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Research Article

Cytotoxic activities of xanthones isolated from *calophyllum depressinervosum* and *calophyllum buxifolium* against snu-1 and ls174t cell lines.

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Abstract

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*A series of xanthones isolated from Calophyllum depressinervosum and Calophyllum buxifolium were studied for their cytotoxicities. Ananixanthone (**1**), caloxanthone B (**2**), caloxanthone I (**3**), caloxanthone J (**4**), xanthochymone B (**5**), thwaitesixanthone (**6**),1,3,5,6-tetrahydroxyxanthone (**7**) and dombakinaxanthone (**8**) were successfully isolated from both Calophyllum species. These eight compoundsand a synthetically derived compound, thwaitesixanthone monoacetate (**9**) were tested for cytotoxicity activities against stomach cancer, SNU-1and LS-174T cell lines. Caloxanthone B (**2**) gave the highest cytotoxic activity against SNU-1 with anIC_{50} valueof 1.47µg/ml. Meanwhile, xanthochymone B (**5**) shows the highest cytotoxic activity against LS-174T cell with an IC_{50} value of 0.45µg/ml. The structure-activity relationship (SAR) studies predicted that the presence and arrangements of furano, pyrano and prenyl substituents in the xanthone skeleton are important towardsthe cytotoxic activity. A molecular docking simulation was performed tomodel the probable binding models of compound **2** into the HER-2(PDB ID: 3PP0) and compound **5** into β-catenin (PDB ID: 1JDH).*

Keywords: Plants; Extracts; Activity; Phytochemistry; Alkaloids; Flavonoids; Glycosides.

Introduction:

The genus *Calophyllum* from the Clusiaceae family comprises 180-200 tree species distributed in the tropical rain forest with some occurring in Malaysia (Cechinel *et al.,* 2009). In Malaysia, this genus is known as *Bintagor* (Corner, 1952). The *Calophyllum* genus has been used in folk medicine to treat peptic ulcer, malaria, tumor, infection, blood pressure, pain and inflammation. In modern medicine, the research on this species has been rising since 1992 due to the discovery of anti-HIV properties possessed by calanolide A from *Calophyllum* leaves (Go´mez-Verjan *et al.,* 2015). The genus *Calophyllum* is abundantly rich with natural productsmainly coumarins, xanthones, flavonoids, chromanones and triterpenes (Ee *et al*, 2011). These types of compounds have significant biological activities such as anti-proliferative, antiviral, chemo-preventive, anti-secretory, crytoprotective, analgesic and antimicrobial properties (Go´mez-Verjan *et al.,* 2015).

Cancer is a leading cause of death worldwide. According to World Health Organization survey in 2015, about 8.8 million cases of deaths due to cancer have been reported (©WHO2017, 2017). Colon and stomach cancer are listed as most common cause of cancer-related death with 774,000 and 754,000 deaths, respectively (©WHO2017, 2017). These cancers are the most common type of cancer disease in both genders (Brand *et al.,* 2000). Less attention

