Phytochemical and in vitro cytotoxicity analyses of wild bean (*Glycine soja*) ethanol extract using laryngeal cancer Hep-2 cells

D A Kusumoningrum and S Dwira*
Department of Chemistry, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia

*E-mail: suryadwira@gmail.com

Abstract: Laryngeal cancer accounts for 0.6% of all cancer-related deaths worldwide. Wild soybean contains natural ingredients that have anti-cancer properties. This study aimed to determine the cytotoxicity of wild bean ethanol extract in inhibiting the growth of epidermal laryngeal cancer (Hep-2) cells for use as adjuvant therapy. Phytochemical analysis and thin-layer chromatography of the wild bean ethanol extract were conducted to identify the compounds contained in the extract. Wild bean ethanol extracts were tested against Hep-2 cancer cells in vitro. Cisplatin was used as a positive control. After incubation for 24 h, the inhibitory activities of the extracts were assessed with the MTT assay, and absorbances were determined with a microplate reader. Based on the phytochemical test results, the wild bean ethanol extract contains alkaloids, flavonoids, tannins, triterpenoids, saponins, and glycosides. The MTT assay showed that the median inhibitory concentration (IC$_{50}$) of the wild bean ethanol extract was 118.061 μg/mL, and there were significant differences ($p < 0.05$) in the inhibitory activity at 800 μg/mL in comparison to the other treatment groups and controls. The IC$_{50}$ value of cisplatin was 78.569 μg/mL. The wild bean ethanol extract had moderate cytotoxicity against Hep-2 laryngeal cancer cells and was less toxic than cisplatin.

1. Introduction
In 2012, there were 8.2 million cancer-related deaths worldwide [1], whereas laryngeal cancer accounted for 0.6% of all cancer-related deaths worldwide in 2014. However, the prevalence of laryngeal cancer is much lower than for cancers of the breast and cervix, and thus is often ignored by society. Of the many cancers of the head and neck region, laryngeal cancer with the histological appearance of squamous cell carcinoma can be quite fatal, even though the 5-year survival rate is relatively high at 60.7% [2-4]. The development of laryngeal cancer is often unnoticed, as new cases are discovered at later stages of the disease (grade III of IV) [5].

At present, available treatment regimens for laryngeal cancer include laser surgery, radiation therapy, and cutting of the vocal cords. However, these treatments do not guarantee full recovery because of various side effects, such as malaise, loss of appetite, permanent voice changes, and permanent stoma [6-8]. Therefore, alternative therapies are needed as adjuvants to current treatment regimens.

The use of herbal extracts, such as wild soybean extract (WSBE), as alternative cancer therapies continues to increase. According to previous studies, WSBE contains flavonoids, which contribute to...
the cytotoxic effects against cancer cells. WSBE is rich in the flavonoid derivative cyanidin-3-glucoside, which was shown to effectively block cell growth in the G2 or M phase of the cell cycle. Other than flavonoids, WSBE contains other ingredients with anti-cancer properties, including saponins, tanins, glycosides, triterpenoids, and alkaloids [9-13].

The extraction of herbal antioxidants is usually performed with methanol or ethanol solutions. Even though neither of these alcohols have significant toxicological effects, ethanol is less toxic and safer to use than methanol [14-16]. WSBE with methanol has anti-carcinogenic effects against Hela-S3 and Raji cervical cancer cells [17]. Unfortunately, the accumulation of methanol and formic acid is toxic.

A previous study found that WSBE inhibited the growth of cancer cells but questioned whether extraction with ethanol conveys cytotoxic effects against laryngeal cancer cells. In addition, the median inhibitory concentration (IC₅₀) of WSBE remains unknown. Therefore, the aim of the present study was to determine the cytotoxic effect of WSBE with ethanol against Hep-2 laryngeal cancer cells.

