A Green Synthesis of Chalcones As an Antioxidant and Anticancer

Elfi Susanti VH, Widiastuti Agustina Eko Setyowati
Department of Chemistry FKIP Universitas Sebelas Maret, Surakarta
Email: elfisusantivh@staff.uns.ac.id

Abstract. Three chalcones (4'-amino-4-methoxy chalcone, 4'-amino-3,4-dimethoxy chalcone and 4'-amino-3,4,5-trimethoxy chalcone) has been synthesized by a green chemistry approach using grinding technique. Antioxidant activity of the chalcones were assessed using 1,1-biphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. Cytotoxicity of chalcones synthesized was evaluated using a tetrazolium (MTT) colorimetric assay against cervical cancer cell line, HeLa. The antioxidant activity test showed that 4'-amino-4-methoxy chalcone had a stronger activity than the 4'-amino-3,4-dimethoxy chalcone and 4'-amino-3,4,5-trimethoxy chalcone, respectively with IC$_{50}$ 58.85, 64.79 and 210.3 µg/mL. These results indicate that there is a relationship between the structure of chalcone with antioxidant activity, the more methoxy groups in the ring B of the chalcone, antioxidant activity is getting smaller. The chalcone synthesized showed cytotoxicity against HeLa cell line with IC$_{50}$ value of 31.75, 36.65, 49.04 µg/mL, respectively. It was observed that the highest cytotoxic activity was found at 4'-amino-4-methoxy chalcone (IC$_{50}$ 31.75 µg/mL). Lower activity was showed by 4'-amino-3,4,5-trimethoxy chalcone with IC$_{50}$ value of 49.04 µg/mL. There is a relationship cytotoxicity with chalcone structure, the more the number of methoxy groups in ring B chalcone, will decrease the activity of cytotoxicity.

1. Introduction
Chalcone (1,3-diphenyl propenon) is known to have a variety of pharmacological activities, such as anticancer [1], antiproliferative [2], antimicrobial [3] and [4], insecticides [5] and antioxidants [6. This compound is the main precursor in the synthesis of a variety of heterocyclic compounds, therefore it is very important to develop the latest strategies in the synthesis of chalcone.

Chalcone can be synthesized by Claisen-Schmidt condensation, the condensation reaction between aromatic aldehydes and ketones to form aromatic ketone α, β-unsaturated. Solvent in the synthesis reaction chalcone derivatives are hazardous to health and not environmentally friendly. One of the principles of green synthesis is the removal of solvent in the synthesis process, can be made by grinding technique. Modification of synthesis of chalcone with grinding techniques ever conducted for the synthesis of chalcone of 2-acetyl-1-naphtol and benzaldehyde [7]. Synthesis using this technique requires no solvent, the reaction time is short (4-8 minutes) and high yield (84-95%). Grinding technique has also been used for the chalcone synthesis of cyclohexanone and benzaldehyde, yield 96-98% [8]. Susanti et al., (2014) have synthesized hydroxy chalcone of hydroxy acetophenone with grinding techniques, giving 70-84% yield [9][10]. However, the grinding technique has never been
used for the synthesis of amino chalcone derivative from methoxy benzaldehyde and amino acetophenone.

Kumar et al., (2008) have synthesized several chalcone derivatives by several methods, i.e reflux, ultrasonic irradiation and grinding techniques [16]. Kumar explained that the synthesis with grinding technique gives better results than using ultrasonic irradiation and reflux, yield 83-92%, 5-80% and 24-89%. Rateb and Zohdi (2009) have synthesized several chalcone derivatives by grinding technique using NaOH catalyst, with good results (yield 71-95%) [14]. Zangade et al. (2011) also have synthesized derivatives of 2-hydroxy chalcone with grinding technique using KOH catalyst, yield 85-94% [7]. Susanti, et al. (2014) have also been synthesized chalcone derivatives of 4-hydroxy acetophenone and veratraldehyde using grinding techniques and provide greater yield (70-84%) compared to the conventional chalcone synthesis (45-75%) [9].

Chalcone activity as anticancer and antioxidant is a topic of interest in recent years. Antioxidants are substances that protect cells from damage caused by free radicals. Some chalcones reported to have antioxidant activity include 2'-hydroxy-2,4'-dimethoxy chalcone and 4'-hydroxy chalcone [11], 2'-hydroxy chalcone, 2'-hydroxy-4-methoxy chalcone, 2'-hydroxy-4-chloro chalcone, 2'-hydroxy-2-chloro chalcone, 2'-hydroxy-2-bromo chalcone dan 2'-hydroxy-3,4,5-trimethoxy chalcone [6].

