual stirring until the polymer-solvent solution became pasty, thick and packed. The fully filled pasty Petri-dish was tapped a few times so that the content became compact and even. This procedure was carried out in the fume cupboard. Subsequently, the packed-in pasty was put immediately inside the desiccators (to minimize the rapid phase disseverment, which could resulted in etching surfaces) and air-dried for 2 days to exhaustively remove all solvent remnants. Finally, the salt leaching process was carried out by means of continuous flow leaching kit [18]. The morphology of the porous 3-D scaffolds is shown in Figure 1.

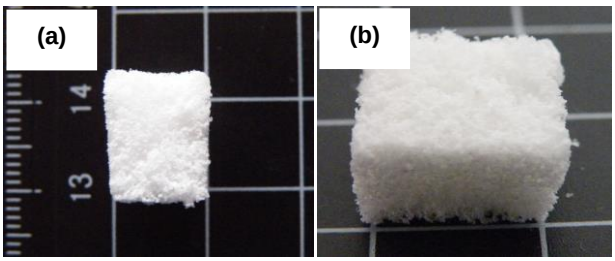


Figure 1 Morphology of polymeric porous 3-D scaffold in rectangular cubes with an approximate size of 10 mm x 10 mm x 5 mm: (a) Aerial view of leached PHBV (4%, w/v) scaffolds, (b) Side view of leached PHBV (4%, w/v) scaffolds. (Adapted from Zubairi, 2013 [15])

2.9 Cell Seeding Efficiency of 3-D Scaffolds

Approximately 1 x 10^5 cells of both lung cancer A549 and normal fibroblast IMR90 were seeded into porous 3-D scaffolds. The cells were allowed to proliferate for 48 hours. After 48 hours of incubation, the extract (IC_{50}) was carefully inserted into the cell seeded 3-D scaffolds and incubated prior to MTS analysis. Cell proliferation was quantitatively assessed based on the changes in the number of metabolically active cells using the cell-permanent compound that was blue in colour and virtually non-fluorescent (MTS analysis). This calorimetric assay measured the reduction of the resazurin compound by the cells into a resorufin product that was red in colour and highly fluorescent. The amount of resorufin produced that was measured by the absorbance was directly proportional to the number of living cells in the culture. During the analysis, the treated 3-D scaffolds were transferred to a new 24-well tissue culture plate. Then, 1.5 ml of DMEM (pre-warmed at 37 °C) was added to each of the cell seeded 3-D scaffolds. Presto Blue® solutions that were thawed for 10 minutes in a water bath at 37 °C were added to each well. The cell seeded 3-D scaffolds with Presto Blue® solution were incubated for over 3 hours at 37 °C and 5% CO_2. Approximately 100 µl of the solution was taken out and transferred into the ELISA 96-well plate. It was then diluted 3x with the cell growth media prior to the absorbance measurement (100 µl of growth media in 3 wells each). 100 µl of the diluted cell solutions was transferred into the new ELISA 96-well plate in 3 replicates with control (empty scaffold without cells). The absorbencies of the produced resofurin were recorded at 490 nm using a 96-well plate reader.

2.10 Statistical Analysis

All statistical calculations were statistically analyzed using one way-ANOVA and this was followed by Tukey's post Hoc-t-test analysis and the significant differences in means were determined at the level of $p<0.05$. All values were expressed as mean ± SD.

3.0 RESULTS AND DISCUSSION

3.1 Phytochemicals Profiles

The HPLC chromatogram profiles (Figure 2) of an aqueous extract exhibited 2 peaks were detected which represent 2 essential terpenoids compounds: (1) asiaticoside and (2) madecassoside. Unlike methanolic extract (chromatogram not shown), only one compound was identified which is madecassoside. Table 2 shows the concentrations of terpenoid compounds present in both *Centella asiatica* extracts. Higher amount of terpenoids were obtained from the aqueous extract as compared to methanolic extract ($p<0.05$).

