Phytochemical content an in vitro toxicity of *Glycine soja* ethanol extract on the A549 Lung cancer line cell

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Abstract. Lung cancer incidence is the highest of all cancers, and its management is still a challenge today. Natural anticancer compounds are being developed, one of which is derived from black soybeans. Black soybean (*Glycine soja*) contains bioactive compounds that are potential anticancer agents. The objective of this research was to determine the secondary metabolite content of black soybean ethanol extract and its toxicity to A549 lung cancer cells. Identification of the content of the extract was performed by thin layer chromatography (TLC) and phytochemical testing. Cytotoxicity testing was performed using an in vitro MTT-assay. The concentration tested were 6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/ml. The TLC results show that there were 6 compounds in the black soybean ethanol extract. Phytochemical test identified flavonoids, alkaloids, saponins, tannins, triterpenoids, and glycosides that have potential as anticancer agents. The extract exhibited moderate cytotoxic activity in A549 cells (IC$_{50}$ = 114.5 µg/ml and the highest extract concentration (800 µg/ml) gave the highest percentage inhibition (83.8%) and significantly different optical density than the other concentrations (p < 0.05). Black soybean ethanol extract has the potential to be developed as a lung anticancer agent.

1. Introduction

Based on Globocan statistical data, lung cancer has the highest incidence and is the number one cause of cancer death in the world, reaching 1.6 million in 2012 [1,2]. Indonesia is no exception, with 25,332 incidences being reported in men [3]. This situation is exacerbated by the fact that Indonesia has one of the highest prevalence of smokers in the world, including 75.2% of all men [4,5]. Thus, effective management and prevention of this disease is required [4].

Good lung cancer management practice involves the removal of cancer tissue or deadly cancer cells with minimal side effects on normal cells. There are several therapies for NSCLC treatment available in Indonesia, including surgery, chemotherapy or radiation, targeted therapy, and immunotherapy [6]. However, each still has deficiencies, especially in terms of side effects from toxicity to normal cells and the development of chemo-resistance to the therapeutic agents [7,8].

The development of an alternative anticancer drug produced from natural ingredients is attracting more attention because they may be relatively safer with regard to side effects and toxicity [9]. Thus, research into natural anticancer compounds that can help to control the growth of cancer cells by inducing apoptosis, inhibiting proliferation, interfering with signal transduction pathways, or that can be used as chemo-preventive agents, is needed [9,10].
Soybeans are one of the major agricultural commodities in Indonesia and are known to contain potentially active anticancer substances [11]. Black soybean are increasingly in demand because they contain a wide variety of nutrients and active substances compared to regular soybeans. In addition to isoflavones, such as genistein and daidzein, black soybean seeds also contain anthocyanins in their black pigments [12]. Anthocyanins have been shown to exhibit anticarcinogenic activity against various cancer cells both in vitro and in vivo [13].

Research on the cytotoxic activity of black soybean pigment extract has shown significant activity in Jurkat T leukemia cells and MCF-7 breast adenocarcinoma cells [14]. Black soy extract has also been shown to inhibit the growth of AGS gastric cancer cells by inducing apoptosis [15]. In addition, a case study in Japan also showed that the regular consumption of soybeans may reduce the risk of lung cancer [16]. These properties of black soybean extracts prompted us to investigate the anticancer activity of the ethanol extract of black soy (Glycine soja) in A549 lung cancer cells in vitro. This study was aimed at developing a potential safe and effective alternative treatment for lung cancer in Indonesia.

2. Methods

Samples were divided into experimental and control groups. The experimental group consisted of extracted black soy ethanol of different concentrations. The purpose of this study was to investigate the potential anticancer activity of the optimal concentration of black soybean extracts on A549 lung cancer cells. Anticancer activity was expressed as IC₅₀, which is the concentration at which 50% inhibition of A549 cell growth/viability is observed.

The Federer formula was used to specify the number of samples, and the sample number in each group was three. Eight different concentrations of black soy ethanol extract were tested, and there was one control group making a total of 9 groups and 27 individual tests.

Black bean seeds that had been dried at 45 °C–50 °C were ground with a grinder. The black soybean powder was placed into an Erlenmeyer flask and suspended in 500 ml of 99% ethanol. The resulting liquid was decanted into a fresh flask, and the sediment was resuspended in 500 ml of ethanol. This process was repeated three times. The extracts were pooled, filtered, and dried using a rotary evaporator. The resulting material was a brown solid.