2. Methods
The experimental design of this study employed Hep-2 laryngeal cancer cells that were treated with variable concentrations of ethanol-derived WSBE or left untreated as control cells. Cytotoxic testing was performed by analyzing the IC₅₀ value of cells and the extent of cell inhibition in each group at 48 h. According to the Federer equation, three samples were assigned to each of the nine groups in this study (eight treatment groups and one control group).

For preparation of the WSBE, wild soybean powder was soaked in 99% ethanol solution for two days. Then, the extracts without dregs were transferred to heat-resistant containers and heated to 57°C–60°C in a rotary evaporator for 2 days to fully evaporate the ethanol. The resulting “rough” extract was later fractionated for analysis.

Compounds contained within the extract were quantified by thin-layer chromatography (TLC) and phytochemical testing. The purpose of TLC was to determine the number of compound types within the extract by separating the components on an inert material. A 1 cm-wide polar strip was used for the TLC experiment. Two lines at a distance of 0.5 cm from the top and bottom of the strip were marked. The extracts were first diluted with ethanol, and then capillary action was initiated at the bottom line using a capillary tube. Subsequently, the strip was inserted into a container containing a mixture of ethyl acetate, formic acid, and hexane at a ratio of 2:1:4. Phytochemical testing was performed to determine the presence or absence of saponins, flavonoids, tanins, alkaloids, glycosides, and triterpenoids.

Before starting the experiment, Hep-2 laryngeal cancer cells were cultured for 6 weeks to confluence in the IPB Primate Animal Study Center of Universitas Indonesia (Jakarta, Indonesia) and then incubated in a culture medium that resembled the internal environment of the human body (humid atmosphere with 5% CO₂ at 37°C). After culturing for 1 week, the cell concentration was determined using a hemocytometer. Briefly, 90 μL of trypan blue was mixed with 10 μL of the cell suspension under a microscope and observed at 80× magnification. The number of cells/mL was calculated by

\[
\frac{1}{4} N \times P \times 10^4 \quad \text{where } N \text{ is the total number of cell in four wells, and } P \text{ is solvent factor}
\]

According to this equation, each well contained 10,000 cells 100 μL of growth media. After inoculation of the wells with the cell suspension, the plate was incubated for an additional 38 h.

Then, 10 mg of the WSBE was mixed into 1 mL of dimethyl sulfoxide (DMSO). After reaching homogeneity, the media was switched with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% streptomycin/penicillin and 10% fetal bovine serum, and mixed until homogenous. The solution was then diluted to eight concentrations for analysis (6.25, 12.5, 25, 50, 100, 200, 400, and 800 μg/mL, respectively).

After the cells reached a confluence of 50%, the test plate was removed from the incubator, the medium was discarded, and triplicate wells were inoculated with 100 μL of the appropriate extract
concentrations. The control wells contained 100 μL of medium only. Afterward, the test plate was incubated for an additional 24 h.

The various treatment groups were as follows: solvent (control) group: Hep-2 laryngeal cancer cells in 100 μL of DMEM and 100 μL of DMSO; media control group: 100 μL of DMEM without Hep-2 laryngeal cancer cells; positive control group: Hep-2 laryngeal cancer cell and cisplatin (at 6.25, 12.5, 25, 50, 100, 200, 400, and 800 μg/mL); negative control group: Hep-2 laryngeal cancer cell and 100 μL of DMEM; and variable groups: Hep-2 laryngeal cancer cells with WSBE at concentrations of 6.25, 12.5, 25, 50, 100, 200, 400, and 800 μg/mL.

After 48 h of incubation, the MTT assay was performed. Briefly, 10 μL of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 5 mg/mL was added to each well. Then, the plate was incubated for 3 h at 37°C to promote the formation of formazan crystals, which were later diluted in ethanol.

