Food and Chemical Toxicology 133 (2019) 110800

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Cytotoxic xanthones isolated from *Calophyllum depressinervosum and Calophyllum buxifolium* with antioxidant and cytotoxic activities

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ARTICLE INFO

Keywords:
Caloxanthone B
K562 cells
Molecular docking
Radical scavenging activities

ABSTRACT

The stem bark of *Calophyllum depressinervosum* and *Calophyllum buxifolium* were extracted and examined for their antioxidant activities, together with cytotoxicity towards human cancer cells. The methanol extract of *C. depressinervosum* exhibited good DPPH and NO scavenging effects. The strongest BCB inhibition and FIC effects were shown by dichloromethane and ethyl acetate extracts of both species. Overall, DPPH, FRAP and FIC assays showed strong correlation with TPC. For cytotoxicity, hexane extract of *C. depressinervosum* possessed the strongest anti-proliferative activities towards SNU-1 cells while the hexane extract of *C. buxifolium* showed the strongest activity towards LS-174T and K562 cells with the IC_{50} values ranging from 7 to 17 μg/mL. The purification of plant extracts afforded eight xanthones, 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**). All the xanthones, except **1** were reported for the first time from both *Calophyllum* species. The xanthones were examined for their cytotoxic effect against K562 leukemic cells. Compounds **1** and **2** showed strong cytotoxicity with the IC_{50} values of 2.96 and 1.23 μg/mL, respectively. The molecular binding interaction of **2** was further investigated by performing molecular docking study with promising protein receptor Src kinase.

1. Introduction

Calopyllum plant belongs to the family of Clusiaceae and this plant genus comprises of 180–200 species, which are widely distributed in the tropical countries (Cechinel et al., 2009). This plant grows as common trees in the lowland and mountain forest. There are around 80 species of *Calophyllum* plants can be found in Malaysia and it is locally known as *Bintagor* (Corner, 1952). *Calophyllum* plants have been used in folk medicine since ancient time to treat peptic ulcer, malaria, tumor, infections, blood pressure, paint and inflammation. In modern medicine, the research interest on this plant has been rising since 1992 due to the discovery of anti-HIV compound, calonolide A from its leaves (Gómez-Verjan et al., 2015). This genus is an important source of natural products, mainly coumarins, xanthones, flavonoids, chromanones and triterpenes (Ee et al., 2011). These compounds possess significant biological activities such as antiviral, chemo-preventive, anti-secretory, cytoprotective, analgesic and antimicrobial properties (Gómez-Verjan et al., 2015). In this study, two *Calophyllum* species, *C. depressinervosum* and *C. buxifolium* were selected for the evaluation of antioxidant and cytotoxic effects due to limited studies that have been conducted on these species. This is the first report on the total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activities of *C. depressinervosum* and *C. buxifolium*, as well as their cytotoxic activities towards human cancer cells.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in the human body by energy transfer processes and enzymatic reactions (Schulz, 2005). An increase in the level of reactive oxygen species will lead to disorders and diseases such as atherosclerosis, neurodegenerative disorder, aging and cancer (Gómez-Verjan et al., 2015). Cancer is a major cause of death worldwide. According to a World Health Organization survey in 2015, about 8.8 million cases of death were reported to be due to cancer (©WHO, 2017). The inhibition

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https://doi.org/10.1016/j.fct.2019.110800
Received 2 May 2019; Received in revised form 24 August 2019; Accepted 30 August 2019
Available online 31 August 2019
0278-6915/

of ROS and other radical molecules might be able to reduce these disorders and diseases. Phenolic compounds are known to have antioxidant properties, which will protect the cells from ROS and RNS. These phenolic compounds usually exhibit anti-oxidative activities *via* several mechanism of actions. The mechanisms are singlet oxygen quencher, hydrogen donating antioxidant, free radical scavenger and metal ions chelator (Öztürk et al., 2007; Ruhomally et al., 2015). These antioxidant activities are commonly exhibited by the plant extracts and correlated to the TPC and TFC of the plant extracts (Saeed et al., 2012). Thus, TPC and TFC of the plant extracts of *C. depressinervosum* and *C. buxifolium* were examined, in addition to the antioxidant activities, which were conducted by using different bioassay methods.

Unfortunately, not all phenolic compounds exhibited antioxidant activity. Some of these compounds such as polyphenols showed pro-oxidant activities (León-González et al., 2015) and increased the ROS and RNS in a cell, in turn lead to mutagenic and carcinogenic activities (Schulz, 2005). However, a recent study has shown that a pro-oxidant molecule can act as a selective cytotoxic agent against cancer cells by increasing the toxicity level of ROS and RNS in cancer cells and lead to the apoptosis of cancer cells (León-González et al., 2015). Phenolic compounds found in the plants are also known to have anti-proliferative properties, probably due to pro-oxidant activity (Hinneburg et al., 2006). Therefore, phenolic compounds are considered important for their dietary roles as both antioxidants and anticancer agents. The molecular mechanisms of cancer involves a wide range of protein tyrosine kinases, which are vital in regulating the signalling pathways in cellular differentiation, metabolism and apoptosis in response to indigenous stimuli. One of the tyrosine kinases, Src is regularly overexpressed in a variety of epithelial and non-epithelial cancers such as lung, breast, colorectal and melanomas. It also plays an important role in tumour progression and metastasis development, unveiling Src as a promising target for cancer.