Identification of compounds in black soy ethanol extract was performed using thin layer chromatography (TLC) and a phytochemical test. The dried extract was dissolved in ethanol and 2–3 drops of extract were dropped to the lower limit of the TLC plate. The plates were run with a solvent consisting of hexane, ethyl acetate, and formic acid at a ratio of 4:2:1 and visualized with UV light.

Black soy ethanol extract was dissolved in 10% DMSO at a concentration of 10 mg/ml. Thus, an extract is formed with concentrations of 10,000 μg/ml and used to prepare dilutions with final concentrations of 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml, and 800 μg/ml in DMEM medium containing 10% fetal bovine serum, streptomycin, and penicillin.

Cells were cultured for 1 week before use and then seeded into 96-well plates at 10⁴ cells per well containing 100 μl of DMEM growth medium and incubated for 24 hours to facilitate cell attachment. Medium was then removed and replaced with 100 μl of extract or control treatment as follows: (1) negative control, 100 μl DMEM medium; (2) positive controls, 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml, and 800 μg/ml of cisplatin; and (3) treatment groups comprising the eight black soy extract dilutions described above.

After incubation for 48 hours, the media were removed and cell viability was measured using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay [17]. The cells were washed with phosphate buffered saline (PBS) and 10 μl of 5 mg/ml of MTT reagent was added to each well. The mixture was incubated for 4 hours at 37 °C. The plate was centrifuged for 5 minutes at a rate of 500 rpm, and the formazan crystals were dissolved in ethanol prior to reading absorbance at 515 nm using an ELISA-reader.
The percentage of living A549 cells in each sample was determined by

\[
\text{Percentage of inhibition} = \frac{OD_{\text{treatment}} - OD_{\text{media control}}}{OD_{\text{cell control}} - OD_{\text{media control}}} \times 100\%
\]

Then, Percentage of A549 cell inhibition was calculated by 100 - % living cell.

Each experiment was conducted three times, and the results were averaged.

Data were analyzed using SPSS software version 21. Data was also processed with Microsoft Excel, where IC\textsubscript{50} was obtained from linear fitting of plots of percentage of inhibition versus log concentration. Normality testing was performed with the Kolmogorov–Smirnov test because the sample number was more than 50. Equality of variance was assessed with Levene’s test and analysis of significance was by one-way ANOVA followed by a post hoc test [18].

3. Results
Using TLC, we found that black soy ethanol extract contained 6 different types of secondary metabolites and their retention factors can be found in Table 1.

Table 1. Retention factors of compounds contained in an ethanol extract of G. soja

| Compound | Distance | Calculation | Retention factors |
|----------|----------|-------------|-------------------|
| A        | 2.7      | 2.7/3.3     | 0.818             |
| B        | 2.4      | 2.4/3.3     | 0.727             |
| C        | 1.9      | 1.9/3.3     | 0.575             |
| D        | 1.5      | 1.5/3.3     | 0.454             |
| E        | 1.3      | 1.3/3.3     | 0.394             |
| F        | 1.1      | 1.1/3.3     | 0.333             |

The results of phytochemical testing of the extract were positive for flavonoids, alkaloids, saponins, tannins, triterpenoids, steroids, and glycosides but were negative for steroid compounds. The average MMT assay absorbance cell control value from the 3 experiments was 0.765 and the media control was 0.147. These were used to calculate the percentage inhibition at each concentration of cisplatin or ethanol extract as described in the Methods section (Tables 2 and 3).

