

1 RESEARCH ARTICLE

2 Antimicrobial, antioxidant and cytotoxic activities of
3 *Garcinia eugenifolia* and *Calophyllum enervosum*

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8 ABSTRACT

9 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
11 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-
14 picrylhydrazyl (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,
17 26.82, 26.82 and 60.97, 30.48, 60.97, 60.97 μ M, respectively. Rubraxanthone and epicatechin exhibited
18 antioxidant activities with IC₅₀ of 0.89 M and 2.6 μ M, 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
20 1.07 μ M.

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

22 Secondary metabolites produced by plants constitute
23 a source of bioactive substances. Nowadays the scien-
24 tific interest on these metabolites has increased due to
25 the search for new drugs from plant origin. Plants of the
26 family Guttiferae are known to be very good sources of
27 such compounds [1].

28 Guttiferae in Malaya includes some well known and
29 important trees. There are four genera of Guttiferae such
30 as *Calophyllum*, *Garcinia*, *Mammea* and *Mesua*. The
31 genus *Calophyllum* (*bintangor*) is considerable as im-
32 portant timber and may reach the largest size. *Garcinia*
33 (*manggis*, *kandis*) is primarily a genus of fruit tree and
34 is frequent as a small wild forest tree [2].

35 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 occurred widely in temperate region. The
39 majority of these plants are trees or shrubs and some of
40 them yield useful timber. Some of these genera and spe-
41 cies are found to be endemic to certain land masses, e.g.
42 *Kielmeyera* is confined to the South Africa continent,
43 *Symphonia* and *Pentadesma* are confined to Africa.

44 Many chemical constituents have been studied from
45 Guttiferaceous plants. The compounds, especially xan-
46 thones are usually found in this family. These results are

47 considerable chemotaxonomic interest. In the review by
48 Sultanbawa [3], 95 xanthenes from Guttiferae have been
49 listed. Since then there has been a steady stream of re-
50 ports in which more than 80 new xanthenes which have
51 been characterized and many known xanthenes re-
52 isolated from ca. 60 species of Guttiferae [4].

53 In this paper, we wish to report the bioactivities of
54 enervosanone (1) cambogin (2), epicatechin (3) and
55 osajaxanthone (4) isolated from *G. eugenifolia* and ru-
56 braxanthone (5) and isocowanol (6) purified from *C.*
57 *enervosum*. Their activities were tested for antimicro-
58 bial, antioxidant and cytotoxicity. Anti platelet activat-
59 ing factor activity of two xanthenes is also reported.

60 MATERIAL AND METHODS

61 General experimental procedures

62 Melting points were determined by using a Leica
63 Gallen III apparatus and were uncorrected. UV spectra
64 were measured on UV-100PC Shimadzu using methanol
65 solution. IR spectra were measured on a FT-IR Perkin-
66 Elmer 1600 as KBr discs. Optical rotations were meas-
67 ured by a Polarimeter Type AA-5. ¹H NMR (400 MHz)
68 and ¹³C NMR (100 MHz) spectra were recorded on a

Table 1. Antibacterial activity of compounds 1-6

Micro-organisms	Inhibition zone in mm (MIC value in $\mu\text{g/mL}$)						standard
	1	2	3	4	5	6	
<i>B. subtilis</i>	12 (0.0125)	8 (0.05)	-	-	9(0.025)	-	-
<i>E. coli</i>	9.5 (0.0125)	-	-	na	9(0.0125)	-	21 (3.9x10 ⁻⁴)
<i>P. aeruginosa</i>	11 (0.0125)	8 (0.05)	-	-	8(0.025)	-	-
<i>S. aureus</i>	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 IC₅₀ of the compounds analyzed by ESR spectrometry

Sample	IC ₅₀	Percent inhibition at 500 $\mu\text{g/mL}$
Epicatechin	2.60 μM	96.5
Rubraxanthone	0.89 M	55.3
Enervosanone	na	42.7
Vitamin C	8.01 μM	97.7
Vitamin E	210.20 μM	95.7

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

Compound	18.2	9.1	4.5	1.8	IC ₅₀ (μM)
		($\mu\text{g/mL}$)			(mean \pm S.D)
Rubraxanthone	76*	56	35	13	18.2 \pm 2.1
Isocowanol	15	11	4	1	227.8 \pm 3.4
Cedrol	75	66	56	47	10.6 \pm 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 FeCl₃ reagent; mp. 206-208 °C,
72 230 mesh and 230–400 mesh). Spots were visualized by 107 lit. 205-206 °C [6].