Phytochemical constituent study was also conducted on the selected *Calophyllum* species, which are *C. depressinervosum* and *C. buxifolium*. A total of eight xanthones, **1**–**8** with the chemical structures as shown in Fig. 1, were successfully isolated from the plant extracts of two species. The cytotoxic effects of these compounds were examined towards human cancer cell line, K562 cells (leukemia) and reported for the first time. Furthermore, in order to elucidate the potential mechanism by which the bioactive compounds induce the cytotoxic activity, molecular docking study was performed by positioning the most cytotoxic compound into the active loop of Src kinase (PDB ID: 2SRC).

2. Materials and methods

2.1. Chemicals

The Roswell Park Memorial Institute (RPMI 1640) and Eagle's Minimum Essential (MEM) media used for the cell culture work were purchased from AMRESCO®. Fetal bovine serum (FBS) and phosphate buffered saline (PBS) were purchased from Thermo Scientific. All chemicals and solvents with high purity grade were purchased from Merck, Sigma-Aldrich and Fisher Scientific for plant extraction, compounds purification and bioassay.

2.2. Plant collection

The stem barks of *Calophyllum depressinervosum* (1.8 Kg) and *Calophyllum buxifolium* (2.2 Kg) were collected from the Sri Aman district in Sarawak, Malaysia and identified by Professor Dr. Rusea Go from biology department, University Putra Malaysia. Voucher specimens of *Calophyllum depressinervosum* (RG5028) and *Calophyllum buxifolium* (RG5020) were deposited in the herbarium of Biology Department, Faculty of Science, University Putra Malaysia.

2.3. Plant extraction and isolation of compounds

The collected plant samples were dried under open air and ground into fine powder. The powdered stem bark of *C. depressinoversum* and *C. buxifolium* were macerated three times with different solvents in the sequence of increasing polarity for 72 h and the solvents are hexane, dichloromethane, ethyl acetate and methanol. The macerated plant sample was then filtered and the filtrates were evaporated under reduced pressure to obtain dry plant extracts of hexane (27 g), dichloromethane (26 g), ethyl acetate (33 g) and methanol (87 g). The same extraction procedure was performed on *Calophyllum buxifolium* and this afforded dry plant extracts of hexane (47 g), dichloromethane (66 g), ethyl acetate (81.3 g) and methanol (103 g).

The plant extracts were subjected to a series of column chromatography separation using silica gel 60 (Merck, 0.040–0.063 mm) and the mobile phase was comprised of hexane, chloroform, ethyl acetate and methanol. Sephadex LH20 was used in the final step of purification of the compounds. Extensive purification of the hexane extract of *C. depressinervosum* resulted in the isolation of ananixanthone (**1**) and caloxanthone B (**2**) while the dichloromethane and ethyl acetate extract gave caloxanthone I (**3**), caloxanthone J (**4**) and xanthochymone B (**5**). On the other hand, purification of the hexane extract of *C. buxifolium* afforded thwaitesixanthone (**6**) while 1,3,5,6-tetrahydroxyxanthone (**7**) and dombakinaxanthone (**8**) were successfully isolated from the dichloromethane and ethyl acetate extracts respectively. The structures of all the xanthone were shown in Fig. 1.

2.3.1. Ananixanthone (1)

Yellow needle crystal; m.p. 168-170 °C (literature 170-171 °C, Joaquim et al., 1998); IR ν_{max} cm^{-1}: 3217, 2918, 1573, 1439, 1221, 1115; UV (EtOH) λ_{max} nm: 334, 319, 253; EIMS [M^+] *m/z*: 378, 363, 323, 305, 152; ^1H and ^{13}C- NMR spectra are consistent with literature (Joaquim et al., 1998).

2.3.2. Caloxanthone B (2)

Yellow needle crystal; m.p. 159-161 °C (literature 160.5 °C, Iinuma et al., 1994); IR ν_{max} cm^{-1}: 3422, 2928, 1586, 1419, 1129; UV (EtOH) λ_{max} nm: 364, 318, 284, 250; EIMS [M^+] *m/z*: 410, 395, 367, 352, 325, 176; ^1H and ^{13}C- NMR spectra are consistent with literature (Iinuma et al., 1994).

2.3.3. Caloxanthone I (3)

Yellow amorphous powder; IR ν_{max} cm^{-1}: 3398, 2918, 1570, 1436, 1126; UV (EtOH) λ_{max} nm: 361, 294, 228; EIMS [M^+] *m/z*: 460, 445, 417, 405, 215, 187; ^1H and ^{13}C- NMR spectra are consistent with literature (Cheng et al., 2004).

2.3.4. Caloxanthone J (4)

Yellow amorphous powder; IR ν_{max} cm^{-1}: 3217, 2928, 1610, 1461, 1298, 1151; UV (EtOH) λ_{max} nm: 382, 274, 233; EIMS [M^+] *m/z*: 462, 447, 419, 391, 215, 188; ^1H and ^{13}C- NMR spectra are consistent with literature (Iinuma et al., 1997).

2.3.5. Xanthochymone B (5)

Yellow gum; IR ν_{max} cm^{-1}: 2978, 1611, 1455, 1295, 1145; UV (EtOH) λ_{max} nm: 338, 264, 218; EIMS [M^+] *m/z*: 378, 363, 335, 279; ^1H and ^{13}C- NMR spectra are consistent with literature (Trisuwan et al., 2014).

