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CHEMICAL CONSTITUENTS AND CYTOTOXIC ACTIVITY OF STEM BARK EXTRACT OF *CALOPHYLLUM INOPHYLLUM*

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ABSTRACTS

Stem bark of *Calophyllum inophyllum* was extracted with aqueous methanol and screened for secondary metabolites. The crude extract was partitioned with *n*-hexane to remove fat-soluble constituents, thereafter a portion of the defatted extract was purified using silica gel column chromatography. Both the crude and defatted extracts were investigated for cyctotoxic activity using Brine shrimp lethality assay. A number of bioactive secondary metabolites were confirmed present in the crude while the chromatographic purification afforded three compounds that were characterized as stigmasta-5, 22-dien-3-O-β-D-glucoside (C-1), macluraxanthone (C-2A) and 1, 5-dihydroxyxanthone (C-2B). LC_{50} for both crude and defatted extract were 56.22 and 183.55 μg/mL, respectively, indicating a good cytotoxic potential.

Key words: *Calophylum inophyllum*; xanthones; secondary metabolites; Brine shrimps

INTRODUCTION

Calophyllum inophyllum Linn. (Guttiferae) is an economically and medicinally important ever-green shrub endemic in tropical regions (Xiao *et al*., 2008). Various parts of the tree have been used traditionally for treatment of diseases and medical conditions such as rheumatism, eye and skin diseases, inflammations, arthritis, microbial infections, lumbago etc (Hay *et al*., 2004). Previous studies showed that *C. inophyllum* is rich in bioactive secondary metabolites, particularly oxygenated xanthones, coumarins and triterpenes (Dharmaratne *et al*., 2002). Twigs of *C. inophyllum* from Hainan, China were reported to contained prenylated xanthones named caloxanthone N and gerontoxanthone C (Xiao *et al*., 2008). Goh and Jantan, 1991, isolated 2-(3-hydroxy-3-methylbutyl)-1,3,5,6-tetrahydroxyxanthone, jacareubin, 6-deoxyjacareubin, 2-(3-methylbut-2-enyl)-1,3,5,6-tetrahydroxyxanthone and 2-(3-methylbut-2-enyI)-1,3,5-trihydroxyxanthone from heartwood of *C. inophyllum* from Malaysia. Caloxanthones A and B, macluraxanthone, 1,5-dihydroxyxanthone and (-)-epicatechin were characterised from root bark extract of *C. inophyllum* collected in Japan (Munekazu *et al*., 1994). Also, eight triterpenoids, 3β, 23-epoxy-friedelan-28-oic acid, friedelin, epifriedelanol, canophyllal, canophyllol, canophyllic acid, 3-oxo-friedelan-28-oic acid and oleanolic acid have been reported from the twigs of *C. inophyllum* obtained in Hainan, China (Yan-Zhi *et al*., 2010). Most of the reported compounds from the plant have been shown to exhibit pharmacological activities such as cytotoxicity (Xiao *et al*., 2008), antimalarial (Hay *et al*., 2004), antimicrobial (Dharmaratne *et al*., 2004), inhibition of HIV-1 reverse transcriptase and HIV-1 replication (Dharmaratne, 2002) and antifungal (Reyes-Chilpa *et al*., 1997). Fatty acid analysis of the seedoil from *C. inophyllum* from Nigeria showed high amounts of unsaturated fatty acids with linoleic and oleic acids as major ones, the oil also had inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus* (Ajayi *et al*., 2008; Adewuyi *et al*., 2014). Unlike other countries such as Japan, France, China, Malaysia etc where extensive secondary metabolite profiling has been done on their indigenous *C. inophyllum*, fewer literature, particularly on isolation and characterization exists on *C. inophyllum* grown in Nigeria, thus, in this study, the chemical constituents of stem bark of *C. inophyllum* grown in Nigeria were investigated; also, solvent extracts of the plants were examined for cytotoxic activity.

MATERIALS AND METHODS

Plant collection and extraction

The stem barks of *C. inophyllum* were collected

in the premises of Bowen University, Iwo. Identification and authentication of the plant were performed by Mr S.A. Gabriel of Herbarium Unit, Department of Botany, Obafemi Awolowo University, Ile-Ife. Upon air-drying and pulverisation of the plant stem bark, 1.0 kg was extracted with aqueous methanol (95%). The methanolic extract was concentrated *in vacuo* to give crude (200 g), which was subsequently partitioned with *n*-hexane to yield defatted extract (70 g). Qualitative determinations of secondary metabolites such as tannins, alkaloids, saponins flavonoids anthraquinones cardiac glycoside and phlobatanins were carried out on the crude extract using standard procedures (Harbone, 1973; Sofowora, 1993; Trease and Evans, 1995).

