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Investigation on anticancer agent against cervical and colorectal cancer cell lines: One-pot synthesis, *in vitro* and *in silico* assays of xanthone derivatives

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ABSTRACT

Cervical or colorectal cancer disease attracts the world's attention due to the high mortality in a year. Because of that, an effective anticancer treatment employing an active anticancer agent is urgently needed. Herein, we reported the synthesis and evaluation of the anticancer activity of hydroxylated xanthones against cervical (HeLa) and colorectal (WiDr) cancer cell lines. The hydroxylated xanthones were successfully synthesized through a one-pot reaction between hydroxybenzoic acid and phenolic derivatives in a 6.60%–46.50% yield. Their chemical structures have been confirmed using Fourier-transforms infrared, nuclear magnetic resonance, and mass spectrometries. From the *in vitro* anticancer investigation, 1,3-dihydroxyxanthone exhibited the strongest anticancer activity against HeLa and WiDr cancer cells among the examined hydroxylated xanthones. The 1,3-dihydroxyxanthone gave half-maximal inhibitory concentration and selectivity index values of 0.086–0.114 mM and 2.690–3.591, respectively. The *in silico* molecular docking studies revealed that 1,3-dihydroxyxanthone interacted with Adenine12, Guanine13, Cytosine14, Arginine503, Glycine504, Lysine505, Isoleucine506, Leucine507, Asparagine520, Alanine521, and Glutamic acid522 through noncovalent forces generating the Gibbs free energy of −6.85 kcal/mol in the active site of Topoisomerase II protein receptor. These results demonstrate that 1,3-dihydroxyxanthone could act as an active anticancer agent against cervical and colorectal cancer cells by inhibiting the Topoisomerase II protein receptor.

INTRODUCTION

Cancer caused 10 million deaths in developed and developing countries in 2020 [1]. World Cancer Research Fund International reported that cervical and colorectal cancers contributed to 342,000 and 935,000 deaths, respectively, in 2020 [2,3]. Therefore, cervical and colorectal cancers have been considered the leading cause of global cancer-related deaths [4,5]. Chemotherapy utilizing an anticancer agent is a widely used treatment to suppress cancer cells' growth; however, some cancer cell lines have been resistant to commercial anticancer agents [6,7]. Numerous chemical compounds have been elucidated from natural sources and/or synthesized through chemical modifications [8–10]. However, their time-consuming isolation process and/or complicated synthesis procedures limit their real applications for large-scale drug production. Moreover, weak anticancer activity and poor selectivity of the new anticancer drugs attract serious concerns in the war on cancer diseases. [11–13].

Xanthone, an oxygenated heterocyclic compound, has been reported as a potential anticancer agent due to its ease of synthesis and good anticancer activity and selectivity [14]. Kostanecki and Nessler [15] reacted ortho-hydroxybenzoic acid and phenol compounds in the presence of acetic acid anhydride and $ZnCl_2$ as the Lewis acid catalyst. On the other hand, Grover *et al*. [16] used $POCl_3$ and $ZnCl_2$ as the Lewis acid catalyst to react with ortho-hydroxybenzoic acid and

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phenolic compounds. Unfortunately, neither method is suitable for all phenolic compounds, especially 1,3-dihydroxybenzene and 1,3,5-trihydroxybenzene. When these phenolic compounds were reacted, the reaction stopped in the formation of benzophenone; thus, the tricyclic xanthone structures were not produced. Thus, researchers are trying to establish a new and efficient synthesis method for xanthone derivatives. Ullman condensation, Friedel–Crafts acylation, Robinson–Nishikawa, Smiles rearrangement, and Tanase synthetic routes have been reported to synthesize xanthones. However, their procedures need several reaction steps; thus, these methods are not categorized as efficient synthesis from the green chemistry point of view [17].

Eaton's reagent consists of a mixture of 10% wt. P_2O_5 in methanesulfonic acid as the solvent. Eaton's reagent has been employed as a Lewis acid catalyst in various cyclization reactions to generate heterocyclic structures of quinolinones, benzimidazoles, and carbazoles [18–20]. Our research group has been using Eaton's reagent for the one-pot synthesis of 1,6-dihydroxyxanthone, 1,3,6-trihydroxyxanthone, 1,3,8-trihydroxyxanthone, and 1,5,6-trihydroxyxanthone [21]. In continuation of our research, we herein synthesized the other hydroxylated xanthones, i.e., 1-hydroxyxanthone, 1,3-dihydroxyxanthone, and 3,6-dihydroxyxanthone, employing Eaton's reagent, in a one-pot synthesis procedure.

Xanthone and its derivatives have been investigated as bioactive compounds, including anticancer, anti-inflammatory, antiviral, antioxidant, antidiabetic, antimicrobial, and antimalarial agents [14]. As the anticancer agent, the unmodified xanthone gave poor anticancer activity against the colorectal cancer cell line with a half-maximal inhibitory concentration (IC_{50}) value of 200 mM. The addition of chloro and bromo substituents at the xanthone structure, resulting in 2-chloroxanthone and 2-bromoxanthone, enhanced the anticancer activity with IC_{50} values of 96.7 and 85.1 mM, respectively. Meanwhile, the addition of a hydroxyl group on the halogenated xanthone exhibited much stronger anticancer activity with IC_{50} values of 0.15 and 0.15 mM for 2-chloro-1,3-dihydroxyxanthone and 2-bromo-1,3-dihydroxyxanthone, respectively [22]. This result clearly demonstrated that the presence of the hydroxyl group is much more critical than halogen groups for the anticancer activity of xanthone derivatives.

