



Research Article

THE RESEARCH EVIDENCE OF ANTIOXIDANT AND ANTI-CANCER ACTIVITY OF GENISTEIN CONTENT IN THE INDONESIAN TRADITIONAL FOOD (ONCOM) ETHANOL EXTRACTResmi Mustarichie¹, Moelyono Moektiwardoyo¹, Jutti Levita¹, Supriyatna¹, Ahmad Muhtadi¹, Anas Subarnas¹ and Linar Z. Udin²¹ Faculty of Pharmacy, Universitas Padjadjaran, Jatinangor, Indonesia.² Indonesian Institute of Sciences, Bandung, Indonesia

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Abstract: Oncom ethanol extracts in our previous study contains genistein which is a potent flavonoid antioxidants and anti-cancer activity. This study is a series of studies to prove that oncom, a traditional Indonesian food, has antioxidant properties, anti-inflammatory and anti-cancer properties. Anti-inflammatory properties are also our previous report. Phytochemical screening in this study showed that ethanol extract contains only condensed flavonoid. Ethanol extract obtained by soxhletation, evaporation and freeze drying tested the antioxidant activity and anti-cancer in vitro by using a 1,1-diphenyl 2-picrylhydrazil (DPPH) and Sulforhodamin B against breast cancer cells (MCF-7), respectively. Operating time for the antioxidant reaction was 40-100 minutes. By using vitamin C as a standard, antioxidant IC₅₀ for oncom ethanol extract was 363.8 ppm. It was found that the addition of HCl to the extract prior DPPH reaction would increase the IC₅₀ value significantly. For in-vitro antiproliferation tests, pure genistein, oncom ethanol extract and cisplatin as a standard were applied. The genistein had IC₅₀ = 15.45 ppm against MCF-7 cells. The oncom ethanol extract had IC₅₀ = 15.79 ppm whereas standard cisplatin gave IC₅₀ = 4.80 ppm. These data indicated that genistein in oncom extract may cause its anti-cancer activity.

Key words: Oncom, flavonoids, DPPH, Sulforhodamin B, MCF-7, anti-cancer, antioxidant

1. INTRODUCTION

Free radicals are chemical molecules that have one or more unpaired electrons that are reactive. This causes the reactive nature of the unstable free radical molecules. To stabilize free radicals by way of trying to get electrons combine with other molecules that lead to other unstable molecules. Transfer electrons to the free radical molecules cause oxidative damage to tissues. This triggers the occurrence of various diseases such as cardiovascular and carcinogenesis¹.

Body has endogenous defense mechanisms against free radicals or antioxidants, but if the amount of excess free radicals, the body needs antioxidants derived from natural sources or synthetic¹. Synthetic antioxidants are now rarely used because it can trigger cancer². Natural antioxidants found in vegetables, fruits, and grains. Hernandez *et al.*³ found it in fruit - fruit contains many vitamins that showed antioxidant activity such as vitamin C, vitamin E, and β-carotene.

Oncom is one of the traditional food of the people especially in the area of Java Island, Indonesia. Based on the fact that there are special treatments for the rest of the remaining peanuts and

remaining making tofu, the research will attempt to uncover whether oncom which is very cheap food still contain isoflavones, especially genistein which is known to have a very good effect on health. The content of isoflavones are potent antioxidants as well as anti-cancer activity. This study reports on the antioxidant activity of ethanol extracts from red oncom using picril-diphenyl-hidrazil (DPPH) and its anti-cancer activity using Sulforhodamin B against MCF-7 method. Anti-inflammatory properties of oncom extract has been verified in our in our previous report⁴.

2. MATERIALS & METHODS**2.1 Materials**

Materials used in this study was the ethanol extract of oncom.

Chemicals used in this study was amyl alcohol, ammonia 10%, DPPH (1,1-diphenyl 2-picrylhydrazil) (Merck ®), ethanol 95% (Bratachem ®), ether, a solution of iron (III) chloride, ethyl acetate, 1% gelatin, 2N hydrochloric acid, reagents Dragendorf, Liebermann-Burchard reagent, reagent Mayer, powdered magnesium, and vitamin C. All reagents, are pro analysis.