2.3.6. 1,3,5,6-Tetrahydroxyxanthone (6)

White amorphous powder; IR ν_{max} cm^{-1}: 3342, 2927, 1644, 1463, 1156; UV (EtOH) λ_{max} nm: 311, 252, 218; EIMS [M^+] *m/z*: 260, 231, 203, 152, 69; ^1H and ^{13}C- NMR spectra are consistent with literature (Jiang et al., 2003).

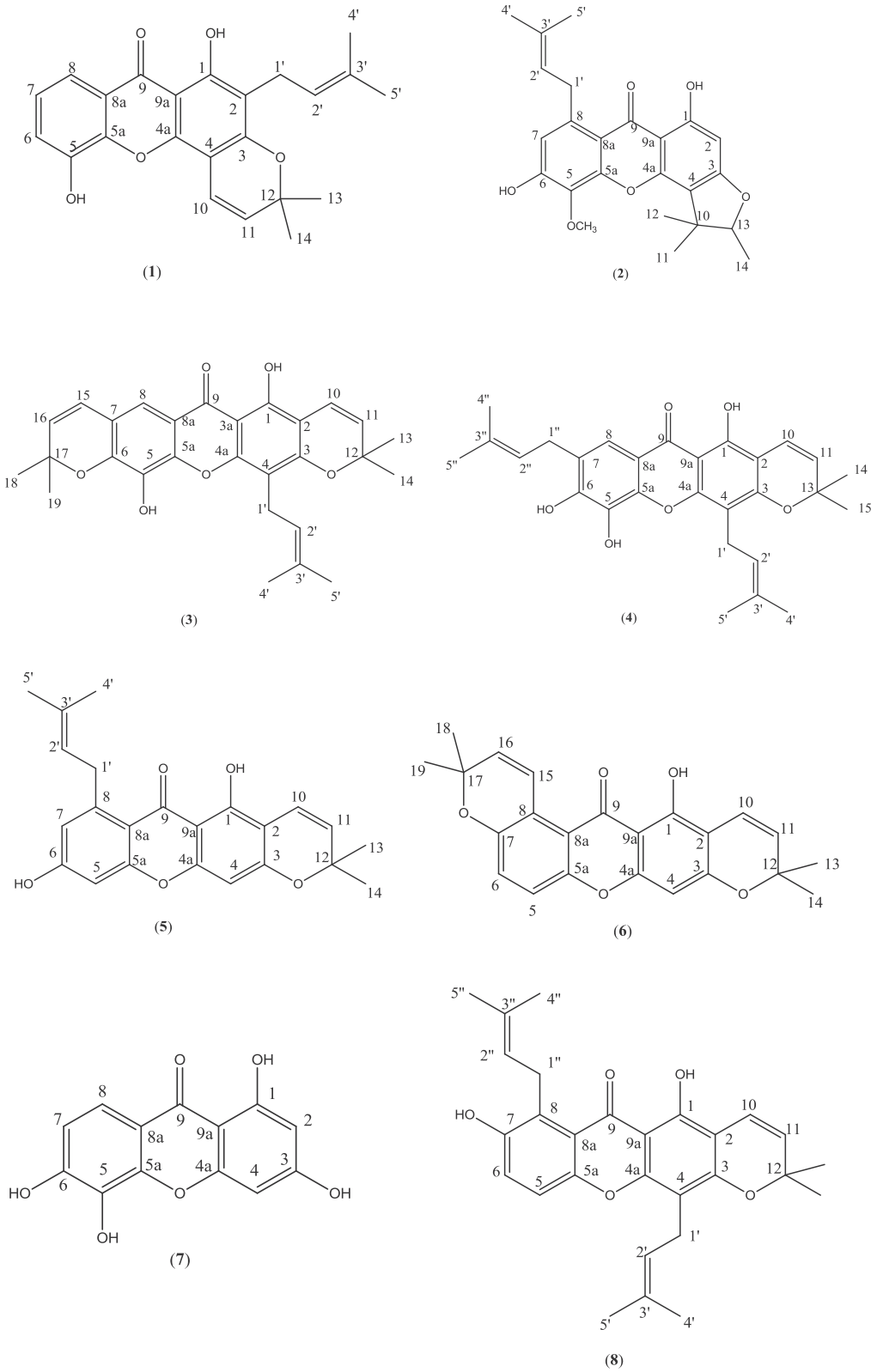

Fig. 1. Structures of ananixanthone (**1**), caloxanthone B (**2**), caloxanthone I (**3**), caloxanthone J (**4**) and xanthochymone B (**5**), thwaitesixanthone (**6**), 1,3,5,6-tetrahydroxyxanthone (**7**) and dombakinaxanthone (**8**).

2.3.7. Thwaitesixanthone (7)

Yellow needle crystal; m.p: 218–221 °C (literature 221-224 °C, Dharmaratne et al., 1986); IR v_{max} cm^{-1}: 3458, 2859, 1731, 1458, 1246 and 1021; UV(EtOH) λ_{max} nm: 372, 322 and 249; EIMS [M^+] *m/z*: 376, 361, 343, 331, 173; ^1H and ^{13}C- NMR spectra are consistent with literature (Jiang et al., 2003).

2.3.8. Dombakinaxanthone (8)

Yellow needle crystal; m.p. 147-149 °C (literature 146-147 °C, Dharmaratne and Wijesinghe, 1997); IR v_{max} cm^{-1}: 3440, 2922, 1606, 1451, 1261, 1134; UV (EtOH) λ_{max} nm: 328, 295, 209; EIMS [M^+] *m/z*: 446, 431, 403, 347, 208, 67; ^1H and ^{13}C- NMR spectra are consistent with literature (Dharmaratne and Wijesinghe, 1997).