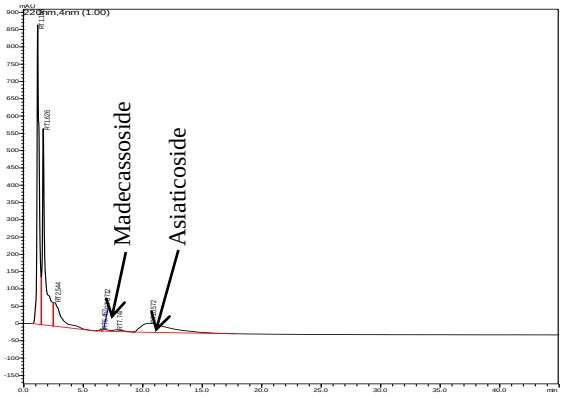


Figure 2 Chromatorgram profiles of triterpenoid compounds in the aqueous extract

The low concentration of the targeted phytochemicals in the methanolic extract was possibly due to the usage of high temperature (greater than 40 °C) during the concentration process by means of rotary evaporator. The thermal degradation might have been the reason of those depletion as the susceptibility of those compounds to evaporation is in the range of 28 to 32 °C [18, 19]. In fact, the concentration of madecassoside in the methanol extract also exhibited minute amount as compared to aqueous extract ($p<0.05$). The finding was in line with Settharaksa *et al.* (2012) [19] as he discovered that an increased heating time had reduced flavonoids, total phenolic contents and DPPH radical scavenging activity. This means that the active compounds were heat labile or easily destroyed to fragments by heat.

Table 2 The concentration of triterpenoid compounds in the *Centella asiatica* extract

Terpenoid compounds	Aqeuous extract (mg/ml)	Methanolic extract (mg/ml)
Asiaticoside	7.50 ± 1.10	-
Madecassoside	0.74 ± 0.24[a]	0.122 ± 0.022[b]
Asiatic acid	-	-

Concentrations that were not *sharing* a *letter* are considered significantly different ($p<0.05$)

Moreover, the absence of asiatic acid in this study was highly due to the different cultivar planting of the plant itself. Zheng and Wang (2001) [20] claim that cultivar and climate can be counted as the elements that are possible to affect the structure of phenolics and bioactive compounds. The triterpene components in *Centella* are not always the same as a result of different locations of the plant and diverse environmental conditions [21] as well as various accessions [22]. Composition of the extracts would be different because of the distinct growing regions, geographical habitats and climates. Thus, for the subsequent analysis, only aqueous extract was used prior to the anti-proliferative study via 3-D cell culture system.

3.2 Effect of Cell Culture Media on Acidic Extract

The results indicated that the pH value of the *Centella asiatica* extract was measured slightly acidic (5.0 ± 0.015). This acidic value was due to the presence of some those triterpene acid compounds such as asiatic acid, madecassic acid, terminolic acid, vanillic acid and succinic acid [23, 24]. However, when this acidic extract was introduced into the culture media, the pH of the media was neutralized by several other compounds that are presented in the culture media ($p>0.05$: data not shown). The culture media contains organic materials such as phosphate buffer saline which is able to ensure the stability of pH medium during culturing. According to Gardner (1969)[25], organic buffers can be used with many cell lines to effectively buffer the pH of the medium. Buffer and media themselves are able to maintain the optimum pH for culturing cells. In fact, the optimal pH range of 7.2 to 7.4 could be maintained by supplementing the medium with sodium bicarbonate and well-regulated amount of CO_2 during the incubation period. Therefore, the usage of any acidic extract for mammalian cell culture would compensate and maintain the optimal pH value as long as those supplementary compounds are well regulated in culture media.

3.3 Antioxidant Activities

3.3.1 Total Phenolic Content

The aqueous extract of *Centella asiatica* in this research possessed a large amount of total phenolic content (TPC) at 10,133 ± 119.30 mg GAE/100 g. Chew *et al*. (2011) [26] believes that the amount of TPC of some selected plants can be categorized into 4 classes, which are high (>5000 mg GAE/100 g), medium high (3000 - 5000 mg GAE/100 g), medium low (1000 - 3000 mg GAE/100 g) and low (<1000 mg GAE/100 g). Based on the results of TPC obtained, we can concluded that *Centella asiatica* aqueous extract provides an excellent antioxidant effect as it contains high in TPC. Several studies revealed that flavonoids, isoflavones, flavones, anthocyanin, catechin and other phenolics were the main

antioxidant activities of plant materials. Terpenoid, an active constituent in *Centella asiatica* is considered as a plant antioxidant. The frozen drying method exercised during the preparation of *Centella asiatica* extract also helped in preserving the phytochemical constituents in the sample compared to performing oven drying [26].

3.3.2 DPPH Free Radical Scavenging Activity

This assay was based on the reduction of DPPH radicals in methanol, which caused an absorbance drop at 515 nm. In this study, the antioxidant activity was expressed as Trolox equivalent per gram of plant material on a dry basis. The DPPH free radical scavenging activity of *Centella asiatica* extract was 230.67 ± 4.04 mg/ml. Asiaticoside, which was present in a high quantity (7.50 mg/ml), might be a significant contributor to the observed antioxidant activity of *Centella asiatica*. The enhancement of the antioxidant activity might be due to the presence of asiaticoside in the extract [27]. Generally, it was expected that extracts with a high amount of polyphenol content should also exhibit an active antioxidant activity [20].

