

SOME BENZOXAZINE AND AMINOMETHYL DERIVATIVES OF EUGENOL: CYTOTOXICITY ON MCF-7 CELL LINE

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Received: 23 Jan 2015 Revised and Accepted: 25 Feb 2015

ABSTRACT

Objective: The objective of this research is to examine *in vitro* cytotoxicity of some benzoxazine and aminomethyl derivatives of eugenol against the MCF-7 cancer cell line.

Methods: 6-Allyl-8-methoxy-3-phenyl-3,4-dihydro-2H-benzo[e][1,3]oxazine, 6-allyl-3-benzyl-8-methoxy-3,4-dihydro-2H-benzo[e][1,3]oxazine, 6-allyl-3-(furan-2-ylmethyl)-8-methoxy-3,4-dihydro-2H-benzo[e][1,3]oxazine, 4-allyl-2-methoxy-6-(phenylaminomethyl)phenol, 4-allyl-2-(benzylaminomethyl)-6-methoxyphenol, and 4-allyl-2-[[furan-2-ylmethyl]amino]methyl]-6-methoxyphenol were subjected to cytotoxicity study using MTT assay and calculate the percent of cell viability.

Results: The obtained LC₅₀ values of the tested compounds on MCF-7 cancer cell line are respectively 96.9±6.84, 26.4±2.68, 21.7±2.90, 58.4±4.42, 29.2±2.39, and 45.7±4.62 µg/ml.

Conclusion: All the tested compounds have cytotoxicity against the MCF-7 cell line.

Keywords: Eugenol, Benzoxazines, Aminomethyl, Cytotoxicity, MCF-7.

INTRODUCTION

According to WHO, there were 8.2 million people worldwide died from cancer and 32.6 million people living with cancer in 2012 [1]. In fact, cancer is the second cause of death after cardiovascular disease [2]. Among women, cancer with the highest prevalence (25.1%), highest mortality (14.7%), and highest 5-year prevalence (36.3%) is breast cancer [1].

One of the methods of choice in cancer therapy is chemotherapy using chemicals that are able to kill or inhibit the growth of cancer cells. Chemotherapy is known for its side effects, such as hair fall, dry mouth, diarrhea, nausea and vomiting, loss of appetite, and fatigue [3]. Therefore, the invention of the more effective and more selective new anticancer substances is very important [4].

We are interested in preparing new bioactive compounds derived from eugenol for two reasons. The first is because in our country

this compound exists as a natural product in large quantity, and the second reason is because this compound has several functionalities, i.e. hydroxyl, methoxy, phenyl, and olefinic groups, giving many possibilities to be converted to bioactive compounds.

The authors have previously reported the preparation of some benzoxazine and aminomethyl compounds derived from eugenol. The authors also reported that some of the obtained compounds showed promising anticancer activity based on brine shrimp lethality test [5]. In this study, the authors wanted to examine the *in vitro* cytotoxicity of the previously synthesized compounds on MCF-7 cell line.

MCF-7 was chosen for this study because the cell line, which was derived from a patient with metastatic breast cancer in 1970, has become a prominent model system for the study of breast cancer as it relates to the susceptibility of the cells to apoptosis [6].

