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## Synthesis and in vitro assay of hydroxyxanthenes as antioxidant and anticancer agents

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In the present work, three hydroxyxanthenes were synthesized in 11.15–33.42% yield from 2,6-dihydroxybenzoic acid as the starting material. The chemical structures of prepared hydroxyxanthenes have been elucidated by using spectroscopic techniques. Afterward, the hydroxyxanthenes were evaluated as antioxidant agents through radical scavenging assay; and anticancer agents through in vitro assays against WiDr, MCF-7, and HeLa cancer cell lines. Hydroxyxanthone 3b was categorized as a strong antioxidant agent ( $IC_{50} = 349 \pm 68 \mu M$ ), while the other compounds were categorized as moderate antioxidant agents ( $IC_{50} > 500 \mu M$ ). On the other hand, hydroxyxanthone 3a exhibited the highest anticancer activity ( $IC_{50} = 184 \pm 15 \mu M$ ) and the highest selectivity ( $SI = 18.42$ ) against MCF-7 cancer cells. From the molecular docking study, it was found that hydroxyxanthone 3a interacted with the active sites of Topoisomerase II protein through Hydrogen bonding with DG13 and  $\pi$ - $\pi$  stacking interactions with DA12 and DC8. These findings revealed that hydroxyxanthenes are potential candidates to be developed as antioxidant and anticancer agents in the future.

Oxidative stress diseases have been receiving great attention due to their harmful, severe, and unfavorable effects on the human body<sup>1</sup>. Several fatal diseases such as cancer, arthritis, cardiovascular, and neurodegenerative diseases are generated by oxidative stress that damages human tissue and organs<sup>2</sup>. Free radicals are reported as the main factor in the production of oxidative stress in the human body. Free radicals are very reactive; thus, they react rapidly with biomolecules such as DNA and affect cell metabolism<sup>3</sup>. Additionally, free radicals are also responsible for cholesterol and atherosclerosis deposition inside the blood vessels<sup>4</sup>. Therefore, researchers are making extensive efforts to prevent and deactivate the free radicals before damaging the cell function<sup>5</sup>.

Usage of antioxidant agents is the most straightforward approach to deactivate the free radicals<sup>6</sup>. Almost all antioxidant agents are composed of phenolic compounds because phenolic compounds are able to generate hydrogen donor and electron delocalization mechanisms to suppress the formation of oxidative stress<sup>7</sup>. Several natural and synthetic antioxidant agents such as chalcone, indole-3-acetamide, cadmium-bismuth microsphere, indazole, and salicylhydrazidehydrazone derivatives have been evaluated; however, their chemical synthesis is not easy, and their antioxidant activity is still unsatisfactory<sup>8–12</sup>.

Almost 80–95% of antioxidant and anticancer agents consist of a heterocyclic structure. Among the heterocyclic compounds, xanthone derivatives with a dibenzo- $\gamma$ -pyrone skeleton exhibit unique physicochemical properties<sup>13–16</sup>. Furthermore, xanthenes display broad pharmacological activities, which are remarkable to be further developed for the drug research<sup>17–21</sup>. Xanthone could be obtained from various plants, such as *Symphonia globulifera* and *Garcinia mangostana*<sup>22,23</sup>. Nevertheless, the isolation process is very complicated to obtain the desired xanthone derivatives in high purity. Furthermore, the isolation and purification of xanthenes require a large volume of organic solvents in a time-consuming process<sup>24</sup>. Therefore, the synthesis of xanthone is much attractive to be developed to afford xanthone derivatives in a higher yield within a faster research period. Moreover, it is possible to obtain xanthone derivatives with specific functional groups at a certain position by using a synthesis reaction, which may not be found in natural sources<sup>18</sup>.

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The synthesis of xanthone was firstly established in 1892 by Michael and Kostanecki by reacting phenol, acetic acid-*o*-hydroxybenzoate, and acetic anhydride. Unfortunately, this method generates an unfavorable side reaction; thus, the obtained yield was very low. Eaton's reagent (a mixture of  $P_2O_5$  and  $CH_3SO_3H$ ) has recently been introduced to give a higher yield for xanthone derivatives<sup>25</sup>. For example, Zhou et al. successfully synthesized 1,3-dihydroxydinitroxanthone and 7-(2,4-diphenyl)-1,3-dihydroxyxanthone in up to 76% yield; however, their antioxidant activity was still lower compared to the commercial antioxidant agents<sup>26</sup>. Because of that, synthesis and modification of other xanthone derivatives are important to find the active antioxidant agents to be used for commercial purposes.

