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Original Article

In Vitro Anti-Amebic Activity of Cage Xanthones from *Cratoxylum sumatranum* Stem Bark Against *Entamoeba histolytica*

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ABSTRACT

Background: Amoebiasis is caused by *Entamoeba histolytica*, which is a pathogenic species living on human colon tissues. The development of new drugs for anti-amebic are still very needed for clinical treatment. **Objective:** This aims to identify the compounds in *Cratoxylum sumatranum* for their anti-amoeba activity. **Materials and Methods:** In this study we used bioactivity-guided isolation and structural analysis to identified anti-amebic compounds from dichloromethane extract of C*ratoxylum sumatranum* stem bark. Their anti-amebic activity was determined by an *in vitro* cell-based assay against *Entamoeba histolytica* and an enzymatic assay on NAD kinase. **Results:** Two known compounds from the cage xanthone groups, namely cochinchinoxanthone (1) and cochinchinone D (2), were isolated. The structures of the cage xanthone compounds were established by extensive spectroscopic data analysis. Compound (1) showed the greatest level of anti-amebic activity both in cell-based and enzymatic assay, yielding IC_{50} values of 4.57 and 12.17 µg/mL, respectively. In contrast, compound (2) yielded IC_{50} values of 5.19 and 12.60 µg/mL, respectively. **Conclusion:** When considering the demonstrated anti-amebic activities, it becomes apparent that these compounds, isolated from C*ratoxylum sumatranum* stem bark, have the potential to be further developed into effective anti-amebic medicine against *Entamoeba histolytica*.

Key words: Amoebiasis, Bioactivity-guided isolation, *Cratoxylum sumatranum*, *Entamoeba histolytica*, NAD kinase.

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INTRODUCTION

The genus *Cratoxylum* consists of tropical plant within the Hypericaceae family which is widespread in tropical forests.[1] Plant from the genus *Cratoxylum* been widely used for ethnomedicinal applications in South East Asia as a treatment of stomachache and diarrhea as well as for the prevention of gastric ulcers.[2,3] Further work was able to isolate xanthone compounds from *C. cochinchinense*, which was reported to have bioactivity as an antioxidant, anticancer agent and protein inhibitor of tyrosine in diabetes.[4-7] Preliminary research screening 22 species of plants from Balikpapan Botanical Gardens, Kalimantan Indonesia, demonstrated that dichloromethane extract from the stem bark of *Cratoxylum sumatranum* (Jack) Bl. has anti-amebic activity against *E. histolytica* cells with an IC_{50} value of 22.07 µg/mL, CC_{50} of 29.69 µg/mL and a Selective Index (SI) of 1.34.[8] Phytochemical studies of *C. sumatranum* obtained xanthone and anthraquinino-benzophenone such as cratoxyarborenones A-F; cratoxyarborequinones A-B; vismione B; 2,4,6-trihydroxybenzophenone 4-*O*-geranyl ether; *δ*-tocotrienol; betulinic acid,[9] and sumatranaxanthone A.[10] Other compounds that have also been isolated are cratosumatranone A-B; pruniflorone N; neriifolone B; isocudraniaxanthone B; 10-O-methylmacluraxanthone; macluraxanthone; 5-O-methyl-2-deprenylrheediaxanthone B; pancixanthone B; pruniflorone M; 5′-demethoxycadensin G; 1,5-dihydroxy-8-methoxyxanthone; 1,5-dihydroxy-6,7-dimethoxyxanthone; 1,3,6-trihydroxy-7-methoxyxanthone; 1,3,5,6-tetrahydroxyxanthone; 1,2,8-trihydroxyxanthone; 2,8-dihydroxy-1-methoxyxanthone; cratoxyarborenone F; 1,7-dihydroxyxanthone; trapezifolixanthone; cochinchinoxanthone; 5-O-methylisojacareubin; 2,4,6-trimethoxybenzophenone; 4-hydroxy-2,6-dimethoxybenzophenone; and annulatomarin.[11]