Fatmasari *et al.* [23] reported the anticancer activity of 1,3,8-trihydroxyxanthone, 1,6-dihydroxyxanthone, and 1,5,6-trihydroxyxanthone against WiDr and HeLa cancer cell lines. These hydroxyxanthones gave the IC_{50} value of 0.209–0.355 and 0.241–0.322 mM against the WiDr and HeLa cancer cell lines, respectively. It was reported that the anticancer activity of the hydroxyxanthones comes through the Topoisomerase II inhibition, thus leading to anti-proliferative and apoptosis mechanisms [23]. The presence of the hydroxyl group is critical to enhancing anticancer activity through the formation of noncovalent interactions with the key amino acid residues at the Topoisomerase II receptor, which is overexpressed in the cervical and colorectal cancer cells [24]. Unfortunately, a comprehensive discussion on the effect of the number and position of the hydroxyl group on the anticancer activity of xanthone has not been available as of today.

Therefore, in the present work, we synthesized the other hydroxylated xanthones (i.e., 1-hydroxyxanthone, 1,3-dihydroxyxanthone, and 3,6-dihydroxyxanthone) and discussed the effect of the number and location of the hydroxyl group on the anticancer activity and selectivity against HeLa and WiDr cancer cells. The most potent anticancer agent was further examined through molecular docking studies against the Topoisomerase II receptor to reveal its mechanism of action as the anticancer agent.

MATERIALS AND METHODS

Chemistry

The 2-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 1,3-dihydroxybenzene, 1,3,5-trihydroxybenzene, and Eaton's reagent were purchased from Merck in proanalytical grade. The synthesis and anticancer activity of 1,6-dihydroxyxanthone, 1,3,6-trihydroxyxanthone, 1,3,8-trihydroxyxanthone, and 1,5,6-trihydroxyxanthone have been reported in the previous work [23]. The infrared spectra of the synthesized compounds were measured in the form of a KBr pellet using a Fourier-transform infrared spectrophotometer (FTIR, Shimadzu Prestige21). The proton and carbon nuclear magnetic resonance (NMR) spectra of the products were analyzed in deuterated methanol with tetramethylsilane as the internal standard using a JNM-ECZ500R/S1 spectrometer. The mass spectra (MS) were recorded on a Shimadzu QP2010S spectrometer. Meanwhile, the 3-D structure of Topoisomerase II was retrieved from a protein data bank (https://www.rcsb. org) with 4G0V as the ID number. The software utilized for molecular docking studies was Gaussian09W, Chimera 1.13.1, AutoDock Tools 1.5.6, and Discovery Studio Visualizer 2019. These software programs were supported by Austrian–Indonesian Center for Computational Chemistry, Universitas Gadjah Mada.

METHODS

### *One-pot synthesis of hydroxylated xanthones*

An equivalent mixture of hydroxybenzoic acid (15 mmol) and hydroxybenzene (15 mmol) was mixed in the presence of Eaton's reagent (8 ml) as the Lewis acid catalyst and solvent. The mixture was then reacted at 80°C. The reaction was monitored by thin-layer chromatography analysis using a mixture of *n*-hexane:ethyl acetate in a 7:3 volume ratio as the mobile phase. After 3 hours, the mixture was cooled to reach room temperature and then poured into cold distilled water (30 ml) to precipitate the product. The crude product was filtered, dried, and purified by preparative thin-layer chromatography using a mixture of *n*-hexane:ethyl acetate in a 7:3 volume ratio as the mobile phase. The yellow spot was taken, rinsed with methanol (10 ml), filtered, and evaporated under a vacuum to obtain the target compound. The product was characterized using FTIR, NMR, and MS analyses. The 1-hydroxyxanthone was obtained from the reaction of 2-hydroxybenzoic acid and 1,3-dihydroxybenzene. The 1,3-dihydroxyxanthone was obtained from the reaction of 2-hydroxybenzoic acid and 1,3,5-trihydroxybenzene.

The 3,6-dihydroxyxanthone was obtained from the reaction of 2,4-dihydroxybenzoic acid and 1,3-dihydroxybenzene. Meanwhile, the 1,3,6-trihydroxyxanthone has been prepared from 2,4-dihydroxybenzoic acid and 1,3,5-trihydroxybenzene, according to the previous work [24].

1-Hydroxyxanthone was obtained as a yellow solid in 6.60% yield. FTIR (cm^{-1}): 3,124 (O─H stretching), 1,613 (C═O), 1,450 (C═C stretching), and 1,119 (C─O stretching) (Fig. S1). ^{1}H-NMR (500 MHz) δ (ppm): 6.79 (1H, *doublet*, J = 7.5 Hz, H2), 7.01 (1H, *doublet*, J = 7.5 Hz, H4), 7.45 (1H, *triplet*, J = 8.5 Hz, H7), 7.57 (1H, *doublet*, J = 8.5 Hz, H5), 7.66 (1H, *triplet*, J = 7.5 Hz, H3), 7.84 (1H, *triplet*, J = 8.5 Hz, H6), and 8.26 (1H, *doublet*, J = 8.5 Hz, H8) (Fig. S2). ^{13}C-NMR (125 MHz) δ (ppm): 108.31, 109.99, 111.42, 119.20, 121.79, 125.54, 126.87, 137.31, 138.41, 157.77, 157.90, 163.17 (12 aromatic carbons), and 183.75 (C═O) (Fig. S3). MS: m/z = 213 ([M+1]$^+$, 15%), 212 ([M]$^+$ and base peak, 100%), 184 (25%), 155 (5%), 128 (30%), and 77 (10%) (Fig. S4).