Xanthone, benzylhydrazone, and quinoxaline derivatives have been reported to give remarkable anticancer activities<sup>16,27,28</sup>. Hydroxyxanthones exhibit good anticancer activity against lung, breast, hepatoma, cervical, colorectal, and other cancer cell lines<sup>16,29</sup>. Hydroxyxanthones could lead to cell apoptosis by stimulating the caspase enzyme<sup>30</sup>. Furthermore, hydroxyxanthones are able to inhibit Topoisomerase II protein by intercalation mechanism on the DNA cleavage sites<sup>31</sup>. This anticancer mechanism occurred as the hydroxyxanthones were constructed by three fused aromatic systems, which were reported as the essential pharmacophoric feature of Topoisomerase II inhibitors<sup>32</sup>. Furthermore, the three fused aromatic structure of hydroxyxanthones is similar to doxorubicin and mitoxantrone as standard DNA intercalators<sup>28</sup>. Therefore, hydroxyxanthones are potential to be further investigated as anticancer agents.

In our previous work, several hydroxyxanthone derivatives, i.e., 1,3-dihydroxyxanthone, 3,4-dihydroxyxanthone, 3,6-dihydroxyxanthone, 1,3,6-trihydroxyxanthone, and 3,4,6-trihydroxyxanthone have been synthesized and evaluated as the anticancer agent against WiDr cancer cell line<sup>33</sup>. In continuation of our previous research, we synthesized other hydroxyxanthone derivatives to give a broader map of their antioxidant and anticancer activities through in vitro and molecular docking studies. In this work, three hydroxyxanthone derivatives, i.e., 1,3,8-trihydroxyxanthone (**3a**), 1,6-dihydroxyxanthone (**3b**), and 1,5,6-trihydroxyxanthone (**3c**) were prepared from 2,6-dihydroxybenzoic acid with phloroglucinol, resorcinol, and pyrogallol, respectively. The chemical structure of these hydroxyxanthones was elucidated using Fourier transform infrared (FTIR) spectrophotometer, mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectrometers. These compounds were evaluated as antioxidant and anticancer agents through in vitro assay using 1,1-diphenylpicryl-2-hydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) methods, respectively. The inhibition mechanism of the most active anticancer agent against Topoisomerase II protein was further studied through a molecular docking study.

## Materials and methods

**Materials.** The chemicals used in the synthesis, i.e., 2,6-dihydroxybenzoic acid ( $C_7H_6O_4$ ), phloroglucinol ( $C_6H_6O_3$ ), resorcinol ( $C_6H_6O_2$ ), pyrogallol ( $C_6H_6O_3$ ), Eaton's reagent ( $P_2O_5$ ,  $CH_3SO_3H$ ), DPPH ( $C_{18}H_{12}N_5O_6$ ), *n*-hexane ( $C_6H_{14}$ ), ethyl acetate ( $C_4H_8O_2$ ), and methanol ( $CH_3OH$ ) were purchased from Merck in pro analytical grade. In addition, butylated hydroxytoluene (BHT,  $C_{15}H_{24}O$ ) and MTT ( $C_{18}H_{17}N_5S$ ) were obtained from Aldrich in pro analytical grade.

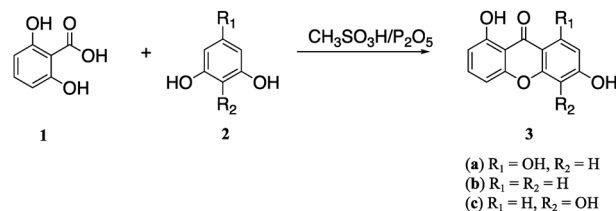
**Preparation of hydroxyxanthones.** A mixture of 2,4-dihydroxybenzoic acid (3.66 g, 15.0 mmol), the substituted phenol (phloroglucinol, resorcinol, and pyrogallol) (15.0 mmol, 1 equiv.), and Eaton's reagent (8.00 mL) was heated at 80–85 °C for 3 h. Then, the mixture was allowed to reach room temperature, and the mixture was poured with cooled water (50 mL). The resulting solid residue was collected by filtration, washed with water until neutral, dried, and then purified by preparative thin layer chromatography. In the preparative thin-layer chromatography, silica was used as the stationary phase, while a mixture of *n*-hexane and ethyl acetate 1:1 v/v was used as the mobile phase to afford the target compound. The products were then characterized using FTIR (Shimadzu-Prestige 21), MS (Shimadzu QP-2010S),  $^1H$ - and  $^{13}C$ -NMR (JEOL JNMCA 500 MHz) spectrometers.