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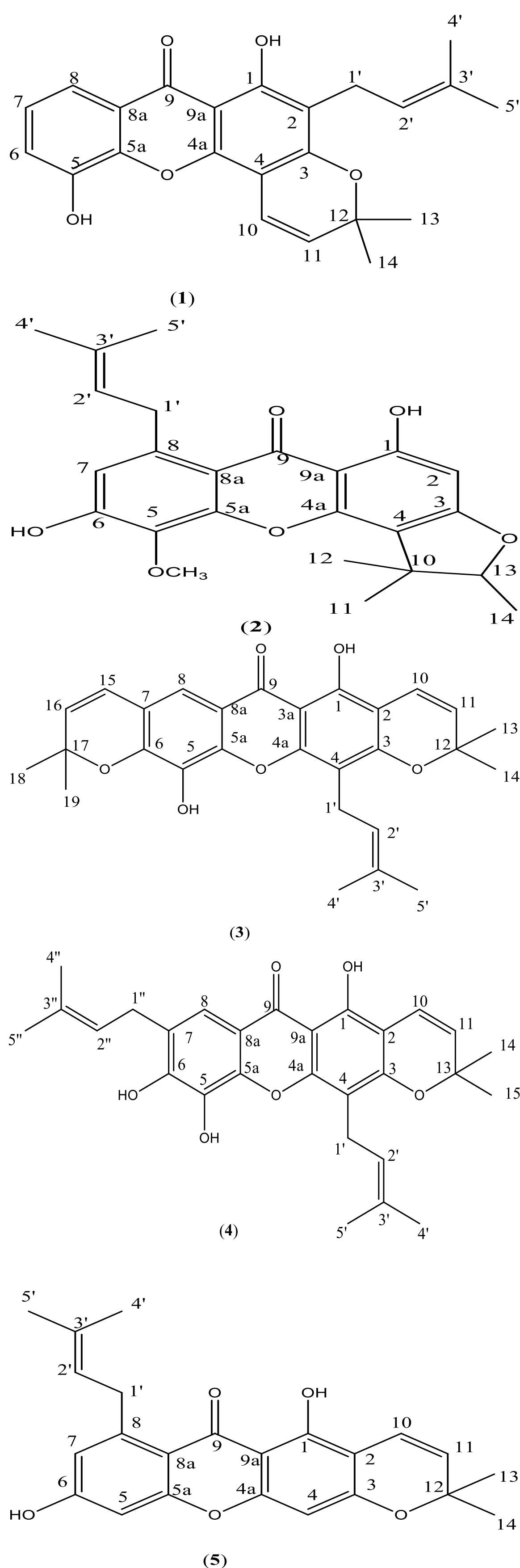
was given to these types of cancer when compared to breast and cervical cancer (Teng and Lu, 2013). Recent study demonstrated that amplification or overexpression about 10–30% of HER2 receptor in HER/EGFR signalling pathway are the cause for the development of stomach cancer. This significant amplication of HER2 has served as a prognostic and predictive biomarker for stomach cancer (Mark *et. al*., 2009). Meanwhile, colon cancer was reported to have a connection with the increase of β-catenin level in Wnt/β-catenin/Tcf signalling pathway, which leads to high proliferation and growth of cell cancer (Fatima *et. al*., 2011).

Based on the above information, two *Calophyllum* species such as *Calophyllum depressinervosum* and *Calophyllum buxifolium* were selected for detail phytochemical and pharmacological investigation towards SNU-1 and LS174T cell lines. A total of eight xanthones, compounds **1-8** (Fig. 1), were successfully isolated from these two *Calophyllum* species. A synthetically derived xanthone, **9** was produced from thwaitesixanthone (**6**). The cytotoxic activitiesof these compounds were then tested against stomach cancer cell line, SNU-1 and colon cancer, LS-174T cell line. The cytotoxic activities as well as structure activity relationship of these compounds are reported here. In order, to construct molecular models that incorporate all the experimental evidences, molecular docking was performed. These models are necessary to obtain a detailed picture of the biologically active compounds at molecular level, thus providing new insights to assist future research in the design and development of chemotherapeutic drugs.