1,3-Dihydroxyxanthone was obtained as a yellow solid in 46.50% yield. FTIR (cm^{-1}): 3,387 and 3,140 (O─H stretching), 1,612 (C═O), 1,458 (C═C stretching), and 1,172 (C─O stretching) (Fig. S5). ^{1}H-NMR (500 MHz) δ (ppm): 6.19 (1H, *doublet*, J = 2.0 Hz, H2), 6.35 (1H, *doublet*, J = 2.0 Hz, H4), 7.39 (1H, *triplet*, J = 8.0 Hz, H7), 7.48 (1H, *doublet*, J = 8.0 Hz, H5), 7.76 (1H, *triplet*, J = 8.0 Hz, H6), and 8.18 (1H, *doublet*, J = 8.0 Hz, H8) (Fig. S6). ^{13}C-NMR (125 MHz) δ (ppm): 95.36, 99.44, 103.89, 118.81, 121.78, 125.26, 126.62, 136.43, 157.50, 159.56, 164.94, 168.14 (12 aromatic carbons), and 181.82 (C═O) (Fig. S7). MS: m/z = 229 ([M+1]$^+$, 15%), 228 ([M]$^+$ and base peak, 100%), 200 (20%), 171 (35%), and 77 (5%) (Fig. S8).

3,6-Dihydroxyxanthone was obtained as a yellow solid in 21.93% yield. FTIR (cm^{-1}): 3,325 (O─H stretching), 1,621 (C═O), 1,458 (C═C stretching), and 1,165 (C─O stretching) (Fig. S9). ^{1}H-NMR (500 MHz) δ (ppm): 6.81 (2H, *doublet*, J = 2.5 Hz, H2), 6.85 (2H, *doublet of doublet*, J = 2.5 and 6.0 Hz, H4), and 8.06 (2H, *doublet*, J = 6.0 Hz, H8) (Fig. S10).
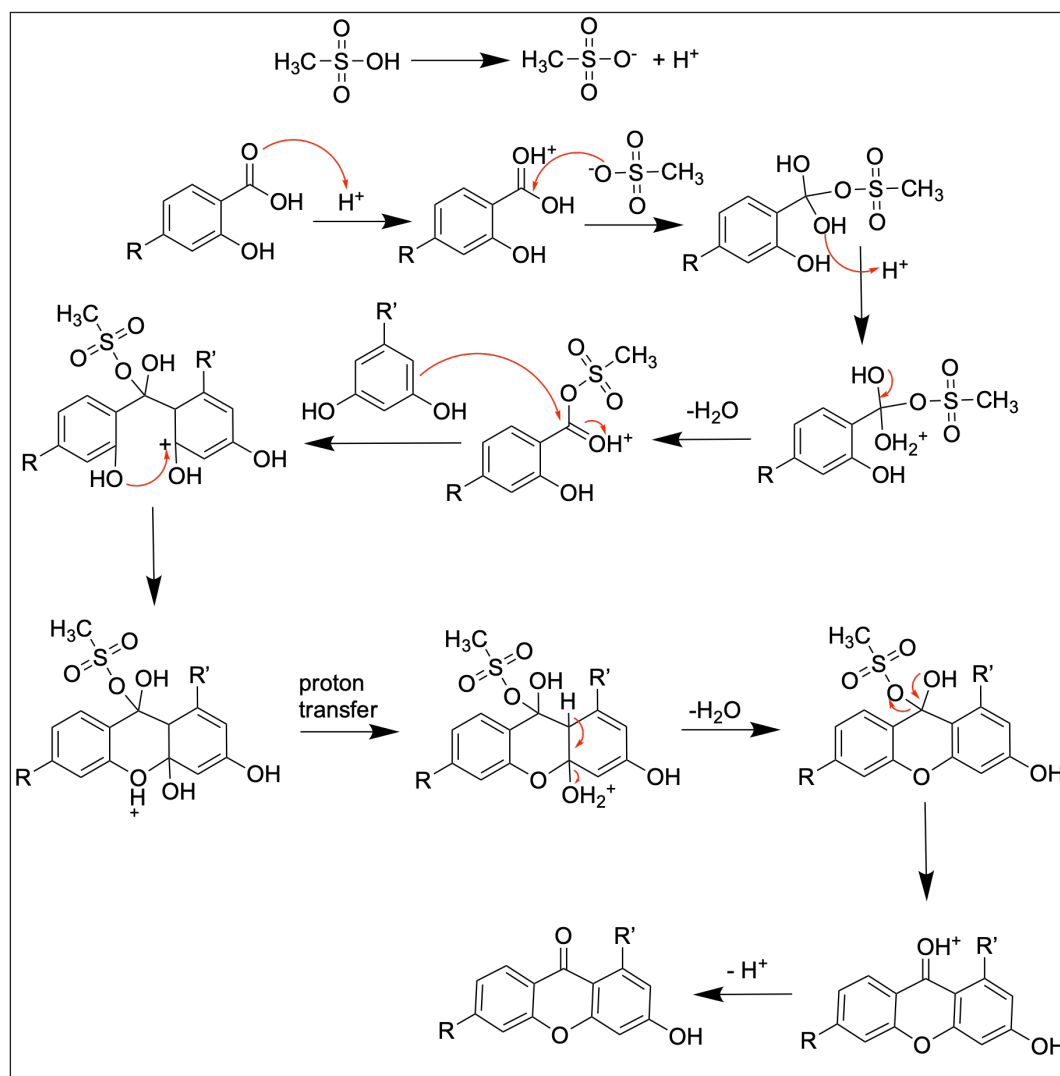
Figure 1. The reaction mechanism in the production of the hydroxylated xanthones.

^{13}C-NMR (125 MHz) δ (ppm): 103.31, 115.92, 115.59, 129.12, 159.88, 165.52 (6 aromatic carbons), and 177.69 (C═O) (Fig. S11). MS: m/z = 229 ([M+1]$^+$, 20%), 228 ([M]$^+$ and base peak, 100%), 200 (70%), 171 (40%), 155 (5%), and 77 (5%) (Fig. S12).

