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Anticancer activity in 2-methoxy-4-((4-methoxy-phenylimino)-methyl)-phenol compound on T47D breast cancer cells

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Abstract. 2-methoxy-4-((4-methoxyphenylimino)-methyl)-phenol compound is a Schiff base compound synthesized from vanillin and p-anisidin. The purpose of this research is to determine the stability of the 2-methoxy-4-((4-methoxyphenylimino) methyl) phenol compound and to test the anticancer activity of 2-methoxy-4-((4-methoxyphenylimino) methyl) phenol in inhibiting T47D breast cancer cells. The stability of the 2-methoxy-4-((4-methoxy-phenylimino)-methyl)-phenol compound was carried out by characterization using chemical tests, identification using FTIR and GC-MS. While the anticancer activity test of compound 2-methoxy-4-((4-methoxyphenylimino) methyl) phenol using the MTT method. The results of re-characterization using chemical tests, identification of FTIR and GC-MS showed the compound was still stable. The IC₅₀ value of compound 2-methoxy-4-((4-methoxyphenylimino) methyl) phenol was 353,038 µg / mL which showed weak activity in inhibiting T47D breast cancer cells.

1. Introduction

Cancer is the second disease that causes death worldwide after cardiovascular disease and more than 70% of cancers occur in developing countries. Deaths due to disease are increasing in 2030 [1]. According to WHO, cancer patients every year increase by around 6.25 million people [2].

Breast cancer is the most common cancer among women in the world with an incidence that increases 1-2% every year [3]. In Indonesia, this cancer is ranked second after cervical cancer with 17.77% incidence cases [4]. So, to reduce the percentage of patients with breast cancer, it is necessary to develop a chemopreventive agent or a compound that can prevent and inhibit the development of cancer.

The breast cancer cell used in this study was T47D. T47D is a continuous cell line isolated from breast ductal tumor tissue in a 54-year-old woman. Continuous cell line is often used in cancer research in vitro because this cancer cell is easy handling, has unlimited replication ability, high homogeneity, and this cancer cell is easily replaced with frozen stock in case of contamination. The cell line is cells subcultured from primary cultures, or cells originating from organs or tissues that are cultured under appropriate hormonal conditions [5].

Schiff base compounds are primary amine condensation products with carbonyl compounds. The main characteristic of this compound structure is that it has the general formula RHC = N-R1, where R and R1 are alkyls, aryl, cycloalkyl or heterocyclic [6]. Imines or Schiff base is also a group of compounds that have important biological roles as antioxidants [7], anti-inflammatory and analgesic agents [8], antifungal [6], antimalarials [9], antibacterial [10]. In addition, Schiff base compounds can also function as anticancer compounds such as studies conducted by Mokhles et al. [11] and Gupta et al. [12].



Research conducted by Mokhles et al. states that the Schiff base compounds resulting from the reaction between salicylaldehyde and 2-amino-4-phenyl-5-methylthiazole have anticancer activity against HepG2, MCF-7, and HCT116 cells with IC_{50} values consecutively $9,22 \mu\text{g mL}^{-1}$, $10,00 \mu\text{g mL}^{-1}$, dan $9,50 \mu\text{g mL}^{-1}$. Other research was also carried out by Gupta et al. (2015) which stated that schiff base product of 2,4-dihydroxybenzaldehyde with various primary amines of butylamine, aniline, and 4-(1H-benzo[d]imidazole-2-yl)aniline had anticancer activity against PC3 cells. The test was carried out by the MTT method which yielded IC_{50} values consecutively $7.43 \mu\text{M}$, $7.15 \mu\text{M}$, and $4.83 \mu\text{M}$. Meanwhile, based on research conducted by Zhao et al.[13], Schiff base compounds derived from dehydroabietilamine have anticancer activity against Hela cells, HepG2, MCF-7, A549, HUVEC. As in the imidazole-2-formaldehyde-dehydroabietilamine compound IC_{50} values consecutively were $3,22 \pm 0,02 \mu\text{M}$, $0,24 \pm 0,01 \mu\text{M}$, $4,28 \pm 0,70 \mu\text{M}$, $3,17 \pm 0,05 \mu\text{M}$ dan $7,67 \pm 0,43 \mu\text{M}$.

