Cytotoxic Activity Assay of N-Hexane Extract of *Solanum nigrum* L. Fruits Fermented by Kombucha against MCF-7 Breast Cancer Cell Line

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Cytotoxic Activity Assay of N-Hexane Extract of *Solanum nigrum* L. Fruits Fermented by Kombucha against MCF-7 Breast Cancer Cell Line

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Abstract. Background: Breast cancer is the second leading cause of cancer-related deaths among women worldwide. Anticancer drugs therapy cause unwanted side effects problems that occur when treatment affects healthy tissues or organs. It is quite essential to find potential herbal as alternative cancer treatments. Glycoalkaloid (β2-solanin), solamargin, solasodine, dan solasonin of *Solanum nigrum* L. are potentially inhibit proliferation of leukemia and tumor cell: hepatic, intestinal, breast and cervic. On the other hand, kombucha as symbiotic culture of bacteria and yeast (SCOBY) has ability to increase activity of substrate by fermentation process. Objective: To determine cytotoxicity activity of n-hexane fruits extract of *Solanum nigrum* L. against MCF-7 breast cancer cell line after fermented by kombucha. Method: n-hexane fruits extract of *Solanum nigrum* L. was fermented for eight days at 25 ºC with 20% of combucha and addition of 20% of sucrose. Citotoxic activity was performed by using MTT(3- (4,5-dimethyltiazole-2-yl) -2,5-diphenyltetrazolium bromide) assay. Phytochemical of n-hexane extract was analyzed by GC-MS. Total acid and total phenol were characterized quantitatively. Results: IC_{50} of n-hexane fruits extract and fermentation product were 544,30 ppm and 1386,39 ppm, respectively. Flavonoids and steroids were identified from the extract and GC-MS shown seven compounds have been extracted from n-hexane fraction. Conclusion: condition of kombucha fermentation required optimization to improve cytotoxic activity of n-hexane fruits extract of *Solanum nigrum* L. against MCF-7 breast cancer cell line.

1. Introduction

Internal factors such as inherited mutations, hormones, and immune conditions and environmental/acquired factors such as tobacco, diet, radiation, and infectious organisms have been reported as cause of cancer [20]. Cancer is associated with absence of apoptosis and lead to uncontrolled cell proliferation [5]. Chemotherapy mechanisms might induced dysfunction of renal in general, abnormality of vasculature or kidneys structures, haemolytic uraemic syndrome and prerenal perfusion deficits [19]. Alternative herbal treatment believed has minimum side effects, less than synthetic anticancer drugs. One of potential plants investigated as traditional alternative for cancer treatment is black night shade (*Solanum nigrum* L.) [24]. Glycoalkaloids β2-solanine of this plants have potential activity to inhibit leukimia cells. Another active compounds from Solanum which able to suppress cell proliferation are solamargine, solasodine, and solasonine [25].

Kombucha is *Symbiotic Colony of Bacteria and Yeast* (SCOBY). Kombucha tea posses antiproliferative properties associated with significant antimicrobial activity and potentially use as traditional treatment for metabolic diseases and various types of cancer [6]. Kombucha contains regenerative enzymes, organic bacteria and macromolecules that help the intestinal flora stimulate the immune system and balance the endocrine system to increase the body's natural ability to heal itself [20].

The main metabolites found in this fermentation are acetic acid, lactic acid, gluconic acid, glucuronic acid, ethanol and glycerol [12] In addition, there are polyphenols which are proven to inhibit gene mutations, proliferation of cancer cells, induce cancer cell apoptosis and increase the ability to terminate metastasis [29].

In this study, we have examined cytotoxic activity of n-hexane fruits extract of *Solanum nigrum* L.) before and after fermented by kombucha, against MCF7 breast cancer cell line.

2. Materials and Methods

2.1 Materials

Fruits of *Solanum nigrum* L. which used for this study were obtained from Research Institute of Spice and Medicinal Plants, Bogor, Indonesia and have been identified at Center for Plant Conservation Botanic Gardens, The Indonesian Institute of Sciences. Kombucha cultures were obtained as small pieces of biosellulose from commercial outlet of wikikombucha, Indonesia.