Table 2. Percentage inhibition of A549 lung cancer cells by G. soja ethanol extract

| Extract Concentration (µg/ml) | Concentration log | Repetition | Median | Average | SD | % inhibition |
|------------------------------|-------------------|------------|--------|---------|----|-------------|
| 6.25                         | 0.796             | 0.737      | 0.738  | 0.724   |    | 5.2         |
|                              |                   | 0.737      | 0.733  | 0.01    |    |             |
| 12.5                         | 1.097             | 0.750      | 0.728  | 0.739   |    | 4.3         |
|                              |                   | 0.739      | 0.739  | 0.01    |    |             |
| 25                            | 1.398             | 0.642      | 0.639  | 0.639   | 0.00| 20.6        |
|                               |                   | 0.638      | 0.638  |         |    |             |
Table 2. Continue

| Concentration (µg/ml) | Concentration log | Repetition | Median | Average | SD | % inhibition |
|-----------------------|-------------------|------------|--------|---------|----|--------------|
|                       |                   | I   | II    | III   |     |              |
| 50                    | 1.699             | 0.539 | 0.545 | 0.525 | 0.539 | 0.536 | 0.01 | 37.0 |
| 100                   | 2.000             | 0.443 | 0.444 | 0.429 | 0.443 | 0.439 | 0.01 | 52.8 |
| 200                   | 2.301             | 0.394 | 0.380 | 0.393 | 0.393 | 0.389 | 0.01 | 60.8 |
| 400                   | 2.602             | 0.340 | 0.338 | 0.343 | 0.340 | 0.340 | 0.00 | 68.7 |
| 800                   | 2.903             | 0.244 | 0.249 | 0.248 | 0.248 | 0.247 | 0.00 | 83.8 |

Table 3. Percentage inhibition of A549 lung cancer cells by cisplatin

| Cisplatin concentration (µg/ml) | Concentration log | Repetition | Median | Average | SD | % inhibition |
|---------------------------------|-------------------|------------|--------|---------|----|--------------|
| 6.25                            | 0.796             | 0.627 | 0.657 | 0.628 | 0.628 | 0.637 | 0.02 | 20.7 |
| 12.5                            | 1.097             | 0.599 | 0.597 | 0.594 | 0.597 | 0.597 | 0.00 | 27.3 |
| 25                              | 1.398             | 0.536 | 0.532 | 0.536 | 0.536 | 0.535 | 0.00 | 37.3 |
| 50                              | 1.699             | 0.484 | 0.492 | 0.491 | 0.491 | 0.489 | 0.00 | 44.7 |
| 100                             | 2.000             | 0.431 | 0.439 | 0.434 | 0.434 | 0.435 | 0.00 | 53.4 |
| 200                             | 2.301             | 0.428 | 0.424 | 0.388 | 0.424 | 0.413 | 0.02 | 56.9 |
| 400                             | 2.602             | 0.334 | 0.341 | 0.336 | 0.336 | 0.337 | 0.00 | 69.2 |
| 800                             | 2.903             | 0.233 | 0.423 | 0.273 | 0.273 | 0.310 | 0.10 | 73.7 |

Graphs of percentage inhibition versus the logarithms of black soy ethanol extract and cisplatin concentrations revealed linear relationships can be seen in Figure 1 and 2.

Figure 1. Percentage inhibition of A549 cells by various concentrations of G. soja ethanol extract
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Figure 2. Percentage inhibition of A549 cells by various concentrations of cisplatin

IC$_{50}$ values (i.e. the concentration producing 50% inhibition of MTT activity) were 114.5 µg/mL for the G. soja extract and 85.5 µg/mL for cisplatin. This difference was significant (P < 0.05) by one-way ANOVA. Levene test results also gave a value of P < 0.05, meaning that there the two groups had different variances. After finding significance using one-way ANOVA test, we followed up with a Tamhane's post-hoc test.

4. Discussion
Black soybean (G. soja) is a versatile plant that has proven to have various health benefits [19]. TLC analysis in this study found 6 types of secondary metabolites in an ethanol extract of black soybeans. This is consistent with the literature, which reports that the secondary metabolite content of black soy is high, with more than 50 bioactive substances [20]. These bioactive substances have been shown to possess many properties, including anticancer properties [21].

Phytochemical tests performed on a black soy ethanol extract found flavonoids, alkaloids, saponins, tannins, triterpenoids, and glycosides. Based on several studies, all these components have potential anticancer properties. Alkaloids isolated from herbs have been shown to be antiproliferative and antimetastatic in various types of cancer both in vitro and in vivo. Additionally, camptothecin and vinblastine from the alkaloid group have been used successfully in anticancer drugs [21]. Furthermore, glycosides have been shown to reduce cancer cell adhesion, cell migration, angiogenesis and tumor invasion [22]. Saponins have also been shown to induce cancer cell apoptosis and prevent invasion or metastasis and angiogenesis [23]. The saponins present in this black soy ethanol extract were triterpenoids.

