

Isolation and Structure Elucidation of Soulatro Coumarin From Stem Bark of *Calophyllum soulattri* Burm F and *In Vivo* Antiplasmodial Activity by Using Mice Infected by *Plasmodium berghei*

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Abstract

The soulatro coumarin compound was isolated and elucidated from the stem bark of *Calophyllum soulattri* Burm F, the samples were collected from Jayapura Papua Irian Island in Indonesia. Isolation process was done by maceration at room temperature in methanol, then partitioned in a mixture of *n* hexane-water (1:1), followed by dichloromethane-water (1:1) and ethyl acetate-water (1:1). A portion of ethyl acetate extract was subjected to column chromatography over silica gel packed and eluted with *n*-hexane a gradient of ethyl acetate to 100% followed by CHCl₃ in MeOH (20:1, 10:1, 5:1, 1:1). Fraction B (CHCl₃ in MeOH 20:1) was subjected to column chromatography over silica gel 300 mesh and eluted with EtOAc-MeOH mixtures of increasing polarity. Fraction with the same R_f value were combined and eluted with EtOAc-MeOH (19:1) showed one spot on TLC. They were combined and evaporated to yield a solid then was recrystallized in mixture of CH₂Cl₂-methanol to give soulatro coumarin compound. The structure was determined by spectroscopic analysis, in particular by 1D and 2D NMR techniques, from these spectra data conclusion that compound is soulatro coumarin. Antimalarial assay was tested against *Plasmodium berghei* parasite as *in vivo* using 18 mice rodent which was infected by *Plasmodium berghei* parasite. The soulatro coumarin showed activity against *P. berghei* with dosage 0.0005867 mM/kg body weight; 0.005867 mM/kg bw; 0.05867 mM/kg bw; 0.5867 mM/kg bw 5.867 mM/kg bw and 58.67 mM/kg bw could inhibit growth rate of parasite = 57.32%; 63.37%; 43.02%; 53.49%; 47.67% respectively.

Keywords: Antiplasmodial activity, coumarin, *Calophyllum soulattri* Burm. F, *in vivo*, Chloroquine, *Plasmodium berghei*.

INTRODUCTION

Calophyllum soulattri Burm F [(local name: Sulatri (Sunda), slatri (Java), malang-malang (Bangka)]. Vernacular Name : bintagor labu (Malaysia). Bintagor lanchar, mintak (Peninsular). *C. soulattri* is a wide spread but in many places rather uncommon tree of fairly small dimensions, growing in lowland or lower montane rain forest or sometimes in swamp forest, up to 1700 m altitude. (Soerianegara *et al.*, 1994). *C. soulattri* distributed in West Java, Central Java, Irian and Kalimantan Island and also distributed in Vietnam, Cambodia, Thailand,

Andaman, Solomon Island, Northern Australia and Malaysia (Suryanegara *et al.*, 1994). In these research the stem bark of *C. soulattri* Burm F (Guttiferae Family) was used and collected from Jaya Pura (Irian Island) in Indonesia.

The bark, roots and latex of *C. soulattri* are used in local as folk medicine. The fruits are edible but sour. The latex may be used to poison dogs.

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A number of medicinal and therapeutic properties have been described to various parts of *C. soulattri*, e.i root and leaves, including the treatment of rheumatism, varicose veins, hemorrhoids and chronic ulcers (Crane *et al.*, 2005).

Root and leaves of *C. soulattri* also used as rheumatic pain. Seed or other species are used for scabies, ringworm, as an analgesic in leprosy, dermatoses, as diuretic, gonorrhea, tumors, migraine and vertigo (Crane *et al.*, 2005). In the Bulolo area in Papua New Guinea (PNG) the plant used for several diseases, such as dry skin, high blood pressure and malaria (Khan *et al.*, 2002). The balsem from the bark of *Calophyllum sp* used as a cicatrisan, infusion or decoction of the leaves has been traditionally used as an eye remedy in Asian (Iinuma *et al.*, 1993). Prenylated xanthenes have been reported to show antibacterial and antifungal activities (Dharmaratne *et al.*, 1997). The anticancer activity of new coumarin was also investigated against HeLa cell line and the new

coumarin showed the most potent as anticancer (Abbas, 2008).

We now report the isolation, structural elucidation and evaluation of the antiplasmodial activity of natural soulatro coumarin isolated from *C. soulattri*, *in vivo* evaluation of the antiplasmodial activity has been done by using mice which it's infected by *Plasmodium berghei*.

The Genus *Calophyllum* is well known as a source of biologically active compound such us xanthenes (Reye-Chipa *et al.*, 1997; Kijjoa *et al.*, 2000; Limuna, M *et al.*, 1993), terpenoids (Cao *et al.*, 1998), neoflavonoid (Dharmaratne *et al.*, 1984). Coumarin (Guilet *et al.*, 2001 and Ito *et al.*, 2003). Xanthenes, terpenoides, neoflavonoid were found in related species.