2.2 Methods

Extraction

Oncom with some wet weight oven dried at temperatures of 105⁰C, extracted by the use of soxhlet apparatus. Soxhletation aimed to attract secondary metabolites present in simplicia. Simplicia was extracted until the entire flow of solvent extracted was marked by a clear extract. The extract obtained was evaporated using a Rotavapor (40-50⁰C) and then followed by evaporation using a water bath (40⁰C) to obtain a thick extract, then the freeze-dried, % yield of the extract calculated with the formula:

$$\% \text{ Yield of extract} = \frac{\text{extract weight (g)}}{\text{original sample wt.(g)}} \times 100\%$$

Phytochemical screening

Phytochemical screening conducted to determine alkaloid, polyphenols, flavonoids, saponins, tannins, quinones, sterols, triterpenoids, and sesquiterpenoid monoterpene content. This test was performed on ethanol extracts of oncom. The method used is according to Farnsworth⁵, Ditjen POM⁶ and Mustarichie *et al.*⁷

Inspection of alkaloids compounds

Materials basified with dilute ammonia, then extracted with chloroform to obtain a filtrate. Hereinafter the filtrate was shaken with 2 mL of 2 N hydrochloric acid. Acid layer was separated, then the filtrate was divided by three, one of which was not given a solution of the reagent and the remaining two were each given a solution of Dragendorff reagent and Mayer. Positive results were indicated by the formation of brownish-red precipitate to Dragendorff and Mayer white precipitate.

Inspection of polyphenol compounds

Material was extracted by heating the mixture using distilled water. To the filtrate was added a solution of reagent iron (III) chloride. Presence of phenolic compounds characterized by the occurrence of black-green-blue to black.

Examination of tannin compounds

Material was extracted by heating the mixture using distilled water. To the filtrate was added 1% gelatin. Presence of tannin compounds

characterized by the occurrence of a white precipitate.

Examination of flavonoid compounds

Material was extracted by heating the mixture using distilled water was then added magnesium metal and hydrochloric acid 2 N. Into the filtrate in a test tube amyl alcohol was added, then shaken vigorously. The presence of flavonoids would cause the red filtrate that could be withdrawn by the amyl alcohol.

Examination of saponin compounds

Material was extracted by heating the mixture using distilled water. Filtrate in a test tube and then shaken vigorously for about 30 seconds. Foaming at least 1 cm and consistent for five minutes and did not disappear after addition of 1 drop of dilute hydrochloric acid showed that the saponin compounds there.

Examination of monoterpene and sesquiterpenoid compounds

Material extracted with ether, then the filtrate was evaporated to dryness. Residue dripped on anisaldehyde-sulfuric acid reagent or reagents 10% vanillin in concentrated sulfuric acid. The formation of the colors indicated the presence of monoterpene and sesquiterpenoid compounds.

Inspection of steroids and triterpenoids compounds

Material was extracted with ether, then the filtrate was evaporated to dryness. Residue dripped on Liebermann-Burchard reagent. Formation of purple color indicated that the group contained triterpenoids, whereas when forming blue-green color indicated the presence of steroid compounds.

Examination of quinone compounds

Material was extracted by heating the mixture using distilled water. Filtrate poured with sodium hydroxide or potassium hydroxide. Formation of red and yellow colors indicated the presence of quinone groups of compounds.

Antioxidant activity assay

Preparation of DPPH solution

DPPH as much as 6 mg dissolved in 200 mL of ethanol to obtain stock solution of DPPH with the concentration of 30 ppm. DPPH solution was made

fresh and kept at low temperature protected from light.

Wavelength measurements

As many as 3 mL DPPH solution dissolved in 2 mL of ethanol, homogenized, and measured at a wavelength of 400-750 nm.

Preparation of DPPH Standard Curve

DPPH solution was diluted to 12, 15, 18, 21, 24, 27 and 30 ppm. DPPH solution was allowed to stand 40 minutes each concentration and measured absorbance at a wavelength of maximum standard curve is then created.

Preparation of test solution and Vitamin C

Condensed extract ethanol from oncom and the comparison of vitamin C weighed as much as 20 mg and dissolved in 50 mL of ethanol in order to get the mother liquor 400 ppm. Mother liquor is then diluted to a concentration.

Determination of DPPH Operating Time

DPPH solution 40 mg / ml mixed into the test sample with a ratio of DPPH: sample (3:2) and placed in a reservoir. Absorbance of this solution were observed at λ 450-650 nm at during a certain time span. The resulting absorbance data is then observed and presented in graphical form.