1,3,8-trihydroxyxanthones (**3a**). The compound **3a** was obtained as a yellow solid in 16.14% yield. m.p. 239–240 °C. FTIR (KBr)  $\nu/cm^{-1}$ : 3448 (O–H), 1612 (C=O), 1418 (C=C), 1296 (C–O–C).  $^1H$ -NMR ( $CD_3OD$ , 500 MHz)  $\delta/ppm$  = 6.09 (1H, d,  $J$  = 2.07 Hz, H7), 6.20 (1H, d,  $J$  = 2.07 Hz, H5), 6.64 (1H, d,  $J$  = 8.50 Hz, H2), 6.78 (1H, d,  $J$  = 8.50 Hz, H4), 7.49 (1H, t,  $J$  = 8.36 Hz, H3).  $^{13}C$ -NMR ( $CD_3OD$ , 125 MHz)  $\delta/ppm$  = 95.8 (C7), 100.0 (C5), 107.9 (C8a), 111.4 (C8b), 115.9 (C4), 128.4 (C2), 137.6 (C3), 157.4 (C4a), 159.4 (C4b), 162.3 (C1), 164.2 (C6), 169.9 (C8), 185.1 (C9). MS  $m/z$  = 244 [M]<sup>+</sup>.

1,6-dihydroxyxanthones (**3b**). This compound was obtained as a yellow solid in 33.42% yield; m.p. 248–249 °C. FTIR (KBr)  $\nu/cm^{-1}$ : 3425 (O–H), 1604 (C=O), 1465 (C=C), 1273 (C–O–C).  $^1H$ -NMR ( $CD_3OD$ , 500 MHz)  $\delta/ppm$  = 6.67 (1H, dd,  $J$  = 2.15 and 8.81 Hz, H7), 6.71 (1H, d,  $J$  = 2.15 Hz, H5), 6.81 (1H, dd,  $J$  = 8.31 and 2.23 Hz, H2), 6.85 (1H, dd,  $J$  = 8.31 and 2.23 Hz, H4), 7.52 (1H, t,  $J$  = 8.31 Hz, H3), 7.99 (1H, d,  $J$  = 8.81 Hz, H8).  $^{13}C$ -NMR ( $CD_3OD$ , 125 MHz)  $\delta/ppm$  = 102.7 (C5), 107.3 (C4), 108.8 (C8b), 110.5 (C2), 113.2 (C8a), 115.4 (C7), 127.9 (C8), 136.6 (C3), 157.2 (C4a), 159.4 (C4b), 162.4 (C6), 167.5 (C1), 181.9 (C9). MS  $m/z$  = 228 [M]<sup>+</sup>.

1,5,6-trihydroxyxanthones (**3c**). This compound was obtained as a yellow solid in 11.15% yield. m.p. 251–252 °C. FTIR (KBr)  $\nu/cm^{-1}$ : 3448 (O–H), 1604 (C=O), 1458 (C=C), 1273 (C–O–C).  $^1H$ -NMR ( $CD_3OD$ , 500 MHz)  $\delta/ppm$  = 6.67 (1H, dd,  $J$  = 8.31 and 2.23 Hz, H4), 6.78 (1H, dd,  $J$  = 8.31 and 2.23 Hz, H2), 6.84 (1H, d,  $J$  = 8.94 Hz, H7), 7.51 (1H, t,  $J$  = 8.31 Hz, H3), 7.97 (1H, d,  $J$  = 8.94 Hz, H8).  $^{13}C$ -NMR ( $CD_3OD$ , 125 MHz)  $\delta/ppm$  = 103.4 (C4), 107.8 (C8b), 109.4 (C2), 110.9 (C7), 113.2 (C8a), 116.6 (C8), 128.3 (C5), 137.0 (C3), 157.7 (C4b), 160.1 (C6), 162.9 (C4a), 169.4 (C1), 182.3 (C9). MS  $m/z$  = 228 [M–16]<sup>+</sup>.