Kalazeyloxanthone has been isolated from the root bark of *C. soulattri* by Dharmaratne (Dharmaratne *et al.* at 1984) (Fig.1)

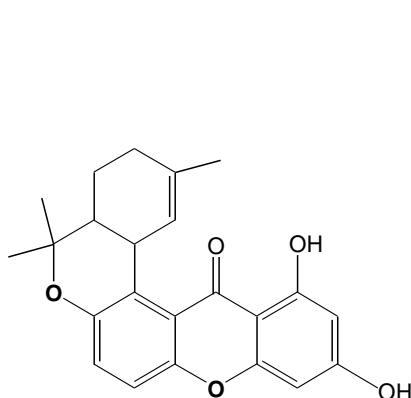


Figure 1. Structure of kalazeyloxanthone

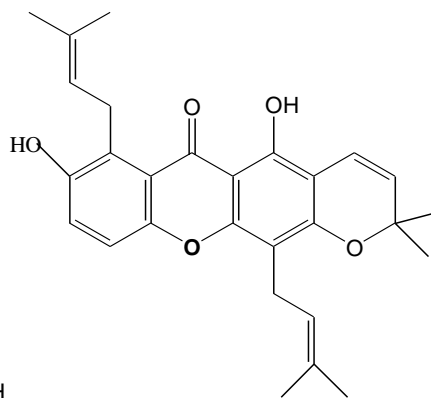


Figure 2. Structure of dombakinaxanthone

Dombakinaxanthone (diprenylated chromenxanthone) has been isolated from the cold hexane extract of the root bark of *C. soulattri* (Dharmaratne *et al.*, 1993) (Fig. 2). Jayapuracoumarin has also been isolated from stem bark of *C. soulattri* that Jayapuracoumarin has antioxidant and antiplasmodial acrivity (Abbas *et al.*, 2009) (Fig. 3). One new

coumarin (aziz coumarin) has also been isolated from stem bark of other species of *Calophyllum*, that coumarin has antimalarial and cytotoxic acrivity (Abbas *et al.*, 2009) (Fig. 4). One kalocoumarin (brasimarin C) have also success isolated from *C. incrasaptum*, that kalocoumarin has antimalarial activity too (Abbas *et al.*, 2009) (Fig. 5).

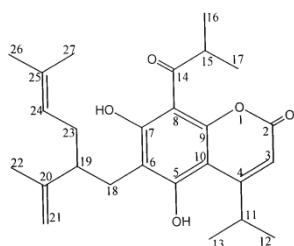
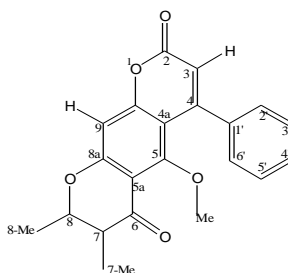


Figure 3. Structure of Jayapuracoumarin



Structure of kalocoumarin

Figure 4. Structures of new coumarin (Aziz coumarin)

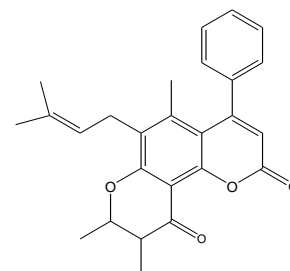
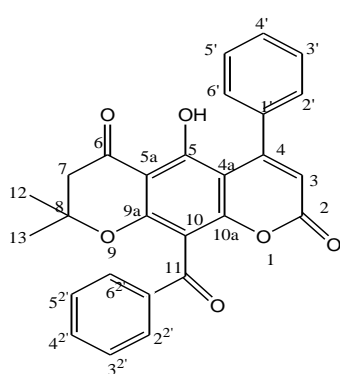


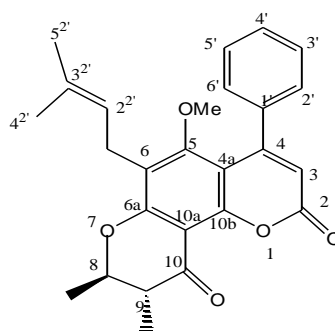
Figure 5. (Brasimarina C)

Three coumarin (teysmanone A, teysmanone B, calanone) and two coumarin (iophyllum C and iophyllum D) have been isolated from *C. teysmannii* and *C. inophyllum* respectively (Cao 1998). Two soulatro coumarine (pyranocoumarin) has been

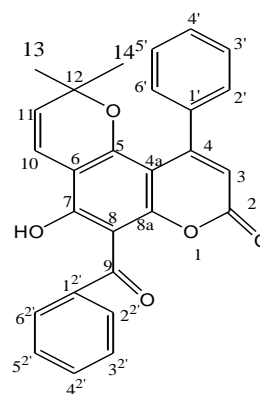
previously reported from stem bark and leaves of *C. soulattri* by Samarawera (1983), and one coumarin (soulatrolide) has also been isolated from stem bark of *C. soulattri* (Fig.6).