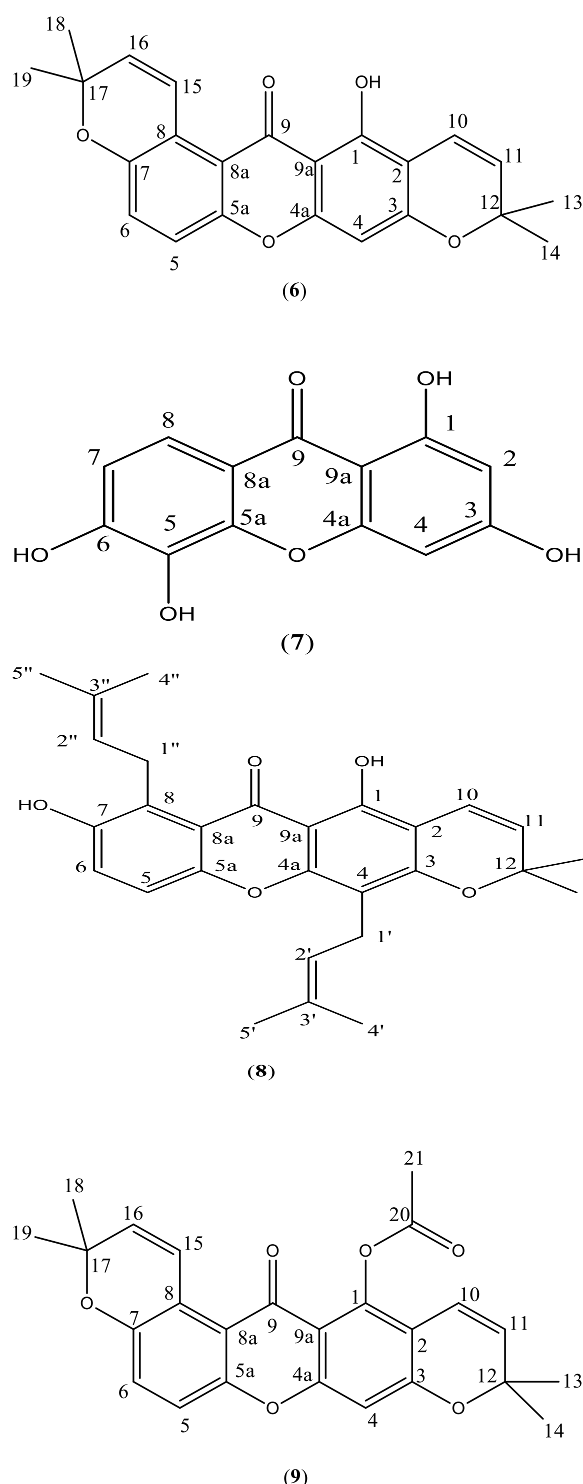


Fig. 1. Structures of ananixanthone (**1**), caloxanthone B (**2**), caloxanthone I (**3**), caloxanthone J (**4**) and xanthochymone B (**5**), thwaitesixanthone (**6**), 1,3,5,6-tetrahydroxyxanthone (**7**) and dombakinaxanthone (**8**) and thwaitesixanthone monoacetate (**9**)

MATERIAL AND METHODS

Chemicals

The Roswell Park Institute (RPMI 1640) and Eagle's Minimum Essential media (MEM) used for cell culture were purchased from AMRESCO®. The fetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from Thermo Scientific. All solvents and chemicals were purchased from Merck, Fisher and Sigma-Aldrich.

Plant Collection

The stem bark of *Calophyllum depressinervosum* (1.8 kg) and *Calophyllum buxifolium* (2.2 kg) were collected from Sri Aman district in Sarawak, Malaysia and was identified by Professor Dr. Rusea Go from the Biology Department, Faculty of Science, Universiti Putra Malaysia. The voucher specimens were deposited in the Herbarium of Biology Department, Faculty of Science, Universiti Putra Malaysia.

Plant Extraction and compound isolation

Both collected stem bark samples were dried in open air and ground into fine powder. The powdered stem bark of *Calophyllum depressinervosum* was macerated three times in three different solvents which were hexane, dichloromethane and ethyl acetate for 72 hours each. The macerated extracts were filtered and evaporated under reduced pressure in a rotary evaporator to remove all the solvents. This resulted in a solidified extract of hexane (27g), dichloromethane (26g) and ethyl acetate (33g). The same extraction procedure was performed on *Calophyllum buxifolium* to provide extracts of hexane (47g), dichloromethane (66g) and ethyl acetate (81.3g). Purification of the *Calophyllum depressinervosum*hexane extract resulted in the isolation of ananixanthone (**1**) and caloxanthone B (**2**). From the semi polar extracts (dichloromethane and ethyl acetate) of

Calophyllum depressinervosum were successfully obtained caloxanthone I (**3**), caloxanthone J (**4**) and xanthochymone B (**5**). The column chromatographic separations on *Calophyllum buxifolium* hexane extract gave thwaitesixanthone (**6**). Meanwhile, 1,3,5,6-tetrahydroxyxanthone (**7**) and dombakinaxanthone (**8**) were successfully isolated from the dichloromethane and ethyl acetate extracts.