Black soya seeds also contain anthocyanin pigments and proanthocyanidins. Anthocyanins are flavonoids and proanthocyanidin is a condensed tannin that is classified as a polymeric flavonoid [24]. Anthocyanin has been shown to have significant anticarcinogenic effects both in vitro and in vivo [13]. In addition, G. soja also contains flavonoids that also have anticancer properties, such as genistein and daidzein. Genistein inhibits the proliferation of A549 lung cancer cells by the induction of cell apoptosis, activation of caspases 3 and 9 and MET proteins, and expression of microRNA-27a [25]. Tannins have also been shown to play a role in preventing proliferation and interfering with the replication of cancer cells [26]. However, each compounds in this extract is not necessarily active as an anticancer agent and further research is need on its fractionated compounds.

In this study, we used a crude extract without fractionation. The purpose was to retain all the soluble bioactive substances in soybeans in the ethanol solvent. Thus, it is likely that the toxicity properties of black soybeans can be optimized. An ethanol extract was used because ethanol is polar, as are the majority of bioactive substances in black soy [27,28]. Methanol, which has been used in other studies involving G. soja extract was not used because it is quite toxic to humans [29].
This study was performed in vitro, therefore, it could be conducted more rapidly and economically than an animal study [30]. The MTT-assay is used as it is a relatively fast and accurate method for living cell quantification [31]. MTT-assays can distinguish between living and dead or senescent cells. However, one drawback is that they are less sensitive in distinguishing between dead and senescent cells [32].

The IC
t
50 of the G. soja ethanol extract on A549 lung cancer cell was 114.5 μg/ml, whereas that of cisplatin was 85.5 μg/ml. Based on the reference value from the National Cancer Institute and the Geran Protocol, strong cytotoxic effects are characterized by IC
t
50 < 21 μg/ml, moderate cytotoxic effects by IC
t
50 of 21–200 μg/ml, and weak cytotoxic effects by IC
t
50 of 201–500 μg/ml; IC
t
50 values > 501 μg/ml are considered to be non-cytotoxic [33]. Thus, the crude ethanol extract of G. soja is moderately cytotoxic; however, its cytotoxic properties are still slightly lower than those of cisplatin.

In an in vitro study, Yongsho et al. obtained EC
50 values from a black soy anthocyanin fraction and studied it against Jurkat-leukemia T cells and MCF-7 breast adenocarcinoma cells at 100–250 μg/ml [14]. In contrast, Zou et al. obtained an IC
50 of 3.69 mg/ml extract against AGS gastric cancer cells [15]. In addition, Atun et al. found that IC
50 from a 6-day fermented black soybean extract against Raji cells was 229.1 μg/ml, whereas in HeLa cells it was 316.2 μg/ml [34].

IC
50 values from previous studies are inconsistent and can be affected by various factors such as the type of solvent used for extraction and the cancer cell types tested. In the study with AGS gastric cancer cells, the extraction solvent was acidified aqueous acetone, whereas in the Raji and HeLa cell study used methanol [15,34]. Literature references on the IC
50 of an ethanol extract of G. soja on A549 lung cancer cells do not exist.

Here, the percentage of inhibition of extract at concentrations of 6.25 and 12.5 μg/ml was below 5%. At 25 μg/ml percentage inhibition was 20.6% and cell progressively died up to a concentration of 800 μg/ml. In contrast to the extract, cisplatin at the lowest concentration (6.25 μg/ml) was able to produce an inhibition percentage of 20.7%. However, at the highest concentrations (800 μg/ml) the extract produced a higher inhibition percentage (83.8%) than cisplatin (73.7%).

Tamhane’s post-hoc test showed that there was a significant difference between control and extracts ranging from 25 to 800 μg/ml. In the cisplatin group, significant differences with the control began at a concentration of 12.5–100 μg/ml and a concentration of 400 μg/ml. Each extract concentration was significantly different with 4-fold concentration up to the highest concentration of 800 μg/ml. Exceptions that 50 μg/ml extract was significant different to the 100 μg/ml extract and that the 100 μg/ml extract was no significantly different to the 400 μg/ml extract, but significantly different to the 800 μg/ml extract.