Based on these studies, an anticancer activity test was carried out on 2-methoxy-4-((4-methoxyphenylimino) methyl) -phenol compounds synthesized by Adawiyah (2017) [14] on T47D breast cancer cells. Before conducting anticancer tests, characterization was first performed using FTIR and GC-MS as well as chemical testing with NaOH solution to ensure the compound was not damaged due to storage.

2. Methods

2.1. Materials

2-methoxy-4-((4-methoxyphenylimino)methyl)phenol Compound that has been stored ± 1 year (Sample A), vanillin, p-anisidine, 2M NaOH, aquades, chloroform, PBS (Phosphate Buffered Saline), trypsin-EDTA, RPMI (Roswell Park Memorial Institute) culture media, DMSO (Dimethyl Sulfoxide), MTT 5 mg / mL (50 mg MTT and 10 mL PBS), SDS (Sodium Dodecyl Sulfate) 10%, HCl 0.1 M.

2.2. Methods

2.2.1 Characterization 2-methoxy-4-((4-methoxyphenylimino)-methyl)-phenol Compound Using Chemistry Test. A chemical test sample of product A was tested in the water solvent. 0.01 gram of sample A product is put into a test tube, then add 5 mL of aquades. The mixture is shaken with a stirring distance of 10 cm. if the product A sample is not completely dissolved, then add 2 M NaOH drops per drop ($\pm 20 \mu\text{L}$) and observe the changes that occur.

2.2.2 Characterization 2-methoxy-4-((4-methoxyphenylimino)-methyl)-phenol Compound Using FTIR. Identification of functional groups of phenol compounds from 2-methoxy-4-((4-methoxyphenylimino)-methyl)-phenol compounds in sample A using FTIR VARIAN type FT-1000 spectrophotometer. Then A sample mixed with KBr and crushed in an agate mortar. Then the mixture is pressed and formed into a pellet, then the pellet is placed in a cell holder in the FTIR instrument and the FTIR spectrum is made in the range of $4000-400 \text{ cm}^{-1}$ wavenumbers.

2.2.3 Characterization 2-methoxy-4-((4-methoxyphenylimino)-methyl)-phenol Compound Using GC-MS. A total of $1 \mu\text{L}$ of sample A dissolved in chloroform at a concentration of 70,000 ppm was injected using a syringe into the KG-SM VARIAN CP-3800 SATURN 2200 injector.

2.2.4 Test of Anticancer Activity. Samples A, Vanillin, and p-anisidin were prepared in a series of concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.63 ppm. Each concentration series is made three times. T47D breast cancer cell suspension (50×10^4 cells/mL) was put into a plate containing 96 wells and incubated for 24 hours. After 24 hours the media was removed and washed with PBS, a series of concentrations of sample A, vanillin, p-anisidin and doxorubicin were added to the well and then incubated for 24 hours. At the end of the incubation, the solution in the dish is discarded and washed with PBS then MTT reagent is added and incubated $37^\circ\text{C}/5\% \text{ CO}_2$ for 2-4 hours.

After formazan salt is formed, 10% SDS stopper and 0.1 N HCl are added. Then the plates were incubated in a dark place at room temperature overnight. After that, the treatment results are read out

the absorbance results using an Elisa reader with a wavelength of 595 nm. Living cells will react with MTT to form a purple color. Then calculate the percentage of living cells with the following equation.

$$\% \text{living cell} = \frac{(\text{absorbance of the treatment} - \text{absorbance of media control})}{(\text{absorbance of negative control} - \text{absorbance of media control})} \times 100\% \quad (1)$$

3. Results and discussion

3.1. Characterization 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol Compound Using Chemistry Test

Chemical tests on sample A were carried out to determine the presence of phenolic groups in sample A. The test was based on the principle of the Bronsted-Lowry acid-base reaction, a reaction involving proton transfer. The compound 2-methoxy-4-((4-methoxyphenylimino)-methyl)-phenol acts as an acid and NaOH acts as a base. The acid-base reaction will produce salts that are soluble in water.