Anticancer activity assay of hydroxylated xanthones

The anticancer activity of hydroxylated xanthones was investigated through *in vitro* assay following the reported procedure employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent [23]. Meanwhile, molecular docking was conducted the same as reported before [23]. Briefly, the 3-D structure of the Topoisomerase II receptor was prepared using Chimera 1.13.1, while the 3-D structure of hydroxylated xanthone was optimized using Gaussian09W with DFT-B3LYP-6,31G as the basis set. The molecular docking of hydroxylated xanthone was conducted in a $50 \times 50 \times 50$ Å grid box with a spacing of 0.375 Å. The minimal and maximal coordinates in the grid box were fixed at $(x, y, z) = (26.124, 79.227, 41.381)$ and $(44.874, 97.977, 60.131)$, respectively, for 100 calculations of Lamarckian genetic algorithms. The final coordinate of hydroxylated xanthone, Gibbs free energy, ligand efficiency, binding constant, and root-mean-square-deviation (RMSD) parameters were retrieved from the calculations using AutoDock Tools 1.5.6. On the other hand, the formed noncovalent interactions between the hydroxylated xanthone and the active site of the Topoisomerase II protein receptor were visualized using Discovery Studio Visualizer 2019 software.

RESULTS AND DISCUSSION

One-pot synthesis of hydroxylated xanthones

In this work, hydroxylated xanthones were synthesized by reacting hydroxybenzoic acid with hydroxybenzene derivatives under acidic conditions. Eaton's reagent facilitates the formation of the cyclic structure of xanthone. At first, methanesulfonic acid donates a proton to the carbonyl group of benzoic acid. Consequently, the electrophilicity of benzoic acid is higher and ready to be attacked by hydroxybenzene. The acylation reaction of hydroxybenzene with activated benzoic acid derivative leads to the formation of a benzophenone structure as the intermediate. Afterward, both hydroxyl groups at the ortho position were eliminated as water molecule to form the tricyclic structure of hydroxylated xanthones, as

shown in Figure 1 [25]. The synthesis scheme of hydroxylated xanthones in this work is shown in Figure 2. In this work, the hydroxylated xanthones have been successfully synthesized as yellow solids in 6.60%–46.50% yield. The lowest yield of 1-hydroxyxanthone (6.60%) was caused by the competition reaction with the formation of 3-hydroxyxanthone. Meanwhile, the other hydroxylated xanthones were obtained in low-to-medium yield (21.93%–46.50%) due to a short reaction time (3 hours), indicating that the reaction might not be optimized yet.

The chemical structures of hydroxylated xanthones have been confirmed by using FTIR, NMR, and also MS. The FTIR data confirmed the presence of O─H hydroxyl, C═O carbonyl, C═C aromatic ring, and C─O ether functional groups on the structure of hydroxylated xanthones. The C=O functional group was observed in 1,612–1,621 cm^{-1} due to the conjugation with two aromatic rings that agreed with the other reported works [23]. The 1-hydroxyxanthone showed seven aromatic protons and twelve aromatic carbons on its NMR spectra. The 1,3-dihydroxyxanthone showed six aromatic protons and twelve aromatic carbons on its NMR spectra, while the 3,6-dihydroxyxanthone showed three types of aromatic proton and six types of aromatic carbon on its NMR spectra due to its symmetrical structure. The presence of C═O carbonyl group of 1-hydroxyxanthone, 1,3-dihydroxyxanthone, and 3,6-dihydroxyxanthone on their FTIR spectra was confirmed by the signal at 183.75, 181.82, and 177.69 ppm, respectively, on the ^{13}C-NMR spectrum. The molecular mass of the hydroxylated xanthones was confirmed by the appearance of molecular ion (M$^+$) at m/z = 212 and 228. These molecular ions became the base peak of MS because of the highly stable hydroxylated xanthones' structure.

Anticancer activity investigation of hydroxylated xanthones

The anticancer activity of hydroxylated xanthones is shown in Table 1, while their chemical structures are displayed in Figure 2. It was found that 1-hydroxyxanthone, 1,3-dihydroxyxanthone, 1,6-dihydroxyxanthone, 3,6-dihydroxyxanthone, 1,3,6-trihydroxyxanthone, 1,3,8-trihydroxyxanthone, and 1,5,6-trihydroxyxanthone gave the IC$_{50}$ values of 1.198, 0.086, 0.322, 0.162, 0.203, 0.277, and 0.241 mM, respectively, against HeLa cancer cells. A lower IC$_{50}$ value represents a higher anticancer activity [26].
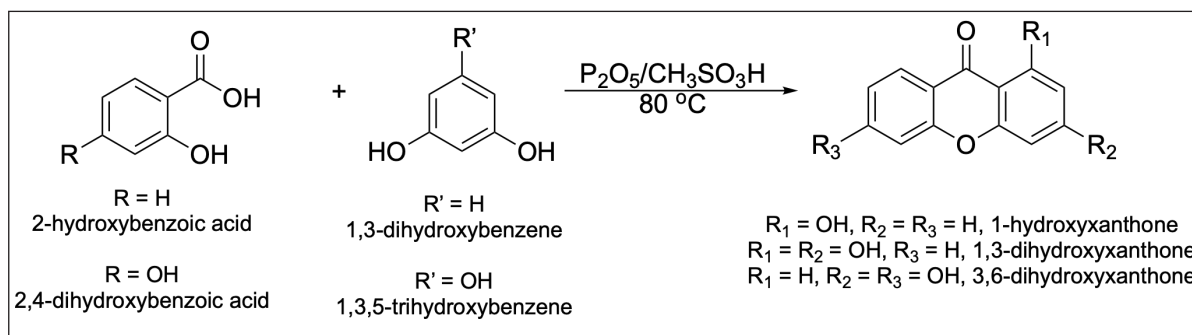

Figure 2. The synthesis scheme of hydroxylated xanthones.

Table 1. Anticancer activity and cytotoxicity of hydroxylated xanthones against HeLa and WiDr cancer cells.