73 UV (254 and 365 nm), FeCl₃ and *p*-anisaldehyde spray-108 The *n*-hexane-EtOAc (1:1) soluble fraction (3.7 g)
74 ing reagent. Streptomycin sulphate standard was pur-109 from 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

76 Plant materials

77 Stem bark of *C. enervosum* and *G. eugenifolia* were 113 tions 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
79 tinggi, West Sumatra, Indonesia in September 1998. 115 sephadex 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. 118 (silica gel): R_f 0.56 in EtOAc-PE (7:3).

83 Extraction and isolation

84 Extraction of the dried and powdered stem bark of 120 The isolated compounds from the stem bark of *G.*
85 *C. enervosum* and *G. eugenifolia* were extracted using 121 *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* (900 123 timicrobial assay was carried out using disc diffusion
88 g) was extracted by soxhlet apparatus for 18 hours with 124 method [7]. The filter-paper discs were impregnated
89 *n*-hexane (2.5 L), ethyl acetate (2.5 L) and acetone (2.5 125 with 10 μL of methanol solution of each sample (1
90 L) 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 $\mu\text{g/disc}$) was used as positive
92 brown 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%). 129 aerobically at 37 °C for 18 h. The diameter of inhibition

94 The EtOAc extract (25 g) was subjected to column 130 zone around each disc was measured and recorded at the
95 chromatography (60 cm length, 6 cm diameter) on silica 131 end of the incubation period.

96 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)

98 1:2) and EtOAc to give four fractions. The *n*-hexane- 134 DPPH radical scavenging test using electron spin
99 EtOAc (2:1) (9.2 g) soluble fraction was purified by 135 resonance was carried out according to the method de-
100 column chromatography (42 cm length, 3.5 cm diame- 136 scribed by Ohtani et al. [8] with a slightly modification.
101 ter) over silica gel 70-230 mesh (90 g) and eluted with 137 The ethanolic solution of the test sample 100 μL (1
102 *n*-hexane-EtOAc (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), 138 mg/mL) was added to 100 μL of DPPH (25.3 mM) in
103 (3:7), (2:8), and EtOAc. The combined 1-20 results

139 ethanol solution. After shaking vigorously for 10 s, the
140 solution was transferred to a flat cell. The ESR spectra
141 were recorded after 40 s. The condition of ESR spec-
142 trometer were set at room temperature, power 1 mW,
143 magnetic field 336.000 ± 5 mT, field modulation width
144 0.5 mT, sweep time 30 sec and time constant 0.03 s.

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

$$149 \text{ Percent scavenging} = \frac{PH_{DPPH} - PH_{sample}}{PH_{DPPH}} \times 100\%$$

151

153

154 PH = peak height of the third and the fifth line signals of
155 DPPH radical

156 The IC_{50} value was determined as the concentration
157 of each sample required to give 50% of scavenging of
158 DPPH. All test and analyses were run in triplicates.

160

$$162 \text{ Percentage inhibition} = \frac{(Tc - Nc) - (Ts - Ns)}{Tc - Nc} \times 100$$

164 Where:

165 Tc and Ts = total binding of control and sample, re-
166 spectively

167 Nc and Ns = non-specific binding of control and
168 sample respectively

169 Cytotoxicity (MTT assay)

170 Cytotoxicity assay was carried out using the 3-(4, 5-
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
174 buffer, 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 6×10^4
177 cells/mL. After reaching confluent (2×10^5 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-
181 centrations of the sample were made with serial dilu-
182 tion. Tamoxifen was used as positive control. Exper-
183 iment was allowed to proceed for 48 hours at 37 °C in
184 humidified 5% CO₂ 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
194 microplate reader. The 50% reduction in cell number
195 relative to the control or IC_{50} was estimate visually.