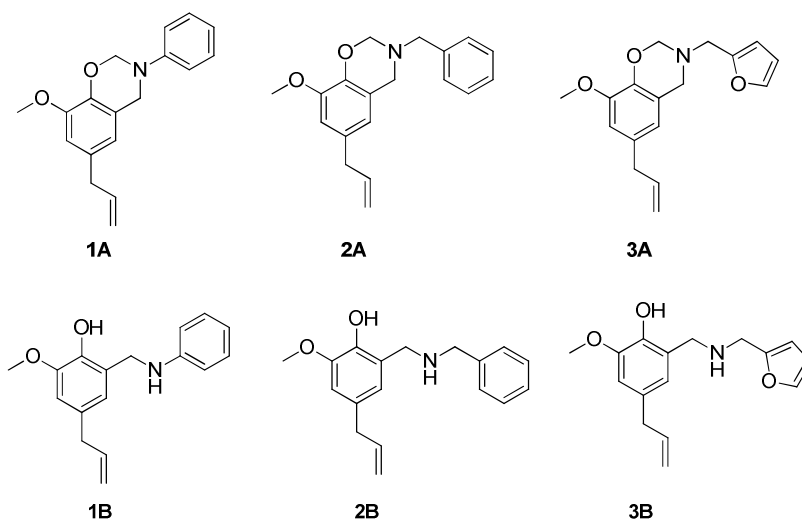


Fig. 1: Structures of testing compounds

MATERIALS AND METHOD

Materials

Test materials are compounds that were prepared as previously reported [5], *i.e.* 6-allyl-8-methoxy-3-phenyl-3,4-dihydro-2H-benzo[e][1,3]oxazine (1A), 6-allyl-3-benzyl-8-methoxy-3,4-dihydro-2H-benzo[e][1,3]oxazine (2A), 6-allyl-3-(furan-2-ylmethyl)-8-methoxy-3,4-dihydro-2H-benzo[e][1,3]oxazine (3A), 4-allyl-2-methoxy-6-(phenylaminomethyl)phenol (1B), 4-allyl-2-(benzylaminomethyl)-6-methoxyphenol (2B), and 4-allyl-2-(((furan-2-ylmethyl)amino)methyl)-6-methoxyphenol (3B) (fig. 1). DMSO (99,5%) was purchased from Sigma Aldrich. MCF-7 cells were obtained from Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University, Yogyakarta. Cell culture was grown on *Rosewell Park Memorial Institute* (RPMI) medium containing 10% (v/v) fetal bovine serum (FBS) (Gibco, Invitrogen, USA) and 1% (v/v) penicillin-streptomycin (Gibco, Invitrogen, USA). Cells were cultivated from *culture dish/flask* using 25% trypsin-EDTA (Gibco, Invitrogen, USA). MTT stock 5 mg/ml solutions were prepared by dissolving MTT (Sigma-Aldrich) in *phosphate buffer saline* (PBS). The MTT work solution was prepared by diluting the stock solution by ten times using DMEM medium. The stopper reagent is a 10% solution of sodium dodecyl sulfate (SDS) (Merck) in 0,1 N HCl (Merck).

Methods

Test solutions

Stock solutions were prepared by dissolving 10 mg of each test compound in 100 µl of DMSO. The stock solutions were then diluted with DMEM media to give 15.625 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125.0 µg/ml, 250.0 µg/ml, and 500.0 µg/ml test solutions. The assay for each concentration was performed in triplicate.

Propagation and harvesting of MCF-7 cells

All the equipments were sterilized prior to use. Cells were taken from the liquid nitrogen vessel, and then were immediately brought to 37 °C using water bath. Cell ampoule was sprayed with 70% ethanol and moved to LAF. The ampoule was opened and cells were moved to a sterile conical tube containing medium. The cell suspension was centrifuged at 650 RPM for three minutes, then supernatant was removed. To the precipitate was added the new medium, and the mixture was slowly homogenized. Cells were cultivated in tissue culture flasks and were incubated at 37 °C under

5% CO₂ flow. After 24 hours, the medium was changed, and the cells were incubated again until becoming confluent and the amount was suitable for assay. Medium was removed, and the cells were washed twice with PBS. After addition of trypsin-EDTA 0.25%, cells were incubated for 3 minutes in CO₂ incubator. The medium was added to the tissue culture dish or flask, and the mixture was re-suspended until the cells were released from the dish wall. The cell suspension was then moved to the new sterile conical tube. Cells were counted with the hemocytometer and cell counter, then cell suspension with the required concentration was prepared. To the suspension was added medium until the cell concentration is 5 x 10³ cells/ml.