Compound	IC_{50} (mM)		
	HeLa	**WiDr**	**Vero**
1-Hydroxyxanthone	1.198	1.111	7.562
1,3-Dihydroxyxanthone	0.086	0.114	0.308
1,6-Dihydroxyxanthone	0.322	0.355	0.308
3,6-Dihydroxyxanthone	0.162	0.786	1.281
1,3,6-Trihydroxyxanthone	0.203	0.141	0.457
1,3,8-Trihydroxyxanthone	0.277	0.254	3.395
1,5,6-Trihydroxyxanthone	0.241	0.209	0.224
Doxorubicin	0.068	-	0.169
Cisplatin	-	0.004	0.150

Table 2. Selectivity index of hydroxylated xanthones as the anticancer agents.

Compound	HeLa	WiDr
1-Hydroxyxanthone	6.315	6.806
1,3-Dihydroxyxanthone	3.591	2.690
1,6-Dihydroxyxanthone	0.957	0.868
3,6-Dihydroxyxanthone	7.902	1.630
1,3,6-Trihydroxyxanthone	2.249	3.236
1,3,8-Trihydroxyxanthone	12.26	13.37
1,5,6-Trihydroxyxanthone	0.929	1.072
Doxorubicin	2.465	-
Cisplatin	-	40.21

The 1-hydroxyxanthone showed the weakest anticancer activity against HeLa cancer cells (IC_{50} = 1.198 mM) due to the presence of a single hydroxyl group. Additional hydroxyl groups in 1-hydroxyxanthone structure, i.e., 1,3-di-hydroxyxanthone, 1,6-dihydroxyxanthone, 3,6-dihydroxyxanthone, 1,3,6-trihydroxyxanthone, 1,3,8-trihydroxyxanthone, and 1,5,6-trihydroxyxanthone, enhanced its anticancer activity (IC_{50} = 0.086–0.322 mM). The addition of a hydroxyl group on the 3-position of 1-hydroxyxanthone, named 1,3-dihydroxyxanthone, exhibited better anticancer activity (IC_{50} = 0.086 mM) compared to the addition of hydroxyl group on the 6-position (IC_{50} = 0.322 mM). It suggested that the 3-hydroxyl group gave a higher anticancer activity enhancement of 1-hydroxyxanthone against HeLa cancer cells compared to the 6-hydroxyl group. A combination of 3-hydroxyl and 6-hydroxyl groups at 3,6-dihydroxyxanthone gave the IC_{50} value of 0.162 mM, in which the IC_{50} value was in between the anticancer activity of 1,3-dihydroxyxanthone (IC_{50} = 0.086 mM) and 1,6-dihydroxyxanthone (IC_{50} = 0.322 mM).

Further addition of hydroxyl group on the 1,6-dihydroxyxanthone (IC_{50} = 0.322 mM), i.e., 1,3,6-trihydroxyxanthone and 1,5,6-trihydroxyxanthone, slightly enhanced its anticancer activity (IC_{50} = 0.203–0.241 mM). In contrast, the additional hydroxyl group on the 1,3-dihydroxyxanthone (IC_{50} = 0.086 mM), i.e.,

1,3,6-trihydroxyxanthone and 1,3,8-trihydroxyxanthone, demarcated its anticancer activity (IC_{50} = 0.203–0.277 mM). As reported before, this result could be explained by stronger intramolecular and/or intermolecular hydrogen bonds between the trihydroxylated xanthones during the anticancer examination [13]. From these data, the anticancer activity order against HeLa cancer cells was as follows: 1-hydroxyxanthone < 1,6-dihydroxyxanthone < 1,3,8-trihydroxyxanthone < 1,5,6-trihydroxyxanthone < 1,3,6-trihydroxyxanthone < 3,6-dihydroxyxanthone < 1,3-dihydroxyxanthone. It meant that 1,3-dihydroxyxanthone exhibited the strongest anticancer activity against HeLa cancer cells with an IC_{50} value of 0.086 mM.

Since the hydroxylated xanthones showed high anticancer activity against HeLa cancer cells, the cytotoxicity evaluation was also performed against non-cancer (normal) cells, i.e., the Vero cell line. The IC_{50} values of hydroxylated xanthones against normal cells are listed in Table 1. The IC_{50} value of hydroxylated xanthones against normal cells is expected to be higher than the HeLa cancer cells. If so, the hydroxylated xanthones prefer to attack HeLa cancer cells rather than normal cells. From Table 1, only 1,6-dihydroxyxanthone and 1,5,6-trihydroxyxanthone gave lower IC_{50} values against Vero cells (0.308 and 0.224 mM) than HeLa cancer cells (0.322 and 0.241 mM). These results indicated that both hydroxylated xanthones were more toxic to normal cells than HeLa cancer cells, which is unfavorable.

The selectivity index is a well-known parameter to determine the toxicity of anticancer agents. An anticancer agent is labeled a toxic drug when the selectivity index value is lower than 2.00 [27]. The selectivity index is calculated by the division of the IC_{50} value against normal cells over the IC_{50} value of cancer cells. The selectivity index of hydroxylated xanthones from the IC_{50} values data against HeLa cancer cells is shown in Table 2. The selectivity indexes of 1-hydroxyxanthone, 1,3-dihydroxyxanthone, 3,6-dihydroxyxanthone, 1,3,6-trihydroxyxanthone, and 1,3,8-trihydroxyxanthone were 6.315, 3.591, 7.902, 2.249, and 12.26, respectively, which were higher than 2.00. Thus, these hydroxylated xanthones are not considered toxic to normal cells.