**In vitro antioxidant assay.** The antioxidant activity of hydroxyxanthones was evaluated by using a DPPH scavenging assay. Each hydroxyxanthone (**3a-c**) was dissolved in methanol at a concentration range from 10 to



**Figure 1.** Synthesis scheme of hydroxyxanthone derivatives.

100  $\mu\text{g mL}^{-1}$ . The solution was then mixed with 100  $\mu\text{g mL}^{-1}$  DPPH in methanol and allowed for 30 min in a dark condition. The absorbance value of the absorption signal at 517 nm was measured using a UV-Vis spectrophotometer (Shimadzu UV 1800). The radical scavenging percentage was calculated using Eq. (1), whereas  $A_{\text{blank}}$  and  $A_{\text{sample}}$  are the absorbances at 517 nm for blank (methanol) and sample (hydroxyxanthone), respectively.

$$\% \text{ Radical scavenging} = (A_{\text{blank}} - A_{\text{sample}}) / (A_{\text{blank}}) \times 100\% \quad (1)$$

Afterward, the radical scavenging percentage was plotted versus the concentration of the antioxidant agent. The half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) value was calculated by fixing the radical scavenging percentage equal to 50%. The in vitro antioxidant assay for each sample was performed in three replications.

**In vitro anticancer assay.** The in vitro anticancer activity of hydroxyxanthones was evaluated by using the MTT method. All cancer and normal (Vero) cell lines were supplied by the Parasitology Laboratory, Universitas Gadjah Mada, Indonesia. The cells were cultured at 37 °C in an incubator containing 5%  $\text{CO}_2$ . Briefly, the WiDr (colorectal cancer), MCF-7 (breast cancer), and HeLa (cervical cancer) cells were suspended in medium with 10% Fetal Bovine Serum. Concisely, the cell lines were added to 96-well plates and cultured in a medium with various concentrations of each hydroxyxanthone 3a–c. The mixture was stored in a  $\text{CO}_2$  incubator. After 24 h, the MTT reagent was added, and the mixture was further incubated for an additional 3 h. Afterward, the sodium dodecyl sulfate (SDS) page was added to the mixture, and the mixture was re-incubated for 24 h in a darkroom. The absorbance value of the solution at 495 nm was measured using an Elisa reader (Benchmark) to calculate viability cells' percentage (Eq. 2).

$$\% \text{ Viability cells} = (A_{\text{blank}} - A_{\text{sample}}) / (A_{\text{blank}}) \times 100\% \quad (2)$$

Afterward, the viability cells' percentage was plotted versus the concentration of the anticancer agent. The  $\text{IC}_{50}$  value was calculated by fixing the viability cells' percentage equals 50%. The in vitro anticancer assay for each sample was performed in three replications.

**Molecular docking study of hydroxyxanthone derivative as an anticancer agent.** The human Topoisomerase II with 4G0V as the protein code was retrieved from the Protein Data Bank. At first, the water molecules were removed from the Topoisomerase II protein structure. Then, the hydrogen atoms were added to build up the structure of the Topoisomerase II protein using AutoDock Tools software. The molecular docking process was performed by using AutoDock Vina software. The validity of the molecular docking study is represented by the root-mean-square deviation (RMSD) value less than 2.0 Å<sup>34</sup>. Finally, the formed interactions between hydroxyxanthone and the active site of Topoisomerase II protein were visualized by using Discovery Studio software.