Ananixanthone (**1**); Yellow needle crystals; MP: 168-170ºC (Lit:- 170-171ºC (Joaquin *et al.,* 1998); IR v_{max} cm^{-1}:3217, 2918 and 1573, 1439,1221 and 1115; EIMS m/z: 378, 363, 323, 305, 152;^{1}H and ^{13}C- NMR spectra are consistent with literature Joaquim *et al.,* 1998.

Caloxanthone B (**2**); Yellow needle crystals; m.p. 159-161ºC Lit. 160.5ºC (Iinuma *et al.,* 1994); IR v_{max} cm^{-1} 3422, 2928, 1586, 1419, 1129; EIMS *m/z*: 410, 395, 367, 352, 325, 176; ^{1}H and ^{13}C- NMR spectra are consistent with literature Iinuma *et al.,* 1994.

Caloxanthone I (**3**); Yellow amorphous powder, IR v_{max} cm^{-1}:3398, 2918, 1570, 1436 and 1126; EIMS *m/z*: 460,445,417,405,215,187. ^{1}H and ^{13}C- NMR spectra are consistent with literature Cheng *et al.,* 2004.

Caloxanthone J (**4**); Yellow amorphous powder, IR v_{max} cm^{-1}:3217, 2928, 1610, 1461, 1298 and 1151;EIMS *m/z*: 462, 447, 419, 391, 215, 188; ^{1}H and ^{13}C- NMR spectra are consistent with literature Iinuma *et al.,* 1997.

Xanthochymone B (**5**); Yellow gum; IR v_{max} cm^{-1}:2978, 1611, 1455, 1295, 1145; EI-MS *m/z*: 378,363,335,279.^{1}H and ^{13}C- NMR spectra are consistent with literature Trisuwan *et al.,* 2014.

Thwaitesixanthone (**6**); Yellow needle crystals; m.p: 218-221ºC Lit: 221-224ºC (Dharmaratne *et al.,* 1986); IR v_{max} cm^{-1}:3458, 2859, 1731, 1458, 1246 and 1021; EIMS *m/z*: 376, 361, 343, 331, 173; ^{1}H and ^{13}C- NMR spectra are consistent with literature Jiang *et al.,* 2003.

1,3,5,6-tetrahydroxyxanthone (**7**); White amorphous powder; IR vmax cm-1: 3342, 2927, 1644, 1463 and 1156; EIMS m/z: 260, 231, 203, 152, 69; ^{1}H and ^{13}C- NMR spectra are consistent with literature Jiang *et al.,* 2003.

Dombakinaxanthone (**8**); Yellow needle crystals; m.p: 147-149ºC Lit: 146-147ºC (Dharmaratne and Wijesinghe,1997); IR v_{max} cm^{-1}:3440, 2922, 1606, 1451, 1261 and 1134;EIMS *m/z*: 446, 431, 403, 347, 208, 67; ^{1}H and ^{13}C- NMR spectra are consistent with literature Dharmaratneand Wijesinghe, 1997.

Structure modification of thwaitesixanthone

A structural modification was carried out on compound **6**. Thwaitesixanthone monoacetate (**9**) was obtained from the acetylation of compound **6** (50mg). The acetylation method was adopted from Ikeya *et al.,* 1991.