After incubation, the optical density (OD) of the wells were determined using a microplate reader plate (λ = 515 nm) to determine the cell density. A graph was created to illustrate the absorbance results of the negative control and media control groups to calculate the percentage of living cells determined by:

\[
\frac{[\text{variable absorbance}] - [\text{media control absorbance}]}{[\text{cell control absorbance}] - [\text{media control absorbance}]} \times 100\%
\]

Then, a second graph was created to compare the log concentrations of living cells, and a linear regression equation was formulated. If the r-value of the graph was larger than the r-value of the equation, 50% was used in the linear regression equation to determine the value of x. Then, the log value of the concentration was determined. The antilog result was the IC₅₀ value.

After all data were collected, a normality test was performed. As there were more than 50 samples, normality was determined with the Kolmogorov–Smirnov test. Normally distributed data were subjected to analysis of variance, whereas data that were not normally distributed were assessed with the Kruskal–Wallis test. Normally distributed data are presented as the mean ± standard deviation (SD), and data not normally distributed are presented as the median and range. All data analyses were performed using SPSS ver. 20.0 software (IBM Corporation, Armonk, NY, USA).

3. Results
To identify the WSBE contents, TLC and phytochemical testing were performed. The TLC results, presented as retention factor (Rf) scores, are shown in Table 1.

|        | Rf Score |
|--------|----------|
| 1      | 0.818    |
| 2      | 0.727    |
| 3      | 0.575    |
| 4      | 0.454    |
| 5      | 0.394    |
| 6      | 0.333    |

Table 1. TLC results
The phytochemical test results are shown in Table 2.

### Table 2. Phytochemical test results

| Group      | WSBE |
|------------|------|
| Alkaloid   | +    |
| Flavonoid  | +    |
| Tannin     | +    |
| Saponin    | +    |
| Triterpenoid| +   |
| Glycoside  | +    |
| Steroid    | -    |

The cell densities after incubated for 38 h were determined using a hemocytometer under a microscope. The concentrations of Hep-2 laryngeal cancer cells were determined with:

\[
\frac{1}{4} N \times P \times 10^4 \quad N = 26.000 \text{ and } P = 10
\]

Thus, the total cells is \(6.5 \times 10^8\) cells/mL.

In this study, the IC\(_{50}\) value is defined as the concentration of the extract that can inhibit the growth of 50% of the Hep-2 laryngeal cancer cells. The negative control absorbance results are shown in Table 3, and the media control absorbance results are shown in Table 4.

### Table 3. Negative control absorbance results

| Variable          | Absorbance result | Mean | Inhibition percentage |
|-------------------|-------------------|------|-----------------------|
|                   | 1                 | 2    | 3                     |
| Negative control  | 0.751             | 0.752| 0.777                 | 0.760 | 0%                   |

### Table 4. Media control absorbance results

| Absorbance result | Mean | Inhibition percentage |
|-------------------|------|-----------------------|
| 1                 | 2    | 3                     |
| 0.129             | 0.138| 0.127                 | 0.135 | 0.130 | 0.132 | 0.133 | 0.133 | 0.132 | 0%                |

The determine the extent of inhibition of cell growth with WSBE and cisplatin, the following equation was formulated to compare differences in the absorbances of the variable groups and negative and positive media control groups.

The absorbance results of the MTT assay of the variable groups are shown in Table 5.

### Table 5. Absorbance results of the variable groups
Concentration (μg/mL) | Absorbance Result | Minimum | Median | Maximum | Mean | SD | % Mean Inhibition
--- | --- | --- | --- | --- | --- | --- | ---
6.25 | 0.739 0.730 0.731 | 0.730 | 0.731 | 0.739 | 0.733 | 0.00 | 4.2
12.5 | 0.723 0.726 0.729 | 0.723 | 0.726 | 0.729 | 0.726 | 0.00 | 5.4
25 | 0.658 0.641 0.640 | 0.640 | 0.641 | 0.658 | 0.646 | 0.01 | 18.1
50 | 0.532 0.523 0.521 | 0.521 | 0.523 | 0.532 | 0.525 | 0.01 | 37.4
100 | 0.434 0.427 0.424 | 0.424 | 0.427 | 0.434 | 0.428 | 0.01 | 52.8
200 | 0.384 0.394 0.379 | 0.379 | 0.384 | 0.394 | 0.386 | 0.01 | 59.6
400 | 0.330 0.333 0.337 | 0.330 | 0.333 | 0.337 | 0.333 | 0.00 | 68.0
800 | 0.229 0.236 0.242 | 0.229 | 0.236 | 0.242 | 0.236 | 0.01 | 83.5

Figure 1 is a graph of the correlation between the log value of the WSBE concentrations and the average rate of cell growth inhibition.