2.4. Total phenolic content (TPC) and total flavonoid content (TFC)

TPC and TFC of the plant extract of *C. depressinervosum* and *C. buxifolium* were determined. For TPC, the method of Folin-Ciocalteu assay by Kahkonen et al. (1999) was used. Gallic acid was used as a standard compound. TPC of the plant extracts was expressed as gallic acid equivalent, GAE (μg of gallic acid/mg of plant extract). Meanwhile, TFC was determined according to the method by Adedapo et al. (2008). Rutin was used as a standard compound and TFC was expressed in Rutin equivalent (μg of RU/mg of plant extract).

2.5. Antioxidant assay

The plant extracts of both *Calophyllum* species were evaluated for their anti-oxidant activities. Five anti-oxidant assay were used, including 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, nitric oxide (NO) scavenging, β-carotene bleaching (BCB), ferrous ion chelating (FIC) and ferric reducing power (FRAP) assay.

DPPH radical-scavenging activities of the plant extracts were determined using the method described by Othman et al. (2014) and ascorbic acid was used as a standard compound. NO scavenging activities of the plant extract were determined using a method by Tsai et al. (2007) and gallic acid was used as a standard compound. BCB assay was conducted according to the protocol reported by Kassim et al. (2013) and butylated hydroxytoluene (BHT) was used as a standard compound. FIC activity of the plant extract were investigated according to Hinneburg et al. (2006) and ethylenediaminetetraacetic acid (EDTA) was used as a standard compound. Lastly, FRAP of the plant extracts was determined according to Öztürk et al. (2007) by constructing a standard curve of gallic acid to obtain the equation for the determination of FRAP. The results of FRAP were expressed in relative to gallic acid equivalent (GAE) (μg of gallic acid/mg of plant extract).

2.6. Cytotoxic assay

The cytotoxic assay was performed by using MTT assay as described by Mosmann (1983). In this work, stomach cancer (SNU-1), colon cancer (LS-174T) and leukemia (K562) cell lines were obtained from Taylor's University. Briefly, SNU-1 and K562 cells were cultured and grown to the log phase in RPMI 1640 medium, together with 10% fetal bovine serum. Meanwhile, LS-174T cell line was cultured in MEM medium with 10% fetal bovine serum. After that, these cells were seeded at a specific concentration in 96-well flat bottom plates. The cell concentration for SNU-1 and LS-174T cells used was 1.5×10^5 cells per mL, while for K562 cells was 5×10^4 cells per mL. A series of concentration of plant extract and pure compounds were introduced into the plate. The plate was incubated for 72 h at 37 °C in a 5% CO_2 humidifier incubator. After that, 20 μL of MTT solution (5 mg/mL) was added into each well. The plate was further incubated for 3 h. The media from each well was then discarded and the same amount of DMSO was added into each well to dissolve the purple formazan crystal. The absorbance of each well was measured at 550 nm using a microplate reader. *cis*-Diammineplatinum (II) chloride (Sigma Aldrich, USA) was used as a standard drug for all the cancer cell lines in this assay.

2.7. 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 structure of Src kinase (PDB ID: 2SRC) was retrieved from the Protein Data Bank (www.rcsb.org). Then, hydrogen atoms were added to the protein structures using AutoDockTools (Sanner, 1999). The docking was performed using AutoDock Vina (Trott and Olsor, 2010). The grid box was set to cover important residues such as Asp404 to Glu432, which were involved in the ligand binding of Src kinase. The grid box was 16.4 by 20.96 by 58.73 Å with 1.0 Å spacing. The grid box spacing was set to be 1.0 Å so that all the residues were available in equal-opportunity zone for ligand binding. The docked complex of protein receptor with the compound with the highest anti-proliferative effect was carefully inspected and analyse using LIGPLOT (Laskowski and Swindells, 2011).

2.8. Statistical analysis

The results obtain from phytochemical analysis and antioxidant assay were represented as mean ± standard deviation of triplicate independent analyses. Meanwhile, the results from cytotoxic assay were represented as mean ± standard error of three independent experiments. Data were analysed using one-way ANOVA by Tukey's post hoc test (SPSS 14.0) to determine the significant differences among samples. Meanwhile, the independent sample *t*-test (SPSS 14.0) was used to determine significant difference between samples and standard drugs. Pearson correlation (SPSS 14.0) was used to determine the relationship between phytochemical analysis with antioxidant and cytotoxic activities. The significance level was set at $p < 0.05$.

Table 1
Total phenolic content (TPC) and Total flavonoid content (TFC) of the plant extracts.

Plant Species	Plant Extract	TPC (μg of GA/mg of plant extract)	TFC (μg of RU/mg of plant extract)
Calophyllum depressinervosum	Hexane	33.12 ± 0.42^a	8.15 ± 0.36^a
	Dichloromethane	56.51 ± 2.35^b	53.66 ± 2.47^c
	Ethyl acetate	57.19 ± 2.52^b	80.35 ± 2.94^d
	Methanol	171.99 ± 3.94^c	24.38 ± 0.49^b
Calophyllum buxifolium	Hexane	35.89 ± 1.10^a	4.02 ± 0.16^a
	Dichloromethane	30.94 ± 1.47^a	244.79 ± 11.95^f
	Ethyl acetate	61.41 ± 0.25^b	105.67 ± 5.35^e
	Methanol	233.56 ± 6.76^d	23.43 ± 0.54^b

Note: (*a-f*) denote a significant difference between sample ($p < 0.05$).