Extract Concentration Orientation

This procedure was performed to determine the minimum concentration of extract that can inhibit the activity of DPPH radical. The extract was diluted to a concentration of 2 ppm, 8 ppm and 16 ppm. Each extract as much as 2 mL plus 3 mL DPPH 30 ppm, shaken, and allowed to stand 40 minutes at room temperature in a dark room. Absorbance was measured at a wavelength of DPPH obtained. The experiments were carried triplets. If it was not be able to measure, the concentration was increased to 20 ppm, 40 ppm and 60 ppm.

Absorbance measurements of samples

Test solutions were diluted from stock solution to 80, 100, 120, 140, and 160 ppm. Test solution to 2 mL of DPPH solution plus as much as 3ml, homogenized, and allowed to stand for 40 minutes at room temperature in a dark room. Absorbance was measured at a wavelength maximum of DPPH. The experiments were carried triplets. As the blank is used 3 mL of ethanol and 2 mL sample.

Vitamin C as a comparison solution was diluted to 4, 5, 6, 7, 8, 9, and 10 ppm. Vitamin C solution of 2 mL of DPPH solution plus as much as 3ml, homogenized, and allowed to stand for 40 minutes at room temperature in a dark room. Absorbance was measured at a wavelength maximum of DPPH. The experiments were carried triplets. Used as a blank solution of 3 mL of ethanol and 2 mL of vitamin C.

Calculation of IC₅₀ values

Percent inhibition of DPPH by antioxidants in the sample was calculated by the formula:

$$\% \text{ Inhibition} = \left[1 - \left(\frac{A_{test}}{A_{control}} \right) \right] \times 100\%$$

where :

A_{test} = absorbance of DPPH solution
in the sample

$A_{control}$ = absorbance of DPPH solution
in ethanol (3 mL DPPH and
2 mL of ethanol)

(Utami *et al.*⁸)

Acquired percent inhibition datas was plotted against the concentration of the extract. These data were evaluated using linear regression. IC₅₀ value describes the concentration of extract required to inhibit 50% DPPH radical activity. IC₅₀ values was obtained from the graph of extract concentration against the percent inhibition of DPPH.

Anti-cancer activity test

Initial extraction of plants for early anticancer screening

The extract was concentrated, the weight of extract was measured, and then vitro anticancer activity determined (Colegate and Molyneux⁹, Kinghorn¹⁰, Farnsworth⁵).

Preparation of cancer cells to test anticancer

Cancer cells used in this study were breast cancer (MCF-7). Breast cancer (MCF-7) was cultured in Modified Eagle Dulbecco's Medium (DMEM) with 10% FBS and cultured at 37°C at a humidity of 95% and 5% CO₂ for 3 days until confluent cell culture was 60-70%. After that the old medium was removed, replaced with new medium and incubated again for 24 hours. The cell culture was then washed with PBS 1-2 times and suspended using trypsin-

EDTA solution. Cells that were suspended coupled with new media.

In vitro anticancer test

Anticancer testing was by the SRB method (Sulforhodamin B) developed by the National Cancer Institute (Likhitwitayawuid *et al.*¹¹). Cells that have been prepared to test as many as 100 GL plus the test sample as many as 10 GL were incubated for 3-4 days at room temperature. After that, cells were fixed with 50% TCA, staining with 0.4% SRB in 1% acetic acid for 30 minutes. SRB color that was not bound was removed with acetic acid 1%, while the bound was extracted with Tris base (pH 10). The intensity of color produced was measured by using ELISA plate reader on the wavelength of 515 nm respectively. Percent viability was calculated as follows:

$$\frac{\text{OD (cell+sample)} - \text{OD (negative control)}}{\text{OD (cells)} - \text{OD (negative control)}} \times 100 = \% \text{ viability}$$

IC₅₀ was calculated by nonlinear regression analysis between percent survival and the concentration (Skehan *et al.*¹²).

3. RESULTS AND DISCUSSION

Extraction

978.710 g oncom oven dried at 105⁰C for 4 h to obtain a fixed weight of 577.046 g. The dried oncom was extracted by using soxhlet apparatus then dried to obtain extracts. The ethanol extract was evaporated, freeze-dried to produce 74.734 g yielded of 12.95% of dry weight. Extraction method used was soxhlet method, because previous studies showed there was no damage of the isoflavones due to heating as indicated by the activity. To obtain a perfect result, the soxhletation process performed till obtaining a clear extract solution. Selection of extraction solvent should be based on current levels of security and ease of evaporative (Ditjen POM¹³) so that 95% ethanol used as solvent in this study. Ethanol is one of 42 solvents which has ability to pull almost all the compounds that exist in simplicia.