2.2 Preparation of Extracts

The plant material were dried by oven at 40°C, until the water content of simplicia less than 10%. Determination of the water content was performed by using gravimetry method. Simplicia was refined using a blender. Refined material then extracted with n-hexane (1:4, w/v) for 2 x 24 h at room temperature. The extract then evaporated using rotary evaporator to obtain the concentrate and stored in a refrigerator to avoid fungal growth, until fermentation is carried out.

2.3 Preparation and Cultivation of Kombucha

The tea medium was prepared by boiling 3 L of water, 10% white sugar (w/v) and 5% of green tea leaves (w/v) and mixed until homogen. The mixture was strained and allowing at room temperature for 15 min and innoculated with kombucha cultures (bioselulose) for the first fermentation. The process was carried out for 8 days at 25ºC in a sterile glass jar covered with sterile gauze and the pH of beverage measured each day using digital pH meter, after calibrated at pH 4 and 7.

After complete the first fermentation, the beverage was added from the previous fermentation as inoculum for fermentation of extracts. 20% of beverage, 200 g of fruits water extracts, and 20 % of sucrose (w/v) were added into a sterile glass jar with addition of water to reach a volume of 200 mL. Fermentation was conducted for 8 days at 25ºC in a sterile glass jar covered with sterile gauze. After finish the second fermentation, 20% (v/v) of the previous beverage were added as inoculum for extract fermentation, with addition of 1,5% (w/v) n-hexane fruits extract, and 20% (w/v) of white sugar. Fermentation of extract was performed under similar conditions.

2.4 Phytochemical Screening

Five grams of plant material was exhaustively macerated and percolated with 80% ethanol or water. The macerate and percolate were combined and evaporated to dryness. The identification of each major chemical group in the 80% ethanol extracts was carried out by TLC on silica gel Merck 60F245 (layer thickness 0.2 mm) as follows: for terpenes and sterols, hexane/ethyl acetate: 1:1 was used as mobile phase and Liebermann-Burchard as reagent, a range of colors are produced after heating sprayed plates for 10 min at 100°C. Alkaloids were detected in the alkaloid fraction obtained by a classical acid/base extraction procedure for alkaloids and analyzed by TLC in chloroform/ methanol/ammonia solution 25% 8:2:0.5 as solvent system, spots were detected after spraying with

Dragendorff's reagent. To detect flavonoids, TLC was developed in n-butanol/ acetic acid/water 4:1:5 (top layer), spots were visualized with 1% aluminium chloride solution in methanol under UV 366 nm. Aqueous extracts were used for the identification of tannins with 1% gelatin solution, saponins by froth test and quinone with 1N NaOH. For steroids/triterpenoids identification, 1 gram sample was added with 20 mL ether and macerated for 2 h then filtered. The filtrate was moistened and dripped with Libermann-Burchard reagent (acetic acid anhydrous : H_2SO_4 = 2:1) [8].

2.5 Determination of Total Phenolic Content

Total phenolic compound contents were determined by the Folin-Ciocalteau method. 0.5 mL solution of plant extracts before and after fermented by kombucha in methanol were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water, and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA). Total flavonoid contents were calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg ml1 in methanol [11].

2.6 Determination of Total Acid Content

10 mL of sample was dissolved in a measuring flask to 250 mL. Afterwards, 50 mL of sample was transfered into Erlen Mayer flask and added with 2-3 drops of Fenolphthalein indicator. Sample then titrated with 0.1 N NaOH solution until the color becomes pink. Total titrated acid (TAT) was measured as percentage of acetic acid [1].