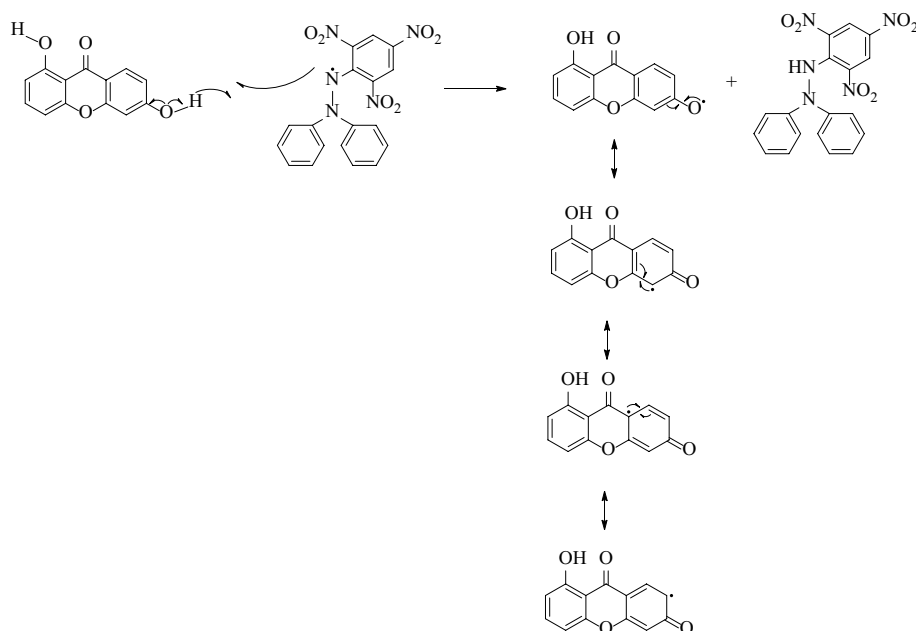
## Results and discussion

**Synthesis of hydroxyxanthone derivatives.** The synthesis scheme of hydroxyxanthone derivatives in this work is shown in Fig. 1. All hydroxyxanthones (3a–c) were prepared through a cyclo-acylation reaction of 2,6-dihydroxybenzoic acid (1) with phenolic derivatives (2a–c). From the spectroscopic elucidation, the correct chemical structure of hydroxyxanthones 3a–c has been confirmed. The FTIR, MS, <sup>1</sup>H-, and <sup>13</sup>C-NMR spectra of hydroxyxanthones 3a–c are depicted in Figs. S1–S12. In general, the FTIR spectra of hydroxyxanthones showed the appearance of O–H hydroxyl groups at 3425–3448  $\text{cm}^{-1}$  while the C=O functional group appeared as a sharp signal at 1604–1612  $\text{cm}^{-1}$ . On the other hand, the C=C aromatic and C–O–C heterocyclic moieties were observed at 1418–1465 and 1273–1296  $\text{cm}^{-1}$ , respectively (Figs. S1, S5, and S9). Another evidence to prove the successful production of hydroxyxanthones was the presence of molecular ion fragments ( $[\text{M}]^+$ ) in their MS spectra (Figs. S2, S6, and S10). Hydroxyxanthones 3a and 3c showed five aromatic protons, while hydroxyxanthone 3b showed six aromatic protons in the range of 6.09–7.99 ppm in their <sup>1</sup>H-NMR spectra (Figs. S3, S7, and S11). Meanwhile, the presence of carbon atoms of the carbonyl group of hydroxyxanthones 3a–c was found at 185.1, 181.9, and 182.3 ppm, respectively, in their <sup>13</sup>C-NMR spectra (Figs. S4, S8, and S12). These spectroscopic data demonstrated that hydroxyxanthones had been successfully synthesized in this work.

**Antioxidant activity assay of hydroxyxanthones.** Antioxidant activity assay of hydroxyxanthones 3a–c was performed by using DPPH free radicals method as the most common procedure. The antioxidant activity of hydroxyxanthones was shown in the term  $\text{IC}_{50}$  value (Table 1). A lower  $\text{IC}_{50}$  value means a higher

| Compound  | IC <sub>50</sub> (μM) |
|-----------|-----------------------|
| <b>3a</b> | 653 ± 53              |
| <b>3b</b> | 349 ± 68              |
| <b>3c</b> | 524 ± 72              |
| BHT       | 40 ± 4                |

**Table 1.** In vitro antioxidant activity of hydroxyxanthone derivatives.



**Figure 2.** The plausible reaction of DPPH free radical with dihydroxyxanthone **3b**.

| Compound    | IC <sub>50</sub> (μM) |          |          |          |
|-------------|-----------------------|----------|----------|----------|
|             | Vero <sup>39</sup>    | MCF-7    | WiDr     | HeLa     |
| <b>3a</b>   | 3395 ± 435            | 184 ± 15 | 254 ± 15 | 277 ± 9  |
| <b>3b</b>   | 308 ± 35              | 450 ± 17 | 355 ± 24 | 322 ± 4  |
| <b>3c</b>   | 224 ± 14              | 419 ± 27 | 209 ± 4  | 241 ± 13 |
| Doxorubicin | 150 ± 3               | 71 ± 6   | 3 ± 0.2  | –        |
| Cisplatin   | –                     | –        | –        | 142 ± 52 |