Phytochemical screening results

Phytochemical screening conducted to determine the class of metabolites contained in ethanol extracts of oncom. Phytochemical screening examination includes group alkaloids, flavonoids, tannins, polifenolat, monoterpenoid and seskuiiterpenoid, steroids and triterpenoids, quinones,

and saponins. The results of phytochemical screening of extracts as follows:

Table 1. Phytochemical screening results of Oncom ethanol extract

Compounds	Oncom Ethanol Extract
Alkaloid	-
Polyphenol	+
Tannin	-
Flavonoid	+
Monoterpenoid and Seskuiiterpenoid	-
Steroid and Triterpenoid	-
Quinone	-
Saponin	-

Description: (+) = detected

(-) = not detected

The results of phytochemical screening of ethanol extract showed phenolic and flavonoid compounds. Phenolic compound identified by reaction with iron (III) chloride produced a blue-black color. Testing a class of flavonoids seen from yellow to red color that can be withdrawn by the amyl alcohol.

Antioxidant Activity Test

Molecules of 1.1-diphenyl-2-pikril-hidrazil (DPPH) free radical is an unstable molecule due to electron delocalisation. Delocalisation is also cause the color purple to give the absorbance at 520 nm in the vicinity of ethanol (Molyneux¹⁴), λ_{max} of 518 nm was applied in this experiment instead. DPPH free radical molecules produced when dissolved in alcohol (Praptiwi *et al.*¹⁵).

Test the antioxidant activity using DPPH free radicals based on the principle of measuring the intensity of the DPPH purple color is directly proportional to the concentration of DPPH. Purple color intensity was measured with a spectrophotometer in the visible region. Purple color

changes to yellow indicating reduced DPPH entirely (Utami *et al.*⁸). When reacted with a compound suspected of having antitoksidan activity, DPPH is reduced so that the color changes to yellow. DPPH free radical reduction activity was measured by calculating the reduction in the intensity of the color purple as measured at a wavelength of maximum absorption is 518 nm DPPH. The maximum wavelength obtained in accordance with the wavelength of maximum absorption DPPH (Leit Ao *et al.*¹⁶).

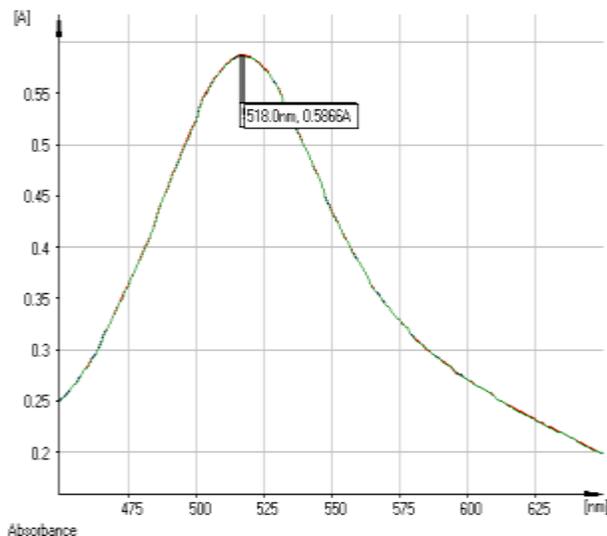


Figure 1 The maximum absorption spectra of DPPH 40 mg / ml

Decrease in intensity of color is caused by the capture of one electron of DPPH radicals by antioxidants that cause the absence of electrons is a chance to resonate. Before testing the antioxidant activity, determination of the operating time in the ethanol solution of DPPH was conducted which aimed to determine the best work of DPPH solution. Determination of operating time DPPH solution was obtained by measuring the absorbance of DPPH solution of 40 mg / ml as measured by changes in time. Changes in absorbance of DPPH solution can be seen through Figure 2.

