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RESEARCH ARTICLE



# <sup>2</sup>Antimicrobial, antioxidant and cytotoxic activities of Garcinia eugenifolia and Calophyllum enervosum

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## 8 ABSTRACT

Chemical investigation of Garcinia eugenifolia and Calophyllum enervosum yielded six compounds. One 10 of these was found to be a novel compound named as enervosanone. Five known compound such as a cambogin, epicatechin, osajaxanthone, rubraxanthone and isocowanol were also isolated. These com-12 pounds were tested for their bioactivity as antimicrobial, antioxidant and cytotoxicity. Antimicrobial assay 13 was performed using disc diffusion method. The antioxidative activity was evaluated using 2,2-diphenyl-1-14picrylhydrazyl (DPPH) method by electron spin resonance. The cytotoxicity was measured by the MTT 15 assay against MCF7 cell line. Enervosanone and rubraxanthone were active against Bacillus subtilis, Es-16 cherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus with MIC value of 26.82, 26.82, 1726.82, 26.82 and 60.97, 30.48, 60.97, 60.97 μM, respectively. Rubraxanthone and epicatechin exhibited respectively. The cytotoxicity assay on MCF7 cell 19 line showed that enervosanone was found to be active in inhibiting cell proliferation of MCF7 with IC50 of 201.07 μM.

21 Keywords: Guttiferae, Garcinia eugenifolia, Calophyllum enervosum, bioactivities

23a source of bioactive substances. Nowadays the scien- 48 Sultanbawa [3], 95 xanthones from Guttiferae have been 24 tific interest on these metabolites has increased due to 49 listed. Since then there has been a steady stream of re-25 the search for new drugs from plant origin. Plants of the 50 ports in which more than 80 new xanthones which have 26 family Guttiferae are known to be very good sources of 51 been characterized and many known xanthones re-27 such compounds [1].

Guttiferae in Malaya includes some well known and 53 29 important trees. There are four genera of Guttiferae such 54 enervosanone (1) cambogin (2), epicatechin (3) and 30 as Calophyllum, Garcinia, Mammea and Mesua. The 550sajaxanthone (4) isolated from G. eugenifolia and ru-31 genus Calophyllum (bintangor) is considerable as im- 56 braxanthone (5) and isocowanol (6) purified from C. 32 portant timber and may reach the largest size. Garcinia 57 enervosum. Their activities were tested for antimicro-(manggis, kandis) is primarily a genus of fruit tree and 58 bial, antioxidant and cytotoxicity. Anti platelet activat-34 is frequent as a small wild forest tree [2].

According to Sultanbawa [3], and Bennet and Lee 36[44], the Guttiferae family contains over 1000 species, 37 mainly restricted to the tropics except to the genus Hy-38*pericum* which occured widely in temperate region. The 39 majority of these plants are trees or shrubs and some of 40them yield useful timber. Some of these genera and spe- 62 41 cies are found to be endemic to certain land masses, e.g. 63 Gallen III apparatus and were uncorrected. UV spectra 42Kielmeyera is confined to the South Africa continent, 64were measured on UV-100PC Shimadzu using methanol 43 Symphonia and Pentadesma are confined to Africa.

45Guttiferaceous plants. The compounds, especially xan- 67 ured by a Polarimeter Type AA-5. <sup>1</sup>H NMR (400 MHz) 46 thones are usually found in this family. These results are 68 and <sup>13</sup>C NMR (100 MHz) spectra were recorded on a

Secondary metabolites produced by plants constitute 47 considerable chemotaxanomic interest. In the review by 52 isolated from ca. 60 species of Guttiferae [4].

In this paper, we wish to report the bioactivities of 59ing factor activity of two xanthones is also reported.