Yield: 45%, Yellow amorphous powder; IR vmax cm-1: 2919, 1597, 1447, 1273, 1072;^{1}H NMR ($CDCl_3$, 500MHz) δ_H:7.94 (d, 1H, *J*=10.30Hz, H-15), 7.16 (d, 1H, *J*=9.15Hz, H-5), 7.09 (d, 1H, *J*=9.15Hz, H-6), 6.65 (s, 1H, H-4), 6.48 (d, 1H, *J*=9.15Hz, H-10),5.74 (d, 1H, *J*=9.15Hz, H-11), 5.72 (d, 1H, *J*=10.30Hz, H-16), 2.49 (s, 3H, H-21), 1.47 (s, 6H, H-13 & H-14), 1.42 (s, 6H, H-18 & H-19) and ^{13}C NMR ($CDCl_3$, 125 MHz)δ_C:177.2 (C-9), 169.4 (C-20), 158.4 (C-3), 157.4 (C-1), 151.0 (C-4a), 149.3 (C-5a), 145.5 (C-7), 132.2 (C-16), 131.2 (C-11), 123.5 (C-6), 121.3 (C-15), 120.0 (C-8), 117.5 (C-5), 117.0 (C-8a), 115.3 (C-10),

111.8 (C-2), 109.7 (C-9a), 101.6 (C-4), 78.1 (C-17), 75.3 (C-12),28.5 (C-18 & C-19), 27.3 (C-13 & C-14), 21.2 (C-21);EIMS m/z: 434, 418, 376, 361,173.

Cytotoxic Assay

The cytotoxic assays were performed using MTT assays as described by Mosmann, 1983. Both stomach cancer SNU-1 and colon cancer LS-174T cell lines for this work were obtained from Taylor's University. The cytotoxicity of the compounds was expressed as IC_{50} values. *Cis*- diammineplatinum (II) chloride was used as standard compound for both cancer cell lines.

Molecular docking

The three-dimensional structure of selected ligand was generated and optimised by using MMFF94s force field in Avogadro software (Halgren, 1999); (Hanwell *et al*., 2012). The crystal structures of HER-2 (PDB: 3PP0, chain A) and β-catenin (PDB: 1JDH, chain A) receptor proteins were retrieved from the Protein Data Bank (www.rcsb.com). Then, hydrogen atoms were added to the protein structures using AutoDockTools (Sanner, 1999). The docking was performed using Auto Dock Vina (Trott and Olsen, 2010). The grid box was set to cover important residues involved in ligand binding, Lys435 and Lys312 in β-catenin, and Lys753 and Glu770 in HER2. The dimension of the grid box is tabulated in Table 1. The grid box spacing was set to be 1.0 angstrom so that all the residues are available in equal-opportunity zone for ligand binding. In β-catenin, two grid boxes were set due to the existence of two active sites (hereafter named Site A and Side B) which are important for β-catenin and Tcf interaction (Liu *et al*., 2006). The complex of the best compound with the protein receptor was carefully inspected and analysed using LIGPLOT (Laskowski & swindells, 2011).

Table 1: The docking parameters

	Colon Cancer (LS174T)		Stomach Cancer (SNU-1)
	β–catenin (site A)	β–catenin (site B)	
x-dimension	20	30	20
y-dimension	20	30	20
y-dimension	20	30	20
X centre	-6.485	-0.707	16.387
Y centre	0.514	11.906	17.394
Z centre	51.129	21.721	26.218

Statistical Analysis

The cytotoxicity test data were represented as mean with standard errors obtained from three independent experiments, in which each experiment was performed in triplicate. The graphs were generated using Microsoft Excel Software (Version 2010). The independent sample T-test was performed by SPSS 14.0 to determine significant difference between the sample and standard drug, *cis*-diammineplatinum (II) chloride. The significance level was set at $p < 0.05$.