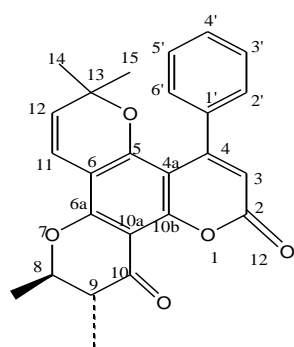
Teymanone A



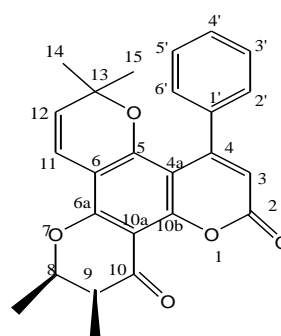
Teymanone B



Calanone



Inophyllum C



Inophyllum E

Figure 6. Structures of some coumarins (Teymanoe A,B, Calanone, Inophyllum C and Inophyllum D)

Two soulatro coumarine (pyranocoumarin) has been previously reported from stem bark and leaves of *C. soulattri* by

Samarawera (1983) and one coumarin (soulatrolide) has been isolated from stem bark of *C. soulattri*. (Fig.7).

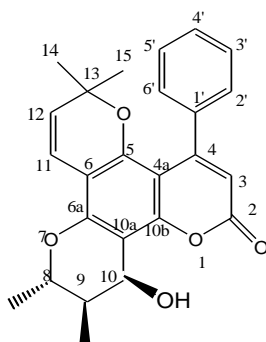


Figure 7. Structure of soulatrolide from *C. Soulattri*

Other author were repoted soulatro coumarin as anticancer (Cao 1998a), anti HIV (Gustafon, 1994) and three new coumarins also has cancer chemopreventive activity (Ito *et al.*, 2003). In this communication, we report the isolation and structure elucidation of a soulatro coumarin from stem bark of *C. soulattri* as antiplasmodial. *C. soulattri* was choised as a source of antiplasmodial drug candidate, because *C. soulattri* content some coumarin compounds, and according to some refferences, coumarin have antiplasmodial activity. Resistance of *plasmodium* to antimalarial drugs and their toxicity are contributing factors to the spread of the disease. This highlights the need to develop quickly more effective and less toxic new antimalarial drugs from *C. soulattri*. The isolation of soulatro coumarin will be explained in the experimental procedures.

MATERIALS AND METHODS

General Experimental Procedures

Melting point was measured on a Fisher Scientific serial 903N0056 apparatus. UV spectra was measuered on a Hitachi UV-VIS spectrophotometer using methanol solution, λ_{\max} in nm : IR spectra were measured on a FTIR Shimadzu Prestige 21 as KBr disc ; ν in cm^{-1} . ^{13}C NMR (150 MHz), ^1H NMR (500 MHz), and 2D NMR experiment (COSY, HMBC, HMQC) spectra were recorded on a Jeol – JNM ECA 500 MHz spectrophotometer, CDCl_3 was used as solvents, TMS as internal standar : δ in ppm, J in Hz. Mass spectrometry analysis was performed on a Mariner Bio Spectrometry, equipped with a pneumatically assisted elecrospray ionization (ESI) source. Silica gel (Merck 200 mesh and 300 mesh)

were used for column chromatography. Precoated silica gel at alluminium plates (silica gel 60 F_{254}) were used for analytical TLC. Spot were visualized by UV (254 and 365 nm), FeCl_3 and 10% H_2SO_4 were used as spraying reagent followed by heating at 110°C .

Plant Material

The stem bark of *C. soulattri* was collected from Irian Island, Indonesia and the voucher specimen was identified and deposited at the Herbarium Bogoriense – LIPI. Bogor, Indonesia.

Extraction and Isolation of Plant Materials

The dried and powdered of the stem bark of *C. soulattri* (1 kg) was maserated by methanol (3 x 2 litre x 7 days), solvent was removed under reduced pressured by rotary evaporator to gave 109,47 g methanol extract, which was then suspended in water and partitioned with *n*-hexane, dichloromethane and ethyl acetate. Each solvent were removed under pressure by rotary evaporator to give *n*-hexane extract (38.39 g, 3.84%), dichloromethane extract (25.05 g, 2.51%) and ethyl acetate extract (25 g, 2.50%).