Table 2
IC_{50} values of DPPH and nitric oxide (NO) radical scavenging activities of plant extracts.

Plant Species	Plant Extract	IC_{50} (μg/mL)	
		DPPH	NO
Calophyllum depressinervosum	Hexane	> 200	> 1000
	Dichloromethane	> 200	634.99 ± 20.56*
	Ethyl acetate	97.93 ± 0.95*	994.87 ± 31.70*
	Methanol	16.02 ± 0.20*	305.95 ± 4.11*
Calophyllum buxifolium	Hexane	> 200	> 1000
	Dichloromethane	> 200	> 1000
	Ethyl acetate	97.05 ± 0.80*	435.95 ± 9.42*
	Methanol	28.60 ± 0.12*	916.67 ± 21.83*
Ascorbic Acid		12.80 ± 0.05	–
Gallic Acid		–	26.62 ± 1.38

(*) denote a significant difference between sample and standard drug ($p < 0.05$). (−) Not tested.

Table 3
β-Carotene bleaching (BCB) and ferrous ion chelating (FIC) activities of the plant extracts.

Plant Species	Plant Extract	BCB (%) At 100 μg/mL	FIC (%) At 500 μg/mL
Calophyllum depressinervosum	Hexane	28.10 ± 1.40*	28.05 ± 0.87*
	Dichloromethane	59.44 ± 2.84*	40.42 ± 0.42*
	Ethyl Acetate	72.89 ± 2.84*	31.61 ± 1.31*
	Methanol	37.52 ± 1.04*	7.55 ± 0.30*
Calophyllum buxifolium	Hexane	44.43 ± 1.76*	27.84 ± 1.65*
	Dichloromethane	63.69 ± 3.15*	37.32 ± 1.60*
	Ethyl Acetate	47.27 ± 1.15*	16.20 ± 0.88*
	Methanol	15.02 ± 0.94*	6.19 ± 0.30*
Butylated hydroxytoluene (BHT)		83.01 ± 2.92	–
Ethylenediaminetetraacetic acid (EDTA)		–	46.43 ± 2.39

(*) denote a significant difference between sample and standard drug ($p < 0.05$). (−) Not tested.

Table 4
Ferric reducing antioxidant power (FRAP) of plant extracts.

Plant Species	Plant Extract	FRAP (μg of GA/mg of plant extract)
Calophyllum depressinervosum	Hexane	0.53 ± 0.02^{a}
	Dichloromethane	$0.94 \pm 0.02^{b,c}$
	Ethyl acetate	2.22 ± 0.09^{d}
	Methanol	6.51 ± 0.05^{f}
Calophyllum buxifolium	Hexane	$0.73 \pm 0.01^{a,b}$
	Dichloromethane	1.17 ± 0.02^{c}
	Ethyl acetate	2.68 ± 0.02^{e}
	Methanol	8.19 ± 0.28^{g}

(*a-g*) denote significant differences between sample ($p < 0.05$).

3. Results

3.1. TPC and TFC

TPC and TFC values of the plant extracts were presented in Table 1. The methanol extract from both *C. depressinervosum* and *C. buxifolium* showed the highest TPC among the plant extracts. Meanwhile, the dichloromethane extract of *C. buxifolium* gave the highest TFC value among the plant extracts for both species.

3.2. Antioxidant assay

The DPPH radical scavenging activities of plant extracts of *C. depressinervosum* and *C. buxifolium* were determined and the results were presented in Table 2. The methanol extract of *C. depressinervosum* exhibited the strongest scavenging activity among the plant extracts from both *Calophyllum* spp. Similarly, the IC_{50} values of NO scavenging activity showed that the same extract exhibited the strongest activities among the plant extracts and hexane extracts from both species showed negligible activities. Meanwhile, the percentage of BCB inhibition exhibited by the plant extracts at the concentration of 100 μg/mL was presented in Table 3. The plant extract that possessed the highest inhibition percentage of 72.89%, which is slightly lower than the standard drug, BHT was the ethyl acetate extract of *C. depressinervosum*. The FIC ability of the plant extract was evaluated at the concentration of 500 μg/mL and it was observed that dichloromethane extracts, which are semi polar extracts showed higher chelation level if compared to other extracts. The strongest chelating effects of 40.42% and 37.32% were shown by the dichloromethane extract of *C. depressinervosum* and *C. buxifolium*, respectively (See Table 3). Lastly, FRAP values obtained from the plant extracts of *Calophyllum* spp. as shown in Table 4 revealed that the methanol extracts possessed significantly stronger reducing power if compared to the other extracts. The FRAP values of the methanol extract of *C. depressinervosum* and *C. buxifolium* are 6.51 and 8.19 μg of GA/mg of plant extract.

3.3. Cytotoxic activity

The plant extracts of *C. depressinervosum* and *C. buxifolium* were evaluated for their cytotoxic activities against three cancer cell lines which are SNU-1 (stomach cancer), LS-174T (colon cancer) and K562 (leukemia) cells. The IC_{50} values for these extracts are summarized in Table 5. The hexane extract of *C. depressinervosum* exhibited the strongest anti-proliferative activity against SNU-1 cells with an IC_{50} value of 9.50 μg/mL while the hexane extract of *C. buxifolium* showed the strongest activity towards LS-174T and K562 cells with the IC_{50} values of 7.88 and 16.72 μg/mL, respectively.