It has been widely reported that flavonoids have potential as positive and negative regulators so that potential as anticancer. One class of flavonoids that have the potential as an anticancer is chalcone and its derivatives [12]. Some chalcones reported to have anticancer activity include 2'-methyl chalcone, 4'-bromo-4,6-dimethoxy chalcone, 4'-phenoxy-2-methoxy-6-flouro chalcone, and 4'-methoxy-3-bromo-6-methoxy chalcone (Upendra, et al, 2014).

Chalcone pharmacological activity generally depends on the number and position of the hydroxy group, methoxy and other groups on the rings A and B, so that the research will be synthesized 3 chalcone derivatives that have variations of methoxy group in ring B of chalcone. This Chalcone will be studied its activities as antioxidant and anticancer. Synthesis of amino chalcone derivatives is very important, as the scientific basis for the development of antioxidant and anticancer, and study the relationship the chalcone structure with antioxidant and anticancer activities.

2. Experimental

2.1. General procedure for synthesis of chalcone

The chalcone were synthesized as mentioned in the reference [9]. A mixture of 4-aminoacetophenones (5 mmol), benzaldehyde (10 mmol) and solid pallete of NaOH (20 mmol) was ground with a mortar and pestle for 15 minutes at room temperature. The obtained solid mixture was diluted with cold water, acidified with cold HCl (10%), and extracted with ether. The ether layer was washed with water, followed by drying over anhydrous Na2SO4 and the solvent was evaporated. The reaction mixture was purified by column chromatography on a silica gel column with hexane:ethyl acetat (6:4) as eluent, followed by recrystallization. Chalcone synthesized were characterization used by GC-MS.

2.2. In vitro antioxidant activity assay

The chalcone synthesized were assessed for antioxidant activity using 1,1-biphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. The DPPH assay was based on the reported methods [10]. Samples were dissolved in methanol and prepared in various concentrations (0, 10, 30, 50 and 70 ppm). Each solution is put into a test tube. Into each tube was added 500 µL solution of 1 mM DPPH in methanol, refined to 5.0 mL, and then incubated at 37°C for 30 minutes. The solution was measured at λ 515 nm. Positive controls use is vitamin C.

2.3. In vitro anticancer activity assay

Cell treatment was performed by adding each of the 100 ul-cell suspensions with a density of 2X104 / 20,000 cells / well (at 96 well plates). Then let stand for 1-2 hours. After that add 100 µL sample with various concentration dose. Incubation in CO2 incubator for 24 hours (5% CO2, 37°C, 98% moisture), after 24 hours seen under a microscope and photographed. Then discard the existing medium (in
reverse way) on the tissue paper (for the attached cell). Add 100 ul MTT (5 mg MTT, 1 ml PFS, 9 ml medium RPMI complete /medium grower) at each well. Incubate for 4-6 hours. Add Stop Solution 100ul at each well, and incubation overnight. Read on ELISA Reader at 550 nm wavelength. The number of living cells is proportional to the absorbance. Calculate live cell percentage and IC<sub>50</sub>.

3. Results And Discussion
Chalcone been synthesized through Claisen-Schmidt condensation reaction of acetophenone and benzaldehyde in the presence of NaOH. Three amino chalcone derivative was synthesized through Claisen-Schmidt condensation reaction between 4-amino acetophenone and benzaldehyde derivatives (Figure 1). Benzaldehyde derivatives used were 4-methoxybenzaldehyde, 3,4-dimethoxybenzaldehyde, and 3,4,5-trimethoxybenzaldehyde.

![Figure 1. Synthesis Scheme of chalcone derivatives](image)

The compounds synthesized in the form of giving a yellow orange color with Mg/HCl, showed positive flavonoids. The yield of the compounds 31.2 to 74.4% (Table 1). Structure of the synthesized compounds were confirmed by GC-MS.

| Compounds                        | Yield (%) |
|----------------------------------|-----------|
| Chalcone 1 (4'-amino-4-methoxy chalcone) | 54.4      |
| Chalcone 2 (4'-amino-3,4-dimethoxy chalcone) | 74.4      |
| Chalcone 3 (4'-amino-3,4,5-trimethoxy chalcone) | 31.2      |

Identification of chalcone with GC-MS gave chromatogram and mass spectra. GC chromatogram for chalcone 1 showed 9 peaks, the most dominant being peak 8 at retention time (tR) 29.09 min with a relative purity of 71.4%. The peak was indicated as chalcone 1, evidenced by the mass spectra (Figure 2) showed the presence of molecular ions (M+) as the peak base corresponding to the molecular weight of chalcone 1 (253). The fragmentation pattern of the Chalcone 1 shows molecular ions (M +) with loss CH<sub>3</sub> radicals gave m/z 238. The fragment m/z 161 with the loss of C<sub>6</sub>H<sub>4</sub>NH<sub>2</sub> radical, while the m/z 92 fragments release the radical C<sub>3</sub>H<sub>6</sub>O<sub>2</sub>.