3.3.3 Ferric Ion Reducing Activity

The ability of the *Centella asiatica* extracts to reduce ferric ions was determined by using the FRAP assay developed by Benzie and Strain (1996) [28]. An antioxidant that was capable of donating a single electron to the ferric-TPTZ (Fe(III)-TPTZ) complex would cause the reduction of this complex into becoming the blue ferrous- TPTZ (Fe(II)-TPTZ) complex which could absorb strongly at 595 nm [29-31]. The antioxidant activities were expressed as the concentrations of antioxidant having a ferric reducing ability equivalent to that of 1 mM of trolox. The result was 127 ± 14.98 mg GAE/100 g sample. The value of FRAP assay was lower than that of the DPPH free radical scavenging activity of *Centella asiatica* extract. The difference in values could be due to different reaction efficiencies of the tested compounds towards these two assays. The lower value of the FRAP assay was possibly because of the presence of some antioxidant compounds such as polyphenols that were reactive towards DPPH free radicals but they did not react efficiently with ferric iron due to steric resistance [13].

3.3.4 Correlation of Antioxidant Activities

The correlation cofficients (R^2) of total phenolics contents, DPPH and FRAP assay of the aqueous extract were shown in Table 3. There was a positive correlation between TPC with the FRAP (R^2 = +0.968) and DPPH (R^2 = +0.835) (p<0.05). Thus, the correlation coefficients (R^2) values of FRAP, DDPH and total phenolics contents showed higher correlation. The higher the total phenolics contents, the higher the antioxidant activities of the extracts exhibited.

Table 3 Correlation coefficients for the relationship between the assays

Antioxidant activity	TPC
DPPH	+0.968[a]
FRAP	+0.835[b]

*p<0.05: Different letters show significant differences as compared to TPC

3.4 Determination of IC_{50}

The aqueous extract of *Centella asiatica* demonstrated a potential anti-proliferative activity against lung cancer cell lines (A549). The IC_{50} value was determined at 5.75 ± 1.0 µg/ml by plotting the log concentration of the *Centella asiatica* extract against the cell response absorbance (%) that produced 50% mortality (Figure 3). The variety of extract concentrations were prepared in 50, 25, 12.5, 6.25 and 3.125 µg/ml by using serial dilution technique. Meanwhile, the extract was also cultured with human fibroblast IMR90 cell lines (control) to ensure that there is no cytotoxic effects on human normal cells. Table 4 shows the terpenoids concentrations in the diluted extracts for the IC_{50} analysis using A549 cells.

Table 4 Terpenoid concentrations in the diluted extracts

Concentration (µg/ml)	Asiaticoside (µg/ml)	Madecassoside (µg/ml)
3.13	0.024 ± 0.003	0.0019 ± 0.0001
6.25	0.047 ± 0.007	0.0048 ± 0.0017
12.5	0.094 ± 0.01	0.0091 ± 0.0028
25.0	0.19 ± 0.03	0.0185 ± 0.0064
50.0	0.39 ± 0.06	0.0375 ± 0.012

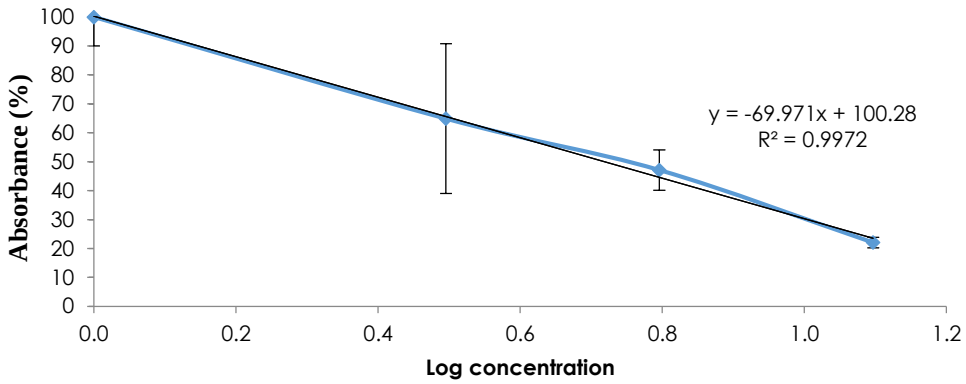


Figure 3 Cell response absorbance (%) of A549 exposed on different concentrations of *Centella asiatica* extract for 48 hours

The cell response (smaller cell growth) against higher concentration was possibly due to the fact that there were several terpenoid compounds that could have the potential of inhibiting A549 cell growth. In fact, the synergistic with the other constituents besides those terpenoids such as phenolic and flavonoids constituents could have also contributed to the inhibition process. The phenolic constituents, especially the flavonoids, have a high antioxidant capacity due to their properties of oxidation-reduction which plays an important role in the adsorption or neutralization of free radicals [32, 33], thus resulting in a raised biological protection. Since these free radicals are involved in the establishment of cancer, the aqueous extract containing higher amount of asiaticoside and other phenolic constituents would be able to help in reducing the number of free radicals (as presented in the antioxidant activity results) and therefore killing those mutated cancer cells.