Abs. sample

$$196 \text{ Cells number after treatment} = \frac{\text{Abs. sample}}{\text{Abs. DMSO}}$$

Abs. DMSO

197 2×10^5 cells/mL

198 Statistical analysis

199 Statistical analyses were performed using Sigma
200 Plot 8.0. Data is presented as means standard error of
201 triplicate samples.

202 RESULTS

203 Enervosanone (1), cambogin (2), epicatechin (3) and
204 osajaxanthone (4) were extracted from *C. nervosum*
205 using soxhlet extractor and purified by chromatographic
206 methods as reported in Taher et al. [10].

207 Extraction of the air dried and powdered of the stem
208 bark of *Garcinia eugenifolia* by soxhlet apparatus with
209 *n*-hexane, ethyl acetate, and acetone successively, af-
210 firmed the *n*-hexane extract as a brown sticky liquid and
211 the ethyl acetate extract as a brown gummy. The ethyl
212 acetate extract of the stem bark of *G. eugenifolia* was
213 submitted to chromatographic separation to afford ru-
214 braxanthone (5) and isocowanol (6).

215 Several bioactivities were tested to these isolated
216 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 gram-
220 negative (*Pseudomonas aeruginosa* and *Staphylococcus*
221 *aureus*) using the disc diffusion method (data is pre-
222 sented in Table 1). The compounds were also tested for
223 their cytotoxicity against breast cancer cells. Their cy-
224 toticity activities are given in Fig. 1.

225 The antioxidant activities of the compounds were
226 tested against DPPH radical and analyze by electron
227 spin resonance spectroscopy. Their activities are given
228 in Table 2 and Fig. 2.

229 DISCUSSION

230 These compounds were identified using physical,
231 chemical and spectroscopic properties. Enervosanone
232 (1), cambogin (2), epicatechin (3) and osajaxanthone (4)
233 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].

236 The purified and characterized compounds were
237 tested for their antimicrobial, citotoxicity, PAF receptor
238 binding and antioxidative activities. As shown in Table
239 1, enervosanone which was found as a new compound
240 in our previous report [10] exhibited strongest activity
241 against four tested bacteria although it is much less ac-
242 tive than the positive control of streptomycin sulfate
243 (MIC of 535.71 nM). While, cambogin was found to be
244 inactive against *S. aureus* and osajaxanthone was found
245 to be inactive against all tested bacteria.

246 In the cytotoxicity assay, enervosanone showed a
247 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_{50} of 1.07 µM.
251 However, IC_{50} value of evervosanone was greater than
252 the positive control, tamoxifen which had the IC_{50} 0.76