Table 5
IC_{50} values of cytotoxic effects of plant extracts towards SNU-1, LS-174T and K562 cells.

Plant Species	Plant Extracts	IC_{50} (μg/mL)		
		SNU-1	LS-174T	K562
Calophyllum depressinervosum	Hexane	9.50 ± 0.07	38.12 ± 0.97*	22.49 ± 0.41*
	Dichloromethane	18.56 ± 0.85*	45.92 ± 0.14*	40.54 ± 1.15*
	Ethyl acetate	> 100	> 100	> 100
	Methanol	21.44 ± 0.40*	> 100	41.88 ± 0.82*
Calophyllum buxifolium	Hexane	18.57 ± 0.10*	7.88 ± 1.52*	16.73 ± 0.17*
	Dichloromethane	20.16 ± 0.10*	16.32 ± 0.59*	47.62 ± 0.49*
	Ethyl acetate	> 100	> 100	> 100
	Methanol	22.01 ± 0.99*	> 100	37.66 ± 3.58*
Cis- Diammineplatinum (II) chloride		9.64 ± 0.59	1.32 ± 0.03	4.08 ± 0.09

(*) denote a significant difference between sample and standard drug ($p < 0.05$).

Table 6
Correlation of TPC and TFC with antioxidant and cytotoxic activity of the plant extracts.

Assays	Correlation	
	TPC	TFC
TPC	–	$r^2(8) = -0.345$, $p = 0.402$
TFC	$r^2(8) = -0.345$, $p = 0.402$	–
FRAP	$r^2(8) = 0.983$, $p = 0.000$	$r^2(8) = -0.252$, $p = 0.548$
DPPH	$r^2(8) = -0.855$, $p = 0.007$	$r^2(8) = 0.235$, $p = 0.575$
BCB	$r^2(8) = 0.646$, $p = 0.108$	$r^2(8) = 0.609$, $p = 0.109$
NO	$r^2(8) = -0.347$, $p = 0.402$	$r^2(8) = 0.121$, $p = 0.775$
FIC	$r^2(8) = -0.829$, $p = 0.010$	$r^2(8) = 0.405$, $p = 0.320$
IC_{50} value of SNU-1	$r^2(8) = -0.134$, $p = 0.751$	$r^2(8) = 0.213$, $p = 0.613$
IC_{50} value of K562	$r^2(8) = -0.173$, $p = 0.682$	$r^2(8) = 0.405$, $p = 0.319$
IC_{50} value of LS-174T	$r^2(8) = -0.644$, $p = 0.402$	$r^2(8) = -0.200$, $p = 0.635$

3.4. Correlation of TPC and TFC with antioxidant and cytotoxic effects of plant extracts

Correlation studies were carried out to determine the role or mechanism of action of phenolic compounds in the plant extracts towards antioxidant and cytotoxic activities. The correlation coefficient of TPC and TFC with all the antioxidant and cytotoxic activities are tabulated in Table 6. The correlation coefficients ranging from 0.8 to 0.9 suggest strong correlations. On the other hand, correlation coefficients ranging from 0.5 to 0.7 suggest moderate correlations while correlation coefficients ranging from 0.1 to 0.4 indicate weak correlations (Lai and Lim, 2011). Strong correlations were observed between TPC with antioxidant assays of DPPH, FRAP and FIC. This indicates that phenolic compounds are responsible for the antioxidant activities of *C. depressinervosum* and *C. buxifolium*. Meanwhile, TFC showed only moderate correlation with BCB assay and weak correlations with all other antioxidant assays, as well as cytotoxicity towards three cancer cells. The results indicate that the antioxidant and cytotoxic activities of the plants are not solely contributed by flavonoids but other types of phenolic compounds.

3.5. Cytotoxicity of the xanthones

Eight xanthones isolated from both *Calophyllum* species were examined for their cytotoxic activities against K562 cell line, which has not been studied previously. *cis*-Diammineplatinum (II) chloride was used as a standard compound. It is a well-known synthetic drug used as a chemotherapeutic in the treatment of ovarian, testicular, head and neck carcinomas (Gately and Howell, 1993). From the literature, *cis*-diammineplatinum (II) chloride showed a strong inhibition activity towards K562 cell line (Kaluderovic et al., 2005). The IC_{50} values of cytotoxic effects against K562 cells for the standard compound, together withthe isolated xanthones are presented in Table 7. Among the xanthones, caloxanthone B (**2**) showed the highest cytotoxic level with an IC_{50} value of 1.23 μg/mL and followed by ananixanthone (**1**) with 2.96 μg/mL. The cytotoxic effects of these compounds are found to be significantly stronger than that of the standard drug, *cis*-diammineplatinum (II) chloride. Interestingly, both compounds were isolated from the non-polar, hexane extract of *C. depressinervosum* that exhibited the strongest cytotoxic activities among the plant extracts. Thus, ananixanthone (**1**) and caloxanthone B (**2**) are some of the important phytochemical constituents that contributed to the cytotoxicity of *C. depressinervosum*.

Table 7
IC_{50} values of cytotoxic effects of compounds towards K562 cells.

Compound	IC_{50} (μg/mL)
Ananixanthone (**1**)	2.96 ± 0.06*
Caloxanthone B (**2**)	1.23 ± 0.03*
Caloxanthone I (**3**)	30.79 ± 0.41*
Caloxanthone J (**4**)	18.43 ± 1.02*
Xanthochymone B (**5**)	6.60 ± 0.50*
Thwaitesixanthone (**6**)	–
1,3,5,6-tetrahydroxyxanthone (**7**)	12.98 ± 0.31*
Dombakinaxanthone (**8**)	7.84 ± 0.03*
cis- Diammineplatinum (II) chloride	4.0 8 ± 0.09

(*) denote significant difference between sample and standard drug ($p < 0.05$).
(−) Not tested.