Column Chromatographic purification

Thirty gram of the defatted extract was chromatographed on silica gel and subjected to gradient elution using dichloromethane (DCM) and methanol in order of increasing polarity to give 122 fractions. Based on TLC profile, the fractions were pooled into 7-sub-fractions (A1-A7). Sub-fraction A-2 (106 mg) eluted by 2.5% methanol in DCM was further purified on silica gel column chromatography using gradient mixture of hexane and DCM to give a white solid **C-1** (26 mg). On standing, sub-fraction A-5 afforded a yellow crystalline solid, (10.6 mg) which had an unresolved band of two spots on TLC, thus, it was taken to be a mixture of two compounds, **C-2A** and **C-2B**.

Brine shrimps lethality Assay

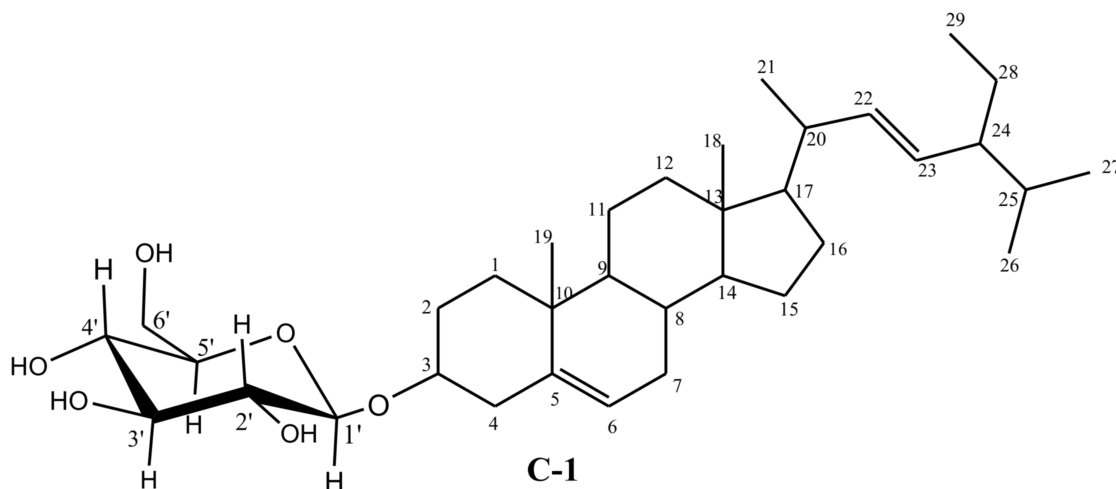
Brine shrimps lethality test as described by Meyer *et al*. (1982) was used to evaluate the cytotoxic potential of the crude and defatted extracts. Briefly, little quantity of brine shrimp (*Artemia salina* Leach) eggs was suspended in sea water and left half-covered for a period of 48 h during which the eggs were hatched into matured *nauplii*. Ten matured *nauplii* each were added to varying concentrations (1000, 100 and 10 μg/mL) of the test samples and were left for 24 h after which the number of survivors were noted and the LC_{50} was computed using Finney programme.

RESULTS AND DISCUSSION

Phytochemical screening of the crude extract indicated the presence of tannins, saponins, flavonoids, anthraquinones and cardiac glycoside while alkaloids and Phlobatanins were absent. The ^{1}H NMR spectrum of C-1 suggested a steroidal compound with a sugar moiety. The anomeric proton was displayed at δ 4.33 (J = 7.8 Hz) indicating a β-linkage while the H-3 of the aglycone appeared as a multiplet at 3.67 ppm. Olefinic protons, H-6 were seen as a singlet at 5.33 ppm and those of the exocyclic double bond (H-22 & 23) were double doublets at δ5.18 and δ5.04. Other steroidal protons resonated upfield (δ0.68-δ2.34) while protons of sugar moiety were observed at 3.00-4.82 ppm (Table 1). Based on comparison with literature (Rail *et al*., 2006; Mahbuba *et al*., 2012), C-1 was characterized as stigmasta-5, 22-dien-3-O-*β*-D-glucoside (Fig 1).