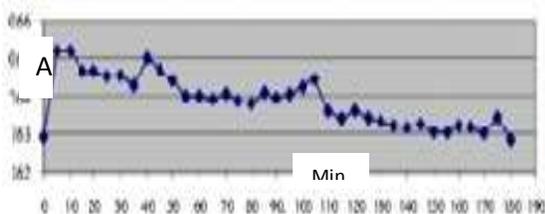


Figure 2. DPPH solution operating time curve .

Figure 2. shows that DPPH can be quite stable over a range of forty to one hundred minutes, so the analysis must be done at that time. In this study, the mother liquor was made from the sample solution. Number concentration series was prepared from the mother liquor. Measurement of free radical activity of DPPH reduction is done by adding 2 ml sample at a certain concentration of DPPH with 3 ml of 40 ug / ml, while for the controls using 3 ml of DPPH and 2 ml of ethanol 95%. Blank on the measurement of the UV-Vis spectrophotometer used 2 ml of the sample with a certain concentration of ethanol and 3 ml of 95%. Use of this form to reduce errors due to measurability of the absorbance of the sample.

Measurements were performed in accordance with the incubation time is 40 minutes for the sample and vitamin C. Measurements were performed 40 minutes after adding DPPH to give time for the sample can reduce free radical activity of DPPH. Use of this form to reduce errors due to measurability of the absorbance of the sample. This test was performed each duplicate. Samples which have antioxidant activity will reduce DPPH (a decrease in the intensity of the color purple). Reduction process is caused by hydrogen atom donation from the test sample to DPPH. This process is illustrated in Figure 3.

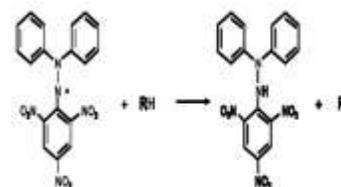


Figure 3. The mechanism of DPPH reduction

In the Figure 3., the antioxidant is symbolized by RH. RH antioxidants will provide H atoms to DPPH radical that binds to the electron deficiency on the DPPH radical was covered. RH to donate electrons to be deprived of one electron (denoted by R.), but R. will stabilize itself so that no radical form.

Tables 2. and 3, respectively show the measurements obtained from Oncom ethanol extract and vitamin C as a comparison.

Table 2. DPPH test results of the oncom ethanol extract

DPPH 0.7885 0.7869 0.7864 0.7873

ppm	A1	A2	A3	A means	% inhibition
40	0.7014	0.7006	0.7006	0.7009	10.97
80	0.6581	0.6574	0.6570	0.6575	16.48
120	0.6232	0.6235	0.6246	0.6238	20.77
160	0.5871	0.588	0.5881	0.5877	25.35
200	0.5504	0.5504	0.5504	0.5504	30.09
320	0.4328	0.4319	0.4332	0.4326	45.05

Table 3. DPPH Test results for vitamin C

DPPH 0.6362 0.6344 0.6323 0.6343

ppm	A1	A2	A3	A means	% inhibition
0.5	0.6180	0.6151	0.6138	0.6156	2.94
1	0.5814	0.5801	0.5793	0.5803	8.52
2	0.5279	0.5315	0.5305	0.5300	16.45
4	0.4600	0.4588	0.4566	0.4585	27.72
6	0.3591	0.3598	0.3586	0.3592	43.38

Parameter interpretation of the results of the test method with DPPH antioxidant activity is the IC₅₀ or Inhibition Concentration 50 i.e where the concentration of the sample can reduce the activity of DPPH by 50% of the initial concentration. Determination of the concentration variation for the test based on the absorbance values obtained when determining the time of incubation with a specific concentration. The absorbance values may indicate

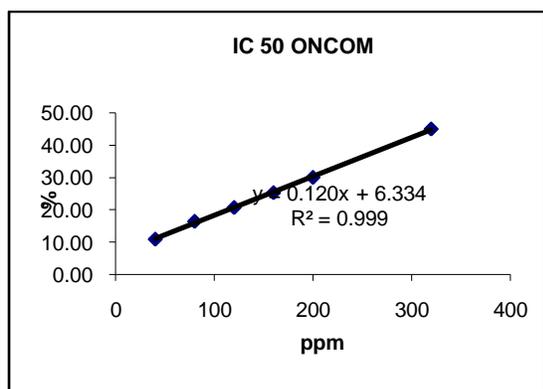


Figure 3. IC₅₀ oncom ethanol extract

By inserting $y = 50$ then the IC₅₀ values obtained for oncom ethanol extracts and IC₅₀ of vitamin C, were 363.8 µg/mL and 6.9 µg/mL, respectively.