Amoebiasis is a common infection in the human digestive tract. The diseaseis caused by *E. histolytica*, which is a pathogenic species found on human colon tissues. Amoebiasis is associated more strongly with poor sanitation and lower socioeconomic status while climate plays a minor role, reflected in its worldwide distribution. This poses major health challenges in China, Southeast Asia, and Western Latin America.[12] In Indonesia, amoebiasis is found in 10% of the population, resulting in 30 deaths annually.[13] Amoebiasis is often caused by the parasite *E. histolytica*, however only circa 10% to 20% of people infected with *E. histolytica* become symptomatic.[14] To treat these infection, metronidazole is usually used for both intestinal and extraintestinal amoebiasis.[15] Although clinical resistance of metronidazole has not been conclusively demonstrated, resistance of *E. histolytica* to treatment with metronidazole in case of amoebiasis has been reported.[16] In addition, it has been shown by *in vitro* assay that this parasite

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is easily adaptable to the metronidazole therapy.[17] Therefore, there is a clear and urgent need for the development of new anti-amebic drugs for clinical treatment.

The isolation and identification of active compounds from the stem bark of *C. sumatranum*, as conducted in this study, was informed by the concept of bioactivity-guided isolation regarding anti-amebic activity.[18] The anti-amebic testing was conducted *in vitro* according to a cell-based assay on inhibition of *E. histolytica* cell growth and an enzymatic assay examining inhibition of NAD kinase activity, which has a role in the metabolic processes of *E. histolytica*.[19]

MATERIALS AND METHODS

General experimental procedures

1D and 2D NMR spectra were recorded on a JEOL (400 MHz), using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal standard. The LC system coupled with Q-TOF/MS (Agilent) was used for HRESIMS analysis. The HPLC (Shimadzu, LC-06) included a detector PDA SPD-M20A, pump LC-6AD and column using silica gel RP-18 shim pack 4.6 x 250 mm (Merck). Optical rotations were measured on a P1000-LED polarimeter. Column chromatography (CC) was carried out on silica gel 60 (Merck). Pre-coated plates of silica gel 60 GF254 were used for TLC analysis. Bioactivity assays were carried out on a GloMax Microplate Multidetection Reader (Promega). All chemicals were analytical grade.

Plant material

The *C. sumatranum* stem barks were collected from Balikpapan Botanical Garden, East Kalimantan Province, Indonesia, in November 2015 and identified by a licensed botanist from Purwodadi Botanical Garden and Indonesian Institute of Sciences, Indonesia. A voucher specimen (No. 0074/IPH.06/HM/XII/2015) of this raw materialis stored at the Herbarium of the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.

Extraction and isolation

The dried stem barks of *C. sumatranum* (400 g) were sequentially extracted at room temperature with hexane (2 L) and dichloromethane (1 L). The removal of solvents in a vacuum produced a yellow-brown, viscous, hexane extract (3.2 g) and a dichloromethane extract (4 g), respectively. The dichloromethane extract (1 g) was subjected to silica gel CC over silica gel 60 and eluted using hexane-ethyl acetate, ethyl acetate, chloroform-methanol, and methanol to give 12 fractions (F1-F12). All fractions were tested for their anti-amebic activity. Fraction F4 (25 mg) showed the strongest activity and was then further separated by semi-preparative HPLC with column reversed silica gel RP-18 and eluted with a gradient flow of MeOH in H_2O (100 to 50%, v/v) at a rate of 1.5 mL/min, to obtain compound 1 (4 mg) and 2 (3 mg), respectively. The results of the isolated compounds were identified based on HRESIMS, 1D and 2D NMR spectroscopic data.

Compound 1

The light yellow solid. HR-MS $[M+Na]^+$ m/z 419.1314 for $C_{23}H_{24}O_6Na$ (calcd. 419.1319). $[\alpha]^{20}_D$ +8 (*c* 0.05, MeOH). UV (MeOH) λmax (nm): 263 and 345. For ^{1}H-NMR and ^{13}C-NMR (400 MHz, $CDCl_3$) spectroscopic data, (Table 2).