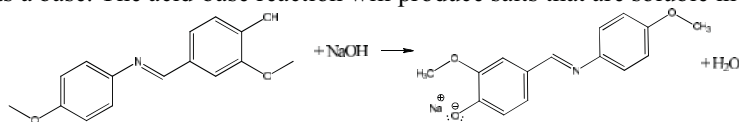


Figure 1. Bronsted-Lowry acid-base reaction.

Schiff base compound 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol is a phenolic compound that has acidic properties and can release H⁺ ions from the hydroxyl group. The OH⁻ ion in NaOH attacks the H⁺ ion in the phenolic group of Schiff base compounds and forms H₂O shown in figure 1. While Na⁺ ions will replace the loose H⁺ ions in Schiff base compounds to form sodium salt that can dissolve in water.

3.2. Characterization 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol Compound Using FTIR

Characterization of sample A by using an FTIR spectrophotometer was performed to determine the functional groups contained in the sample. Spectra analysis has been obtained compared to initial characterization before storage. It aims to find out that sample A is still stable and not damaged due to storage shown in figure 2.

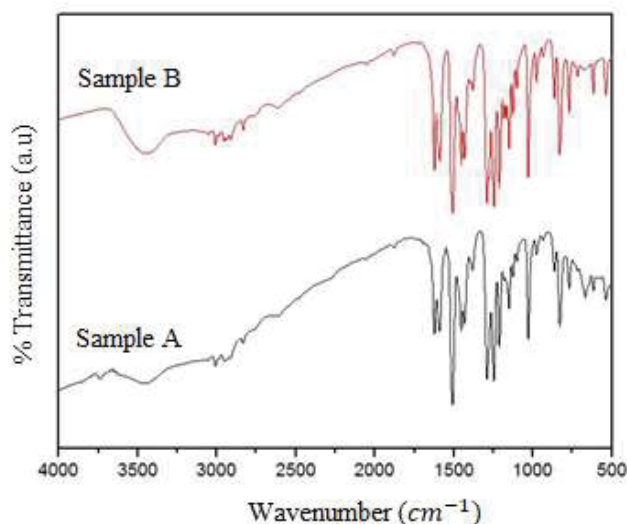


Figure 2. Results of FTIR spectra of 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol after being stored for ± 1 year (A) and 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol synthesized beginning (B).

Table 1. Results of FTIR identification of 2-methoxy-4-((4-methoxyphenylimino)-methyl) phenol compound.

Functional Groups	Wave Number (cm ⁻¹)	
	Sample A	Sample B
-OH <i>stretch</i>	3449	3451
C _{sp2} -H <i>stretch</i> aromatic	3009	3009
C _{sp3} -H <i>stretch</i> alifatic	2954	2950
Overtone aromatic	2050-1879	2050-1880
C=C aromatic	1623 dan 1508	1623 dan 1506
-C=N- <i>stretch</i>	1590	1590
Ph-O-C asymmetric	1247	1246
C-O <i>stretch</i> phenol	1212	1212
Ph-O-C symmetric	1028	1029
-CH ₂ <i>bend</i> aromatic	829	831

Sample A : 2-methoxy-4-((4-methoxyphenylimino)-methyl)-phenol compound after being stored for ± 1 year.