### **MATERIAL AND METHODS**

### 61 General experimental procedures

Melting points were determined by using a Leica 65 solution. IR spectra were measured on a FT-IR Perkin-Many chemical constituents have been studied from 66 Elmer 1600 as KBr discs. Optical rotations were meas-

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Table 1. Antibacterial activity of compounds 1-6

	Inhibition zone in mm (MIC value in $\mu g/mL$ )									
Micro-organisms	1	2	3	4	5	6	standard			
B. subtilis	12 (0.0125)	8 (0.05)	-		9(0.025)	-				
E. coli	9.5 (0.0125)	-	-	na	9(0.0125)	-	$21 (3.0 \times 10^{-4})$			
P. aeruginosa	11 (0.0125)	8 (0.05)	-		8(0.025)	-	21 (3.9×10)			
S. aureus	11.5 (0.0125)	8 (0.05)	-		8(0.025)	-				

-: No activity against the tested bacteria; na.: not available; MIC: minimum inhibition concentration; standard: streptomycin sulfate.

Table 2. The IC<sub>50</sub> of the compounds analyzed by ESR spectrometry

$IC_{50}$	Percent inhibition at 500 µg/mL		
2.60 μM	96.5		
0.89 M	55.3		
na	42.7		
8.01 μM	97.7		
210.20 μM	95.7		
	2.60 μM 0.89 M na 8.01 μM 210.20 μM		

Table 3. % inhibition by xanthones on PAF receptor binding to platelet at various concentrations and their values

Compound	18.2	9.1 (μg/mL)	4.5	1.8	$IC_{50} (\mu M)$ (mean ± S.D)
Rubraxanthone	76*	56	35	13	18.2±2.1
Isocowanol	15	11	4	1	227.8±3.4
Cedrol	75	66	56	47	10.6±1.2

Data represent mean  $\pm$  SD of three independent experiments performed in triplicate; \*p<0.05 as compared with cedrol (Student's test).

69 Varian Unity INOVA spectrophotometer, using TMS as 104 were crystallized with PE and EtOAc to give rubraxan-70 internal standard. EIMS were recorded on a Varian 105 thone (5.78 g, 0.6425%) as yellow needles which exhib-71 mass spectrometer at 70 eV. CC: silica gel (Merck 70–106 ited positive test with FeCl<sub>3</sub> reagent; mp. 206-208 °C, 72230 mesh and 230-400 mesh). Spots were visualized by 107lit. 205-206 °C [6].

73UV (254 and 365 nm), FeCl<sub>3</sub> and *p*-anisaldehyde spray-108. The *n*-hexane-EtOAc (1:1) soluble fraction (3.7 g) 74ing reagent. Streptomycin sulphate standard was pur-109from the EtOAc extract was purified by column chro-75 chased from Oxoid (Hampshire,UK). 110 matography (35 cm length, 2.5 cm diameter) on silica 111gel 230-400 mesh (60 g) using PE-EtOAc (9:1), (8:2),

### 76 Plant materials

Stem bark of C. enervosum and G. eugenifolia were 113tions 1-6 and 7-26. Purification of the combined frac-78 collected from Tilatang Kamang, 6 km East of Bukit-114 tions 7-26 was followed by column chromatography on 79tinggi, West Sumatra, Indonesia in September 1998.115sephadex LH-20 and eluted with MeOH afforded iso-80 The voucher specimen, MT-03 and MT-04, respectively 116 cowanol (160 mg, 0.017 %) as yellow needles which 81 are deposited at the Herbarium of Universitas Andalas 117 reacted positively with FeCl3 reagent; mp. 89-93°; TLC 82(ANDA), Padang, Indonesia.

### 83 Extraction and isolation

Extraction of the dried and powdered stem bark of 120 85*C. enervosum* and *G. eugenifolia* were extracted using <sup>121</sup> eugenifolia and *C. enervosum* were tested against *B.* 86 soxhlet extractor. C. enervosum was extracted as previ-122 subtilis, E. coli, P. aeruginosa and S. aureus. The an-87 ously reported by Taher, et al. [5]. G. eugenifolia (900<sup>123</sup>timicrobial assay was carried out using disc diffusion 88g) was extracted by soxhlet apparatus for 18 hours with<sup>124</sup> method [7]. The filter-paper discs were impregnated 89n-hexane (2.5 L), ethyl acetate (2.5 L) and acetone (2.5<sup>125</sup> with 10 μL of methanol solution of each sample (1 90L) successively. Each solvent was removed in vacuo by 126 mg/mL) and allowed to evaporate at room temperature. 91 rotary evaporator. The *n*-hexane afforded the extract as 127 Streptomycin sulfate (30 µg/disc) was used as positive 92brown sticky liquid (6 g, 0.66%) and the EtOAc extract 128 control. The discs were then incubated on the plate 93 gave a brown gummy (36.1 g, 4.01%).