MTT assay


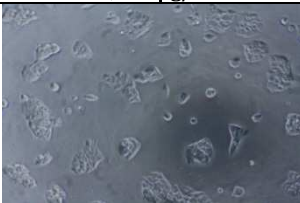
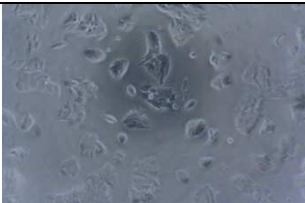

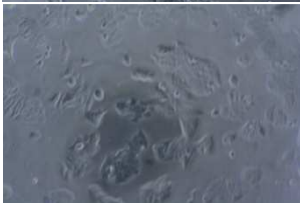
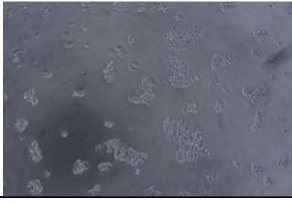
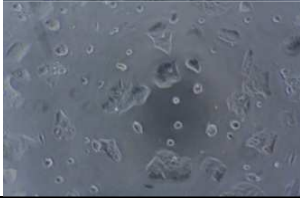
The assay was carried out following Mossman method [7] with modification in stopper reagent. MCF-7 cells were distributed into 96 well plates, then were incubated for 24 hours under CO₂. Test solutions in the series of concentrations were added, then the mixtures were incubated again for 24 hours. At the end of incubation, to each well was added MTT in PBS, then incubation was continued for 6 hours at 37 °C until formazan was formed. MTT reaction was stopped by addition of stopper reagent (sodium dodecylsulfate) followed by overnight incubation at room temperature [8]. Absorbance was read with an ELISA reader at 595 nm. The absorbance was converted to percentage of living cells (cell viability).

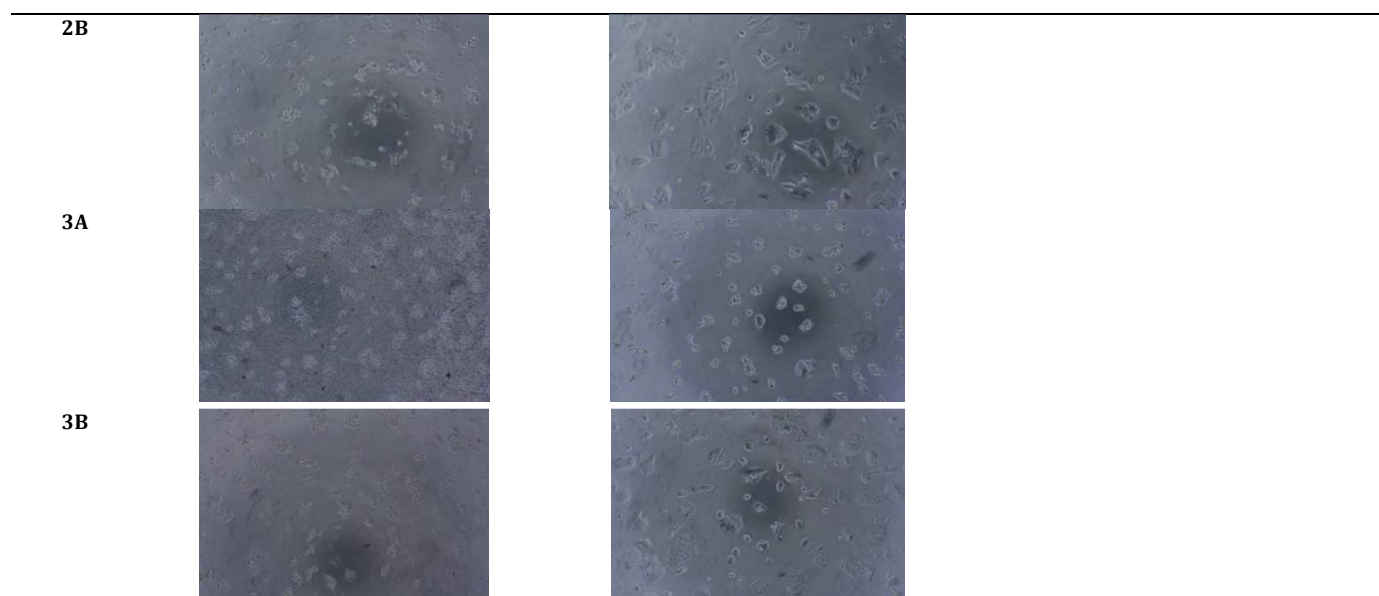
RESULTS AND DISCUSSION

Cytotoxicity assay used in this research is a colorimetric method based on the ability of the mitochondrial dehydrogenase enzyme to convert MTT, a yellow water insoluble substrate, to a dark blue formazan that is also water insoluble [9]. The formed formazan is proportional to the amount of living cells since the conversion of MTT can only happen in living cells [7]. This method was chosen because it is quick, sensitive, accurate, and is applicable for large amount of sample [8]. This method is proven to be more reliable compared to cell counting using hemocytometer [10].