**Table 2.** In vitro anticancer activity of hydroxyxanthone derivatives.

antioxidant activity of hydroxyxanthones. Although all hydroxyxanthones gave a lower antioxidant activity than BHT as the positive control, the trihydroxyxanthones (compounds **3a** and **3c**) gave higher IC<sub>50</sub> values than the dihydroxyxanthone **3b**. It shows that a higher amount of hydroxyl groups on the xanthone structure gave a lower antioxidant activity due to a stronger hydrogen bonding among the hydroxyl groups as previously reported<sup>35,36</sup>. Dihydroxyxanthone **3b** exhibited the strongest antioxidant activity (IC<sub>50</sub> = 349 ± 68 μM). Since the hydroxyl groups were located far from each other, the DPPH free radicals could easily attack the hydroxyl groups of dihydroxyxanthone **3b**, yielding a strong antioxidant activity. This phenomenon was in agreement with the previous report<sup>37</sup>. The plausible reaction mechanism of DPPH free radicals with dihydroxyxanthone **3b** is shown in Fig. 2. This reaction mechanism generated a product with high resonance stability which could be responsible for the high antioxidant activity of dihydroxyxanthone **3b**.

**Anticancer activity assay of hydroxyxanthones.** Evaluation of the anticancer activity of hydroxyxanthones was conducted through the MTT assay. The anticancer activity of hydroxyxanthones was represented in the term of IC<sub>50</sub> value (see Table 2). A lower IC<sub>50</sub> value reflects a stronger anticancer activity of hydroxyxantho-

| Compound | MCF-7 | WiDr  | HeLa  |
|----------|-------|-------|-------|
| 3a       | 18.42 | 13.39 | 12.25 |
| 3b       | 0.686 | 0.870 | 0.959 |
| 3c       | 0.535 | 1.072 | 0.931 |

**Table 3.** Selectivity index of hydroxyxanthone derivatives as anticancer agent.

| Compound                      | IC <sub>50</sub> (μM) | SI    | Reference |
|-------------------------------|-----------------------|-------|-----------|
|                               | WiDr                  |       |           |
| 1,3,8-trihydroxyxanthone (3a) | 254 ± 15              | 13.39 | This work |
| 1,6-dihydroxyxanthone (3b)    | 355 ± 24              | 0.870 |           |
| 1,5,6-trihydroxyxanthone (3c) | 209 ± 4               | 1.072 |           |
| 1,3-dihydroxyxanthone         | 836 ± 109             | 2.230 | 33        |
| 3,4-dihydroxyxanthone         | 1255 ± 105            | 1.160 |           |
| 3,6-dihydroxyxanthone         | 786 ± 146             | 1.630 |           |
| 1,3,6-trihydroxyxanthone      | 384 ± 93              | 1.540 |           |
| 3,4,6-trihydroxyxanthone      | 38 ± 11               | 66.39 |           |

**Table 4.** Comparison of in vitro anticancer activity of hydroxyxanthone derivatives against WiDr cancer cell line.

nes. Even though the anticancer activity of hydroxyxanthones was weaker than either doxorubicin or cisplatin as the positive control, the anticancer activity of hydroxyxanthones was still categorized as medium activity.

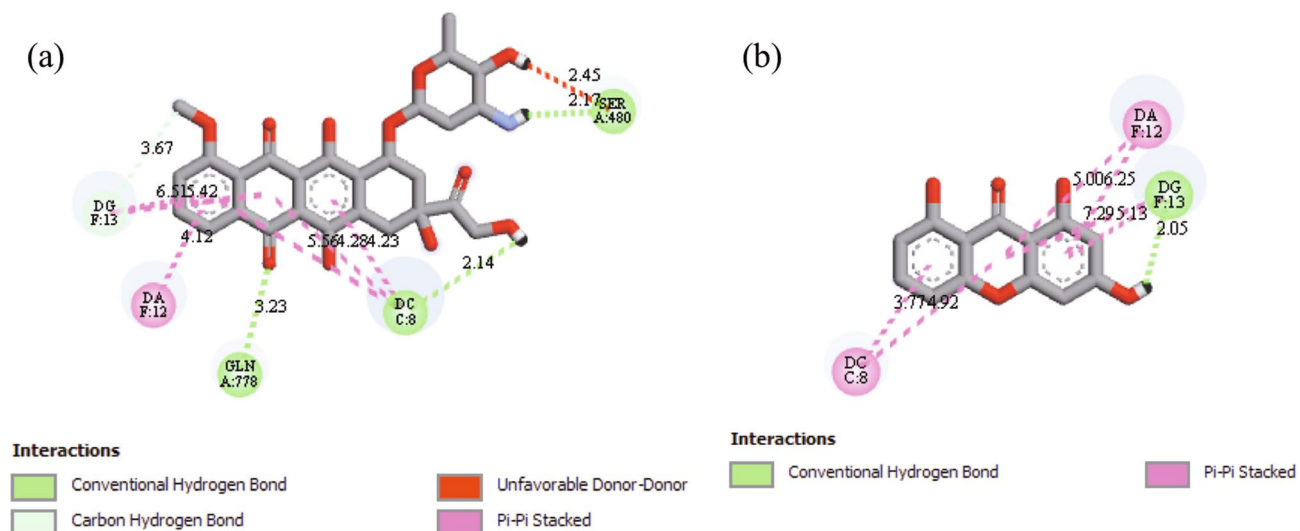
Among the prepared hydroxyxanthones, hydroxyxanthone **3a** was found as the best anticancer agent against the MCF-7 cell line (IC<sub>50</sub> = 184 ± 15 μM) while hydroxyxanthone **3c** was the most active anticancer agent against WiDr (IC<sub>50</sub> = 209 ± 4 μM) and HeLa (IC<sub>50</sub> = 241 ± 13 μM) cell lines. These results demonstrated that trihydroxyxanthones were more active as the anticancer agent than dihydroxyxanthone **3b**. However, trihydroxyxanthone **3c** was more toxic to normal Vero cell lines (IC<sub>50</sub> = 224 ± 14 μM) rather than either MCF-7 (IC<sub>50</sub> = 419 ± 27 μM) or HeLa (IC<sub>50</sub> = 241 ± 13 μM) cell lines. In contrast, trihydroxyxanthone **3a** was more toxic to MCF-7 (IC<sub>50</sub> = 184 ± 15 μM), WiDr (IC<sub>50</sub> = 254 ± 15 μM), and HeLa (IC<sub>50</sub> = 277 ± 9 μM) cancer cell lines rather than normal Vero cell line (IC<sub>50</sub> = 3395 ± 435 μM), which was remarkable.