From these data, the order of toxicity was 1,3,8-trihydroxyxanthone < 3,6-dihydroxyxanthone < 1-hydroxyxanthone < 1,3-dihydroxyxanthone < 1,3,6-trihydroxyxanthone < 1,6-dihydroxyxanthone < 1,5,6-trihydroxyxanthone. This order might suggest that 1,3,8-trihydroxyxanthone was the best anticancer agent with the lowest toxicity to normal cells. However, both sensitivity (low IC_{50} value) and selectivity (high selectivity index) shall be carefully considered in the design and development of anticancer agents. The selectivity index of 1,3,8-trihydroxyxanthone (12.26) was higher than 1,3-dihydroxyxanthone (3.591); however, its anticancer activity (IC_{50} = 0.277 mM) was lower than 1,3-dihydroxyxanthone (IC_{50} = 0.086 mM). Furthermore, the IC_{50} value of 1,3-dihydroxyxanthone (IC_{50} = 0.086 mM) was close to doxorubicin (IC_{50} = 0.068 mM) as the standard cancer drug. In contrast, it shall be noted that the 1,3-dihydroxyxanthone was 1.5 times less toxic than doxorubicin, which was remarkable.

On the other hand, 1-hydroxyxanthone, 1,3-dihydroxyxanthone, 1,6-dihydroxyxanthone, 3,6-dihydroxyxanthone, 1,3,6-trihydroxyxanthone, 1,3,8-trihydroxyxanthone, and 1,5,6-trihydroxyxanthone exhibited an IC_{50} value of 1.111, 0.114, 0.355, 0.786, 0.141, 0.254, and 0.209 mM, respectively, against WiDr cancer cells. The trend for the anticancer activity of hydroxylated xanthones against the WiDr cancer cells was similar to the HeLa cancer cells. The 1-hydroxyxanthone showed the weakest anticancer activity (IC_{50} = 1.111 mM) against WiDr cancer cells due to the presence of a single hydroxyl group. Additional hydroxyl groups in 1-hydroxyxanthone structure, i.e., 1,3-dihydroxyxanthone, 1,6-dihydroxyxanthone, 3,6-dihydroxyxanthone, 1,3,6-trihydroxyxanthone, 1,3,8-trihydroxyxanthone, and 1,5,6-trihydroxyxanthone, increased the anticancer activity (IC_{50} = 0.114–0.786 mM).

The addition of a hydroxyl group on the 3-position of 1-hydroxyxanthone, named 1,3-dihydroxyxanthone, also exhibited better anticancer activity (IC_{50} = 0.114 mM) against WiDr cancer cells compared to the addition of hydroxyl group on the 6-position (IC_{50} = 0.355 mM). It showed that the presence of the 3-hydroxyl group yielded a higher anticancer activity enhancement of 1-hydroxyxanthone compared to the 6-hydroxyl group. Further addition of hydroxyl group on the 1,6-dihydroxyxanthone (IC_{50} = 0.355 mM), i.e., 1,3,6-trihydroxyxanthone and 1,5,6-trihydroxyxanthone, slightly enhanced its anticancer activity (IC_{50} = 0.141–0.209 mM) against WiDr cancer cells.

In contrast, the additional hydroxyl group on the 1,3-dihydroxyxanthone (IC_{50} = 0.114 mM), i.e., 1,3,6-trihydroxyxanthone and 1,3,8-trihydroxyxanthone, suppressed its anticancer activity (IC_{50} = 0.141–0.254 mM). These data could be explained by stronger intramolecular and/or intermolecular hydrogen bonds between the hydroxylated xanthones during the anticancer examination which agreed with the reported literature [23]. From these data, the order of anticancer activity against WiDr cancer cells was 1-hydroxyxanthone < 3,6-dihydroxyxanthone < 1,6-dihydroxyxanthone < 1,3,8-trihydroxyxanthone < 1,5,6-trihydroxyxanthone < 1,3,6-trihydroxyxanthone < 1,3-dihydroxyxanthone. It meant that 1,3-dihydroxyxanthone exhibited the strongest anticancer activity against WiDr cancer cells with an IC_{50} value of 0.114 mM.

From Table 1, only 1,6-dihydroxyxanthone gave a lower IC_{50} value against the normal cell line (0.308 mM) than the WiDr cancer cell line (0.355 mM). Table 2 reveals that three hydroxylated xanthones, i.e., 1,6-dihydroxyxanthone, 3,6-dihydroxyxanthone and 1,5,6-trihydroxyxanthone were not qualified as safe anticancer agents because their selectivity index values were less than 2.00. In contrast, 1-hydroxyxanthone, 1,3-dihydroxyxanthone, 1,3,6-trihydroxyxanthone, and 1,3,8-trihydroxyxanthone yielded a selectivity index of 6.806, 2.690, 3.236, and 13.37, respectively. Therefore, they are considered safe anticancer agents that are nontoxic to normal cells.

The order of toxicity was 1,3,8-trihydroxyxanthone < 1-hydroxyxanthone < 1,3,6-trihydroxyxanthone < 1,3-dihydroxyxanthone < 3,6-dihydroxyxanthone < 1,5,6-trihydroxyxanthone < 1,6-dihydroxyxanthone. Even

though the selectivity index of 1,3,8-trihydroxyxanthone (13.37) was higher than 1,3-dihydroxyxanthone (2.690), however, its anticancer activity (IC_{50} = 0.254 mM) was lower than 1,3-dihydroxyxanthone (IC_{50} = 0.114 mM). Furthermore, Tables 1 and 2 show that 1,3-dihydroxyxanthone gave a comparable IC_{50} value and higher selectivity index than doxorubicin as the positive control. Therefore, as the 1,3-dihydroxyxanthone had fulfilled the requirement for low toxicity against normal cells, 1,3-dihydroxyxanthone was selected as the best candidate for an anticancer agent in this study.