From these results have shown that the IC₅₀ values of extract which means it has very little antioxidant activity compared to vitamin C. It should be added that the low polarity compounds having functional groups containing oxygen less than the

the amount of % inhibition at a concentration of a sample so that it can be used as reference for the determination of the concentration variation. IC₅₀ values obtained using linear regression equation expressing the relation between the concentration on the x axis with DPPH reduction activity (expressed as % inhibition) on the y axis. IC₅₀ calculation results of the ethanol extract and vitamin C oncom can be seen in Figure 3 and 4.

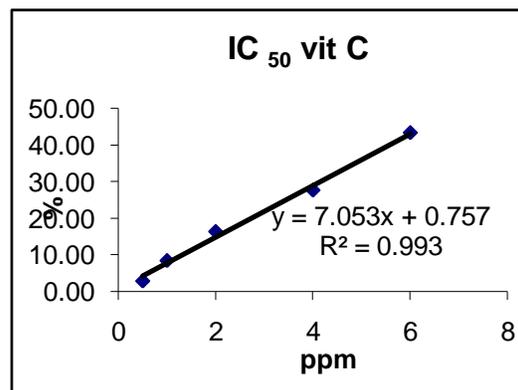


Figure 4. IC₅₀ vitamin C

compounds that have a high polarity that tends to be less reactive.

In the extracts of phenolic compounds, flavonoids and tannins responsible for antioxidant activity. Phenolic compounds by itself is not active as an antioxidant, the substitution of alkyl groups at positions 2, 4 and 6 can increase the electron density of hydroxyl groups that increase the activeness of the radical (Kusumaningati¹⁷). In addition, Jayaprakasha

*et al.*¹⁸ stated that the hydroxyl group of phenol compounds, metoksil and ring substituents on the phenyl diketone 1.3 had a very significant role in the ability as an antioxidant. Presence of β -diketone group led to a hydrogen atom donor to neutralize free radicals. The more free hydroxyl groups which can donate hydrogen reduction of the more well to do against DPPH. Amadou *et al.*¹⁹ reported increased concentrations of phenolic compounds in soybean after fermentation products and contain various amounts of phenolic compounds have been shown to have antioxidant capabilities. In the present study oncom ethanol extract, the presence of phenolic compounds and flavonoids in chemical screening, proving that the possibility of antioxidant properties obtained only due to isoflavonoid compounds as well as reported by Lee *et al.*²⁰. In this study, we found that the addition of acid to the ethanol extract prior mixing with DPPH reagent increased the IC₅₀ of the ethanol extract of oncom significantly. It increased about 50 % . The acid plays an important role in the flavonoids which contains benzopyranon ring system, therefore it will produce its reduced form, flavinium. This finding confirmed the fact that although oncom is made via several steps of processes (e.g., washing, drying, boiling, and fermentation), the flavonoids in the food is not completely degraded. This finding would support, however, our assumption that in the body, genistein content in oncom whenever it was consumed, increase its concentrations due to acidification in our stomach.

Anti-cancer Activity Test

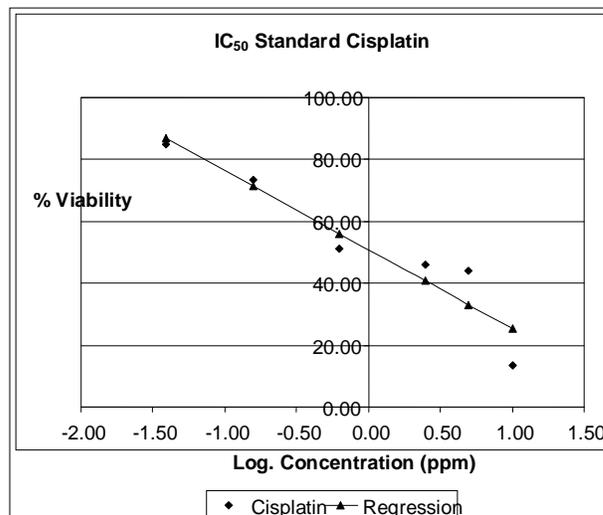
The bioactivity test used anticancer compound cis-Diammineplatinum (II) dichloride (cisplatin) as the standard. From the observation of anticancer bioactivity tests, the oncom ethanol extract was potent in inhibiting the growth of breast cancer cells (MCF-7) because this extract had IC₅₀ close to standard anticancer compounds (cisplatin).

