In vivo anticancer activity of benzoxazine and aminomethyl compounds derived from eugenol

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Abstract

Background: Indonesia is the world's primary producer of clove. In order to find new utilization for clove and new biologically active compounds, eugenol, the main constituent of clove, has been converted to its derivatives.

Objective: This study aims to examine *in vivo* anticancer activity of benzoxazine and aminomethyl compounds derived from eugenol.

Methods: Fibrosarcoma was induced by injection of benzo(a)pyrene solution. The test compounds were given per oral at 20, 40, and 80 mg/Kg body weight, once a day for 30 days.

Results: As a result, all the tested compounds showed activity in reducing the cancer incidence rate. All the tested compounds were also found to reduce tumor weight. Benzoxazine derivatives gave slightly better activity compared to aminomethyl derivatives. The strongest activity was exhibited by 6-allyl-3-(furan-2-ylmethyl)-8-methoxy-3,4-dihydro-2H-benzo(e)(1,3)oxazine.

Conclusions: All four benzoxazine and aminomethyl compounds derived from eugenol that were tested exhibited anticancer activity in mice fibrosarcoma.

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Introduction

Indonesia is the number one producer of the world's clove. According to FAO, 134,792 tones of clove were produced in Indonesia in 2019.[1] In Indonesia, clove is mostly used as an ingredient in 'kretek' cigarettes. As the demand for clove is continuously decreasing due to increasing public awareness of the negative effects of tobacco smoking on health, the authors are interested to find a way to increase the economic value of clove by finding new utilization of clove by taking advantage of the major content of clove oil, namely eugenol, as starting material for the production of new compounds which are expected to have biological activity, especially as anticancer.[2]

Eugenol is known to have pharmacological effects such as abirritation, anesthetic effect, antioxidant function, antibiotic, anti-insect, and anticancer activities, *etc*.[3,4] Efforts to increase the biological activity of eugenol by derivatization have been reported. Upadhyaya *et al*. (2014) reported the synthesis of the eugenol-copper complex to increase its fungicidal activity.[5] Pisano (2007) studied the anti-proliferative and pro-apoptotic activity of eugenol-related biphenyls on malignant melanoma cells.[6] Sudarma *et al*. (2009) added sulfonyl and amino groups to the aromatic ring,[7] while Hidalgo (2009) studied the antioxidant capacity of eugenol derivatives.[8]

Cancer is one of the ten major causes of death globally in 2019, the sixth after ischemic heart disease, stroke, chronic obstructive pulmonary disease, lower respiratory infections, and neonatal conditions.[9] Among many kinds of cancer, breast cancer is the one with the highest incidence rate, highest death rate, and highest 5-year incidence rate among women.[10] One of the most common methods to treat cancer is chemotherapy. However, chemotherapy is notorious for its adverse side effects, such as constipation, diarrhea, alopecia, loss of appetite, thrombocytopenia, anemia, neutropenia, mucositis, nausea, vomiting, neuropathy, *etc*.[11] Therefore, finding new anticancer agents with higher effectivity and selectivity will have a great impact on cancer therapy.[12]

In an effort to find new anticancer substances, using similar transformation of phenols previously reported by Takeda scientists,[13-15] the authors have transformed eugenol to its new benzoxazine and aminomethyl derivatives (Figure 1, with R = methyl, ethyl, phenyl, benzyl, and furfuryl). The authors have also carried out preliminary anticancer screening by using the brine shrimp

lethality test and found that some of the derivatives were promising.[2] The study was followed by the in vitro cytotoxicity examination of compounds that the BST test indicated cytotoxicity, *i.e.* benzoxazine and aminomethyl derivatives with R = phenyl, benzyl, and furfuryl. The results showed that all the compounds have cytotoxic activity against the MCF7 breast cancer cell line.[16,17]

In this paper, we would like to report the results of in vivo anticancer activity examination of benzoxazine and aminomethyl derivatives of eugenol, *i.e.* 6-allyl-3-benzyl-8-methoxy-3,4-dihydro-2H-benzo(e)(1,3)-oxazine (4A), 6-allyl-3-(furan-2-ylmethyl)-8-methoxy-3,4-dihydro-2H-benzo(e)(1,3)oxazine (5A), 4-allyl-2-(benzylaminomethyl)-6-methoxyphenol (4B), and 4-allyl-2-{((furan-2-ylmethyl)-amino)-methyl}-6-methoxyphenol (5B) in mice.