![Figure 2. Mass spectra of chalcone 1](image)
GC chromatogram for chalcone 2 shows 5 peaks, with peak 5 as the dominant peak at retention time (tR) 30.6 min and relative purity 8.68%. The peak is indicated as chalcone 2, shown from the mass spectra (Figure 3) of the presence of molecular ions (M +) as a peak base corresponding to the molecular weight of chalcone 2 (283). The fragmentation of the Chalcone 2 shows molecular ions (M +) with loss CH$_3$ radicals gave m/z 268. The fragment m/z 253 with the loss of OCH$_3$ radical. The loss of C$_6$H$_2$NH$_2$ radical gave fragment m/z 191.

Figure 3. Mass spectra of chalcone 2

Chalcone 3 gave a GC chromatogram (Figure 6), where peak 7 at retention time (tR) of 32.8 minutes and relative purity of 19.35% was suspected as chalcone 3, shown from the mass spectra (Figure 4) showing molecular ions (M +) At 313 as a peak base corresponding to the molecular weight of chalcone 3. The dominant peak at retention time of 16.2 was the peak of the 3,4,5-trimethoxy benzaldehyde, showing the reactants have not been fully synthesized. The fragmentation of the Chalcone 3 shows molecular ions (M +) with loss CH$_3$ radicals gave m/z 298. The fragment m/z 282 with the loss of OCH$_3$ radikal.

Figure 4. Mass spectra of chalcone 3

The antioxidant activity of chalcone synthesized exhibited good antioxidant properties, with the strongest being observed in Chalcone 1 (Table 2). However, all the synthesized compounds were less potent than vitamin C as the reference. The potencies for the antioxidant activity of the test compounds to the reference drug are in the following order: Vitamin C > Chalcone 1 > Chalcone 2 > Chalcone 3.

Table 2. The antioxidant activity of chalcone synthesized

| No | Compounds                                      | IC$_{50}$ (µg/mL) | Categories |
|----|-----------------------------------------------|-------------------|------------|
| 1  | Chalcone 1 (4'-amino-4-methoxychalcone)        | 58.85             | strong     |
| 2  | Chalcone 2 (4'-amino-3,4-methoxychalcone)      | 64.79             | strong     |
| 3  | Chalcone 3 (4'-amino-3,4,5-trimethoxychalcone) | 210.27            | weak       |
| 4  | Vitamin C                                      | 2.59              | Very strong|
The antioxidant activity of the three chalcone showed that chalcone 1 and 2 have strong antioxidant activity with IC\textsubscript{50} values 58.85 and 64.79 µg/mL respectively, chalcone 3 has a weak antioxidant activity. These results also indicate that there is a relationship between the structure of chalcone with antioxidant activity. The more methoxy groups in the ring B of the chalcone, antioxidant activity is getting smaller. Chalcone 3 with three methoxy groups in ring B provides antioxidant activity decrease is very large.

The chalcone synthesized showed cytotoxicity against HeLa cell line with IC\textsubscript{50} value of 31.75, 36.65, 49.04 µg/mL, respectively (Table 3). It was observed that the highest cytotoxic activity was found at chalcone 1 (IC\textsubscript{50} 31.75 µg/mL). Lower activity was showed by Chalcone 3 with IC\textsubscript{50} value of 49.04 µg/mL. There is a relationship cytotoxicity with chalcone structure, the more the number of methoxy groups in ring B chalcone, will decrease the activity of cytotoxicity.

Table 3. Cytotoxic Activity of Chalcone synthesized on HeLa Cell

| No | Compounds | IC\textsubscript{50} (µg/mL) |
|----|-----------|-----------------------------|
| 1  | Chalcone 1 (4'-amino-4-methoxy chalcone) | 31.75 |
| 2  | Chalcone 2 (4'-amino-3,4-methoxy chalcone) | 36.65 |
| 3  | Chalcone 3 (4'-amino-3,4,5-trimethox chalcone) | 49.04 |

4. Conclusion

In summary, a series of novel chalcones have been synthesized by grinding technique. Their in vitro antioxidant and cytotoxic activities against HeLa cell line were also reported. On this basis, the SAR analysis and potential antioxidant and anticancer antitumor activities of these chalcone are under investigation. 4'-amino-4-methoxychalcone with a substitution of methoxy at position 4 at ring B of chalcone were the most effective antioxidant and cytotoxic compounds. These results may be helpful for the design of future antioxidant and anticancer reagents, and offer potential application in the discovery of anticancer drugs.

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