RESULTS

Cytotoxic activity evaluation

All the seven xanthones isolated from *Calophyllum depressinervosumand Calophyllum buxifolium* together with its synthetically derived compound **9** were screened for cytotoxic activity against two cancer cell lines (SNU-1 and LS-174T). The general chemical structure of xanthone is made up of three rings with a carbonyl group attached to the middle oxygenated heterocyclic ring. *Cis*- diammineplatinum (II) chloride was used as positive control for both cancer cells. *Cis*- diammineplatinum (II) chloride is a well-known synthetic inorganic drug used as a chemotherapeutic agent in the treatment of ovarian, testicular, and head and neck carcinomas (Gately and Howell, 1993). From the literature, this control compound showed a strong activity

towards both cell lines (Yoo *et al.,* (2004 Otto *et al.,* (2004). The IC_{50} values of the seven isolated xanthones and compound **9** together with the positive control against SNU-1 and LS-174T are tabulated in Table 2. Among all the xanthones, caloxanthone B (**2**) gave the highest cytotoxic activity against SNU-1 cell line. Meanwhile, forthe LS174T cell line, xanthochymone B (**5**) gave the highest cytotoxic activity compared to the other xanthones. It is interesting to highlight that the cytotoxic activity possessed by xanthochymone B (**5**) is better than the positive control, *cis*-diammineplatinum (II) chloride. The remaining xanthone compounds showed either moderate or weak activities against both cell lines, as listed in Table 2. The cytotoxic activity for thwaitesixanthone monoacetate (**9**) towards SNU-1 was not performed due to insufficient amount of the sample.

Table 2: Cytotoxic activities of xanthones toward SNU-1 cell line (stomach cancer) and LS-174T cell line (colon cancer).

Compound	IC_{50} µg/ml	
	SNU-1 cell	LS-174T cell
Ananixanthone (**1**)	8.97 ± 0.11	7.48 ± 0.08*
Caloxanthone B (**2**)	1.47 ± 0.01*	4.02 ± 0.18*
Caloxanthone I (**3**)	2.90±0.10*	15.93 ± 0.24*
Caloxanthone J (**4**)	4.83±0.04*	9.36 ± 0.23*
Xanthochymone B (**5**)	6.94±0.07*	0.45 ± 0.01*
Thwaitesixanthone (**6**)	NA	NA
1,3,5,6-tetrahydroxyxanthone (**7**)	7.89±0.38	14.10 ± 0.91*
Dombakinaxanthone (**8**)	14.53±0.53*	5.80 ± 0.14 *
Thwaitesixanthone Monoacetate (**9**)	NA	16.63±1.53*
cis- Diammineplatinum (II) chloride	9.64 ± 0.59	1.32 ± 0.03

* NA= not available

* denotes significant difference between sample and positive control, *cis*-diammineplatinum (II) chloride ($p<0.05$).

Molecular Docking Study

In an attempt to gain better insight into the molecular interaction of the most active compounds, caloxanthone B (2) with HER2 protein and xanthochymone B (5) with β-catenin, molecular docking was performed. A visual inspection using LIGPLOT analysis oncaloxanthone B (**2**) dockedonto HER2 protein revealed that two intermolecular hydrogen bonds were observed between two hydroxyl groups and Asp863 (3.21Å) and Asp 808 (3.26Å) respectively. A hydrophobic interaction was observed between Lys753 and the carbon belonging to the prenyl moiety. The hydrophobic properties of the methoxyl, prenyl and furano substituents in caloxanthone B (**2**) contributed to the ligand stabilisation by forming hydrophobic contact with the surrounding residues such as Thr798, Thr862, Gly729, Ser728, Arg849, Cys805, Leu726, Ala751, Leu852 and Val732.The binding interaction diagram for HER2-caloxanthone B (**2**) complex isshown in Fig. 2.