Materials and Methods

Materials

Commercially available materials were used as received. Test compounds, *i.e.* 6-allyl-3-benzyl-8-methoxy-3,4-dihydro-2H-benzo(e)(1,3)oxazine(4A), 6-allyl-3-(furan-2-ylmethyl)-8-methoxy-3,4-dihydro-2H-benzo(e)(1,3)oxazine (5A), 4-allyl-2-(benzyl-aminomethyl)-6-methoxyphenol (4B), and 4-allyl-2-{((furan-2-ylmethyl)-amino)-methyl}-6-methoxyphenol (5B) were prepared following procedures written in the previous paper (2). Balb/C mice were purchased from Pusat Veterinaria Farma Surabaya.

Anticancer test procedure

Ninety-one male mice that have been adapted for study, were randomly divided into 13 groups. Group I was the negative control and only given CMC-Na, group II was given the test compound 4A in a dose of of 20 mg/Kg BW, group III was given the test compound 4A in a dose of 40 mg/Kg BW, group IV was given the test compound 4A in a dose of 80 mg/Kg BW, group V was given test compound 5A in a dose of 20 mg/Kg BW, group VI was given test compound 5A in a dose of 40 mg Kg BW, group VII was given the test compound 5A in a dose of 80 mg/Kg BW, group VIII was given the test compound 4B in a dose of 20 mg/Kg BW, group IX was given test compound 4B in a dose of 40 mg/Kg BW, group X was given test compound 4B dose of 80 mg/Kg BW, group XI was given test compound 5B dose of 20 mg/Kg BW, group XII was given test compound 5B dose of 40 mg/Kg BW, group XIII was given test compound 5B doses 80 mg/ Kg BW in a frequency of once every day for one month. Once every two days (five times in ten days) the mice were induced with 0.3 mg of benzopyrene in 0.2 ml of olive oil by subcutaneous injection in the scapular region. All mice were maintained on the same diet for more than two months. Macroscopic observation of cancer growth was done every day for two months.

Results

Mice body weight during the experiment

The results of body weight observation on all the treated and control groups for two months period are shown in Table 1 and

Table 1. Mice body weight during an experiment (n=7).

Week	Sample and dose											
	5B20	5B40	5B80	5A20	5A40	5A80	4B20	4B40	4B80	4A20	4A40	4A80
1	26.96 ± 2.18	27.90 ± 1.50	27.90 ± 2.59	23.73 ± 2.89	26.20 ± 1.34	22.86 ± 2.23	25.69 ± 3.66	27.88 ± 1.54	25.19 ± 1.64	23.48 ± 2.87	22.67 ± 1.75	24.02 ± 2.59
2	28.41 ± 1.78	29.34 ± 1.81	29.10 ± 2.43	25.66 ± 3.59	28.33 ± 1.47	24.40 ± 1.75	26.73 ± 3.69	28.05 ± 1.41	26.81 ± 1.73	25.13 ± 4.15	23.96 ± 3.19	24.17 ± 3.29
3	29.76 ± 2.29	30.53 ± 1.50	29.67 ± 2.14	28.63 ± 5.73	28.50 ± 2.81	25.45 ± 1.36	27.94 ± 3.70	28.93 ± 1.47	27.60 ± 1.47	26.00 ± 4.54	25.29 ± 3.43	25.85 ± 3.60
4	30.49 ± 2.96	30.36 ± 1.84	29.86 ± 3.36	28.20 ± 5.12	29.60 ± 4.67	26.79 ± 1.97	28.89 ± 3.90	29.08 ± 2.78	28.59 ± 2.31	26.40 ± 2.35	27.93 ± 3.97	26.17 ± 3.11
5	31.21 ± 3.17	30.69 ± 2.14	31.17 ± 3.81	29.66 ± 4.57	28.80 ± 5.51	28.70 ± 2.49	29.14 ± 4.32	29.55 ± 2.89	29.11 ± 2.31	27.60 ± 2.65	28.37 ± 4.00	26.67 ± 2.99
6	32.81 ± 3.48	30.97 ± 2.88	31.16 ± 3.45	29.80 ± 5.04	29.13 ± 5.70	29.80 ± 2.85	29.70 ± 4.09	30.05 ± 2.31	30.11 ± 2.43	27.05 ± 2.00	29.19 ± 3.42	27.32 ± 3.43
7	33.59 ± 3.73	31.21 ± 3.88	31.83 ± 3.29	29.77 ± 3.81	29.03 ± 5.99	29.03 ± 2.90	32.24 ± 3.99	31.43 ± 2.71	30.23 ± 3.08	27.98 ± 1.40	28.27 ± 3.11	27.73 ± 2.52
8	33.07 ± 4.72	30.96 ± 2.57	29.87 ± 2.16	29.16 ± 6.40	28.66 ± 5.78	28.56 ± 5.68	31.79 ± 3.66	31.68 ± 1.84	30.56 ± 3.48	29.35 ± 1.10	26.99 ± 3.14	29.00 ± 2.68