3.5 2-D Cell Morphology

The cells were observed after 24 hours of incubation to visualise its physical changes microscopically (Figure 4). It was observed that all control cells with treatment were morphologically exhibited as a normal cell (Figure 4a and c). Meanwhile, normal fibroblast cells treated with the extract did not exhibit any changes as well but in fact showing a normal sign of cell growth (Figure 4d). However, for the A549 cells with the extract, a dark and disorienting micro-environment was observed clearly with scattering clumpy and fragmented suspension cells (Figure 4b). Moreover, the shape of the A549 cells was found to have shrunk and become smaller and the cells lost their asymmetry membrane as compared to the untreated cells [23]. These cells were treated with IC_{50} concentration of *Centella asiatica* extracts (5.75 ± 1.0 µg/ml) and they showed some severe inhibition effects and possibly leads to apoptosis. Cells undergoing apoptosis are identified by cell shrinkage, loss of adhesion with adjacent cells, the formation of cytoplasmic vacuoles, chromatin condensation and apoptotic body [34, 35]. All those symptoms were clearly observed for A549 throughout the treatment. Thus, the extract have shown a good sign of inhibiting cancer cell growth without compromising any other normal cell growth by giving the good impression of facilitating the growth of a normal cell.

3.6 Cell Responses in 2-D and 3-D

The efficacy of both extracts on both cancerous (A549) and normal fibroblast (IMR90) cells were examined through a 3-D culture and conventional 2-D models (75 mm^2 T-flask). The IC_{50} of 5.75 ± 1.0 µg/ml was utilized in the 3-D cell culture system. Figure 5 shows the normalized cell response absorbance (%) in the 2-D and 3-D models. The A549 cell response (MTS assay) after 3 days of incubation exhibited a good sign of mortality for both models ranging from 55 to 70% as compared to control (p<0.05). However, mortality of the cancerous cells in this 3-D micro-environment was observed to be relatively irresponsive after 3 days of exposure. This might be due to the fact that A549 requires a longer period of time during the treatment to ensure that the cells are more stable and able to adapt into its new 3-D micro-environment [15]. Moreover, any cell phenotypes which are involved in a 3-D micro-environment require a prolonged exposure (greater than 14 days) for adaptation and dynamic morphological changes is needed as the cell-to-cell surface contact is abundantly becoming large with the increase of their biosignalling capability (due to the secretion of their own extracellular matrix compounds) [36].