95chromatography (60 cm length, 6 cm diameter) on silica<sup>131</sup>end of the incubation period. <sup>96</sup>gel 70-230 mesh (200 g) and eluted with the gradient 132 Antioxidant assay (electron spin resonance (ESR) 97 solvent system of *n*-hexane-EtOAc (3:1, 2:1, 1:1, and 133 spectroscopy method) 981:2) and EtOAc to give four fractions. The n-hexane-

112(7:3), (6:4), (5:5), (4:6), (3:7), (2:8) as eluent gave frac-

118(silica gel):  $R_f 0.56$  in EtOAc-PE (7:3).

### 119 Antimicrobial assay (disc diffusion method)

The isolated compounds from the stem bark of G. 129 aerobically at 37 °C for 18 h. The diameter of inhibition The EtOAc extract (25 g) was subjected to column<sup>130</sup>zone around each disc was measured and recorded at the

99EtOAc (2:1) (9.2 g) soluble fraction was purified by 134 DPPH radical scavenging test using electron spin 100 column chromatography (42 cm length, 3.5 cm diame-135 resonance was carried out according to the method de-101ter) over silica gel 70-230 mesh (90 g) and eluted with 136 scribed by Ohtani et al. [8] with a slightly modification. 102n-hexane-EtOAc (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), 137 The ethanolic solution of the test sample 100 µL (1 103(3:7), (2:8), and EtOAc. The combined 1-20 results138 mg/mL) was added to 100 µL of DPPH (25.3 mM) in

### Antimicrobial, antioxidant and cytotoxic activities of Garcinia and Calophyllum enervosum

139ethanol solution. After shaking vigorously for 10 s, the 198 Statistical analysis

140 solution was transferred to a flat cell. The ESR spectra

142trometer were set at room temperature, power 1 mW, 200Plot 8.0. Data is presented as means standard error of 141 were recorded after 40 s. The condition of ESR spec-143 magnetic field  $336.000 \pm 5$  mT, field modulation width

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1440.5 mT, sweep time 30 sec and time constant 0.03 s.

145The scavenging effect of DPPH was calculated by fol-202 147 lowing formula:

$$\frac{PH_{DPPH} - PH_{sample}}{PH_{DPPH}} \times 100\%$$

154PH = peak height of the third and the fifth line signals of 206 methods as reported in Taher et al. [10]. 155 DPPH radical

157 of each sample required to give 50% of scavenging of 209 *n*-hexane, ethyl acetate, and acetone successively, af-158DPPH. All test and analyses were run in triplicates.

<sup>160</sup>  
<sup>162</sup>Percentage inhibition = 
$$\frac{(Tc - Nc) - (Ts - Ns)}{Tc - Nc} \times 100$$

Tc and Ts = total binding of control and sample, re- $^{214}$  braxanthone (5) and isocowanol (6). spectively

sample respectively

### 169 Cytotoxicity (MTT assay)

Cytotoxicity assay was carried out using the 3-(4, 5-<sup>2</sup> 171 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 172(MTT) assay according to Mosmann [9]. MCF7 cell line 173 was cultured in DMEM/F-12 with 15 mM HEPES 174buffer, L-glutamine and pyridoxine hydrochloride sup-175 plemented with 10% fetal bovine serum and 1% penicil-176 lin- streptomycin in 96-well plate at density of  $6 \times 10^4$ 177 cells/mL. After reaching confluent  $(2x10^{\circ} \text{ cells/ mL})$ , 178 the cells were treated with the sample. The sample was 179 dissolved with dimethylsulfoxide (DMSO) at the final 180 concentration of DMSO was 0.1% (v/v). Different con-229 181 centrations of the sample were made with serial dilu-182tion. Tamoxifen was used as positive control. Experi-183ment was allowed to proceed for 48 hours at 37 °C in 184humidified 5% CO<sub>2</sub> atmosphere. At the end of this pe-185 riod, supernatants were discarded. To minimize the in-186 terference of residue of the supernatant, the adherent 187 cells were washed two times with phosphate buffer sa-188 line (PBS) then 20 µl of MTT stock solution (5 mg/ml) 189 was added to each well and the plates were further incu-190 bated overnight at 37 °C. Dimethyl sulfoxide (100 µL) 191 was added to each well to solubilize the water-insoluble 192 purple formazan crystals. After 1 hour, the absorbance 193 was measured at 570 and 655 nm (reference) with  $a_{242}^{2+1}$  use that the positive control of streptomycin sulfate 194 microplate reader. The 50% reduction in cell number 243 (MIC of 535.71 nM). While, cambogin was found to be 195 relative to the control or  $IC_{50}$  was estimate visually.