Table 4 shows data taken from standard cisplatin. IC₅₀ calculated from this data shown Fig.5.

Table 4. Data IC₅₀ standard cisplatin

Concentrations (ppm)	Log Conc. (ppm)	% viability
10.0000	1.00	13.59
5.0000	0.70	43.98
2.5000	0.40	46.11
0.6250	-0.20	51.24
0.1563	-0.81	73.49
0.0391	-1.41	84.88

$Y = -25.50 \log X + 50.85$ (R = 0.94)



IC₅₀ = 4.80 ppm

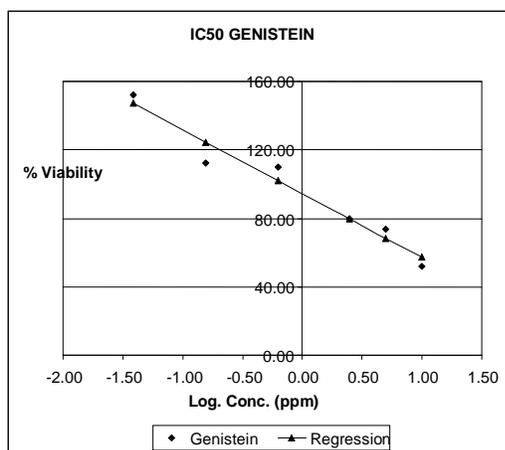
Fig. 5. IC₅₀ Cisplatin

Anticancer bioactivity test observations using pure genistein as a reference showed in Table 5 and calculated IC₅₀ was 15.45 ppm (Fig. 6).

Table 5. Data IC₅₀ Genistein

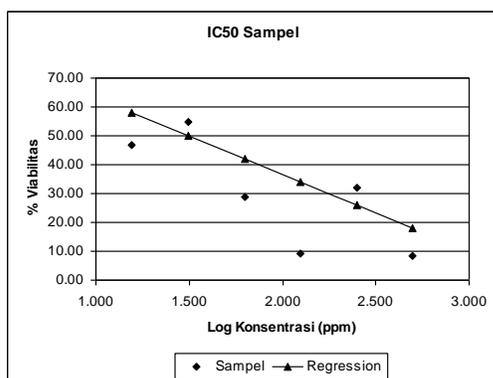
Concentrations (ppm)	log Conc. (ppm)	% viability
10.0000	1.00	51.79
5.0000	0.70	73.49
2.5000	0.40	79.62
0.6250	-0.20	109.93
0.1563	-0.81	112.07
0.0391	-1.41	151.96

$Y = -37.39 \log X + 94.47$ (R = 0.96)

Fig. 6. IC₅₀ GenisteinTable 6. Data IC₅₀ Oncom ethanol extract

Concentrations (ppm)	Log Conc. (ppm)	% viability
1000	3.000	8.41
500	2.699	31.99
250	2.398	9.34
125	2.097	28.66
62.5	1.796	54.98
31.25	1.495	46.95

$$Y = -26.67 \log X + 89.98 \quad (R = 0.90)$$

Fig. 7. IC₅₀ Oncom ethanol extract

IC₅₀ of 15.79 ppm was found for oncom ethanol extract (Tabel 6. and Fig. 7.). The result indicates that the extract has anti-cancer activity against MCF-7 cells. This means that the oncom ethanol extract has a fairly selective cytotoxic activity. This also indicated genistein and daedzein (isoflavones) content in oncom may cause this activity as so far no other secondary metabolite found in oncom by phytochemical screening. Chang *et al.*²¹ reported that daedzein and genistein were the two isoflavones detected in fermented foods by fungi.

CONCLUSION AND SUGGESTIONS

Antioxidant activity of the oncom ethanol extract much less compared to vitamin C but the addition of acid prior to DPPH determination increases its IC₅₀ significantly. Antioxidant activity of compounds in this study were phenolic flavonoids supported by its phytochemical screening.

Anti-cancer activity of the oncom ethanol extract is fairly selective cytotoxic activity against MCF-7.

Further investigation may be performed to isolate and elucidate active compounds from the oncom in order to find as to whether there is other secondary metabolites present and study its interaction with their targets.

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