In a study by Rayapolu et al. a fraction of peptides from soybeans containing high levels of oleic acid had a 68% inhibitory effect on lung cancer cells, where a significant percentage of inhibition of these cells occurred at high concentrations of 600–1000 μg/ml [35]. In this study, the inhibitory effect of G. soja ethanol extract on A549 cell was 68.7% at concentration a 400 μg/ml.

The percentage inhibition of G. soja ethanol extract increased constantly with concentration and the 800 μg/ml extract group was significantly different to all the other extract concentrations. However, the 800 μg/ml concentration had not reached saturation, so further research is needed to determine the concentration range that produces maximum toxicity.

In contrast to the extract, cisplatin at a concentration of 800 μg/ml has no significance in any treatment. Thus, cisplatin at 800 μg/ml may have been saturating that there was no significant change compared to the other concentrations. One of the triplicate data points of the cisplatin 800 μg/ml group was quite different from the other two. Variations in the cisplatin data can be attributed to several factors: concentration saturation, outer well conditions that vary due to edge effects, insoluble formazan crystals and bubbles on the surface of the media [36]. Further research is needed to determine the actual cause.

It is essential to use parameters with exact control in order to optimize conditions and minimize confounding effects. The parameters in question are: cell density, culture medium, optimal concentration and duration of the MTT assay incubation time. Fresh culture medium should also be
used to avoid depletion of nutrients and to prevent the treatment from affecting cell metabolism. By controlling these parameters, the MTT-assay can provide an accurate and reliable quantification of living cells [37]. In this study, we attempted to control the parameters of the MTT-assay by conditioning temperature and the use of isolated devices in the culture laboratory.

Cell density is an important factor that can alter various parameters such as cell viability, toxicity and apoptosis. Number of cells/well maximum of 30,000–40,000 [32]. In this study, we used $10^4$ cells per well, which was the number expected to produce an optimal absorbance value between 0.75 and 1.25 [39], so that the data obtained would be in the linearly quantifiable 0.05–2 range [39]. Consequently, the absorbances of the extract and cisplatin treated samples were between 0.2 and 0.8. The absorbance of the blanks, containing only medium and lacking cells, was lower than treatments and controls and lay between 0.14 and 0.15. Thus, the possibility of the medium being contaminated by cells, bacteria or fungi was eliminated [38].

The incubation time of the MTT cytotoxicity assay also needs to be considered since the cells should not reach confluence before the end point of the test because this may result in a medium saturation and result in lower viability [32]. This is particularly important in cells with high proliferation rates because reaching excess confluence can cause cells to withdraw from the cell cycle or even die [32]. In this study, the cell incubation time used was 24 hours to avoid super-confluence.

The recommended wavelength for detecting formazan dissolved in DMSO is 520–600 nm [36]. The most commonly used wavelength is 570 nm with a reference at 630 nm [39]. Another study conducted on A549 cancer cell used a wavelength of 570 nm at low cell counts ($4 \times 10^3$ cells/well) and 510 nm for the larger numbers of cells [40].

In this study, DMSO was used as a formazan solvent and read with wavelengths of 450 and 515 nm. Readings at 570 nm wavelength could not be made because of the limitations of available ELISA-reader. The results of absorbance readings at 450 nm did not show any inter-concentration differences and so could not be used. With a sufficiently large number of cells, that is the $10^4$ cells/well used in this study, the signal at 515 nm was sufficient to detect the effects of both the extract and cisplatin.

5. Conclusion
This study demonstrated that a crude ethanol extract of *G. soja* contains flavonoids, alkaloids, saponins, tannins, triterpenoids, and glycosides. This extract decreased the viability of A549 lung cancer cells *in vitro* in a dose-dependent manner. The cell viability loss for this cell line after treatment with 800 μg/ml of the extract was significantly different from those after treatment with all other lower concentrations tested.