Molecular docking studies of hydroxylated xanthones

To reveal the mechanism of action of 1,3-dihydroxyxanthone as an anticancer agent, molecular docking studies were conducted against the Topoisomerase II receptor, a protein that plays a pivotal role in the multiplication of cancer cells [28]. The Topoisomerase II receptor was selected as this receptor was overexpressed in 86 cervical and colorectal cancer patients [29]. The first step in the molecular docking of 1,3-dihydroxyxanthone was locating the 1,3-dihydroxyxanthone in the active site of the Topoisomerase receptor in the same position as mitoxantrone as the native ligand. The 1,3-dihydroxyxanthone had a similar structure to mitoxantrone, thus 1,3-dihydroxyxanthone is expected to be able to bind in the active of the Topoisomerase II receptor (Fig. 3).

The whole complex structure of 1,3-dihydroxyxanthone in the active site of the Topoisomerase II receptor is shown in Figure 4a. To simplify, the active site of Topoisomerase II is focused on in Figure 4b. Figure 4b shows that the hydroxyl and carbonyl groups of 1,3-dihydroxyxanthone interacted with Guanine13, Cytosine14, Lysine505, and Alanine521 through the hydrogen bonds. This result confirmed the importance of 1-hydroxyl and 3-hydroxyl groups as described in the *in vitro* assay. The 1,3-dihydroxyxanthone also interacted with
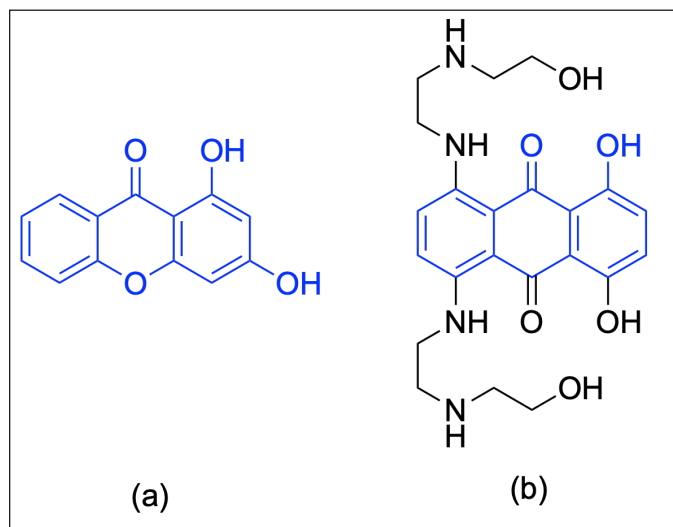
**Figure 3.** The chemical structure of (a) 1,3-dihydroxyxanthone and (b) mitoxantrone.
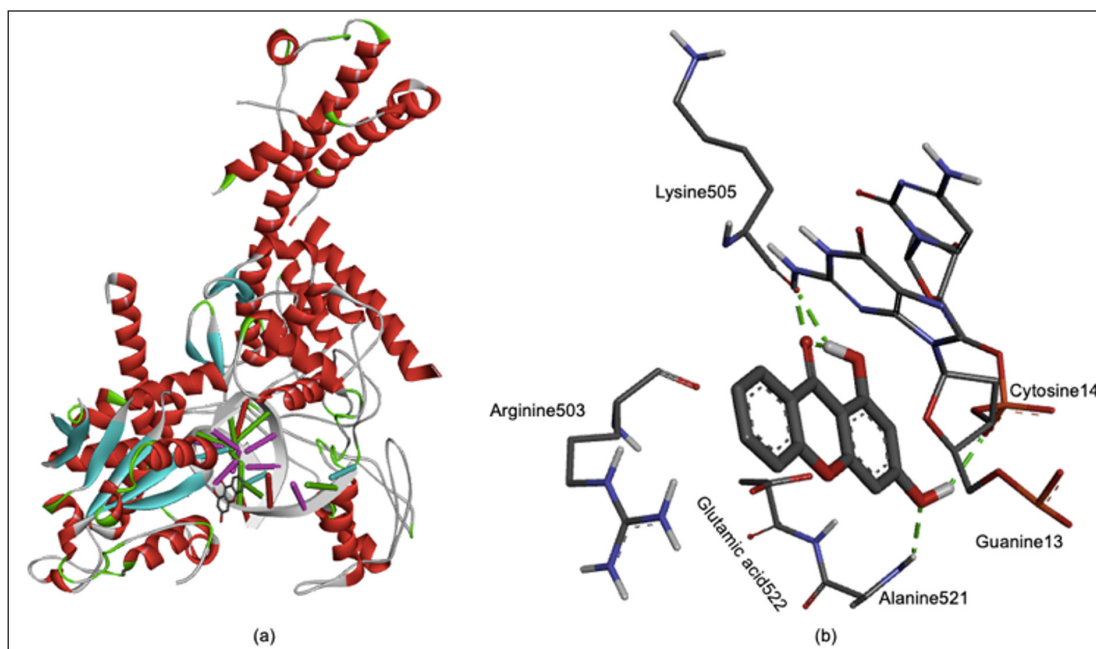
Figure 4. (a) Whole and (b) focused on three dimensional-structure of 1,3-dihydroxyxanthone in the active site of Topoisomerase II.

Arginine503 and Glutamic acid522 through noncovalent interactions, as shown in Figure 4b.

The more detailed noncovalent interactions of 1,3-dihydroxyxanthone in the active site of Topoisomerase II are displayed in a 2-D contour (Fig. 5 and Table 3). The 1,3-dihydroxyxanthone interacted with Guanine13 through its carbonyl group, with Lysine505 through its 1-hydroxyl group, and with Cytosine14 and Alanine521 through its 3-hydroxyl group with the hydrogen bond distance in a range of 3.87–7.01 Å. The six-membered rings of 1,3-dihydroxyxanthone interacted with Glutamic acid522 through pi-anion interaction with a distance of 5.62 and 7.05 Å, respectively. Instead of a hydrogen bond with Guanine13 through its carbonyl group, the 1,3-dihydroxyxanthone also interacted with Guanine13 through pi-pi T-shape interaction with a distance of 4.26 and 4.95 Å, respectively. Furthermore, the aromatic ring of 1,3-dihydroxyxanthone interacted with Arginine503 and Alanine521 amino acid residues through pi-alkyl interactions with a distance of 4.97 and 6.72 Å, respectively. The van der Waals interactions with Adenine12, Glycine504, Isoleucine506, Leucine507, and Asparagine520 are also observed in Figure 5.