The ethyl acetate extract (25 g) was separated by column chromatography on silica gel 70-230 mesh (Merck 800 g) by using *n*-hexane a gradient of ethyl acetat to 100% followed by CHCl_3 in MeOH (20:1, 10 :1, 5:1, 1:1). Frction B from (CHCl_3 in MeOH 20:1) was subjected to column chromatography (60 cm x 6 cm i.d) on silica gel 300 mesh (Merck code 1.07734) eluting by EtOAc : MeOH with a step gradien elution). Faction with the same R_f valeus were combined and eluted with EtOAc-MeOH (19:1) and showed one spot on

TLC than was recrystallized by using CH_2Cl_2 and methanol, to produce coumarin compound (500 mg) as pale yellow powder with mp 83-84°C (from fraction 7a). Molecular weight 426.11 Identically by comparison of $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and MS data with literature values (Cao *et al.* 1998b)

Determination of the Antimalarial Activity. (Trager and Jensen, 1976 and Fotie *et al.*, 2003)

P. berghei was maintained continuously in culture on human erythrocytes as described by Trager and Jensen (Trager and Jensen, 1976). Antimalarial activity of the soulatro coumarin compound was determined *in vivo*. In brief soulatro coumarin 2,5 mg (BW 426,11) was dissolved in 100 μl DMSO to give 58,670 mM as stock solution and kept at -20°C until used. The DMSO concentration never exceeded 0.1% and did not inhibit the parasite growth (Fotie, 2004). Antimalarial activity was done *in-vivo* against *P. berghei* using mice strain Swiss, Groups of 18 mice strain Swiss, weighing 20-24 g (2-3 month old) were used. The animals had free access to standard commercial diet and water ad libitum in a 12 h/12 h light-dark cycle at 22°C and must be acclimation by giving antibiotic for 3 days before be used. Mice were injected by 100 μl *P.bergei* parasite (pi), inoculation for 10-14 days, take blood from the vena of mice's tail after 2 days, calculated amount of parasite, if total amount of parasites in the mice blood under 20 %, take blood from the vena of mice's tail after 3 or 4 or 5 or 6 or 10 days inoculation, calculated amount of parasite, if total of parasite in the mice blood up to 20 %, its mean those mice was positive infected by *P.berghei* parasite, than all mice already be used for antipasmodial evaluation. Inject six

dosages of soulatro coumarins to the mice each 100 μl : 0.0005867 mM/kg body weight; 0.005867 mM/kg body weigh; 0.05867 mM/kg body weight; 0.5867 mM/kg body weight, 5.867 mM/kg body weight and 58.670 mM/kg body weight. Take blood from the vena of mice's tail after zero day (D0), one day (D1), two days (D2), three days (D3) and four days (D4) inoculation, were then calculated % parasites by making a blood smear, fixed with methanol and stained with Giemsa stain. Number of the infected red blood cells were counted under the microscope. Injected the chloroquine to the mice 2,5 mg/kg body weight, cloroquine was used as a positive control.

The concentration response parasite growth data were analyzed by a linear regression function using the Sigma-plot 2000 computer program to determine the 50% inhibitory concentration (IC_{50}). The IC_{50} value is defined as the concentration of compound producing 50% parasite growth inhibition relative to untreated control (without give the soulatro coumarins sample)

RESULTS AND DISCUSSION

Coumarin compound gave a positive reaction with FeCl_3 reagent in methanol (-blue spot) (that mean coumarin contain OH groups, the OH with FeCl_3 become $\text{Fe}(\text{OH})_3$ blue spot. This structure was established by spectroscopic means. In the LC-MS spectrum (Fig. 11), showed the molecular ion $(\text{M}+1)^+$ at $m/z = 427.11$ and molecular ion $(2\text{M})^+$ at $m/z = 852.48$ and also $(2\text{M} + \text{Na})^+$ at $m/z = 874.48$, corresponding to the molecular formula of $\text{C}_{25}\text{H}_{30}\text{O}_6$ and molecular weigh 426.11 (Fig. 8c).