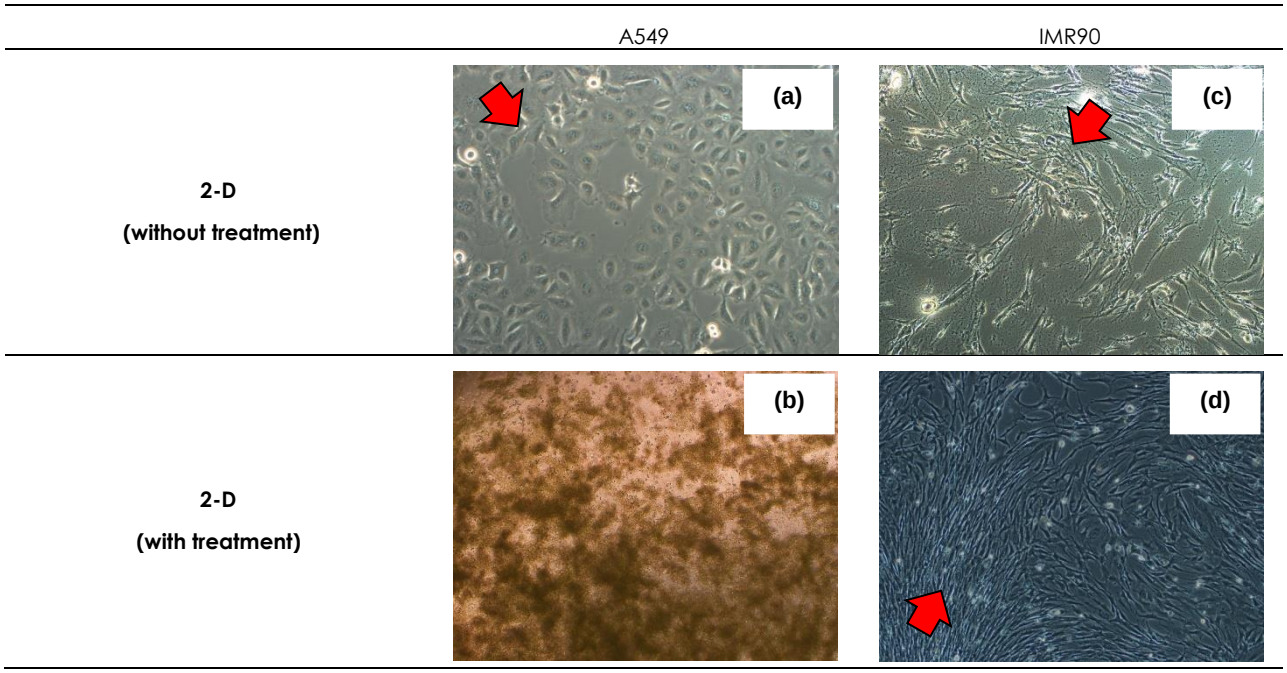


Figure 4 Cell morphology of A549 and IMR90 cells with and without treatment of *Centella asiatica* extract: (a) A549 lung cancer cell without treatment; (b) A549 lung cancer cell became clumpy and suspended in media, exhibited cell death; (c) IMR90 normal fibroblast cell; (d) IMR90 highly cell proliferation of post-treatment. Red arrow indicates live cells

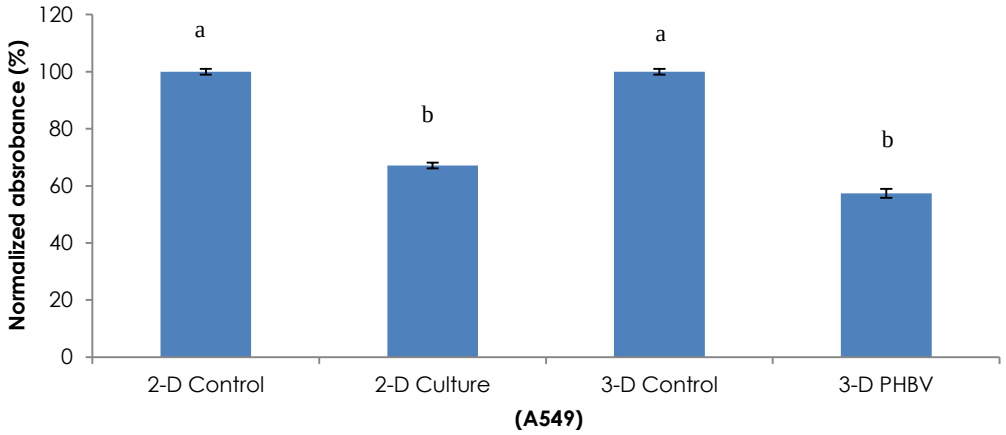


Figure 5 *In situ* growth kinetics of A549 cells treated with the extract on 2-D and 3-D models for 3 days of incubation. MTS assay was used to measure the number of cells metabolically active. The cultures were performed in triplicate (n = 3). Cultures that are not sharing *a* letter *are* significantly different (p<0.05)

Meanwhile, Figure 6 shows the *in situ* growth kinetics (%) of normal fibroblast IMR90 using 2-D and 3-D cell culture systems. The extract was exposed to a normal fibroblast IMR90 to observe its effects on normal cells. The treated 3-D culture produced a higher normalized IMR90 cell response as compared to control 3-D culture (p<0.05). However, the treated 3-D model exhibited a smaller decline of cell response as compared to the conventional treated 2-D culture (p<0.05) as the adaptation period was essentially required whenever the cells were exposed to a new 3-D micro-environment. The devaluation of the cell response of a treated 3-D model seems to demonstrate that the cell requires a longer time to adapt to its new 3-D micro-environment. In addition, when any cells are shifted to a new environment (e.g. 2-D to 3-D micro-environment), the design and architecture of cell-to-cell adhesion mechanism changed dramatically as the expression of cell adhesion molecules and cytoskeleton proteins started to behave like on its nature environment *in vivo* [37]. Therefore, the cell growth for the both treated models was identified to be almost 2-fold greater than that of the untreated models (p<0.05),

thus indicating that the extract did not possess any possible threat to a normal and healthy cell, but in fact it facilitated the growth of normal cell.