Compound 2

The yellow solid. HR-MS $[M+Na]^+$ m/z 449.1440 for $C_{24}H_{26}O_7Na$ (calcd. 449,1426). $[\alpha]^{20}_D$ -48 (*c* 0.05, MeOH). UV (MeOH) λmax (nm): 265 and 348. For ^{1}H-NMR and ^{13}C-NMR (400 MHz, $CDCl_3$) spectroscopic data, (Table 2).

Anti-amebic cell based assay

The cells of HM-1:IMSS (clone 6) *Entamoeba histolytica* strain kindly provided by Prof. T. Nozaki (University of Tokyo) were cultivated in Bisate-Iron-Serum (BI-S) medium (Sigma) that was supplemented with 10% (v/v) bovine serum (Sigma) and 1% (v/v) Diamond Vitamin-Tweena solution (JRH Biosciences, USA) at 37 °C. The cells were conditioned for 2 days to reach a confluence of 80%. The *Entamoeba histolytica* cells were seeded in 98-well plates. 200 µL of cells and BI-S medium were added into each well, then the wells were incubated 2 hours at 35.5 °C. After 2 hours of incubation, they were replaced with mixtures of medium and sample (2.5 µL of extract or isolate), then incubated for 24 hours. The medium was replaced with 10% WST-1 reagent (Roche, Germany) in warmed OPTI-MEM medium (Gibco-Life Technologies). After wards, they were incubated for 30 minutes at 37 °C and absorbance was measured at 560 nm using a GloMax reader. The percent inhibition of cells growth in each samples was calculated by comparison to the control, the Probit analysis with SPSS statistical was used to determine the 50% inhibitory concentration (IC_{50}).

Anti-amebic enzymatic assay

The Master Mix solution was prepared (a mixtures of ATP, INT media, tris HCl, miliQ water and NAD kinase/NO1 enzyme). 96 µL/well of the Master MIX solution was added to 96 well-plates. Subsequently, each well was amended with 5 µL of sample (extract or isolate), 14 µL of NADH solution and tris HCl with pH 7.5. The plates were then incubated for 10 minutes at room temperature, and absorbance was measured at 490 nm using a GloMax reader. The percent inhibition of NAD kinase enzyme samples was calculated by comparison to the control, the Probit analysis with SPSS statistical was used to determine the 50% inhibitory concentration (IC_{50}).

Cytotoxicity assay

The cytotoxicity of the samples was assessed via MTT assay.[20] In brief, Huh7.it cells in 96-well plates were treated with serial dilutions of samples and control for 48 hours. The Dulbeco's Modified Eagle Medium (GIBCO Invitrogen, USA) medium was replaced with MTT reagent containing that medium and incubated for 4 hours. The MTT solution was removed and 100 µL/well of DMSO 100% was then added for dissolution. The absorbance at 560 nm was measured using a GloMax reader. The percentages of cell viability was calculated by comparison to the control and 50% cytotoxic concentration (CC_{50}) values were ascertained.

RESULTS

Fractionation of dichloromethane extract from *C. sumatranum* stem bark obtained twelve fractions. The results of the activity tests of each fraction, regarding their anti-amebic potential, with both cell-based and enzymatic assays (Table 1). From twelve fractions tested, there were four factions that displayed anti-amebic activity in the cell-based assay inhibiting *E. histolytica* cell growth, namely fractions F3, F4, F6 and F8.

The fraction F4, which had the strongest anti-amebic activity, was further fractionated using semi-preparative HPLC (Figure 1). From this, two compounds (1) and (2) were obtained.

Compound (1) was obtained as a yellow solid. Identification via ^{1}H-NMR and ^{13}C-NMR, demonstrated that compound (1) was composed of 1,3-dihydroxy-5,7-diprenyl cage xanthone, namely cochinchinoxanthone.[5] The HMBC and HMQC data from 2D NMR analysis showed a correlation between two hydroxy groups at δ 12.47 (singlet) bound to position C-1 and δ 6.99 (singlet) bound to position C-3. The correlations between H-11 with C-6 and C-8, and also H-16 with C-5 and C-10 indicated that two prenyl groups are each connected

Tabel 1: The anti-amebic activity results from 12 fractions of *Cratoxylum sumatranum* stem bark dichloromethane extract.