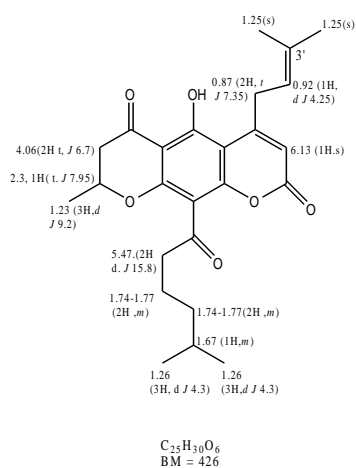


Figure 8a, 1H - NMR
Chemical shift of soulatro coumarin

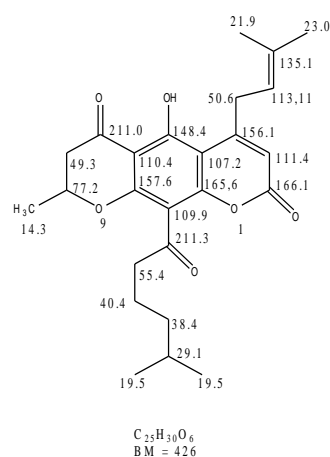


Figure 8b. ^{13}C - NMR
Chemical Shift of soulatro coumarin

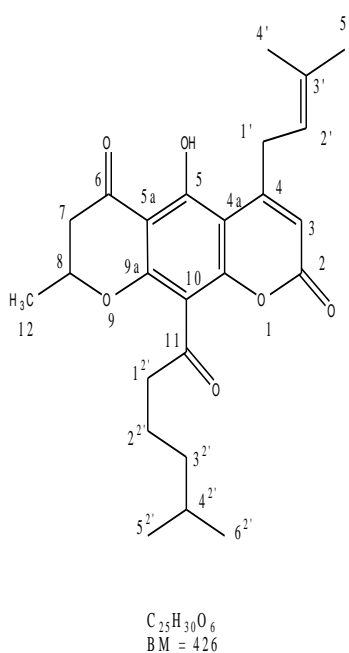


Figure 8c. Structure of soulatro coumarin

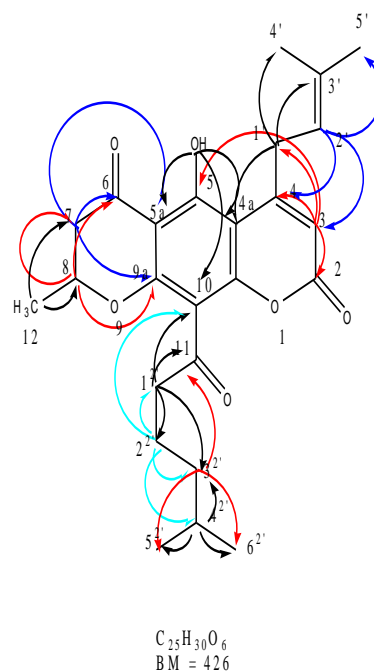


Figure 9. HMBC correlation of new soulatro coumarin

Table I. ^1H and ^{13}C NMR spectral data for soulatro coumarin

Carbon	^{13}C -NMR	^1H -NMR	HMBC correlation
1			
2	166.1		
3	111.4	6.13 (1H,s)	C-2, C-4, C-1'
4	156.1		
4a	107.2		
5	148.4		
5a	110.4		
6	211.0		
7	49.3	4.06 (2H, t J = 6.7)	C-5 _a , C-6, C-9 _a
8	77.2	2.34 (1H, t J = 7.95)	C-7, C-6, C-9 _a
9	-	-	-
9a	157.6		
10	109.9		
10a	165.6		
11	211.3		
12	14.3	1.23 (3H, d J = 9.25)	C-7, C-8
1'	50.6	0.92 (2H, d J = 4.25)	C-3', C-4', C-4 _a
2'	113.10	0.87 (1H, t J = 7.35)	C-3, C-4, C-5'
3'	135.1	-	
4'	21.9	1.25 (3H,s)	C-1',C-2',
5'	23.0	1.25 (3H,s)	C-1',C-2',
12'	55.4	5.47 (2H, d J = 15.8)	C-10, C-11, C-22'- C-32'-
22'	40.4	1.74-1.77 (2H, m)	C-10, C-12'- C-32', C-42'-
32'	38.4	1.74-1.77 (2H, m)	C-11, C-12'- C-52', C-62'-
42'	29.1	1.67 (1H, m)	C-32'- C-52', C-62'-
52'	19.5	1.26 (3H, d , = 4.3)	C-42'- C-32', C-22'-
62'	19.5	1.26 (3H, d , J = 4.3)	C-42'- C-32', C-22'-

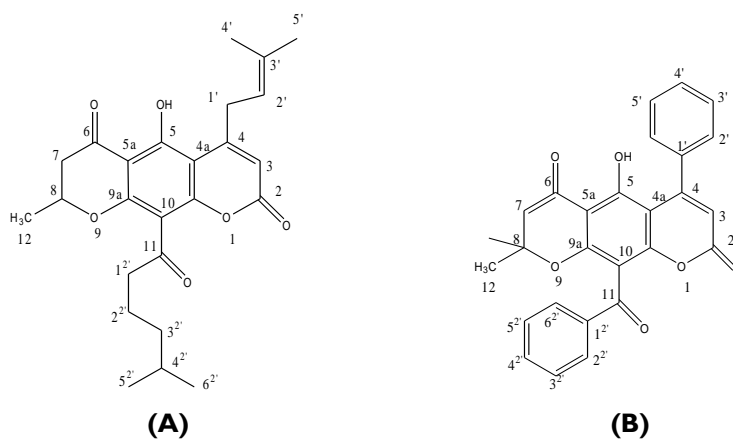


Figure 10. Structure of Soulatro coumarin and teysmanone. (A) Soulatro coumarin from stem bark *C. soulattri*, (B) Coumarin (teysmanone) from reference (Cao 1998)

Table II. Differenties of ^{13}C NMR spectral data of new soulatro coumarin and other coumarin (teysmanone Cao 1998^b)