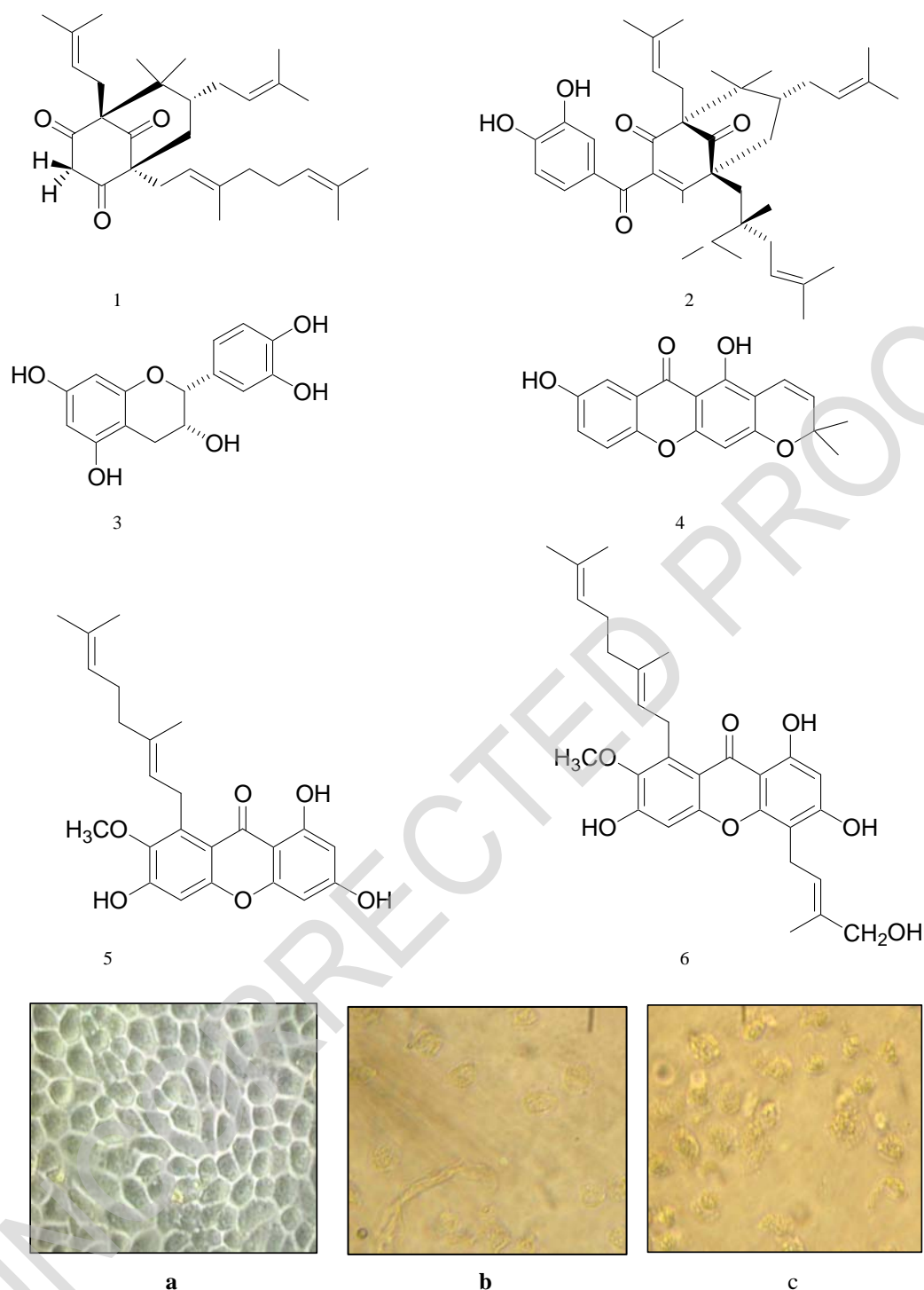


Fig. 1. Morphology of human breast cancer (MCF7). (a) Confluent cells (untreated), (b) After 48 hour treatment with enervosanone (IC_{50} 1.07 μ M), (c) After 48 h treatment with μ M tamoxifen (IC_{50} 0.76 μ M). Different concentrations of the sample were made with serial dilution. Tamoxifen was used as positive control. Experiment was allowed to proceed for 48 hours at 37 °C in humidified 5% CO_2 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 μ 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 μ 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.

253 μ M (SD \pm 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 (cosmeceuticals) 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