Fractions	IC_{50} (µg/mL)	
	Cell-based	Enzymatic
F1	n.a	n.a
F2	n.a	n.a
F3	23.82	17.45
F4	9.25	20.87
F5	n.a	21.16
F6	28.67	16.92
F7	n.a	n.a
F8	39.95	19.35
F9	n.a	14.77
F10	n.a	23.59
F11	n.a	28.92
F12	n.a	27.11

n.a = not active

Tabel 2: ^{1}H and ^{13}C NMR data of compounds (1 and 2) in $CDCl_3$ (400 MHz, δ in ppm, J in Hz).

No.	compound (1)		Reference [5] (300 MHz, $CDCl_3$)		compound (2)		Reference [4] (300 MHz, $CDCl_3$)	
	δ_H J(Hz)	δ_C	δ_H J(Hz)	δ_C	δ_H J(Hz)	δ_C	δ_H J(Hz)	δ_C
1	-	165.3	-	165.3	-	165.5	-	160.5
2	6.01 (d, 1H, *J*=2.8)	96.8	6.01 (d, 1H, *J*=2.8)	96.8	6.01 (d, 1H, *J*=2.4)	97.0	6.03 (d, 1H, *J*=2.1)	95.5
3	-	165.2	-	165.2	-	165.2	-	167.9
4	6.02 (d, 1H, *J*=2.8)	95.3	6.02 (d, 1H, *J*=2.8)	95.3	6.04 (d, 1H, *J*=2.4)	95.3	6.05 (d, 1H, *J*=2.1)	96.9
4a	-	160.7	-	160.7	-	161.1	-	164.5
5	-	83.7	-	83.7	-	84.2	-	83.8
6	-	203.1	-	203.1	-	201.4	-	201.0
7	3.49 (m, 1H)	46.9	3.49 (m, 1H)	46.9	-	84.8		84.5
8	7.42 (d, 1H, *J*=8.8)	133.8	7.42 (d, 1H, *J*=8.8)	133.8	7.44 (s, 1H)	135.8	7.44 (s, 1H)	133.3
8a	-	135.4	-	135.4	-	134.2	-	132.3
9	-	178.8	-	178.8	-	178.7	-	178.0
9a	-	102.7	-	102.7	-	101.3	-	100.5
10	-	90.5	-	90.5	-	88.9	-	88.5
11	2.30 (m, 1H) 2.34 (m, 1H)	25.2	2.30 (m, 1H) 2.34 (m, 1H)	25.2	2.32 (d, 1H, *J*=4.4) 2.36 (d, 1H, *J*=4.4)	30.1	2.35 (d, 1H, *J*=13.2) 1.59 (d, 1H, *J*=13.2)	29.9
12	2.43 (d,1H, *J*=9.6)	48.8	2.43 (d,1H, *J*=9.6)	48.8	2.51 (d, 1H, *J*=9.6)	49.5	2.50 (d, 1H, *J*=9.6)	49.3
13	-	84.4	-	84.4	-	84.1	-	83.8
14	1.28 (s, 3H)	29.1	1.28 (s, 3H)	29.1	1.30 (s, 3H)	29.1	1.31 (s, 3H)	28.9
15	1.67 (s, 3H)	30.4	1.67 (s, 3H)	30.4	1.65 (s, 3H)	30.5	1.66 (s, 3H)	30.3
16	2.60 (d, 2H, *J*=8.8)	29.1	2.60 (d, 2H, *J*=8.8)	29.1	2.62 (d, 2H, *J*=9.6)	29.2	2.63 (d, 2H, *J*=7.5)	28.9
17	4.41 (t, 1H, *J*=7.6)	118.3	4.41 (t, 1H, *J*=7.6)	118.3	4.41 (t, 1H, *J*=7.6)	118.0	4.43 (t, 1H, *J*=7.5)	117.9
18	-	133.9	-	133.9	-	132.1	-	135.5
19	1.38 (s, 3H)	25.6	1.38 (s, 3H)	25.6	1.38 (s, 3H)	25.7	1.40 (s, 3H)	25.5
20	1.10 (s, 3H)	16.9	1.10 (s, 3H)	16.9	1.10 (s, 3H)	17.0	1.13 (s, 3H)	16.9
1-OH	12.47 (s, 1H)	-	12.47 (s, 1H)	-	12.38 (s, 1H)	-	12.39 (s, 1H)	-
3-OH	6.99 (s, 1H)	-	6.99 (s, 1H)	-	6.33 (s, 1H)	-	6.26 (s, 1H)	-
7-OCH_3	-	-	-	-	3.63 (s, 3H)	54.2	3.62 (s, 3H)	53.84