Table 2. Influence of test compounds on tumor incidence rate, tumor inhibition rate, tumor weight, and tumor decrease.

Sample	Incidence Rate (%)	Inhibition Rate (%)	Weight (gram)	Decrease (%)
4A20	71	29	0.72	82.86
4A40	57	43	0.50	88.09
4A80	57	43	0.36	91.43
5A20	57	43	1.17	72.14
5A40	57	43	0.90	78.57
5A80	57	43	0.27	93.57
4B20	57	43	2.37	43.57
4B40	57	43	1.99	52.62
4B80	57	43	0.90	78.57
5B20	57	43	2.11	49.74
5B40	57	43	1.71	59.29
5B80	57	43	0.51	87.86
Benzopyrene	100	0	4.20	0.00

Figure 2. Influence of test compounds on tumor incidence and inhibition rates, tumor weight, and tumor decrease

The results of the observation on tumor incidence rate, tumor inhibition rate, tumor weight, and the results of tumor decrease calculation are shown in Table 2. Tumor incidence and inhibition rates are also presented graphically in Figure 3, while the influence of test compounds on tumor weight is shown in Figure 4, while the influence on the decrease of tumor weight is shown in Figure 5.

Discussion

Influence of test compounds on body weight

In this study, the weight of mice of all the treated and control groups were recorded eight times in the period of two months. As presented in Table 1 and Figure 2, mice in all groups show weight gain. It can also be seen that the administration of all test compounds at any concentration had no different effect on weight gain in mice. This means that the tumor growth gave no effect on the body weight of mice, or in other words, the larger the tumor formed, the slimmer the bodies of mice so that totally there was no obvious effect of test compounds on the growth of body weight.

Influence of test compounds on cancer incidence rate

As presented in Table 2 and Figure 3, the tumor incidence rate in mice of a control group is 100%. Group of mice given compound 4A with a dose of 20 mg/Kg BW experienced 71% tumor incidence. Surprisingly all other groups showed the same incidence rate, *i.e*. 57%. These data indicate that all the test compounds gave a significant effect in reducing tumor incidence compared to the control group, but there was no significant difference in incidence rate between the test compound and the dose of 20, 40, and 80 mg/Kg BW.

Compared to the previous results by Ekowati *et al*. (2012), the tumor growth inhibition rates shown by all the test compounds are higher than that of ethyl *p*-methoxycinnamate (EPMC) but are lower than that of celecoxib.[18] Since the incidence rate only shows whether or not the tumor is formed, ignoring the differences in the size of the tumor, it can be said that the incidence rate data could not fully describe the anti-tumor activity of the test compound.