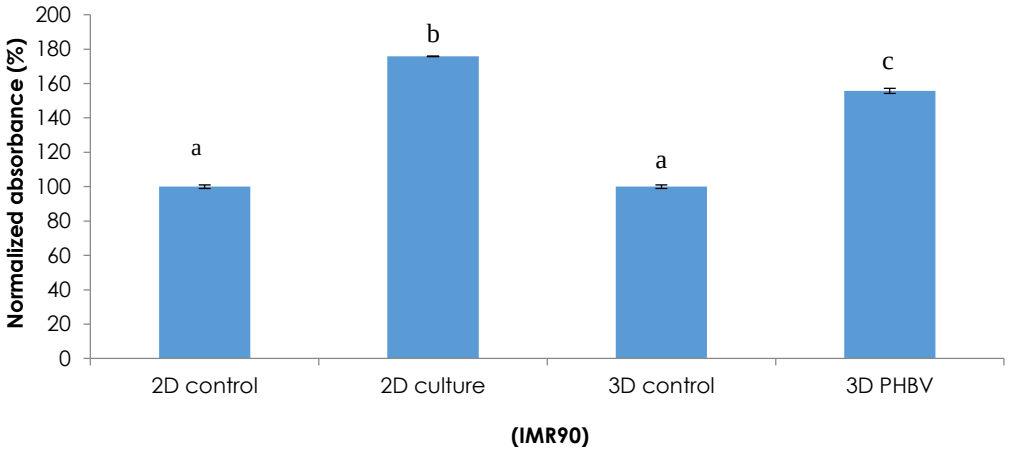


Figure 6 *In situ* growth kinetics of IMR90 cells treated with the extract on 2-D and 3-D models for 3 days of incubation. MTS assay was used to measure the number of cells metabolically active. The cultures were performed in triplicate (n = 3). Cultures that do not share *a* letter *are* significantly different (p<0.05)

4.0 CONCLUSION

The antioxidant capacities of the *Centella asiatica* aqueous extract exhibited a good correlation to its high total phenolic content and its anticancer properties. Those results suggested that these potent antioxidant and anti-proliferation properties were entirely justified due to the high concentration of phenolic and terpenoids constituents in the extract. Moreover, the anti-proliferative activity of both 2-D and 3-D models was equally the same (p>0.05: 3 days incubation - adaptability issues in a new micro-environment), and both models exhibited a good sign of mortality ranging from 55 to 70% as compared to the control (without treatment) (p<0.05). In fact, when the normal fibroblast cell (IMR90) was exposed to the extract, both 2-D and 3-D models exhibited almost 2-fold greater cell numbers than that of the untreated models (p<0.05), thus indicating that the extract did not possess any possible threat to a normal and healthy cell, but in fact it facilitated the cell growth. Thus, the *Centella asiatica* aqueous extract is considered as a one of the botanicals extracts that has the potential to become a good natural therapeutic agent in promoting inhibition of cancerous cell without spoiling normal cell's growth.

Acknowledgement

The authors would like to thank the Ministry of Science, Technology and Innovation (MOSTI) and Ministry of Higher Education (MOE) Malaysia for providing financial support to this project (06-01-02-SF1271, FRGS/2/2013/TK04/UKM/03/1 and GGPM-2013-078).

References

[1] Yoo, H. H., Park, J. H. and Kwon, S. W. 2007. *In vitro* Cytotoxic Activity Of Some Korean Medicinal Plants On Human Cancer Cell Lines: Enhancement In Cytotoxicity By Heat Processing. *Phytotherapy Research*. 21(9): 900-903.

[2] Singh, S., Gautam, A., Sharma, A. and Batra, A. 2010. *Centella asiatica* (L.): A Plant With Immense Medicinal Potential But Threatened. *International Journal of Pharmaceutical Sciences Review and Research*. 4(2).

[3] Roopesh, C., Salomi, K. R., Nagarjuna, S. and Reddy, Y. P. 2011. Diuretic Activity Of Methanolic And Ethanolic Extracts Of *Centella asiatica* Leaves In Rats. *International Research Journal of Pharmacy*. 2(11): 163-165.

[4] Coldren, C. D., Hashim, P., Ali, J. M., Oh, S.-K., Sinskey, A. J. and Rha, C. 2003. Gene Expression Changes In The Human Fibroblast Induced By *Centella asiatica* triterpenoids. *Planta Medica*. 69(8): 725-732.

[5] Babu, T., Kuttan, G. and Padikkala, J. 1995. Cytotoxic And Anti-Tumour Properties Of Certain Taxa Of Umbelliferae With Special Reference To *Centella asiatica* (L.) Urban. *Journal of Ethnopharmacalogy*. 48(1): 53-57.