2.7 GC-MS analysis of steroidal glycoalkaloids aglycones derivatives

Steroidal glycoalkaloid aglycones (SGAA) were obtained by dissolving dried plant materials (10 g) and standards (20 µg each) separately in 2 mL of 1 M HCl in methanol and heated for 3 h at 70 °C. The free aglycones were liberated from the hydrolysate by adding 2 mL of 25% ammonia and extracted with 2 mL of dichloromethane after a few minutes. After vigorous mixing and 5 min., of centrifugation, the dichloromethane layer was removed with a pipette. The aglycon extracts were then evaporated to dryness. For derivatization, 20 µL of Trimethylsilylimidazole (TMS) and dry acetonitrile (50 µL) were added to each sample and standard (solasodine and Solanidine). The mixtures were heated at 60 °C for 15 min, cooled and 1 µL of each solution was injected into the GC-MS system. The SGAA derivatives were determined under the analytical conditions recommended by Laurila et al., (1999) for Solanum species operating at an ionization voltage of 70 eV (EI mode) with ion source temperature of 180°C using split sampling mode and an oven temperature of 180 to 285°C heated at 7.5°C min-1. Injector and detector temperatures were 285°C. Helium was used as the carrier gas (flow rate = 0.5 mL min-1). Identification of the aglycones in the plant materials was based on the GC/MS spectra of TMS derivatives of authentic standards and on reports of GC and MS glycoalkaloid aglycon data [16].

2.8 Cytotoxic Activity Assay

2.8.1 Cell Thawing

5 mL of 10% FBS, 0.5 mL of 1% penicillin-streptomycin and 44.5 mL RPMI 1640 (Roswell Park Memorial Institute) was transfered into conical tube and homogenized. Afterwards, 5 mL of complete media added into a T25 flask and breast cancer cell line MCF-7 for cultivated. The cap of tube was loosened then incubated in a 5% CO_2 incubator [4].

2.8.2 MTT Assay

The breast cancer cell line MCF7, at a density of 1 x 10^4 cell/ml, was seeded in 96 well microplates. 200 µL of n-hexane fruits extract of *Solanum nigrum* L. before and after fermented by kombucha were added to the cell cultures at concentrations of (10–500 µg/ ml). 1,25% of DMSO was used as solvent

for extract before fermentation and aquabidest as solvent for extract after fermentation.The cells were then incubated for 48 h in 5% CO_2 incubator at 37°C and 100 µL of 5 mg/mL MTT solution was added to each well, 4 h before the end of the incubation time. Afterwards, 100 µL DMSO was added as stopper. Cell survival was evaluated with ELISA reader at 550 nm. All data are presented as the mean values of triplicates. Cisplatin (5-250 µg/ ml) was used as positive control and negative control consist of the cell (without sample) and complete medium as a blanco [2, 3, 4].

3. Results and Discussion

3.1 Kombucha Growth Curves

Growth curves were determined from tea kombucha cultures and *Solanum nigrum* L. kombucha. During fermentation (figure 1), tea kombucha shown lag phase at the 1st and 2nd day. This stage characterized by a slightly growth of culture. Afterwards, the lag phase happened at the 3rd and the 4th day of fermentation. The tea kombucha colonies increase dramatically in this periode of time. Furthermore, the culture started to the stationair phase at the 9th day of fermentation and this step has been continued for five days. Optical density value were up and down in this phase, and in the 9th day, the OD became lower and lower, which indicated the dead phase until the 11th day. In this stage, the nutrient in the tea media was exhausted and the metabolism waste accumulated become toxic against colonies of kombucha [22].