Abs. sample

196Cells number after treatment =

Abs. DMSO

 $1972 \times 10^5$  cells/mL

Statistical analyses were performed using Sigma

### RESULTS

Enervosanone (1), cambogin (2), epicatechin (3) and 04 osajaxanthone (4) were extracted from C. enervosum 05 using soxhlet extractor and purified by chromatographic

Extraction of the air dried and powdered of the stem The IC<sub>50</sub> value was determined as the concentration<sup>208</sup>bark of *Garcinia eugenifolia* by soxhlet apparatus with 210 forded the *n*-hexane extract as a brown sticky liquid and 211 the ethyl acetate extract as a brown gummy. The ethyl 12 acetate extract of the stem bark of *G. eugenifolia* was 213 submitted to chromatographic separation to afford ru-

Several bioactivities were tested to these isolated Nc and Ns = non-specific binding of control and<sup>216</sup> compounds such antimicrobial, antioxidant, anti platelet 217 activating factor and cytotoxicity. Antimicrobial assay 218 was tested against two strain bacteria, gram-positive 219(Bacillus subtilis and Escherecia coli) and gramonegative (Pseudomonas aeruginosa and Staphylococcus aureus) using the disc diffusion method (data is presented in Table 1). The compounds were also tested for their cytotoxicity against breast cancer cells. Their cytotocity activities are given in Fig. 1.

> The antioxidant activities of the compounds were tested against DPPH radical and analyze by electron 7 spin resonance spectroscopy. Their activities are given 28in Table 2 and Fig. 2.

### DISCUSSION

These compounds were identified using physical, chemical and spectroscopic properties. Enervosanone (1), cambogin (2), epicatechin (3) and osajaxanthone (4) 33 were identified as reported in Taher et al., [5]. Rubrax-234 anthone (5) and isocowanol (6) were elucidated by 235 comparing data with literature [6].

The purified and characterized compounds were 37 tested for their antimicrobial, citotoxicity, PAF receptor 238 binding and antioxidative activities. As shown in Table 391, enervosanone which was found as a new compound 40 in our previous report [10] exhibited strongest activity 41 against four tested bacteria although it is much less ac-244 inactive against S. aureus and osajaxanthone was found 245 to be inactive against all tested bacteria.

In the cytotoxicity assay, enervosanone showed a x247 significant cytotoxic effect against MCF7 by affecting 248 cell proliferation and changing the cell morphology 249(Fig. 1). Enervosanone was found to be active in inhibit-250 ing cell proliferation of MCF7 with IC<sub>50</sub> of 1.07  $\mu$ M. 251 However, IC<sub>50</sub> value of everyosanone was greater than 252 the positive control, tamoxifen which had the  $IC_{50}$  0.76

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Fig. 1. Morphology of human breast cancer (MCF7). (a) Confluent cells (untreated), (b) After 48 hour treatment with enervosanone ( $IC_{50}$  1.07  $\mu$ M), (c) After 48 h treatment with  $\mu$ M tamoxifen ( $IC_{50}$  0.76  $\mu$ M). Different concentrations of the sample were made with serial dilution. Tamoxifen was used as positive control. Experiment was allowed to proceed for 48 hours at 37 °C in humidified 5% CO<sub>2</sub> atmosphere. At the end of these periods, supernatants were discarded. To minimize the interference of residue of the supernatant, the adherent cells were washed two times with phosphate buffer saline (PBS) then 20  $\mu$ l of MTT stock solution (5 mg/ml) was added to each well and the plates were further incubated overnight at 37 °C. Dimethyl sulfoxide (100  $\mu$ L) was added to each well to solubilize the water-insoluble purple formazan crystals. After 1 hour, the absorbance was measured at 570 and 655 nm (reference) with a microplate reader.