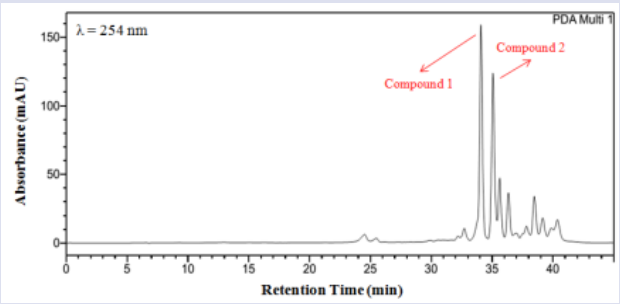

Figure 1: HPLC chromatogram of fraction 4.

with carbon at the C-5 and C-7 positions. Hience, the structure of the compound was determined as xanthone having 1,3-dihydroxy-5,7-diprenyl substituents. The HMBC data also provided information concerning the correlation between C-7 and C-12. This demonstrated a connection between C-7 and C-12 by a bridge formed from a methylene group on C-11. The HR-MS analysis for compound (1) gave a positive ion $[M+Na]^+$ with m/z 419.1314, the molecular formula being $C_{23}H_{24}O_6Na$.

Compound (2) was a solid with light yellow coloring. Identification via ^{1}H-NMR and ^{13}C-NMR, indicted that compound (2) was 1,3-dihydroxy-7-methoxy-5,7-diprenyl cage xanthone, namely cochinchinone D.[4] The correlation between HMBC and HMQC shows connection between C-7 and C-12 by a bridge formed from a methylene group at C-11, similar to that which occurs in the chemical structure of compound (1). According to the analysis of HMBC correlation, differences in the chemical structure between compound (1) and (2) stemmed only from the proton at position C-7 which was replaced by methoxy group (-OCH_3). The HPLC profile demonstrated that the presence of the methoxy group substituent in the structure of the cage xanthone resulted in differing polarity between the two compounds. The peaks of compounds (1) and (2) were close to each other, with retention times of 34 and 35.3 minutes, respectively (Figure 1, 2 and 3). The HR-MS analysis indicated that compound (2) gave a positive ion $[M+Na]^+$ with m/z 449.1440, with the molecular formula being $C_{24}H_{26}O_7Na$.

Here, we report that compounds (1) and (2), purified from fraction F4 demonstrated strong anti-amebic activity. The results of anti-amebic activity in cell-based and enzymatic assays with cytotoxic properties between compounds (1) and (2) compared to fraction F4 (Table 3).

DISCUSSION

The stem bark of *C. sumatranum* were sequentially extracted using hexane and dichloromethane. According to the anti-amebic activity assay, dichloromethane extract had a higher selectivity index (SI) value than hexane extract.[8] Therefore, the fractionation of dichloromethane extract of *C. sumatranum* stem bark was carried out using Column Chromatography (CC). Following fractionation, we obtained 12 fractions, each of which was tested for anti-amebic activity by cell-based and enzymatic assays.