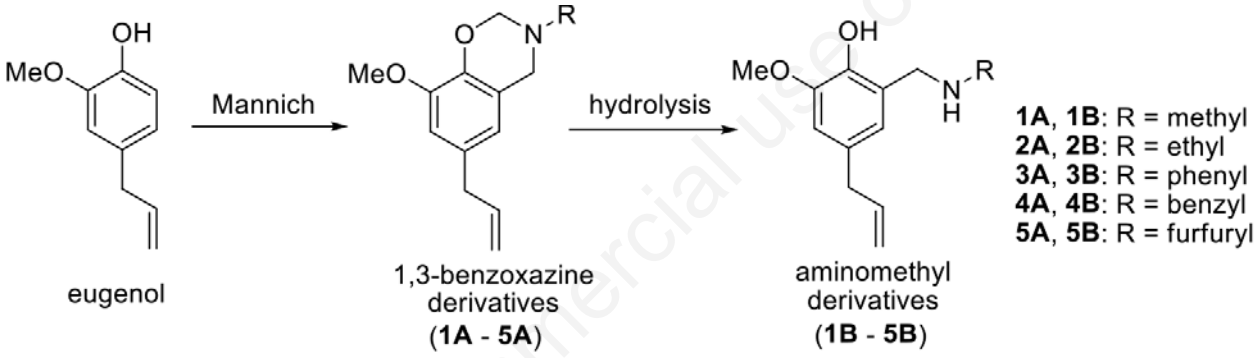

Figure 1. Transformation of eugenol to its benzoxazine and aminomethyl derivatives.

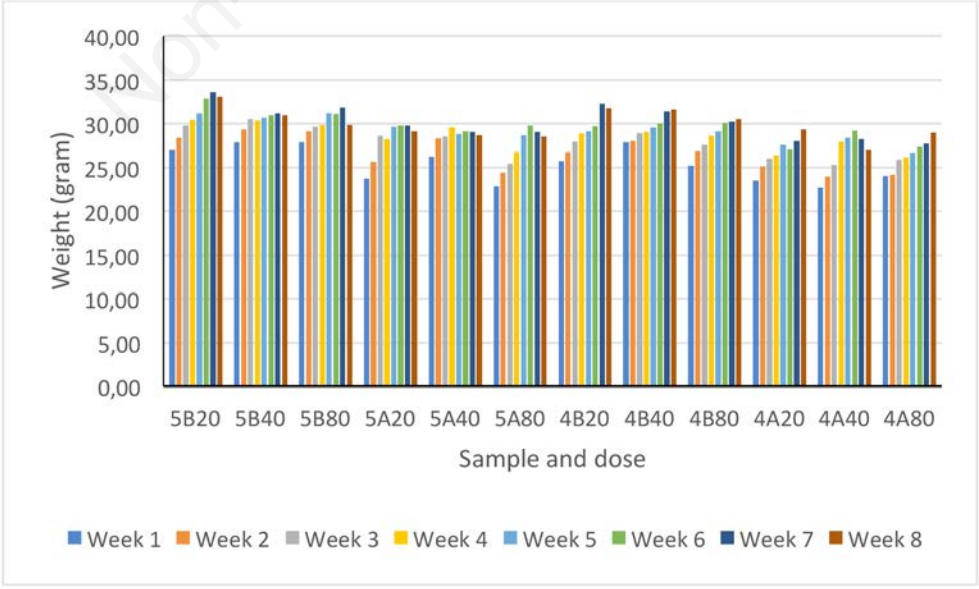

Figure 2. Mice body weights during an experiment.

Influence of test compounds on tumor weight

Figure 4 shows that the weight of the tumor in the entire groups of test compounds were smaller than in the control group. It is also obvious that for every test compound there was a relationship between the dose and the weight of the tumor, *i.e*. the higher dose has the lower tumor weight. The lightest tumors were obtained at compound 5A administration at a dose of 80 mg/Kg BW. Statistical analysis showed that there was no significant difference in median tumor weights between groups of test compounds, but there was a significant difference between the control group and test groups. The effect of test compounds appears more clearly by looking at the data percent decrease in tumor weight (Figure 5). The meaning of decrease in tumor weight is the ratio between the difference in tumor weight of the control group and a group of test compounds against the tumor weight of the control group. Compound 5A at 80 mg/Kg BW showed the highest decrease in tumor weight, *i.e*. 94%.