 $\mu$ M (SD ± 0.005). Hence, it is indicated that ener-259 There is now increasing interest in the antioxidant 254 vosanone was less active than the control of tamoxifen. 260 activity of phytochemical present in the diet, in health 255 Based on the cell morphology, it was proposed that 261 food supplement (nutraceutical), and in topical prepara-256 enervosanone active against human breast cell cancer by 262 tions of cosmetic (cosmaceuticals) from environmental 257 inhibiting the cell proliferation. However, the mecha-263 exposure. A simple and rapid estimation of hydroxyl 258 nism of action of enervosanone is still not understood. 264 and superoxide anion radical scavenging activities can

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Fig. 2. Scavenging activity of, (b) epicathechin + DPPH 25.3 mM (percent inhibition: 96.5 %), (c) rubraxanthone + DPPH 25.3 mM (percent inhibition: 55.3%), (d) enervosanone + DPPH 25.3 mM (percent inhibition: 42.7%), (e) Vitamin C + DPPH 25.3 mM (percent inhibition: 97.7%), (f) Vitamin E + DPPH 25.3 mM (percent inhibition 95.7%) against (a) DPPH (25.3 mM) free radical by ESR spectrometry. The ethanolic solution of the test sample 100  $\mu$ L (1 mg/mL) was added to 100  $\mu$ L of DPPH (25.3 mM) in ethanol solution. After shaking vigorously for 10 s, the solution was transferred to a flat cell. The ESR spectra were recorded after 40 s.

265 measured by using ESR system [11]. In determining275 others tested samples, enervosanone and rubraxanthone 266 antioxidant activity of the compounds, Electron Spin276 showed no significant antioxidant activity (Fig. 2). 267 Resonance method was applied. The results showed that 277 Inhibitory effects of rubraxanthone and isocowanol 268 epicatechin exhibited the best antioxidant property<sup>278</sup> on platelet-activating factors (PAF) binding to rabbit 269 against DPPH radicals with IC<sub>50</sub> of 2.60  $\mu$ M and percent<sup>279</sup> platelet was reported in Jantan et al. [12]. The IC<sub>50</sub> of 270 inhibition of 96.5 at concentration of 500  $\mu$ g/mL (Table<sup>280</sup> compounds at various concentrations are given in Table 271 2). Its activity was almost similar to the antioxidant ac-<sup>282</sup> i.e., as the concentration of the compound increased the 272 tivity of the standard, vitamin C and Vitamin D with<sup>283</sup>% inhibition increased. The results revealed the struc-273 IC<sub>50</sub> of 8.01 and 210.20  $\mu$ M, respectively (percent inhi-284 ture-activity relationship between rubraxanthone and 274 bition of 97.7 and 95.7 at 500  $\mu$ g/mL, respectively). The<sub>285</sub> isocowanol on the PAF receptor binding. Compound

286 which inhibit the specific binding between PAF and 3126. 287 receptors found in a variety of cell membrane including 313 288 those from platelets, have been extensively sought to  $be^{3147}$ . 289 used as leads in the development therapeutic agents in 290 variety of inflammation, respiratory, immunological and  $_{3178}$ . 291 cardiovascular disorders [12].

As the conclusion, the different types of chemicals<sup>319</sup> 293 isolated from Garcinia eugenifolia and Calophyllum<sup>320</sup> 294 enervosum exhibited different activities. The most inter-3219. <sup>295</sup>esting compounds were enervosanone, rubraxanthone<sup>322</sup><sub>323</sub> 296 and epicatechin that exhibited the antimicrobial, inhibi-32410. 297tory effects on platelet-activating factors binding and 325 298 antioxidative activities, respectively.

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