Of the twelve tested fractions, the fraction F4 had an displayed the lowest IC_{50} value in the cell-based assay (9.25 µg/mL). In the enzymatic assay, the fraction F4 also showed inhibitory activity against NAD kinase with an IC_{50} value of 20.87 µg/mL. NAD kinase is a key enzyme responsible for regulating the NAD(H)/NADP(H) concentrations within cells. NAD enzymes play an important role in

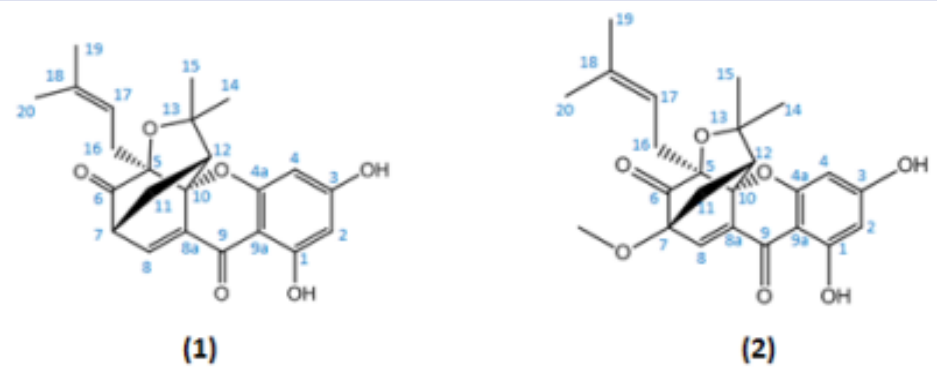

Figure 2: Chemical structures of isolated compounds (1 and 2) from *C. sumatranum*.

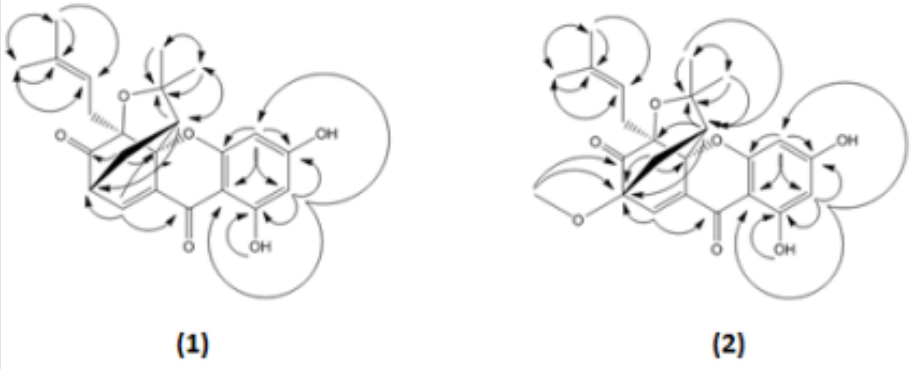

Figure 3: HMBC correlation of compounds (1 and 2).

Table 3: Anti-amebic activity (IC_{50}) and cytotoxicity (CC_{50}) of compound 1 and 2 compared to fraction F4.

Sample	IC_{50} (µg/mL)		CC_{50} (µg/mL)	SI
	Cell-based	Enzymatic		
Fraction F4	9.25	20.87	31.05	3.36
Compound 1	4.57	12.17	48.83	10.68
Compound 2	5.19	12.60	67.69	13.04

ATP synthesis in the metabolic system of amoeba and are hence critical to their functioning. This enzyme is also involved in maintaining the intracellular redox potential and helping parasites respond to oxidative stress. If the action of this enzyme is inhibited, the cell's metabolic system will be hampered thereby killing the amoeba.[19]

The fraction F4 was purified to obtain compounds (1) and (2), which have anti-amebic activity. We were able to identify these, compounds as cochinchinoxanthone (1) and cochinchinone D (2). Both of these cage xanthone compounds are commonly found in the genus *Cratoxylum*. Other cage xanthone compounds such as cochinchinone C and cochinchinoxanthone A-D are reported to have been isolated from *C. cochinchinense*.[4-5] In this study, we report the presence of compound (2) for the first time in *C. sumatranum*.