Good anticancer drug candidates must be selective, i.e., toxic only to cancer cells but safe for normal cell lines<sup>38</sup>. The selectivity index (SI) was a critical parameter to describe selective anticancer activity. The SI value was calculated by dividing the IC<sub>50</sub> of Vero cell lines with IC<sub>50</sub> of cancer cells of hydroxyxanthone **3a–c**. A higher SI value describes a more selective anticancer agent. The SI value of hydroxyxanthones **3a–c** is listed in Table 3. Compound **3a** was found as the most selective anticancer agent due to its highest SI value against MCF-7, WiDr, and HeLa cancer cell lines among the other hydroxyxanthones in this work.

Table 4 compares the anticancer activity of hydroxyxanthones **3a–c** and the previously reported hydroxyxanthones against WiDr cancer cell line. Compared to the other hydroxyxanthone derivatives, dihydroxyxanthones (IC<sub>50</sub> = 355–1255 μM) gave higher IC<sub>50</sub> values than trihydroxyxanthones (IC<sub>50</sub> = 38–384 μM). Among the dihydroxyxanthones, the order of the anticancer activity against the WiDr cancer cell line was 1,6-dihydroxyxanthone > 3,6-dihydroxyxanthone > 1,3-dihydroxyxanthone > 3,4-dihydroxyxanthone. Meanwhile, the order of the anticancer activity of trihydroxyxanthones was 3,4,6-trihydroxyxanthone > 1,5,6-trihydroxyxanthone > 1,3,8-trihydroxyxanthone > 1,3,6-trihydroxyxanthone. From the reported quantitative structure–activity relationship (QSAR) study, we found that the IC<sub>50</sub> value of hydroxyxanthone as the anticancer agent against WiDr cancer cell line depends on the net atomic charge on carbon atoms, dipole moment, and octanol/water partition coefficient<sup>33</sup>. Furthermore, the SI values of trihydroxyxanthones against WiDr cells (SI = 1.072–66.39) are higher than the dihydroxyxanthones (SI = 0.870–2.230), demonstrating that trihydroxyxanthones are more potential anticancer drug candidates than dihydroxyxanthones.

**Molecular docking study on the inhibition of topoisomerase II protein by hydroxyxanthones.** The experimental in vitro assay found that hydroxyxanthone **3a** exhibited the strongest and the most selective anticancer activity among the other hydroxyxanthones in this work. Therefore, a further investigation was conducted for hydroxyxanthone **3a** through a molecular docking study to reveal the inhibition mechanism on the active site of Topoisomerase II protein. Topoisomerase II protein is selected as the target protein since Topoisomerase II serves a pivotal role in the DNA replication and transcription of cancer cells. Doxorubicin is an anticancer agent with strong inhibitory activity towards Topoisomerase II protein. Doxorubicin consists of two fused aromatics with a carbonyl structure, which is similar to the prepared hydroxyxanthone **3a**.