[6] Gurfinkel, D. M., Chow, S., Hurren, R., Gronda, M., Henderson, C., Berube, C., Hedley, D. W. and Schimmer, A. D. 2006. Disruption Of The Endoplasmic Reticulum And Increases In Cytoplasmic Calcium Are Early Events In Cell Death Induced By The Natural Triterpenoid Asiatic Acid. *Apoptosis*. 11(9): 1463-1471.

[7] Park, B. C., Bosire, K. O., Lee, E.-S., Lee, Y. S. and Kim, J.-A. 2005. Asiatic Acid Induces Apoptosis In SK-MEL-2 Human Melanoma Cells. *Cancer Letters*. 218(1): 81-90.

[8] Lee, Y. S., Jin, D.-Q., Kwon, E. J., Park, S. H., Lee, E.-S., Jeong, T. C., Nam, D. H., Huh, K. and Kim, J.-A. 2002. Asiatic Acid, A Triterpene, Induces Apoptosis Through Intracellular Ca 2+ Release And Enhanced Expression Of p53 in HepG2 Human Hepatoma Cells. *Cancer Letters*. 186(1): 83-91

[9] Tang, X.-L., Yang, X.-Y., Jung, H.-J., Kim, S.-Y., Jung, S.-Y., Choi, D.-Y., Park, W.-C. and Park, H. 2009. Asiatic Acid Induces Colon Cancer Cell Growth Inhibition And Apoptosis Through Mitochondrial Death Cascade. *Biological and Pharmaceutical Bulletin*. 32(8): 1399-1405.

[10] Shimizu, T. 2014. Cell Sheet-Based Tissue Engineering For Fabricating 3-Dimensional Heart Tissues. *Circ J*. 78: 2594-2603.