The cochinchinoxanthone (1) and cochinchinone D (2) isolated from *C. sumatranum* stem bark were also reported for the first time to have anti-amoebic activities. These xanthone compounds have anti-amebic activity as demonstrated by cell-based and enzymatic assays. This is supported by previous research, indicating that other xanthone compounds such as α-mangostin isolated from *Garcinia mangostana* (Clusiaceae) also exhibit anti-amebic activity.[21]

The anti-amebic activity of compound (1) yielded IC_{50} values of 4.57 µg/mL (cell-based) and 12.17 µg/mL (enzymatic), CC_{50} of 48.83 µg/ml, and SI value of 10.68. Meanwhile, compound (2) yielded IC_{50} values 5.19 µg/mL (cell-based) and 12.60 µg/mL (enzymatic), CC_{50} of 67.69 µg/mL, and SI value of 13.04. When comparing these levels of anti-amebic activity, it is apparent that isolated compounds derived from *C. sumatranum* stem bark are the more active compared to the mother fraction (F4). This is likely due to the compounds showing SI values greater than the fractions. Therefore, it can be concluded that the compounds are comparatively safe to be developed as an amoebiasis treatment.

CONCLUSION

Two known compounds from *C. sumatranum*, belongings to cage xanthone groups, namely cochinchinoxanthone (1) and cochinchinone D (2) were isolated. Both of these compounds provided anti-amebic activity against *E. histolytica* in cell-based and enzymatic assays. These compounds isolated from C. *sumatranum* stem bark have the potential to be developed as an anti-amebic medicine against *E. histolytica*.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts interest.

ACKNOWLEDGEMENTS

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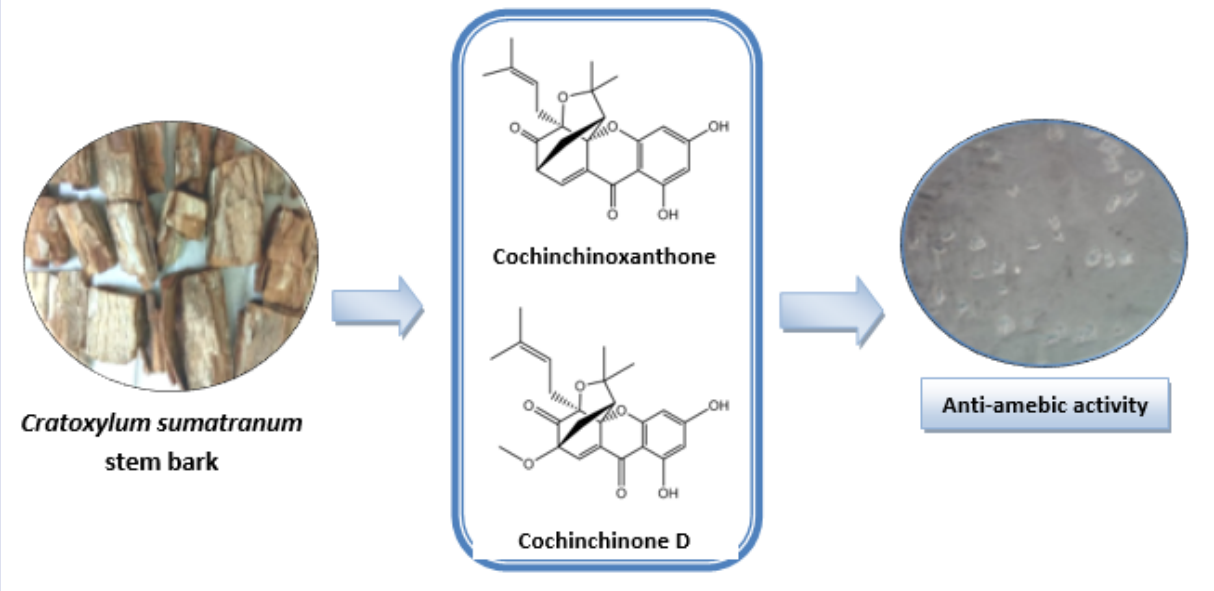
GRAPHICAL ABSTRACT



SUMMARY

Two compounds from the cage xanthone groups, namely cochinchinoxanthone and cochinchinone D, have been isolated from *Cratoxylum sumatranum* stem bark. All compounds showed anti-amebic activity in cell-based and enzymatic assays.

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