The formed interactions from the molecular docking study are displayed in Fig. 3. Several interactions were observed between hydroxyxanthone **3a** with the DNA chains of Topoisomerase II, such as hydrogen bond with DG13, as well as π–π stacking with DA12 and DC8. These kinds of interactions have been reported to be critical for the inhibitory activity against Topoisomerase II protein. Etoposide, a standard Topoisomerase inhibitor, was



**Figure 3.** Visualization of the intermolecular interactions of (a) doxorubicin and (b) hydroxyxanthone **3a** with the active sites of topoisomerase II.

reported to generate hydrogen bond interactions with Asp463 and DG13 on the active site of Topoisomerase II<sup>40</sup>. On the other hand, the binding interaction of the anticancer agent with DG13 was reported as pivotal interaction as this interaction stimulates the formation of DNA damage that is toxic to cancer cells<sup>41</sup>. Similar binding modes with DA12 and DC8 through  $\pi$ - $\pi$  stacking were also reported for the most active benzoxazole and olivacine derivatives as the anticancer agents<sup>40,42</sup>.

On the other side, doxorubicin interacted to the active site of Topoisomerase II through hydrogen bond (Ser480, DC8, Gln778), carbon-hydrogen bond (DG13), and  $\pi$ - $\pi$  stacking (DG13, DA12, DC8) interactions. Compared to doxorubicin, hydroxyxanthone **3a** generated no interaction with Ser480 and Gln778. Therefore, the theoretical binding energy and binding constant of hydroxyxanthone **3a** were lower than doxorubicin. The binding energy and binding constant of hydroxyxanthone **3a** with Topoisomerase II were  $-8.0$  kcal mol<sup>-1</sup> and  $1.362$   $\mu$ M, respectively, which were weaker than doxorubicin ( $-8.9$  kcal mol<sup>-1</sup>,  $0.299$   $\mu$ M). Nevertheless, it is worthy to note that hydroxyxanthone **3a** was able to disturb the function of Topoisomerase II protein, thus increasing the cell apoptosis, which led to the death of cell cancers as previously reported<sup>31</sup>.

## Conclusions

Three hydroxyxanthenes (**3a-c**) have been successfully synthesized from 2,6-dihydroxybenzoic acid and phenolic compounds through an acylation reaction using Eaton's reagent. The chemical structures of hydroxyxanthenes have been clearly elucidated by using FTIR, MS, <sup>1</sup>H-, and <sup>13</sup>C-NMR. The in vitro antioxidant assay showed that trihydroxyxanthenes **3a** and **3c** gave higher IC<sub>50</sub> values than dihydroxyxanthone **3b** due to stronger hydrogen bonds. It was found that dihydroxyxanthone **3b** exhibited the strongest antioxidant activity (IC<sub>50</sub> =  $349 \pm 68$   $\mu$ M). On the other hand, trihydroxyxanthenes gave higher anticancer activity than the dihydroxyxanthone one. Trihydroxyxanthone **3a** exhibited higher toxicity against MCF-7 (IC<sub>50</sub> =  $184 \pm 15$   $\mu$ M), WiDr (IC<sub>50</sub> =  $254 \pm 15$   $\mu$ M), and HeLa (IC<sub>50</sub> =  $277 \pm 9$   $\mu$ M) cancer cell lines rather than normal Vero cells (IC<sub>50</sub> =  $3395 \pm 435$   $\mu$ M). Consequently, trihydroxyxanthone **3a** yielded the highest selectivity index (12.25–18.42) among the prepared hydroxyxanthenes in this work. The molecular docking study reveals that hydrogen bond and  $\pi$ - $\pi$  stacking interactions were observed between trihydroxyxanthone **3a** with the DNA chains of Topoisomerase II. The trihydroxyxanthone **3a** was able to disturb the function of Topoisomerase II protein. These findings are important for the future development of anticancer agents based on hydroxyxanthone derivatives.

## Data availability

The datasets generated during and/or analyzed in the current study are available from the corresponding author upon a reasonable request.

Received: 20 September 2021; Accepted: 14 January 2022

Published online: 27 January 2022

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### Acknowledgements

The financial support from KEMENDIKBUD-RISTEK through the World Class Research grant for the budget year 2021–2023 is greatly acknowledged.

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### Competing interests

The authors declare no competing interests.

### Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-022-05573-5>.

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