Structure-activity relationships

The data on tumor weight decreases (Figure 5) show that compounds 4A and 5A give a greater decrease in tumor weight compared to compounds 4B and 5B. Compounds 4A and 5A are benzoxazine compounds, whereas compounds 4B and 5B are aminomethyl compounds. Benzoxazine compounds are lacking hydroxyl and secondary amine groups, therefore they are theoretically more lipophilic; while the aminomethyl compounds have a

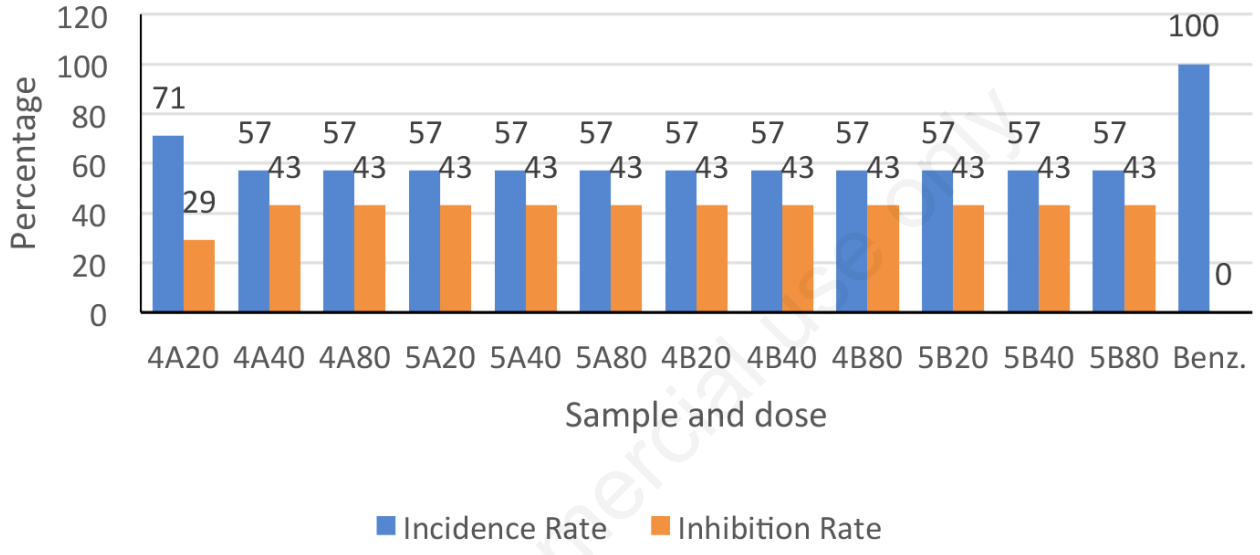

Figure 3. Incidence rate and inhibition rate.