Sample B : 2-methoxy-4-((4-methoxyphenylimino)-methyl)-phenol compound in initial synthesis (2017)

The re-characterization results on the FTIR spectrophotometer showed that in table 1 and figure 2, the wavenumber values obtained were slightly different from the initial characterization. The difference in the IR spectra before storage with the re-characterization is seen in the typical widening intensity of the -OH group in the area of 3400-3450 cm⁻¹ shown in table 1. However, the difference obtained from each functional group is not too significant and there are only a few differences in wavenumbers with the initial characterization before storage. This shows that 2-methoxy-4-((4-methoxyphenylimino)-methyl)-phenol compound is structurally unchanged and the compound is not damaged during storage.

3.3 Characterization 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol Compound Using GC-MS

The characterization results using GC-MS have produced 3 peaks in the chromatogram results as shown in Figure 3 in sample A. The first peaks appear at a retention time of 9.319 minutes and 0.86% level. While the second peak appeared at a retention time 14.990 minutes and 4.87% level. The third peak appeared at a retention time of 26.791 minutes and 94.267% levels. This shows that the third peak has the most dominant level than the results of the other peaks.

Based on the results of the sample A chromatogram that has been obtained, these results have differences with the results of the sample chromatogram B. The difference in the chromatogram lies in the number of chromatograms obtained, which are 3 peaks in the re-characterization, and 2 peaks in the initial characterization with different retention times. This can be caused by differences in the tools used, in sample A using KG-SM VARIAN CP-3800 SATURN 2200. While sample B using KG-SM QP-2010S / Shimadzu. In addition, in sample A, the peaks of the chloroform solvent were excluded or removed so that the small peaks that appeared could be read. Whereas in sample B the chloroform solvent was included in the final result so that the small peaks produced were not readable at retention time and the area.

Sample B levels reached 97.66%, while the results of the characterization of sample B decreased to 94.267%. The decrease in product yields accompanied by the appearance of another peak which is a reactant as shown in Figure 3 in sample A can be caused due to a backlash event on the results of the synthesis product. The back reaction can occur due to the hydrolysis of the 2-methoxy-4- ((4-methoxyphenylimino) methyl) -phenol compound which can be caused by storage with a long period of time accompanied by high humidity.