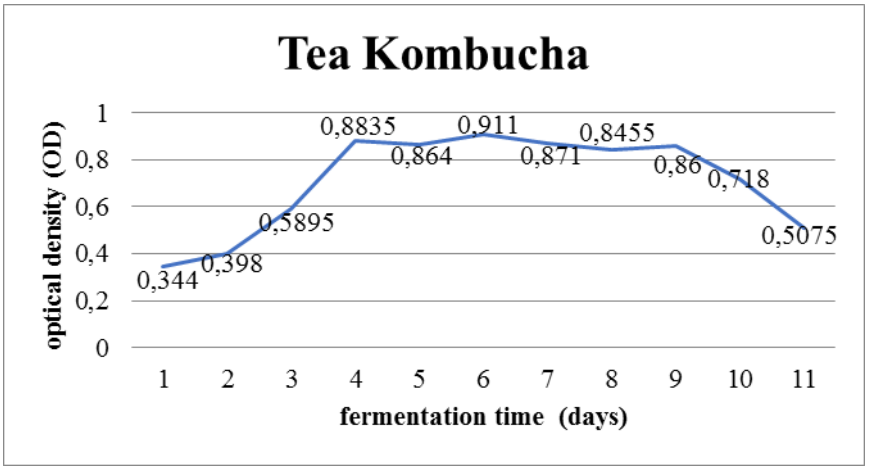

Figure 1. Growth curves of tea kombucha during fermentation

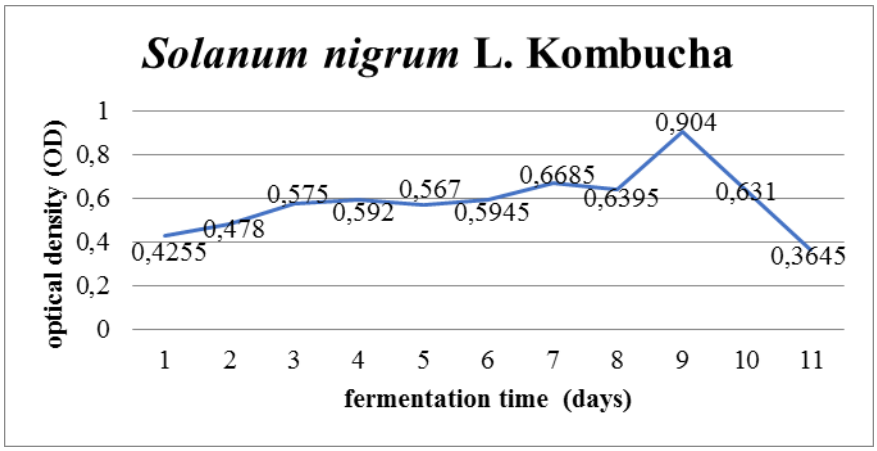

Figure 2. Growth curves of *Solanum nigrum* L. kombucha during fermentation

In contrast, adaptation phase of *Solanum nigrum* L. kombucha shown slower than tea kombucha, at 8 days of fermentation. This condition probably caused by different media used in fermentation. Since the first day, optical density slightly increase and down in the 5th day. On the other hand, logaritmic phase occurs on the 9th day, while tea combucha almost came into dead phase. It has been reported that tea is the optimum media for kombucha, while fruits extract of Solanum was something new which is required adaptation time of kombucha colonies to produce metabolic enzimes in the media. In addition, in figure 2, stationaire phase was not occurs. This circumstance associated with the availability of the nutrient. After passed the long adaptation step, colonies were consumed a large number or nutrient to produce energy in order to survive in the new media. Consequently, after logaritmic phase, the colonies immediately entered the dead phase [22].

3.2 Phytochemical Screening

The results of phytochemical screening as described in table 1, there are some phytochemical constituents contents in the sample, before and after fermentation, they were flavonoids, saponin and steroids/triterpenoids, and the contents were similar under both condition.

Table 1. Phytochemical screening of n-hexane fruits extract of *Solanum nigrum* L.