Table 1: Comparison of ^{1}H NMR data of isolated compounds with literature values

Position	C-1	Stigmasterol	C-2A	Macluraxanthone	C-2B	1, 5-dihydroxyxanthone
1	1.80 (2H, m)	1.83 (1H, m) 1.08 (1H, m)	13.94 (OH)	13.62 (br s, OH)	12.65 (br s, OH)	12.64 (br s, OH)
2	1.41 (2H, m)	1.49 (H, m) 1.82 (1H, m)	-	-	6.83 (1H, d, *J* 8.0	6.83 (1H, d, *J* 8.0
3	3.67 (1H, m)	3.53 (1H, m)	-	-	7.75 (1H, t, *J* 8.25	7.75 (1H, t, *J* 8.0
4	2.34 (1H, m) 2.18 (1H, m)	2.28 (1H, m) 2.24 (1H, m)	-	-	7.11 (1H, d, *J* 8.0	7.11 (1H, d, *J* 8.4
5	-	-	-	-		
6	5.33 (1H, s)	5.35 (1H, d, *J* 4.7)	-	-	7.38 (1H, d, *J* 7.8)	7.37 (1H, dd, *J* 8.0, 6.0)
7	2.10 (1H, m) 1.50 (1H, m)	1.98 (1H, m) 1.53 (1H, m)	7.14 (1H, d, *J* 9.0	6.95 (1H, d, *J* 8.4)	7.32 (1H, d, *J* 7.9)	7.30 (1H, d, *J* 8.0)
8	1.47 (1H, m)	1.46 (1H, m)	7.50 (1H, d, *J* 9.0)	7.88 (1H, d, *J* 8.4)	7.62 (1H, d, *J* 7.9)	7.61 (1H, dd, *J* 8.0, 2.0)
9	0.90 (1H, m)	0.94 (1H, m)	-	-		
10	-	-	-	-		
11	1.48 (1H, m) 1.49 (1H, m)	1.45 (1H, m) 1.48 (1H, m)	6.61 (1H, d, *J* 9.6)	6.69 (1H, d, *J* 10)		
12	1.18 (1H, m) 1.78 (1H, m)	1.15 (1H, m) 1.97 (1H, m)	5.74 (1H, d, *J* 10.2)	5.86 (1H, d, *J* 10)		
13	-	-	-	-		
14	0.96	1.00	1.42 (3H, s)	1.48 (3H, s)		
15	1.12 (1H, m) 1.52 (1H, m)	1.06 (1H, m) 1.55 (1H, m)	1.42 (3H, s)	1.48 (3H, s)		
16	1.22 (1H, m) 1.64 (1H, m)	1.27 (1H, m) 1.71 (1H, m)	-	-		
17	1.26 (1H, m)	1.13 (1H, m)	1.69 (3H, s)	1.77 (3H, s)		
18	0.76 (3H, s)	0.70 (3H, s)	1.69 (3H, s)	1.77 (3H, s)		
19	1.03 (3H, s)	1.01(3H, s)	6.38 (1H, dd, *J* 17, 10	6.40 (1H, dd, *J* 10.2, 10.8		
20	1.98 (1H, m)	2.04 (1H, m)	4.94 (1H, d, *J* 17.4) 4.82 (1H, d, *J* 10)	4.93 (1H, d, *J* 17) 4.85 (1H, d, *J* 10.2)		
21	0.79 (1H, d, *J* 5.1)	0.84 (1H, d, *J* 5.1)				
22	5.18 (1H, dd)	5.15 (1H, dd)				
23	5.04 (1H, dd)	5.02 (1H, dd)				
24	1.62 (1H, m)	1.53 (1H, m)				
25	1.47 (1H, m)	1.44 (1H, m)				
26	0.69 (3H, d)	0.74 (3H, d)				
27	0.68 (3H, d)	0.75 (3H, d)				
28	1.18 (1H, m)	1.185(1H, m)				
29	0.79 (3H, m)	0.80 (3H, m)				
1'	4.23 (1H, d, *J* 7.7)					
2'	3.45 (1H, m)					
3'	3.16 (1H, m)					
4'	3.48 (1H, m)					
5'	3.12 (1H, m)					
6'	3.18 (2H, m)					