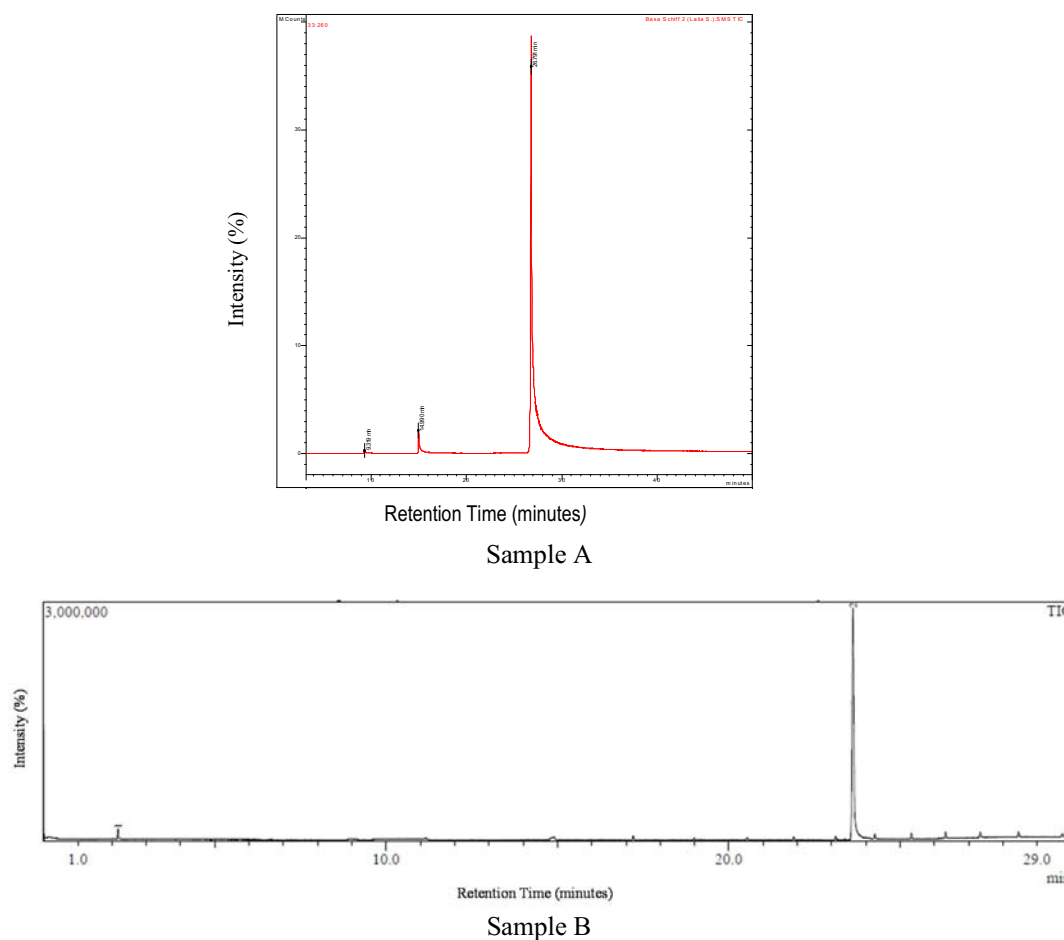


Figure 3. Spectra Result of chromatogram from sample A of Schiff base compound after ± 1 year of storage and Initial Characterization of Sample B before storage.

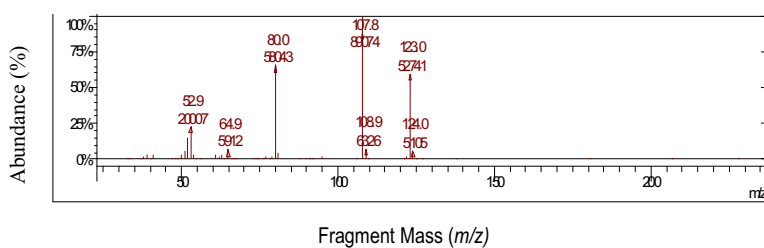


Figure 4. Peak 1 Mass Spectra of Sample A.

The results of peak 1 mass spectra analysis in sample A are shown in Figure 4. The peak mass spectra 1 in sample A has a molecular ion with m/z 123 whose value is equal to the molecular weight of p-anisidin, so peak 1 is thought to be a p-anisidin compound. p-anisidin (C_7H_9NO) is a reactant of the compound in sample A.

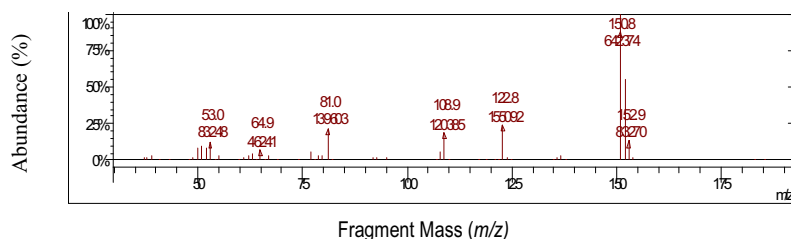


Figure 5. Peak 2 Mass Spectra of Sample A.

The results of peak 2 mass spectra analysis in sample A are shown in Figure 5. Peak mass spectra 2 have m/z 152 molecular ions whose value is equal to the molecular weight of vanillin, so peak 2 is thought to be a vanillin compound which is a reactant of the 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol compound.