Carbon	^{13}C -NMR new coumarin (Soulatro coumarin)	^{13}C -NMR Coumarin (teysmanone) (Reffence, Cao 1998)
1	-	-
2	166.1	158,9
3	111.4	113.1
4	156.1	152.5
4a	107.2	101.1
5	148.4	149.9
5a	110.4	106.3
6	211.0	115.4
7	49.3	47,2
8	77.2	79,5
9	-	-
9a	157.6	157.6
10	109.9	109.9
10a	165.6	106,4
11	211.3	191.9
12	14.3	28.0
1'	50.6	135.9
2'	113.10	127.5
3'	135.1	130.1
4'	21.9	130.6
5'	23.0	130.1
12'	55.4	137.6
22'	40.4	129.6
32'	38.4	128,5
42'	29.1	135.3
52'	19.5	128.5
62'	19.5	129.6

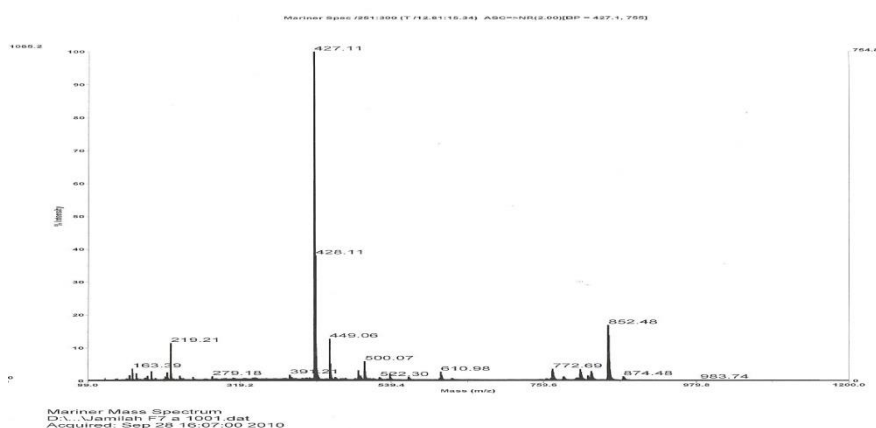


Figure 11. LC-MS spectra of soulatro coumarin compound (Column C18 with 80% methanol-water as eluent)

In the IR spectrum, a absorption bands attributable to an OH (3352 cm^{-1} sharp) and C=O groups from nucleus coumarin (1697 and 1390 cm^{-1} for C-O), aromatic ring (C=C) at

1604 and 1558 cm^{-1} and C-H streching methyl group at 2964 and 2872 cm^{-1} and C-H bending methyl at 850 , 889 and 933 cm^{-1} (Fig. 12).

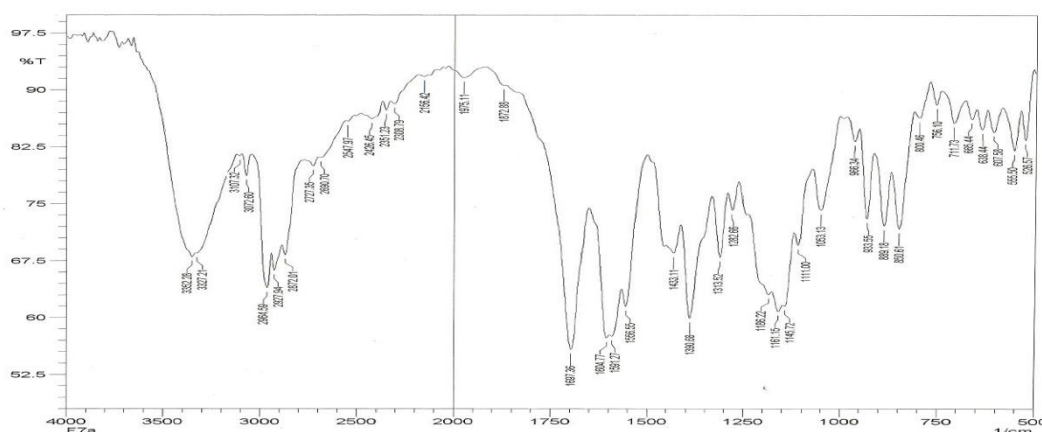


Figure 12. IR spectra of soulatro coumarin compound in KBr pellet

UV absorption maximum at λ 221.5 nm with absorbance 0.394 typical of an oxygenated coumarin and at λ 257.0 nm with absorbance 0.205 and at λ 331.0 nm with absorbance 0.590 (C 10 ppm in methanol). These UV spectrum showed

the typical absorptions of a prenylcoumarin nucleus at $\lambda_{\text{max}} = 221.5$; 257.0 and 331.0 nm (Fig. 13). UV spectra of soulatro coumarin compounds is similar with Ref^a (λ_{max} 228 & 286 and 324 nm, Cao 1998^b, Ito *et al.*, 2003) .