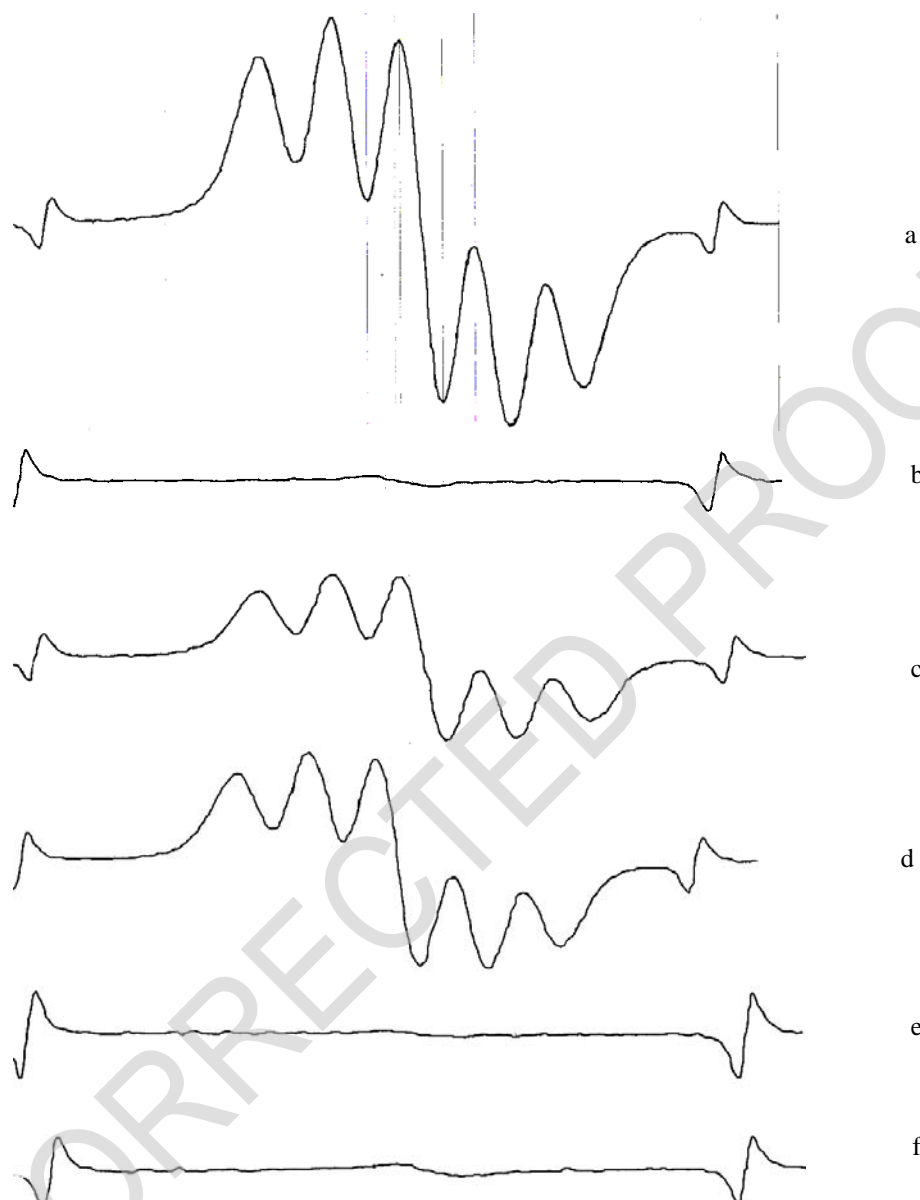


Fig. 2. Scavenging activity of, (b) epicatechin + DPPH 25.3 mM (percent inhibition: 96.5 %), (c) rubraxanthone + DPPH 25.3 mM (percent inhibition: 55.3%), (d) enervosanone + DPPH 25.3 mM (percent inhibition: 42.7 %), (e) Vitamin C + DPPH 25.3 mM (percent inhibition: 97.7%), (f) Vitamin E + DPPH 25.3 mM (percent inhibition 95.7%) against (a) DPPH (25.3 mM) free radical by ESR spectrometry. The ethanolic solution of the test sample 100 μ L (1 mg/mL) was added to 100 μ 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.

measured by using ESR system [11]. In determining others tested samples, enervosanone and rubraxanthone antioxidant activity of the compounds, Electron Spin showed no significant antioxidant activity (Fig. 2). Resonance method was applied. The results showed that Inhibitory effects of rubraxanthone and isocowanol epicatechin exhibited the best antioxidant property on platelet-activating factors (PAF) binding to rabbit platelet was reported in Jantan et al. [12]. The IC_{50} of compounds at various concentrations are given in Table 3. These compounds showed dose-dependent responses, i.e., as the concentration of the compound increased the activity of the standard, vitamin C and Vitamin D with % inhibition increased. The results revealed the structure-activity relationship between rubraxanthone and isocowanol on the PAF receptor binding. Compound

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

292 As the conclusion, the different types of chemicals
293 isolated from *Garcinia eugenifolia* and *Calophyllum*
294 *enervosum* exhibited different activities. The most inter-
295 esting compounds were enervosanone, rubraxanthone
296 and epicatechin that exhibited the antimicrobial, inhibi-
297 tory effects on platelet-activating factors binding and
298 antioxidative activities, respectively.

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