References
[1] WHO. Lung Cancer Estimated Incidence, Mortality and Prevalence Worldwide in 2012. 2013 [cited 25 Agustus 2017]. Available from: http://globocan.iarc.fr/old/FactSheets/cancers/lung-new.asp
[2] Islami F, Torre L A and Jemal A 2015 Global trends of lung cancer mortality and smoking prevalence *Transl. Lung. Cancer. Res.* 4(4) 327-38
[3] World Health Organization. Cancer Country Profile. 2014 [cited 25 Agustus 2017]. Available from: http://www.who.int/cancer/country-profiles/idn_en.pdf
[4] WHO. WHO report on the global tobacco epidemic: country profile Indonesia. 2017 [cited 25 Agustus 2017]. Available from: http://www.who.int/tobacco/surveillance/policy/country_profile/idn.pdf
[5] Zheng W, et al 2014 Burden of total and cause-specific mortality related to tobacco smoking among adults aged ≥45 years in Asia: a pooled analysis of 21 cohorts *PLos. Med.* 11(4) e1001613
[6] Ahmad A and Gadgeel S M 2016 Lung Cancer and Personalized Medicine: Novel Therapies and Clinical Management. Switzerland: Springer; p203-15
[7] Falk S and Williams C 2010 The Facts: Lung Cancer. United States: Oxford University Press; p.3-82
[8] Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, Snyder N and Sarkar S 2014 Drug resistance in cancer: an overview Cancers (Basel). 6(3) 1769-92
[9] Greenwell M and Rahman P K S M 2015 Medicinal Plants: their use in anticancer treatment Int. J. Pharm. Sci. Res. 6(10) 4103-12
[10] Desai A G, Qazi G N, Ganju R K, Tamer M, Singh J, Saxena A K, Bedi Y S, Taneya S C and Bhat H K 2008 Medicinal plants and cancer chemopvention Curr. Drug. Metab. 9(7) 581-91
[11] Lim TK 2012. Edible Medicinal and Non-Medicinal Plants: Volume 2, Fruits. London: Springer; p634-40
[12] Choug M G, Baek I Y, Kang S T, Han W Y, Shin D C, Moon H P and Kang K H 2001 Isolation and Determination of Anthocyanins in Seed Coats of Black Soybean J. Agric. Food. Chem. 49(12) 5848-51
[13] Wang L S and Stoner G D 2008 Antocyanins and their role in cancer prevention Cancer Letters. 269 281-90
[14] Kim Y, Kim D, Woo S, Kim H, Lee Y, Kim H, Ko K and Lee S 2008 Antioxidant activity and cytotoxicity on human cancer cells of anthocyanin extracted from black soybean Korean J. Crop Sci. 53(4) 407-12
[15] Zou Y and Chang S K C 2011 Effect of Black Soybean Extract on the Supression of the Proliferation of Human AGS Gastric Cancer Cells via the Induction of Apoptosis J. Agric. Food. Chem. 59(9) 4597-05
[16] Wakai K, Ohno Y, Genka K, Ohmine K, Kawamura T, Tamakoshi A, Lin Y, Nakayama T, Aoki K and Fukuma S 1999 Risk Modification in lung cancer by a dietary intake of preserved foods and soyfoods: findings from a case-control study in Okinawa, Japan Lung Cancer. 25(3) 147-59
[17] Cancer Chemoprevention Research Center Fakultas Farmasi UGM 2012 Uji Sitotoksisitas Metode MTT. Jogjakarta: Cancer Chemoprevention Research Center Fakultas Farmasi UGM; p3-7
[18] Dahlan S 2016 Statistik untuk Kedokteran dan Kesehatan: Deskriptif, Bivariat, dan Multivariat. 6th ed. Jakarta: Epidemiologi Indonesia; P30-67
[19] Phommalath S, Teraishi M, Yoshikawa T, Saito H, Tsukiyama T, Nakazaki T, Tanisaka T and Okumoto Y 2014 Wide genetic variation in phenolic compound content of seed coats among black soybean cultivars Breed. Sci. 64(4) 409-15
[20] Ng T B, Cheung R C F and Wong J H 2013 Biologically active constituents of soybean. In: A Comprehensive Survey of International Soybean Research: Genetic, Physiology, Agronomy and Nitrogen Relationship. Intech; p240-54
[21] Mbagwu F N, Okafor V U and Ekeanyanwu J 2011 Phytochemical screening on four edible legumes (Vigna subterranea, Glycine max, Arachis hypogea, and Vigna uniguiculata) found in eastern Nigeria Afr. J. Plant Sci. 5(6) 370-72
[22] Aminin D L, Menchinskaya E S, Pisliagin E A, Silchenko A S, Avilov S A and Kalinin V I 2015 Anticancer activity of Sea Cucumber Triterpene Glycosides Mar. Drugs. 13(3) 1202-23
[23] Man S, Gao W, Zhang Y, Huang L and Liu C 2010 Chemical study and medical application of saponins as anticancer agents Fitoterapia. 81(7) 703-14
[24] Hagerman AE. Condensed Tannin Structural Chemistry. 2002 [cited 25 oktober 2017]. Available from: https://www.users.miamioh.edu/hagermae/Condensed%20Tannin%20Structural%20Chemistry.pdf
[25] Yang Y, Zang A, Jia Y, Shang Y, Zhang Z, Ge K, Zhang J, Fan W and Wang B 2016. Genistein inhibits A549 human lung cancer cell proliferation via miR-27a and MET signaling Oncol. lett. 12 2189-93
[26] Sieniawska E and Baj T 2016 Chapter 10: Tannins. In: McCreath SB, Delgoda R. Pharmacognosy. USA: Academic Press; p199-232.27. Yalavarthi C and Thiruvengadarajan V S 2013 A review on identification strategy of phyto constituents present in herbal plants J. Res. Pharm. Sci. 4(2) 123-40