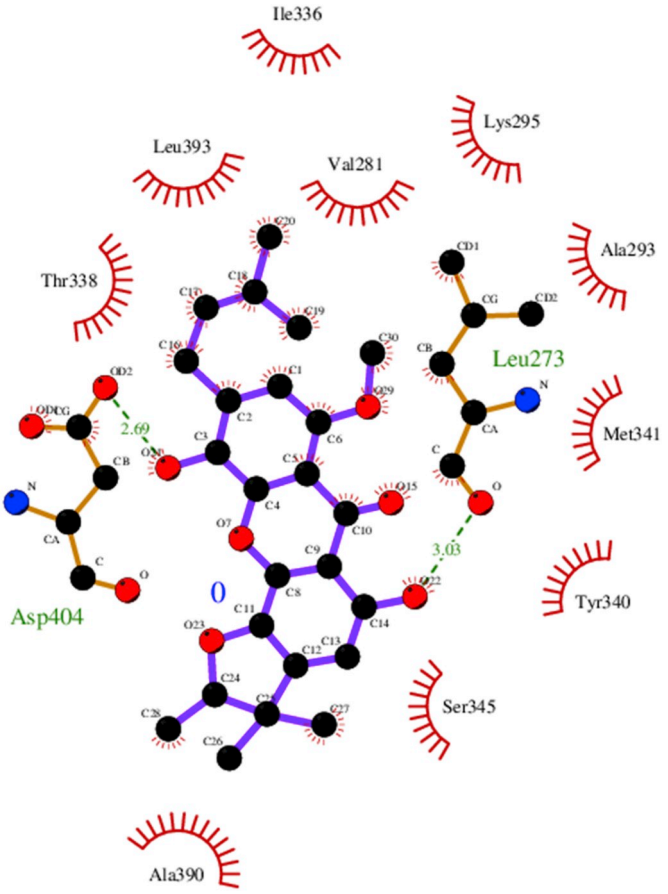

Fig. 2. Two-dimensional diagram of binding interaction of the docked structure of caloxanthone B (**2**) with Src (PDB: 2SRC) protein receptor using LIGPLOT (24).

3.6. Molecular docking studies

Molecular docking study was conducted to gain a better insight of the molecular binding interaction of the most cytotoxic compound, caloxanthone B (**2**) with Src protein kinase, which has been identified as a promising target for the therapy of cancer. The crystal structure of receptor, Src protein kinase (PDB ID: 2SRC) was used in this study. A visual inspection on the docked position of **2** in the activation loop of Src protein kinase (see Fig. 2) revealed that two intermolecular hydrogen bonds present in the docked complex. The hydrogen bonds were observed on two different hydroxyl groups of **2** with the residues of Leu273 (3.03 Å)and Asp404 (2.69 Å) in the activation loop. Meanwhile, a huge number of residues were observed to assist in the binding of the compound through hydrophobic interactions. These residues include Thr338, Leu393, Ile336, Val281, Lys295 Ala293, Met341, Tyr340, Ser345 and Ala390, which are located surrounding the activation loop.

4. Discussion

Plant extracts are rich sources of phenolic compounds, which are important antioxidant agents due to the presence of hydroxyl group. In this study, methanol extracts of both *Calophyllum* species, *C. depressinervosum* and *C. buxifolium* reflected the highest TPC values, which are similar with another species of the same plant, *C. rubiginosum* (Saeed et al., 2012; Taher et al., 2010). The lack of specificity of Folin-Ciocalteau on the phenolic compounds may results in the deviation of the TPC values, besides the choice of plant extraction method. Moreover, side reactions may occur in between the phenols and reducing sugars or acids (Saeed et al., 2012). Therefore, an additional specification test is highly recommended to be carried out to determine specific phenolic compounds that are present in the plant extract. In this case, TFC values of the plant extracts were obtained. Flavonoid is one of the phenolic compounds occurring in nature with a specific skeleton of $C_6C_3C_6$. These compounds are well known for their potential antioxidant and anticancer properties (Xu et al., 1999).

The mechanism of action in a DPPH assay is based on the ability of the free radical to react with a hydrogen donor such as phenol. The antioxidant activity was measured by the decrease in absorbance as the DPPH free radical receives an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule (León-González et al., 2015). Strong correlation between TPC and DPPH results observed in this study is probably due to the presence of low molecular weight phenolic compounds in the plant extracts (Öztürk et al., 2007), besides having phenolic compounds with hydrogen donor properties. On the other hand, a weak correlation between TFC and DPPH results deduced that flavonoids that are present in these species possess weaker hydrogen donor properties.

The principle of FRAP is based on reduction of Fe^{3+} complex to Fe^{2+} complex, which is intensely blue in colour, by any antioxidant compound in an acidic medium (Antolovich et al., 2002). Meanwhile, FIC assay measures the chelation reaction of ferrozine with Fe^{2+}, which gives a purple coloured complex. This reaction will be reduced in the presence of other chelating agents, leading to a decrease in the intensity of purple colour complex due to the competition of chelation effects. Antioxidant compounds present in the plant extract will form coordination complexes with the metal ions, at which chelation reaction happens and subsequently inhibit the transfer of electrons. As a result, the oxidation reaction is arrested and no free radicals are produced. In correlation studies, similar to DPPH results, the FRAP and FIC results showed strong correlations with TPC of the plant extracts of *C. depressinervosum* and *C. buxifolium*. The phenolic compounds in the plant extracts possess both ion chelating and reducing effects, besides DPPH radicals scavenging properties. The common phenolic compounds present in the other species of *Calophyllum* include coumarins, xanthones and flavonoids (Li et al., 2007, 2016; Mah et al., 2012). Thus, these compound are deduced to be the main contributors of their antioxidant effects.