Phytochemical constituents	**Before fermentation**	**After fermentation**
Alkaloids	-	-
Flavonoids	++	++
Saponin	+	+
Tannins	-	-
Quinon	-	-
Steroids/Triterpenoids	+++	++

3.3 Fermentation Process

Fermentation of n-hexane extract requires sonication process, since characteristics of n-hexane extract is non polar, while the fermentation media is polar water. Consequently, it was quite difficult to dissolve extracts in fermentation media. Sonication was carried out to reduce the extract particles using ultrasonic waves. The extract used is 1.5% (w/v) with sucrose which is 20%. At this stage, a solid or SCOBY-shaped kombucha was not used. The distilled water was dissolved with addition of 20% sucrose (w/v) and transfered into a sterile vial. Afterwards, 1.5% of extract (w/v) was added to be sonicated until it was relatively soluble. Furthermore, 20% liquid kombucha starter was added. Fermentation process was carried out under room temperature (25 °C) of the extract solution, since colonies of kombucha were not able to live at high temperatures. The vial was closed with a clean batis cloth to allow CO_2 formed in the fermentation process come out of the jars. Vials should not be opened to minimize contamination and the vials should not be shaken before the fermentation process runs for 8 days. After 8 days, the baby kombucha formed was separated from the media. The fermentation product was stored in a refrigerator in order to stop fermentation process.

3.4 Evaluation of Fermentation Results

The mean pH of n-hexane extract was 5,52 and pH of fermentation was 2,73. This decrease of pH indicated the fermentation process was successful. Kombucha has a pH of around 2.5 [6], while maximum pH of good fermentation products was 4.5. Under a low pH, fermentation process was naturally avoid the contamination from other pathogenic microorganisms that grow in fermentation products [13]. According to the results obtained, the fermentation of n-hexane extract with kombucha meets the requirements as a good beverage.

Total phenolic of extract was determined by the Folin-Ciocalteu method. This reagent is a mixture of Phosphotungstate and Phosphomolybdic Acid which will be reduced by phenolic ions in alkaline conditions. A blue molybdenum-tungsten complex will be formed which can be absorbed at a

wavelength of 700-750 nm [7]. Total phenol obtained was measured in mgGAE / g extract units. By calculating the phenol level formula, phenol extract levels were counted at 6.015 mgGAE / g extract and phenol content after fermentation at 2,465 mgGAE / g extract.

The decrease of phenolic content in fermentation results might be influenced by the presence of acids which formed from the fermentation process, thereby reducing pH. Phenolic compounds formation depends on pH value. Phenol is formed in acidic conditions at pH 4-5 [14], while the pH of fermentation results is below the pH range that is suitable for phenol formation. In addition, it can be influenced by the kombucha microbial metabolism which is not optimum yet. Consequently, during fermentation, the secondary metabolites was not significantly increase [29].

Determination of total acid by titrimetric method were done to evaluate the successful of fermentation. This process was characterized by the presence of acids formed from the metabolic process of microorganisms. In kombucha cultures, acetic acid is one of the acids formed during fermentation. Although the presence of acid can be detected by its distinctive aroma, it is also necessary to calculate the percentage of acids. There are special standards for the acid content that can be contained in a fermented product such as yogurt which is 0.5 - 2.0% [10].

Acetic acids, gluconate acids, glucoronic acids, L-lactic acids, malic acids, malonic acids, pyruvate acids and usnic acids are organic acids which are produced from microbial activity with sugar addition during fermentation. Acetic acid is the most dominant organic acid and produced by acetic acid bacteria through ethanol oxidation. A decrease of pH is followed by an increase in the concentration of organic acids produced during the fermentation process by bacteria and yeast [18].

Acetic acids has a greater cytotoxic effects than other organic acids. Organic acid molecules enter the cell wall and reduce intracellular pH. Then the metabolic pathway of the cell will be directed to the excess efflux of protons (H +) produced by acids. The energy used for this process increases the stress of cell metabolism, causing cell death [27].

3.5 GC-MS analysis of steroidal glycoalkaloids aglycones derivatives

Since n-hexane extract is non-volatile, it was required derivatization process to modify molecular functional groups with reagents. The aglycone can be analyzed below 280°C and if it exceeds of 280°C, half of the aglycone can be damaged. There are 3 types of derivatization reactions namely silylation, acylation and alkylation. The derivatization used is silylation by removing the functional groups –OH, -SH or –NH and adding silyl groups [16].