In vitro anticancer activity of the test compounds against MCF-7 cells was carried out by treating 1x10⁴ cells with test solutions. Pictures in table 1 show cell morphologies that were observed by using an inverted microscope under 400 x magnifications. It is obvious that all of the compounds tested gave significant influence on the cell morphology.

Table 1: Influence of test compounds on cell morphology after 24 hours of incubation

Sample	125 µg/ml	15,625 µg/ml	Control
1A			
1B			
2A			



Percentage of living cells (cell viability) after treatment of test compounds in different concentrations are given in table 2. Cell viabilities were converted from absorbances of formazan formed after MTT treatment. It is obvious that cell viability decreased as the concentration of test compounds increased.

Table 2: MCF-7 cell viability (%) after treatment with different concentrations of test compounds

Compound	MCF-7 viability (%) on different concentrations					
	500 µg/ml	250 µg/ml	125 µg/ml	62.5 µg/ml	31.25 µg/ml	15.625 µg/ml
EU	-0.337±0.34	86.389±5.69	105.737±1.25	130.671±13.47	108.661±1.75	110.686±1.85
1A	23.593±2.59	26.059±4.08	45.351±8.34	56.293±10.13	70.778±5.52	87.034±6.27
1B	25.490±3.71	15.244±1.91	19.671±3.41	18.722±1.11	73.055±8.21	85.515±8.17
2A	-8.476±0.29	0.380±1.51	-0.696±1.47	3.099±1.44	49.209±7.02	76.154±4.37
2B	1.071±1.85	0.189±0.47	-0.756±0.43	1.512±0.76	60.176±2.51	80.529±8.34
3A	-1.414±1.26	0.471±0.50	1.145±0.46	6.465±1.40	51.246±3.19	55.017±10.63
3B	-0.875±0.30	0.943±2.21	-1.077±0.50	24.242±0.14	81.077±6.31	88.889±18.27

Table 3: IC₅₀ of test compounds on MCF-7

Test compound	IC ₅₀ (µg/ml)
Eugenol	>250
1A	96.9±6.84
1B	58.4±4.42
2A	26.4±2.68
2B	29.2±2.39
3A	21.7±2.90
3B	45.7±4.62

After calculation by probit analysis using the statistical program, IC₅₀ of all the test compounds were obtained as given in table 3. Although a virtual screening showed that eugenol might have anticancer activity [11], it did not show cytotoxicity at concentration 250 µg/ml in our experiment. On the contrary, all the tested compounds showed cytotoxicity with IC₅₀ below 100 µg/ml, indicating that all the compounds are suitable for further investigation as anticancer [12].

From the present results (table 3), it can be seen that for compound having aromatic moiety attached directly to N atom, i.e. the aminomethyl derivative (1B) is more active compared to benzoxazine derivative (1A). On the contrary, compounds having an alkyl spacer between aromatic moiety and N atom, i.e. the benzoxazine derivatives (2A and 3A) have slightly higher toxicity compared to the aminomethyl derivatives (2B and 3B). However, further study with more derivatives is required for making generalizations.

CONCLUSION

All of the tested benzoxazine and aminomethyl derivatives of eugenol showed *in vitro* anticancer activity on MCF-7 cancer cell

culture. Among the compounds tested, highest cytotoxicity was shown by 6-allyl-3-(furan-2-ylmethyl)-8-methoxy-3,4-dihydro-2H-benzo[e][1,3]oxazine (compound 3A).

ACKNOWLEDGMENT

This research was funded by Indonesian Directorate General of Higher Education (DIKTI) through Penelitian Unggulan Perguruan Tinggi scheme fiscal year 2014.

CONFLICT OF INTERESTS

Declared None

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