[27] Visht S and Chaturvedi S 2012 Isolation of Natural Products. [cited 25 oktober 2017]. Available from: https://www.researchgate.net/file.PostFileLoader.html?id=5507f9a2f079ed793c8b4567&assetKey=AS%3A273735901220868401442275132294

[28] Korabathina K. Methanol toxicity. [ditasiti 25 oktober 2017]. Tersedia pada: https://emedicine.medscape.com/article/1174890-overview#a6

[29] Institute for Laboratory Animal Research 1999 Summary of Advantages and Disadvantages of In Vitro and In Vivo Methods. Monoclonal antibody production. National Academy of Sciences.

[30] Rao A V and Gurfinkel D M 2000 The bioactivity of saponins: triterpenoid and steroidal glycosides Drug. Metabol. Drug. Interact. 17(1-4) 211-35

[31] Gasque K C, Alahj L P, Oliveira R C and Magalhaes A C 2014 Cell density and solvent are critical parameters affecting formazan evaluation in MTT assay Braz. Arch. Biol. Technol. 57 (3) 381-5

[32] Aghaei M, Ghanadian M, Faez F and Esfandiary E 2015 Cytotoxic activities of Euphorbia kopetdaghi against OVCAR-3 and EJ-138 cell lines J. Herb. Med. Pharmacol. 4 49-52

[33] Atun S, Ariangrum R, Yoshiaki T and Masatake N 2009; Phenolic Content and Cytotoxic Properties of Fermented blakc soybeans (Glycinne soja) Extract on Human Hela-SE and Raji Cell Lines. Pure and Applied Chemistry International Conference. 689-91

[34] Rayaprolu S J, Hettiarachchy N S, Chen P, Kannan A and Mauromostakos A 2013 A. Peptides derived from high oleic acid soybean meals inhibit colon, liver, and lung cancer cell growth Food Res. Int. 50(1) 282-88

[35] Dojindo. Duraliq MTT stable solution technical manual. 2010 [cited 25 Oktober 2017]. Available from: https://www.researchgate.net/file.PostFileLoader.html?id=57bd5f5edc332db99a46a994&assetKey=AS%3A398530612088836%401472028510915

[36] Sylvester P W 2011 Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability Methods Mol. Biol. 716 157-68

[37] ATCC. MTT Cell Proliferation Assay. 2011 [cited 25 oktober 2017]. Available from: https://www.atcc.org/~media/DA5285A1F52C41E4864C966FD78C9A79.ashx

[38] Riss T L, Moravec R A, Niles A L, Duellman S, Benink H A, Worzella T J and Minor L 2016 Cell Viability Assays [cited 25 Oktober 2017]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK144065/pdf/Bookshelf_NBK144065.pdf

[39] Plumb J A, Milroy R and Kaye S B. 1989 Effects of the pH Dependence of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide-Formazan Absorption on Chemosensitivity Determined by a Novel Tetrazolium-based Assay Cancer Res. 49 4435-40