C-1

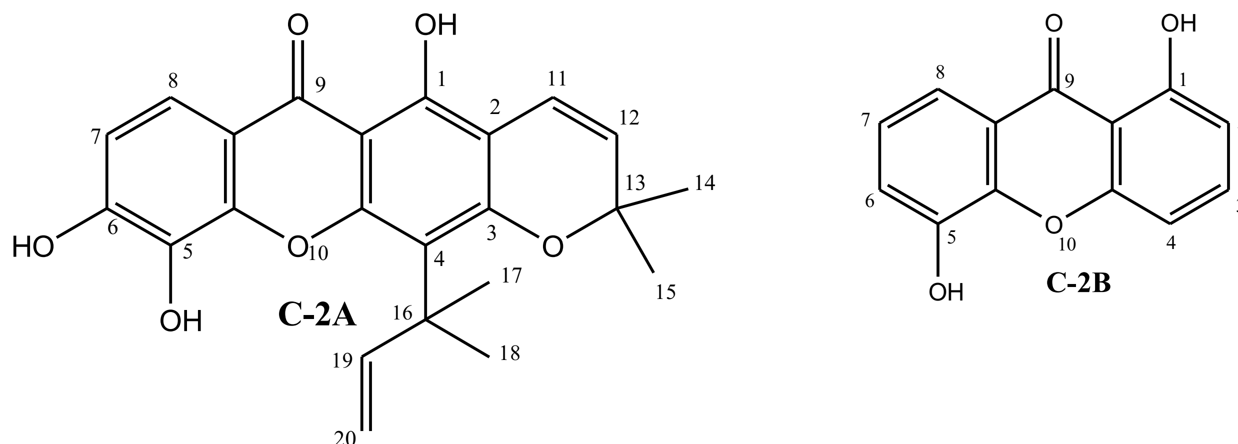


Fig.1: Isolated compounds from *C. inophyllum*

The ^{1}H NMR spectrum of the mixture of C-2A and C-2B was typical of an oxygenated xanthone. Integration of peaks in proton NMR experiment indicated that the components of the mixture were in ratio 1:3 (C-2B:C2-A). Compound C-2A had intense peaks upfield and resonances for olefinic protons which suggested a prenylated moiety. Only two pairs of ortho-coupled aromatic protons at position 7 (δ6.95) and 8 (δ7.50), and 11(δ6.69) and 12 (δ5.86) were observed, which suggested a substituted aromatic system. Chemical shifts of the olefinic protons were between 4.85-6.40 ppm and those of methyls resonated as two intense peaks at 1.42 and 1.69 ppm.

The ^{1}H - ^{1}H COSY showed correlations for H7-H8, H11-H12 and H20a/H20b-H19. Methyl protons at positions 14 and 15, and those at 17 and 18 resonated as two intense singlets at 1.42 and 1.69 ppm respectively. The spectroscopic data of C-2A agreed with those reported for macluraxanthone obtained from the root and stem bark of *C. inophyllum* from Malaysia and Japan, thus, C-2A was identified as macluraxanthone (Fig. 1) (Munekazu *et al.*, 1994; Gwendoline *et al.*, 2011). Compound C-2B which co-crystallised with macluraxanthone (C-2A) had clusters of proton resonances in the aromatic region, also in a manner typical of oxygenated xanthone. All the protons were aromatic and ortho coupled while the hydroxyl group peaks were suppressed at 12.65 and 10.50 ppm (Table 1). The ^{1}H - ^{1}H COSY showed cross-links for H2-H3, H4-H3 and H7-H8. On comparison, the proton resonances of C-2B matched exactly the values reported for **1, 5-dihydroxyxanthone** (Fig. 1) also from the root bark of Japanese *C. inophyllum* (Munekazu *et al.*, 1994).

The LC_{50} as calculated by Finney program at 95% confidence limit for both crude and defatted extract were 56.22 and 183.55 μg/mL respectively. According to Tawaha (2006), plant extract having LC_{50} > 200 ppm are considered highly active, thus, both crude and defatted extracts were cytotoxic. However, lower activity exhibited by the defatted extract compared to crude implied that some of the pharmacologically active constituents of the plant extract were considerably fat-soluble. Caloxanthone N and gerontoxanthone isolated from Chinese *C. inophyllum* were reported to exhibit high cytotoxic activity on chronic myelogenous leukemia cell line (K562) (Xiao *et al.*, 2008). Similarly, brasixanthone B, C and D from *Calophyllum brasiliensis* were found to elicit a good cancer chemopreventive activity (Chihiro *et al.*, 2002).

CONCLUSION

C. inophyllum is rich in bioactive secondary metabolites particularly oxygenated xanthones and coumarins. Macluraxanthone and 1, 5-dihydroxylxanthone partially characterised in this study had been reported for the same plant native to some Asian and European countries, however, reports on glycosylated steroids from Calophyllum species are very scanty; specifically, to our knowledge, stigmasta-5, 22-dien-3-O-β-D-glucoside isolated in this study has not been

previously reported in *C. inophyllum*. Brine shrimp lethality assay detects substances toxic to zoologic system, therefore it is non-specific for any physiological action thus a more specific bioassay might be necessary to confirm or otherwise the cytotoxic activity of the plant extract.

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