Table 2. Concentration of chemical compounds of *S. nigrum* determined by GC-MS

No.	RT	Chemical compounds	% Area
1.	18.420	Ethylene glycol	65.339
2.	18.649	1,3-Benzenedicarboxylic acid	4.426
3.	21.529	9-Octadecenethioic acid, 12-hydroxy-, S-t-butyl ester	2.333
4.	22.064	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	1.346
5.	23.052	1,4-Benzenedicarboxylic acid, bis(2-hydroxyethyl) ester	6.663
6.	31.969	2,6-Diamino-4,4'-di-tert-butylbiphenyl	10.880
7.	32.813	7,7-Bis(methylthio)-6-methyl-1-(2-thienyl)-2,4,6-heptatriien-1-one	1.999

HCl was added in the preparations to precipitate the alkaloids by forming salt in acidic conditions. Aglycones are released from their hydrolysates by adding alkaline ammonia which and then extracted with dichloromethane. Dichloromethane (MTC) functions as a solvent from free aglycones. The solvent was then evaporated and the sample was added with derivatization reagents namely

Trimethylsilylimidazole (TMS) and Trimethylchlorosilane (TMCS). GC-MS was carried out by used HP-5MS capillary column with a length of 30 m, an inner diameter of 0.25 mm, and a thickness of 0.25 µm film layer, namely phenyl methyl cyclosan. The carrier gas used is Helium with a pressure of 100 kpa and a flow rate of 1 µL / minute. The detector used is MS (Mass Spectrum) and split injection technique. Injector temperature of 250°C with an initial temperature of 40°C, a temperature rise rate of 10 °C / minute, and a final temperature of 280 ° C. The results of GC-MS extract of n-hexane can be seen in table 2.

1,2-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester has been known to have anticancer activity, based on the tests of 1,2-Benzenedicarboxylic acid isolates, bis (2-ethylhexyl) esters obtained from the *Pervivirus* thevetia against various cancer cells using the MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromid) method [23].

3.6 Cytotoxic Activity Assay

Cytotoxic activity of n-hexane fruits extract of *S. Nigrum* before and after fermented by kombucha was determinated based on color changes of tetrazolium salt which indicated reduction process. MTT or 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide is a yellow substance that will produce blue formazan when reduction occurs. The process occurs in the cytoplasm, mitochondria and cell membranes.

Reduction activity depends on the intracellular concentration of NADH and NADPH. The abundance of nucleotide cofactors was followed by the presence of extracellular glucose. Therefore, the poor state of the culture cell media may induce MTT absorbance identified as low value, because of the low sugar concentration. Test substances or samples that interfere with these enzymes or with glycolysis was able to change the level of MTT reduction and affected the results of cell calculations. Based on this metabolic process, formazan crystals were formed like dark purple needles. Formazan crystals well dissolved with the addition of organic solvents, including alcohol and dimethyl sulfoside [28].

After analyzed, the IC_{50} value of n-Heksana extract before fermentation was calculated at 544.30 ppm and the IC_{50} value after fermentation was 1386.39 ppm. Fermentation of kombucha was not effectively yet in increase the cytotoxic activity for some reasons. From the kombucha growth curves, it has been known that fermentation time required to be extended, since *S.nigrum* kombucha has a longer time of adaptation than tea kombucha, before start the logaritmic phase. In addition, the concentration of sucrose in the media was also necessary to be increased, in order to provide optimum nutrient which important in the metabolism process of the colonies. Furthermore, the starter in fermentation should be completed with the solid pellicle (SCOBY) of kombucha, not only the beverage [12]. Another factor that might be influenced this result is the used of water solvents that are less able to perfectly dissolved the compound, even though the sample was seemed to be soluble in water. DMSO is a universal solvent because it can transport compounds through the skin [9].

4. Conclusions

We have examined the effects of kombucha fermentation of n-hexane fruits extract of *S. nigrum* L. towards cytotoxic activity against breast cancer cell line MCF-7 using MTT method. We have found that fermentation process was required optimization conditions, in order to provide optimum results of secondary metabolites which have cytotoxic properties.

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