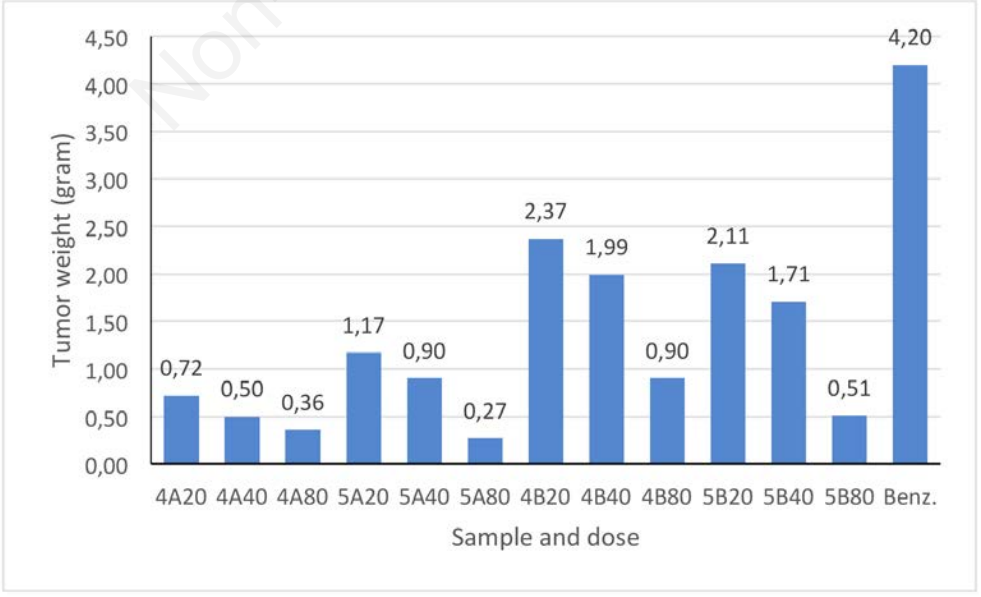

Figure 4. Influence of test compounds on tumor weight.

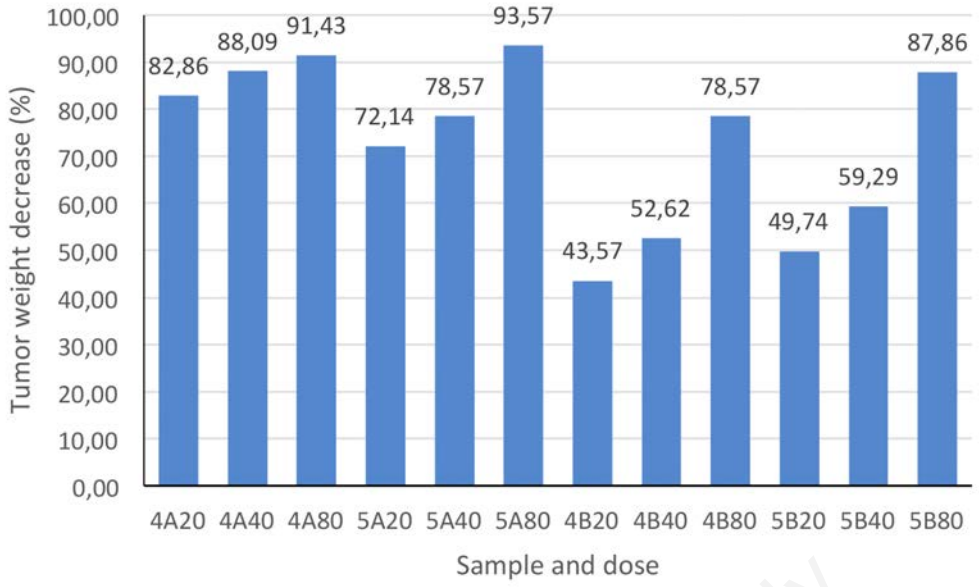

Figure 5. Influence of test compounds on a decrease in tumor weight.

free Hydroxyl (OH) and Secondary Amine (NH) groups that make them more hydrophilic. The fact that benzoxazine compounds are more active than aminomethyl compounds is probably related to their higher lipophilicity causing them more easily penetrate cell walls. It is well known that the tumor-inducing property of benzopyrene is caused by its susceptibility to oxidation into compounds that are carcinogenic. The fact that the benzoxazine compounds, which are theoretically more resistant to oxidation, are more active than the aminomethyl compounds, which are more susceptible to oxidation, leads to the speculation that the anticancer activity of these compounds is not a result of their work through an antioxidant mechanism.

Speculations that the test compound works not through an antioxidant mechanism are strengthened by the observation during an experiment that there were mice that had been showing signs of tumor formation and then experienced tumor shrinkage, so there is a possibility that the test compounds work through the mechanism of apoptosis. This is in accordance with the results of Zao *et al*. (2009) who reported a benzoxazine compound induces vascular endothelial cell apoptosis in the presence of fibroblast growth factor-2 by elevating NADPH oxidase activity and reactive oxygen species levels.[1]

Conclusions

In conclusion, all four benzoxazine and aminomethyl compounds derived from eugenol that were tested showed activity in reducing incidence rate and tumor weights. The highest activity was shown by compound 5A.

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