[11] Härmä, V., Virtanen, J., Mäkelä, R., Happonen, A., Mpindi, J.-P., Knuuttila, M., Kohonen, P., Lötjönen, J., Kallioniemi, O. and Nees, M. 2010. A Comprehensive Panel Of Three-Dimensional Models For Studies Of Prostate Cancer Growth, Invasion And Drug Responses. *PLOS One*. 5(5): e10431.
[12] Babykutty, S., Padikkala, J., Sathiadevan, P., Vijayakurup, V., Azis, T., Srinivas, P. and Gopala, S. 2009. Apoptosis Induction Of *Centella asiatica* On Human Breast Cancer Cells. *African Journal of Traditional, Complementary and Alternative Medicines*. 6(1): 9-16.
[13] Wong, S. P., Leong, L. P. and William Koh, J. H. 2006. Antioxidant Activities Of Aqueous Extracts Of Selected Plants. *Food Chemistry 2006*. 99(4): 775-783.
[14] Brand-Williams, W., Cuvelier, M. and Berset, C. 1995. Use Of A Free Radical Method To Evaluate Antioxidant Activity. *LWT-Food Science and Technology*. 28(1): 25-30.
[15] Zubairi, S. I., Bismarck, A., Koutinas, A., Panoskaltsis, N. and Mantalaris, A. 2013. *Development Of Biomimetic PHB and PHBV Scaffolds For A Three Dimensional (3-D) In Vitro Human Leukaemia Model*. Imperial College London.
[16] Zubairi, S. I., Bismarck, A., Koutinas, A., Panoskaltsis, N. and Mantalaris, A. 2013. Surface Treatment of Poly (3-Hydroxybutyric Acid)(PHB) and Poly (3-Hydroxybutyric-co-3-Hydroxyvaleric Acid)(PHBV) Porous 3-D Scaffolds With An Improved Thickness To Enhance Cell-Biomaterial Adhesion and Interactions. *Polymer. 1*. 2: 3.
[17] Zubairi, S. I., Koutinas, A., Bismarck, A., Panoskaltsis, N. and Mantalaris, A. 2011. Poly (3-hydroxybutyric acid)(PHB) and poly (3-hydroxybutyric-co-3-hydroxyvaleric acid)(PHBV) Porous Three-Dimensional Scaffolds With An Improved Thickness As Cell Growth Supporting Materials. *Advances in Environmental Biology*. 8(10): 925-932.
[18] Aizad. S., Yahaya. B. H. and Zubairi S. I. 2014. Fabrication of 3-D Polymeric Scaffold: The Development of Continuous Flow Salt Leaching Kit Coupled with Ion Conductivity Detection. *Regenerative Research*. 3(2): 163-166.
[19] Siripongvutikorn, S. 2012. Flavonoid, Phenolic Contents And Antioxidant Properties Of Thai Hot Curry Paste Extract And Its Ingredients As Affected Of Ph, Solvent Types And High Temperature.
[20] Zheng, W. and Wang, S. Y. 2001. Antioxidant Activity And Phenolic Compounds In Selected Herbs. *Journal of Agricultural And Food Chemistry*. 49(11): 5165-5170.
[21] James, J. T. and Dubery, I. A. 2009. Pentacyclic Triterpenoids From The Medicinal Herb, Centella asiatica (L.) Urban. *Molecules*. 14(10): 3922-3941.
[22] Zainol, N., Voo, S., Sarmidi, M. R. and Azizi R. A. 2008. Profiling of *Centella asiatica* (L.) Urban Extract. *The Malaysian Journal of Analytical Sciences*. 12(2): 322-327.
[23] Joshi, K. and Chaturvedi, P. 2013. Therapeutic Efficiency Of *Centella asiatica* (L.) urb. An Underutilized Green Leafy Vegetable: An Overview. *International Journal of Pharma & Bio Sciences*. 4(1): 135-149.
[24] Jian, P., Guiqing, K., Chuanxun, Y., Beibei, Z., Risheng, J. and Yuan, Y. 2007. Separation And Determination Of Madecassic Acid In Extracts Of *Centella asiatica* Using High Performance Liquid Chromatography With β-Cyclodextrin As Mobile Phase Additive. *Chinese Journal of Chromatography*. 25(3): 316-318.
[25] Gardner, R. S. 1969. The Use Of Tricine Buffer In Animal Tissue Cultures. *The Journal Of Cell Biology*. 42(1): 320-321.
[26] Chew, Y. L., Chan, E. W. L., Tan, P. L., Lim, Y. Y., Stanslas, J. and Goh, J. K. 2011. Assessment Of Phytochemical Content, Polyphenolic Composition, Antioxidant And Antibacterial Activities Of Leguminosae Medicinal Plants In Peninsular Malaysia. *BMC Complementary And Alternative Medicine*. 11(1): 12.
[27] Shukla, A., Rasik, A. M. and Dhawan, B. N. 1999. Asiaticoside-induced Elevation Of Antioxidant Levels In Healing Wounds. *Phytotherapy Research*. 13(1): 50-54.
[28] Benzie, I. F. and Strain, J. 1996. The Ferric Reducing Ability Of Plasma (FRAP) As A Measure Of "Antioxidant Power": the FRAP Assay. *Analytical Biochemistry*. 239(1): 70-76.
[29] Escarpa, A. and González, M. 2001. Approach To The Content Of Total Extractable Phenolic Compounds From Different Food Samples By Comparison Of Chromatographic And Spectrophotometric Methods. *Analytica Chimica Acta*. 427(1): 119-127.
[30] Tulipani, S., Mezzetti, B., Capocasa, F., Bompadre, S., Beekwilder, J., De Vos, C. R., Capanoglu, E., Bovy, A. and Battino, M. 2008. Antioxidants, Phenolic Compounds, And Nutritional Quality Of Different Strawberry Genotypes. *Journal of Agricultural and Food Chemistry*. 56(3): 696-704;
[31] Singleton, V. L., Orthofer, R. and Lamuela-Raventos, R. M. 1999. Analysis Of Total Phenols And Other Oxidation Substrates And Antioxidants By Means Of Folin-Ciocalteu Reagent. *Methods in Enzymology*. 299: 152-178.
[32] Basile, A., Ferrara, L., Del Pezzo, M., Mele, G., Sorbo, S., Bassi, P. and Montesano, D. 2005. Antibacterial And Antioxidant Activities Of Ethanol Extract From Paullinia Cupana Mart. *Journal of Ethnopharmacology*. 102(1): 32-36.
[33] Pittella, F., Dutra, R. C., Junior, D. D., Lopes, M. T. and Barbosa, N. R. 2009. Antioxidant And Cytotoxic Activities Of Centella asiatica (L) Urb. *International Journal Of Molecular Sciences*. 10(9): 3713-3721.
[34] Buttke, T. M. and Sandstrom, P. A. 1994. Oxidative Stress As A Mediator Of Apoptosis. *Immunology Today*. 15(1): 7-10.
[35] Wyllie, A. 1984. The Biology Of Cell Death In Tumours. *Anticancer Research*. 5(1): 131-136.
[36] Cukierman, E., Pankov, R. and Yamada, K. M. 2002. Cell Interactions With Three-Dimensional Matrices. *Current Opinion In Cell Biology*. 14(5): 633-640.
[37] Lee, J. M., Mhawech-Fauceglia, P., Lee, N., Parsanian, L. C., Lin, Y. G., Gayther, S. A. and Lawrenson, K. 2013. A Three-Dimensional Microenvironment Alters Protein Expression And Chemosensitivity Of Epithelial Ovarian Cancer Cells *In Vitro*. *Laboratory Investigation*. 93(5): 528-542.