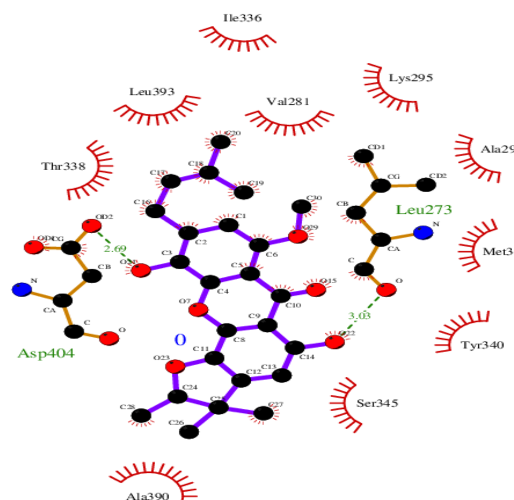


Fig. 2. Two-dimensional diagram of binding interaction of the docked structure of caloxanthone B (**2**) bound to HER2 (PDB: 3PP0) protein receptor using LIGPLOT (Laskowski & Swindells, 2011).

In colon cancer, the Wnt/β-catenin/Tcf signalling pathway plays an important role in cancer proliferation and self-renew. An alternative way to modify the signalling is by disrupting the interaction between two proteins along this pathway: β-catenin and Tcf4. There are two active sites on β-catenin, Site A and Site B. Site A of β-catenin contains the key important amino acid Lys312, while Site B contains amino acid Lys435, which is important in the interaction between the protein and the TCF4 protein (Graham *et al.,* 2001). The binding of small molecules isolated from *Calophyllum* species will block the active site of β-catenin, thus disrupting the protein-protein interaction. The highest cytotoxic activity possessed by xanthochymone B (**5**) againstthe colon cancer cell line, in which β-catenin level is elevated, inspired us to perform molecular docking on the compound. A visual inspection of the docked xanhochymone B (**5**) into Site A of β-catenin revealed two intermolecular hydrogen bonds with Lys312 (2.81Å) (3.01Å). The stabilisation of the compound was also contributed via hydrophobic contracts between the pyrano and prenyl moieties of the compounds with residues Val346, Try306, Gly30, Gln309, Ser351 and Val349 of β-catenin. The attachmentsof prenyl and pyrano groups at different heterocyclic ring increase the cytotoxic activities against LS174T cell line. In Site B, two intermolecular hydrogen bonds were seen between the two hydroxyl oxygen atoms and Arg474 (3.21Å) and Gln482 (3.18Å). However, there was no interaction between the compound and the important residuesLys 435. Meanwhile, the hydrophobic interactions were formed between the ligand and the residues Leu519, Ile579, Asn516, Arg515, Ser473, Ala478, His475, Glu479 and Gln476. The binding interaction of the docked xanthochymone B (**5**) onto β-catenin (PDB:-1JDH) isshown in Fig. 3.