BCB assay measures the ability of an antioxidant to inhibit lipid peroxidation. β-Carotene and linoleic acid undergo a rapid discoloration in the absence of an antioxidant. The free linoleic acid radical was formed due to the loss of hydrogen atom from one of its methylene groups and this hydrogen atom will reduce the double bond of β-carotene, subsequently changing the orange colour of β-carotene (Öztürk et al., 2007). The antioxidant compounds in the plant extracts will inhibit the reduction of β-carotene and retaining its orange colour. The weaker correlations observed between BCB results and both TPC and TFC of *Calophyllum* plant extracts imply that most of the phenolic compounds involve in the mechanism of reactions in the DPPH scavenging, ion chelating and ion reducing activities instead of lipid peroxidation inhibition.

Each antioxidant assay measures the activity based on different mechanism of reaction, leading to a wide range of antioxidant effects. Similarly, the cytotoxic activity of each compound has a different mechanism of action towards specific types of cancer cells. Furthermore, the differences in cytotoxic activities from their TPC and TFC are due to a mixture of phytochemical constituents present in the plant extracts. These plant extracts contain diverse types and classes of compounds that have cytotoxic properties, leading to the changes in bioactivities caused by synergism and antagonism effect among these compounds (Kassim et al., 2013). Thus, a wide range of anti-proliferative effects against different cancer cell lines are deduced to be contributed by different phytochemical constituents present in the plant extracts of *Calophyllum* species,. However, one of these constituents that showed good cytotoxic properties is xanthone that was isolated as major class of compound from *C. depressinervosum* and *C. buxifolium*. Previous studies have proven that xanthones isolated from natural products possessed moderate to strong cytotoxic effects towards various cancer cells, including SNU-1, LS174T and K562 cells (Mah et al., 2012; Teh et al., 2013). The cytotoxic effect of the xanthones present in *Calophyllum* have been once again proven in this study.

Xanthones are found to be commonly present in this plant and these compounds have been claimed to possess a wide range of biological activities, including cytotoxicity. Thus, isolation of these highly bioactive potential phytochemical constituents from *Calophyllum* plants were carried out subsequently and resulted in a total of eight xanthones **2–8**. The structure of these xanthones were confirmed by the 1D- and 2D-NMR, MS, IR and UV. Furthermore, the cytotoxic effects of the xanthones towards K562 cells, together with the molecular interactions of the most cytotoxic xanthone with Src protein kinase, which is overexpressed in cancer, were reported for the first time. Activation loop of Src protein kinase plays an important role in activating the protein. The activation loop is made up of residues from 404 to 432 (Xu et al., 1999). A previous research by Seeliger et al. (2009) has shown that the binding of imatinib, which is a kinase inhibitor drug, in this activation loop deactivated the Src protein kinase and inhibit the cell proliferation. *In-vivo* and *in-vitro* studies have shown that imatinib inhibited the proliferation by reducing cells expression of Bcr-Abl, thus decreasing cell cancer population (Druker et al., 2002). In order to explore the molecular binding in the activation loop of Src protein kinase by the most cytotoxic compound, caloxanthone B (**2**), a molecular docking was performed using the crystal structure of Src protein kinase (PDB ID: 2SRC) as the receptor. Two intermolecular hydrogen bonds were observed between the hydroxyl groups of **2** with Leu273 and Asp404 of the kinase. In addition, the methoxyl, prenyl and furanyl moieties in **2** contributed to the hydrophobic interactions with other residues as favourable non-bonded interactions. A study by Xu et al. (1999) showed that the interaction between a ligand and residue Asp404 or Lys 295 of Src protein kinase deactivated the protein kinase by inhibiting the salt bridge formation between Lys295-Glu310. In relation to the docking results mentioned above, it is hypothesised that **2** involved in the deactivation of Src protein kinase through the residues binding in the activation loop.

5. Conclusion

This study demonstrates that *Calophyllum depressinervosum* and *Calophyllum buxifolium* possess good antioxidant effect, as well as cytotoxic activities towards SNU-1, LS174T and K562 cancer cells. Purification of the plant extracts of these species afforded eight xanthones and caloxanthone B (**2**) was found to be the most cytotoxic xanthone towards K562 cells. It was further studied for its molecular binding interaction with a promising target Src protein kinase and the results suggested the involvement of hydrogen bondings and hydrophobic interactions. The outcomes of the study revealed that *C. depressinervosum* and *C. buxifolium* are potential plants to be further studied on the antioxidant properties by using ROS enzymes while caloxanthone B (**2**) is a potential lead compound for anti-cancer agent, at which the studies on detailed mechanism of cytotoxicity with molecular dynamic simulation are highly recommended to be conducted in due course.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors acknowledge financial support from Taylor's University under Taylor's Research Grant Scheme (TRGS) (TRGS/ERFS/2/2018/SBS/019). The Sarawak Biodiversity Centre (SBC) is acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110800.

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