Figure 1. Correlation between the log value of the WSBE concentration and the average rate of cell growth inhibition (%).

The following linear equation \( y = 39.844x - 32.561 \) with \( R^2 = 0.9786 \) was calculated from the graph in Figure 1 and was used to determine the IC\( _{50} \) value of the WSBEs to inhibit the growth of Hep-2 laryngeal cancer cells. There use is antilog (2.072) \( \approx 118.061 \) μg/mL.

In addition to the WSBEs, the cells were treated with cisplatin at the same concentrations (positive control groups). Table 6 shows the absorbance results of the MTT assay and the average rate of inhibition of the positive control group.

Table 6. Absorbance results of the positive control group

| Concentration (μg/mL) | Absorbance Result | Minimum | Median | Maximum | Mean | SD | % Mean Inhibition |
|---|---|---|---|---|---|---|---|
| 6.25 | 0.622 0.644 0.634 | 0.622 | 0.634 | 0.644 | 0.633 | 0.01 | 20.2 |
| 12.5 | 0.572 0.570 0.605 | 0.570 | 0.572 | 0.605 | 0.582 | 0.02 | 28.3 |
| 25 | 0.521 0.596 0.525 | 0.521 | 0.525 | 0.596 | 0.547 | 0.04 | 33.9 |
| 50 | 0.471 0.491 0.474 | 0.471 | 0.474 | 0.491 | 0.479 | 0.01 | 44.8 |
| 100 | 0.422 0.441 0.428 | 0.422 | 0.428 | 0.441 | 0.430 | 0.01 | 52.5 |
| 200 | 0.372 0.387 0.426 | 0.372 | 0.387 | 0.426 | 0.395 | 0.03 | 58.1 |
| 400 | 0.319 0.333 0.347 | 0.319 | 0.333 | 0.347 | 0.333 | 0.01 | 68.0 |
Then, a graph was created to identify the correlation between the log value of the cisplatin concentration and the average rate of cell growth inhibition (Figure 2).

![Graph](image)

**Figure 2.** Correlation between the log value of the cisplatin concentration and the average rate of cell growth inhibition

The following linear equation $y = 28.627x - 4.2553$ with $R^2 = 0.9846$ was calculated from the graph in Figure 2 and used to determine the IC$_{50}$ value of cisplatin to inhibit the growth of Hep-2 laryngeal cancer cells. Thus, the IC$_{50}$ value is antilog ($1.895$) $\approx 78.569$ µg/mL.

4. Discussion

In this study, WSBE samples were extracted with ethanol solvent and then fractionated to identify individual compounds, especially flavonoids[18-19], saponins, tanins, glycosides, triterpenoids, and alkaloids [9-13]. All six compounds were confirmed as constituents of the WSBE by TLC and phytochemical testing.

Eight different concentrations of the WSBE were produced (6.25–800 µg/mL). To simplify the concentration process, concentrations were increased by twofold. The concentrations were based on a study conducted by Yongsho et al. which reported that anthocyanin conveyed cytotoxic effects at concentrations of 100–250 µg/mL. In that study, rough extract was not used, but rather the fraction that contained cyanidin-3-glucoside and delphinidin-3-glucoside, and concluded that the half maximal effective of the wild soybean fraction was 13.25 µg/mL [20].

In contrast to the study by Yongshi et al. rough extract without further fractionation was used in the present study to obtain any bioactive compounds that were soluble in ethanol so that the results of the cytotoxicity test were appropriate.