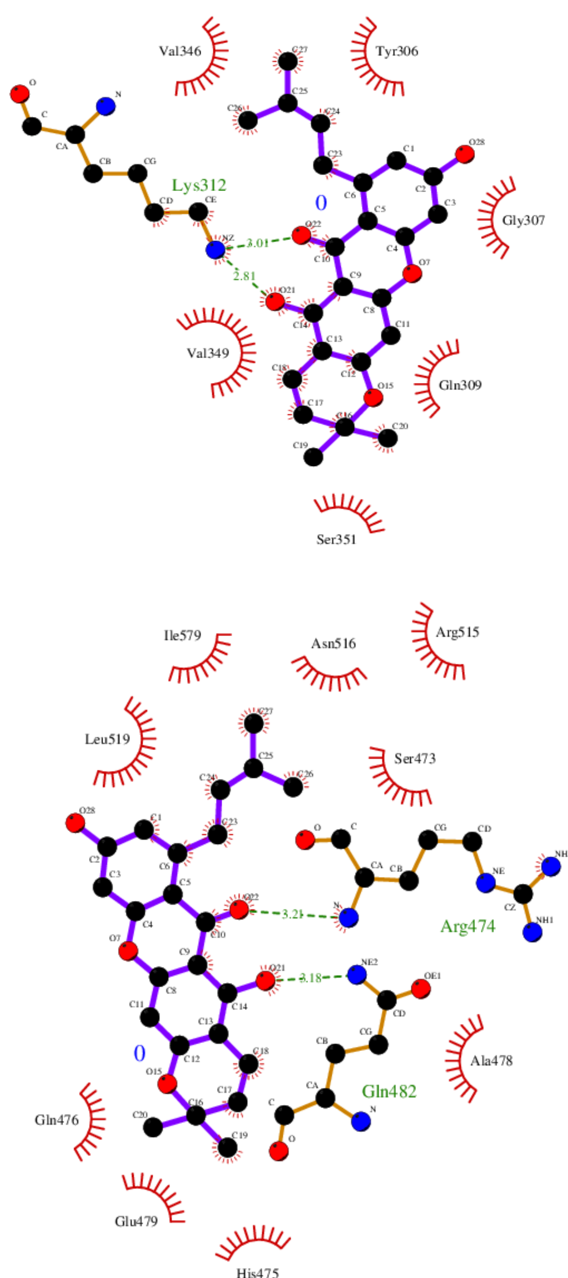


Fig. 3. Two-dimensional diagram of binding interaction of the docked structure of xanhochymone B (**5**) with β-catenin (PDB: 1JDH) protein receptor.

A: between xanhochymone B (5) and residues at Site A. B: Between analysis of xanhochymone B (5) and residues at Site B.

DISCUSSION

Structure-activity relationships of xanthones were studied to describe the structural requirement for the SNU-1 inhibitor. In the SNU-1 cell line assay, caloxanthone B (**2**) showed the highest cytotoxic activity compared to the other compounds. The presence of methoxyl, furano and prenyl substituents allows the compound to make molecular interactions with the protein cancer cell line, thus inhibiting the cell proliferation causing apoptosis. The extra one hydroxyl and prenyl moieties located at C5 and C7 respectively, make caloxanthone J (**4**) more active than dombakinaxanthone (**8**). Meanwhile, the presence of two pyrano and one prenyl substituents in caloxanthone I (**3**) may contribute to the strong cytotoxic activity towards SNU-1 cell line. This may be due to the high binding interaction between caloxanthone I (**3**) with the amino acid sequence of protein. The optimum orientations of compound **5** in the active site of the protein also lead to the tighter binding interaction.

Eight xanthones were tested for their cytotoxic activities towards LS174T. The structure activity relationships of these xanthones were studied to identify the structural requirement for inhibition of colon cancer cell line. Ananixanthone (**1**), caloxanthone B (**2**), dombakinaxanthone (**8**), caloxanthone J (**4**) and xanthochymone B (**5**) demonstrate strong cytotoxic activities towards LS174T because of the presence of one furan substituent in the xanthone skeleton. Meanwhile,thwaitesixanthone monoacetate (**9**) and caloxanthone I (**3**) have two pyrano groups and they showed moderate activities against LS174T. The bulky skeletons of these compounds make them unable to fit into the active sites and interact with the important residues of the protein. A simple xanthone, 1,3,5,6-tetrahydroxyxanthone (**7**) which contain only a hydroxyl substituent gave a moderate activity because of the less binding interaction between the compound and the responsible protein in LS174T. The prenyl and pyrano moieties in one heterocyclic ring decrease the cytotoxic activity as shown by ananixanthone (**1**). However, when a prenyl and pyrano group were located in a different heterocyclic ring, for example as in xanthochymone B (**5**), a significant increase in the cytotoxic activity against LS174T was observed.

In summary, caloxanthone B (**2**) showed strong inhibition against SNU-1 while xanhochymone B (**5**) strongly inhibitsLS-174T compared to the other xanthones tested. It is observed that the types of substituent attachment and arrangement in a xanthone skeleton play a major role in the ligand-protein interaction. The results from molecular docking showed that both compounds can bind well onto the respective targeted protein by making interactions with one or more residues located at the active site, thus altering the protein activity. Detail *in-vitro* and *in-vivo*work on these two compounds are needed to design and develop potential lead compounds for candidates of anticancer drugs.

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