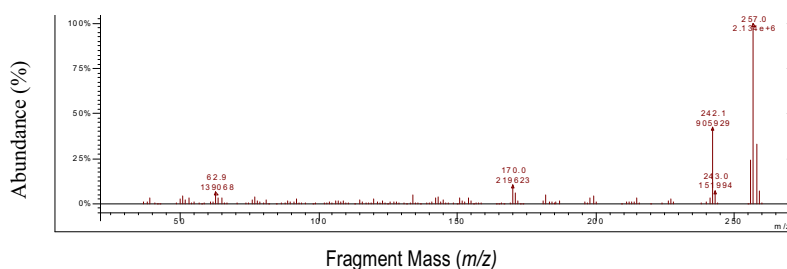


Figure 6. Peak 3 Mass Spectra of Sample A.

The peak mass spectra 3 in Figure 6. shows that the value of m/z 257 with an abundance of 100% is molecular ion and base peak. The molecular ion value of m/z 257 corresponds to the molecular weight of the 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol compound.

3.4. Anticancer Activity Test

Anticancer activity testing is carried out to determine the ability of 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol compound to inhibit the growth of breast cancer cells. Testing was carried out in vitro against T47D breast cancer cells with the MTT method. The sample concentration used 1000; 500; 250; 125; 62.5; 31.25; 15.63 ppm.

Table 2. IC₅₀ values for Anticancer Activity Test.

Sample	IC ₅₀ (µg/mL)
2-methoxy-4-((4-methoxyphenylimino)methyl)phenol (sample A)	353,038
Vanillin	845,134
p-Anisidin	176, 642
Doxorubicin	0,627

Based on Table 2. shows that sample A has weak activity in inhibiting T47D breast cancer cells with IC₅₀ 353,038 µg/mL, and vanillin has lower activity compared to sample A with IC₅₀ 845,134 µg/mL, and p-anisidin is quite active in inhibits T47D breast cancer cells with IC₅₀ 176,642 µg/mL. The positive control results of Doxorubicin showed that the compound was very active in inhibiting T47D breast cancer cells with IC₅₀ 0,627 µg/mL. This is based on the US National Cancer Institute when IC₅₀ values of ≤20 µg/mL show very toxic results, IC₅₀ values of 21-200 µg/mL indicate sufficiently toxic or

moderately active. When IC_{50} values of 201-500 $\mu\text{g/mL}$ indicate weak oxidation, and when $IC_{50} \geq 500$ $\mu\text{g/mL}$ show non-toxic results.

Schiff base compounds are generally biosynthetic alkaloid compounds [15], where alkaloids can inhibit the activity of T47D breast cancer cells in the G1 phase by increasing p53 which is a protein that inhibits cancer cells [16]. The effect of anticancer activity from sample A can be influenced by the phenolic group who are in the position of para who can provide greater activity. As research conducted by Mohamed et al. [17] shows that the compound 4-[(3-Ethoxy-4-hydroxy-benzylidene)-amino]-benzenesulfonamide which is also a Schiff base compound which has a phenolic group in position para can be active in inhibiting breast cancer cells with an IC_{50} value of 96 μM . Whereas the compound 4-[(2-Hydroxyphenyl)methylidene]amino}benzenesulfonamidewhich has a phenolic group in the ortho position has an IC_{50} value of 101 in breast cancer cells.

The activity of p-Anisidin compound in inhibiting T47D breast cancer cells cannot be used in the development of further chemopreventive agents. This is because the p-Anisidin compound can actually cause cancer if it enters the body (Merck Chemicals). In addition, based on research conducted by Yoshida et al. [18] that the p-anisidin compound is a nephrotoxic compound against 344 male Fischer rats, which means that the p-anisidin compound can cause kidney damage if it enters the body.

4. Conclusion

Based on the re-characterization of 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol compound using chemical test, identification with FTIR and GC-MS showed that the Schiff base compound was stable, and was not damaged by storage. The results of the anticancer activity of 2-methoxy-4-((4-methoxyphenylimino)methyl)phenol compounds have weak activity in inhibiting T47D breast cancer cells with IC_{50} values of 353,038 $\mu\text{g/mL}$.

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