Ethanol was chosen as the solvent as it was able to dissolve the bioactive compounds contained in black soybeans [18]. Moreover, ethanol is less toxic and safer to use than other solvents, such as methanol and acetone, applied in previous studies of Raji cells and human gastric carcinoma (AGS) cells [17,19]. Although a much safer solvent than ethanol, water is only capable of dissolving polar compounds, which would exclude the nonpolar compounds contained in black soybeans, resulting in unreliable cytotoxicity test results [21].

In the present study, the MTT assay was used to assess the cytotoxicity of WSBE to Hep-2 laryngeal cancer cells. The data from MTT assay showed that a higher OD value indicated a larger percentage of living cells. The OD values were then used to formulate an equation to determine the extent of inhibition. As shown in Table 6, cell inhibition was increased as the sample concentration was increased. However, the increase in inhibition was not consistent with the largest increase from a concentration of 25 to 50 µg/mL. The smallest inhibition percentage was observed at a concentration
of 6.25 μg/mL (20.9%) and the largest at a concentration of 800 μg/mL (86.4%). A graph was then produced of the log values of the extract concentrations against the extent of cell growth inhibition (as the percentage of living cells) to formulate a linear equation. The concentrations of the extracts were converted to a logarithmic equation because the changes in extract concentrations were not linear. The linear equation was used to calculate the IC_{50} value.

According to National Cancer Institute guidelines, the IC_{50} value obtained in this study of 118.06 μg/mL is moderately cytotoxic [22]. This finding is different from that of previous cytotoxicity studies of wild soybean skin extract with acidified aqueous acetone solvent against AGS gastric cancer cells, which reported an IC_{50} value of 3.69 mg/mL with an extract concentration of 1–5 μg/mL [19].

There were various factors that may have influenced the results of this study. First, the number of living cells at the start of the study was too large to inhibit cell apoptosis, as inhibition of apoptosis of highly confluent cells occurs via the activation of the Hippo pathway, which inhibits tyrosine kinase c-Abl, resulting in damage to the DNA and subsequent low cytotoxic activity of the extract [23]. Second, the presence of insoluble formazan also affected the results of the research because a greater number of living cells will form greater amounts of formazan. As a result, the OD value was lower.

DMSO is a good solvent for formazan, as this compound is not easily soluble. However, the formazan dissolved in DMSO could be wasted when removing the culture medium, resulting in less formazan and a subsequent increase in the OD value [24]. Third, the selection of wavelengths to assess absorbance was determined by the color of the substance to be read. Hence, reading at an inappropriate wavelength would result in incorrect absorbance values because the light waves were not absorbed optimally, or the substance could absorb other light waves instead. In this study, formazan was used because this substance absorbs light waves of yellow to orange (wavelength of 550–600 nm). Therefore, a wavelength of 515 nm was chosen so that some light was reflected, and the other part was absorbed. As a result, the OD values obtained were less than optimal [25].

The results of the Tamhane post hoc test showed that, statistically, the OD values of the variable group of 800 μg/mL were statistically different from those of the other variable groups, suggesting that saturation had not been achieved at an extract concentration of 800 μg/mL. Therefore, further research is warranted to determine the concentration with the maximum cytotoxicity.

Saturation was observed in the positive control group (200 μg/mL), but there were no statistically significant differences in comparison with all other positive control groups, indicating that the addition of cisplatin had no significant effect.

Cytotoxicity analysis was conducted to determine the IC_{50} values of the extracts. However, since cancer cells more actively divide and metabolize, the substrate would certainly be more quickly absorbed by cancer cells. Therefore, further studies are needed to confirm the effect of WSBE on normal and large cells as well as the sensitivity of the extract.

5. Conclusion
A rough WSBE was found to contain flavonoids, tannins, triterpenoids, saponins, alkaloids, and glycosides. The WSBE conveyed moderate cytotoxic effects to Hep-2 epidermal laryngeal cancer cells. The higher extract concentration, the greater the toxicity.

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