The 1,3-dihydroxyxanthone was located in a final coordinate of $(x, y, z) = (34.415–36.444, 84.007–93.235, 48.450–53.379)$. With the aforementioned noncovalent interactions above, the 1,3-dihydroxyxanthone generated four types of energy. The first energy consisted of hydrogen bonds, van der Waals, de-solvation, and electrostatic energy with a total of −7.47 kcal/mol. The other energies were final total internal energy, torsional free energy, and unbonded system with a value of −0.67, +0.60, and −0.67 kcal/mol, respectively. The Gibbs free energy (−6.85 kcal/mol) was calculated by and summarizing all energies. Meanwhile, the ligand efficiency, binding constant, and RMSD values were 0.40, 9.56×10^{-6} mol/l, and 0.15 Å, respectively. These results demonstrated
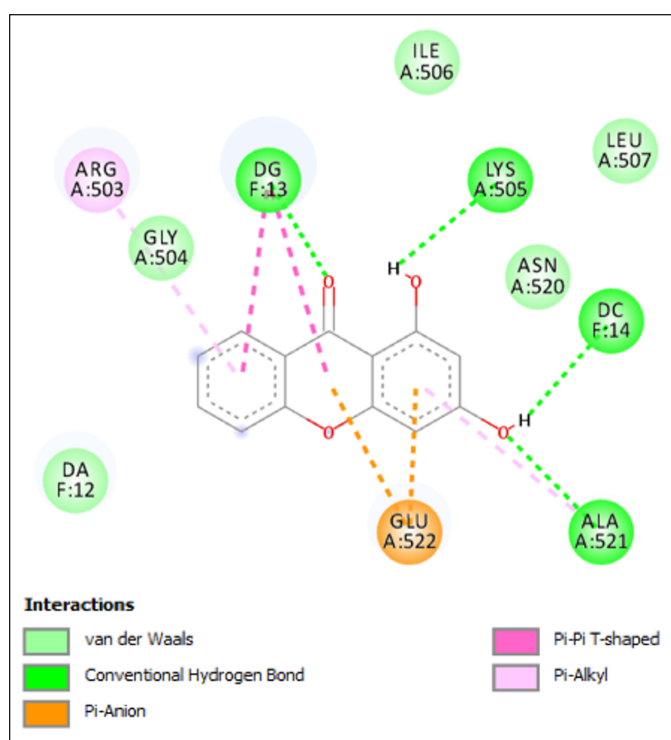
Figure 5. Non-covalent interactions of 1,3-dihydroxyxanthone in the active site of Topoisomerase II.

that 1,3-dihydroxyxanthone interacted well with key amino acid residues in the active site of the Topoisomerase II receptor, thus, disturbing the protein's function in the multiplication of cancer cells. It was reported that disturbance of Topoisomerase II receptors' function led to apoptotic cancer cell death [23]. Furthermore, Negri *et al.* [30] and Zhao *et al.* [31] reported

Table 3. Observed interactions of 1,3-dihydroxyxanthone in the active site of Topoisomerase II receptor.

Interaction	Amino acid/ nitrogen base	Distance (Å)	Donor/acceptor
Hydrogen bond	Guanine13	4.53	Carbonyl
	Cytosine14	7.01	3-Hydroxyl
	Lysine505	6.64	1-Hydroxyl
	Alanine521	3.87	3-Hydroxyl
Pi-anion	Glutamic acid522	5.62	Aromatic ring of 1,3-dihydroxy
		7.05	Tetrahydropyran-one ring
Pi-pi T-shaped	Guanine13	4.26	Tetrahydropyran-one ring
		4.95	Phenyl ring
Pi-alkyl	Arginine503	4.97	Phenyl ring
	Alanine521	6.72	Aromatic ring of 1,3-dihydroxy

that Topoisomerase II inhibition decreased the cancer cells' viability, as well as the rate of deoxyribonucleic acid synthesis in the cancer cells. This result could be the reason for the strong anticancer activity of 1,3-dihydroxyxanthone with IC_{50} values of 0.086 and 0.114 mM against HeLa and WiDr cancer cell lines.

CONCLUSION

The hydroxylated xanthones have been successfully synthesized through a one-pot reaction in 6.60%–46.50% yield. Seven hydroxylated xanthones have been evaluated as anticancer agents against HeLa cervical and WiDr colorectal cancer cells through *in vitro* MTT and *in silico* molecular docking studies. The addition of the 3-hydroxyl group gave a stronger anticancer activity than the addition of the 1-hydroxyl group against both cancer cells. Among the investigated hydroxylated xanthones, 1,3-dihydroxyxanthone exhibited the strongest anticancer activity with IC_{50} and selectivity index values of 0.086–0.114 mM and 2.690–3.591, respectively. Moreover, 1,3-dihydroxyxanthone exhibited comparable anticancer activity to doxorubicin with less toxicity to normal cells. From the molecular docking studies, 1,3-dihydroxyxanthone interacted with amino acid and nitrogen base residues through non-covalent interactions. These findings demonstrated that 1,3-dihydroxyxanthone could disturb the function of Topoisomerase II on the multiplication of cancer cells and lead to the death of cervical and colorectal cancer cells.

LIST OF ABBREVIATIONS

FTIR: Fourier-transforms infrared spectrophotometer; IC_{50}: Half-maximal inhibitory concentration; MS: Mass spectra; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR: Nuclear magnetic resonance; RMSD: Root-mean-square-deviation.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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