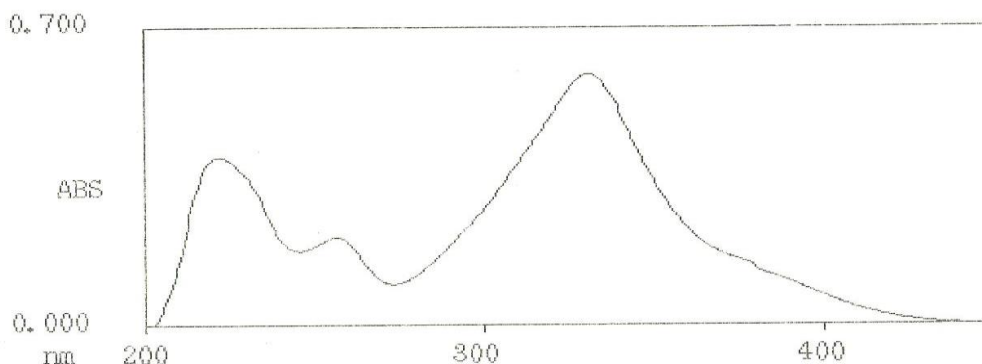


Figure 13. UV spectra of soulatro coumarin compound in methanol as solvent

The ^1H -NMR spectrum (500 MHz in CDCl_3) revealed signal assignable to a prenyl group at C-4, and additional one proton singlet at δ 6.13 (1H,s) specific at position C-3. Proton singlet at δ 6.13 correlated to the C-atom at δ 111.4 in the HMQC spectrum, The HMBC spectrum proton singlet at δ 6.13 showed long-range correlation to C-signals at δ 165.6 (C-10a), 156.1 (C-4), 148.4 (C-5) and 50.6 (C-1'). The C-atom at 50.6 (C-1') was also correlated to the aliphatic proton at C-3', C-4' and C-5', The correlation between proton at C-3 and carbon at C-2, C-4, C-1' and C-2') allowed the position of prenyl group at C(4) (Table I and Fig. 10) which confirmed that the isoprenyl

group was attached at C-4 of the coumarin moiety

HMBC-2D data illustrate the existence of a single isoprenyl group substitution at position C-4 and one hydroxyl at position C-5 and one carbonyl at C-6, that new compound were given name soulatro coumarin. The ^1H -NMR and ^{13}C -NMR spectral data of new soulatro coumarin as in Table I, Fig. 8a and 8b. The structure of the new soulatro coumarin was showed in Fig. 8c and HMBC correlation of a new soulatro coumarin was showed in Fig. 9.

The ^{13}C -NMR spectrum revealed an aliphatic ketone (δ 211.3), a conjugated lactone (δ 116.1), a monosubstituted methyl (δ 14.3, 1x

Me), a fully substitute benzen ring bearing three oxygen moities δ 157,6, 109,9, 165,6 148,4, 110,4,107,2) . These data (Table I) suggested that compound from *C. soulatri* as a new coumarin (soulatro coumarin). A

comparison of soulatro coumarin with teymanone (Cao *et al.*, 1998a) showed considerable differences of both chemical shifts of ^{13}C -NMR (Table II).

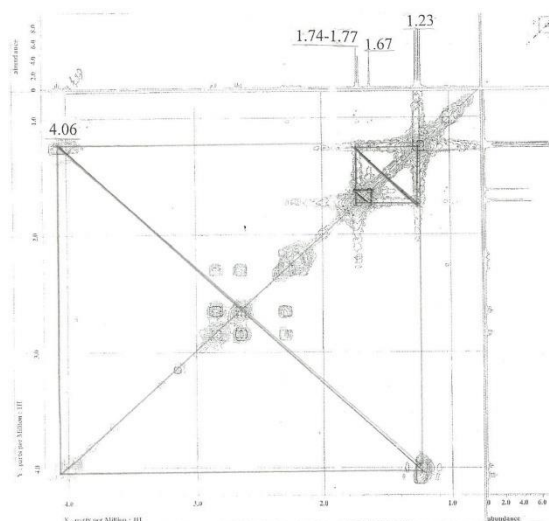


Figure 14. COSY spectrum of new soulatro coumarin compound

The COSY spectrum (Fig. 14) showed the connection of a doublet signal at δ 4.06 and a doublet signal at δ 1.23 ($J = 9.2$ Hz), other connectivity could be shown between a multiplet signal at δ 1.74-1.77 and doublet signal at 1.26 ($J = 4,3$), also other connectivity

could be shown between a multiplet signal at δ 1.74-1.77 and multiplet signal at 1.67

Soulatro coumarin have antiplasmodial activity with inhibiton growth rate of paracites but were not depend of samples concentrations (Table III). Chloroquine was used as positive control (Table IV and Fig. 15) .

Table III. Result of antiplasmodial activity of soulatro coumarin compound as *in vivo*

Soulatro coumarin Concentration (mM)	Parasetemia (%)					Growth rate (%)
	Mean DO	Mean DI	Mean D2	Mean D3	Mean D4	
0.0005867	3.575	5.000	4.300	4.225	4.175	100.00
0.005867	3.575	5.000	2.465	4.450	4.150	57.32
0.05867	3.575	4.100	2.725	2.900	3.150	63.37
0.5867	3.575	3.275	1.850	3.400	4.325	43.02
5.8670	3.575	1.775	2.300	3.650	4.450	53.49
58.670	3.575	2.950	2.050	2.175	3.600	47.67

The result of antiplasmodial activity of the sample of soulatro coumarin are recorded in Table III. The sample soulatro coumarin showed activity against *P. berghei* with dosage 0.0005867 mM/1 kg bw; 0.005867 mM/1 kg bw; 0.05867 mM/1 kg bw; 0.5867 mM/1 kg bw

5.867 mM/1 kg bw and 58.67 mM/1 kg bw could inhibite growth rate of parasite 57.32%; 63.37%; 43.02%; 53.49%; 47.67% respectively. Soulatro coumarin shown activity against *Plasmodium berghei*, but it is not dose dependent,

Table IV. Growth rate inhibition of *Plasmodium* parasite by six dosage of the chloroquine

Chloroquine Concentration n (mM)	Parasetemia (%)					Growth rate (%)		IC ₅₀ (mMol)
	DO	DO	Mean D0	D2	D2	Mean D2	Mean D2	
3×10^{-7}	0.45	0.5	0.475	3.7	4.0	3.85	100.00	3×10^{-6}
3×10^{-6}	0.45	0.5	0.475	2.3	2.8	2.55	66.23	
3×10^{-5}	0.45	0.5	0.475	0.3	0.8	0.55	14.29	
3×10^{-4}	0.45	0.5	0.475	0.08	0.09	0.085	2.21	
3×10^{-3}	0.45	0.5	0.475	0.03	0.04	0.035	0.91	
3×10^{-2}	0.45	0.5	0.475	0.01	0	0.005	0.13	

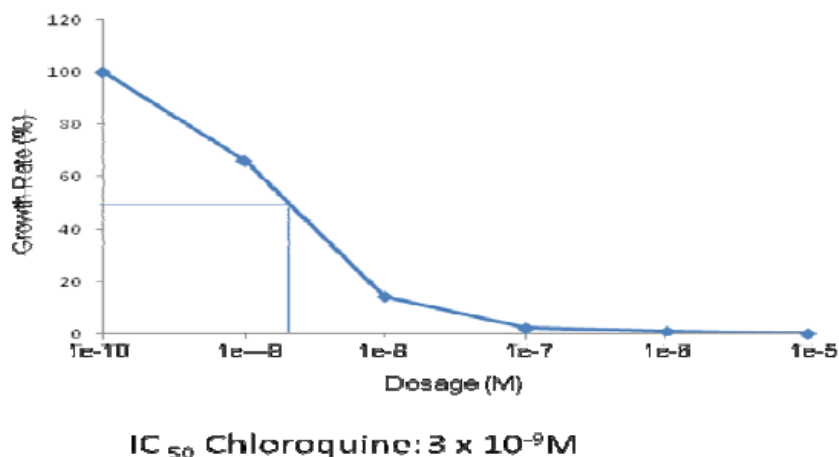


Figure 15. Effect of six dosage of chloroquines in growth rate inhibition of *Plasmodium* parasite (%)

The chloroquine showed activity against *Plasmodium* with dosage 3×10^{-7} , 3×10^{-6} , 3×10^{-5} , 3×10^{-4} , 3×10^{-3} and 3×10^{-2} could inhibit growth rate of parasite 0%; 33.77%, 85.71 %, 97.79 %, 99.09 % and 99.87 % respectively. Growth rate inhibition of *Plasmodium* parasite by chloroquines are depend of dosage of chloroquines. The chloroquine displayed significant inhibition of growth of *Plasmodium* parasite with IC₅₀ = 3×10^{-9} Mol or = 3×10^{-6} mM or = 0,03 μ M, but growth rate inhibition of *Plasmodium* parasite by soulatro coumarin are note depend of dosage of soulatro coumarin. Chloroquine have shown the best growth rate inhibition of *Plasmodium* parasite, with IC₅₀= 3×10^{-9} M or = 0.03 μ M indicating that chloroquine are the most potent compound compared with benzophenon compounds.

CONCLUSION

As a conclusion, new soulatro coumarin showed moderate activity on a chloroquino-resistant strain of *Plasmodium berghei*. The natural approaches developed here to isolate

coumarin structures, in association with the biological evaluation, is providing interesting improvement of the antiplasmodial activity. Firstly, the position of the hydroxyl groups at C-5 appears to be important as indicated of activity observed in the present report. Secondly, the substitution by a 1,1-dimethylallyl chain, appear to be factors for good activity. So far, the antiplasmodial potency of solatro coumarin correlated well with its ability to inhibit *in vivo* heme polymerization, suggesting that this compound exerts its antiplasmodial action by preventing hemozoin formation (Ignatuschenko *et al.*, 1997). IC₅₀ values were obtained from douplet experiments.

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