

## 1 RESEARCH ARTICLE

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

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6 Received February 23, 2007; Revised May 2, 2007; Accepted June 26, 2007

7 This paper is available online at <http://ijpt.iums.ac.ir>

## 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  $\mu$ M, respectively. Rubraxanthone and epicatechin exhibited  
 18 antioxidant activities with IC<sub>50</sub> of 0.89 M and 2.6  $\mu$ 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 IC<sub>50</sub> of  
 20 1.07  $\mu$ M.

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

22 Secondary metabolites produced by plants constitute 47 considerable chemotaxonomic interest. In the review by  
 23 a source of bioactive substances. Nowadays the scien- 48 Sultanbawa [3], 95 xanthonenes 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 xanthonenes which have  
 26 family Guttiferae are known to be very good sources of 51 been characterized and many known xanthonenes re-  
 27 such compounds [1]. 52 isolated from ca. 60 species of Guttiferae [4].

28 Guttiferae in Malaya includes some well known and 53 In this paper, we wish to report the bioactivities of  
 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 55 osajaxanthone (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-  
 33 (*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]. 59 ing factor activity of two xanthonenes is also reported.

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

## 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. <sup>1</sup>H NMR (400 MHz)  
 68 and <sup>13</sup>C 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 <sup>-4</sup> )
<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 IC<sub>50</sub> of the compounds analyzed by ESR spectrometry

Sample	IC <sub>50</sub>	Percent inhibition at 500 $\mu\text{g/mL}$
Epicatechin	2.60 $\mu\text{M}$	96.5
Rubraxanthone	0.89 M	55.3
Enervosanone	na	42.7
Vitamin C	8.01 $\mu\text{M}$	97.7
Vitamin E	210.20 $\mu\text{M}$	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 <sub>50</sub> ( $\mu\text{M}$ )
		( $\mu\text{g/mL}$ )			(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  $\text{FeCl}_3$  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),  $\text{FeCl}_3$  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  $\text{FeCl}_3$  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  $\mu\text{L}$  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)

99 EtOAc (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-  
101 ter) over silica gel 70-230 mesh (90 g) and eluted with 136 scribed by Ohtani et al. [8] with a slightly modification.  
102 *n*-hexane-EtOAc (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), 137 The ethanolic solution of the test sample 100  $\mu\text{L}$  (1  
103 (3:7), (2:8), and EtOAc. The combined 1-20 results 138 mg/mL) was added to 100  $\mu\text{L}$  of DPPH (25.3 mM) in

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  $336.000 \pm 5$  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  $6 \times 10^4$   
177 cells/mL. After reaching confluent ( $2 \times 10^5$  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  $37^\circ\text{C}$  in  
184 humidified 5%  $\text{CO}_2$  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  $\mu\text{l}$  of MTT stock solution (5 mg/ml)  
189 was added to each well and the plates were further incu-  
190 bated overnight at  $37^\circ\text{C}$ . Dimethyl sulfoxide (100  $\mu\text{L}$ )  
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  $2 \times 10^5$  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  $\mu\text{M}$ .  
251 However,  $IC_{50}$  value of evervosanone was greater than  
252 the positive control, tamoxifen which had the  $IC_{50}$  0.76

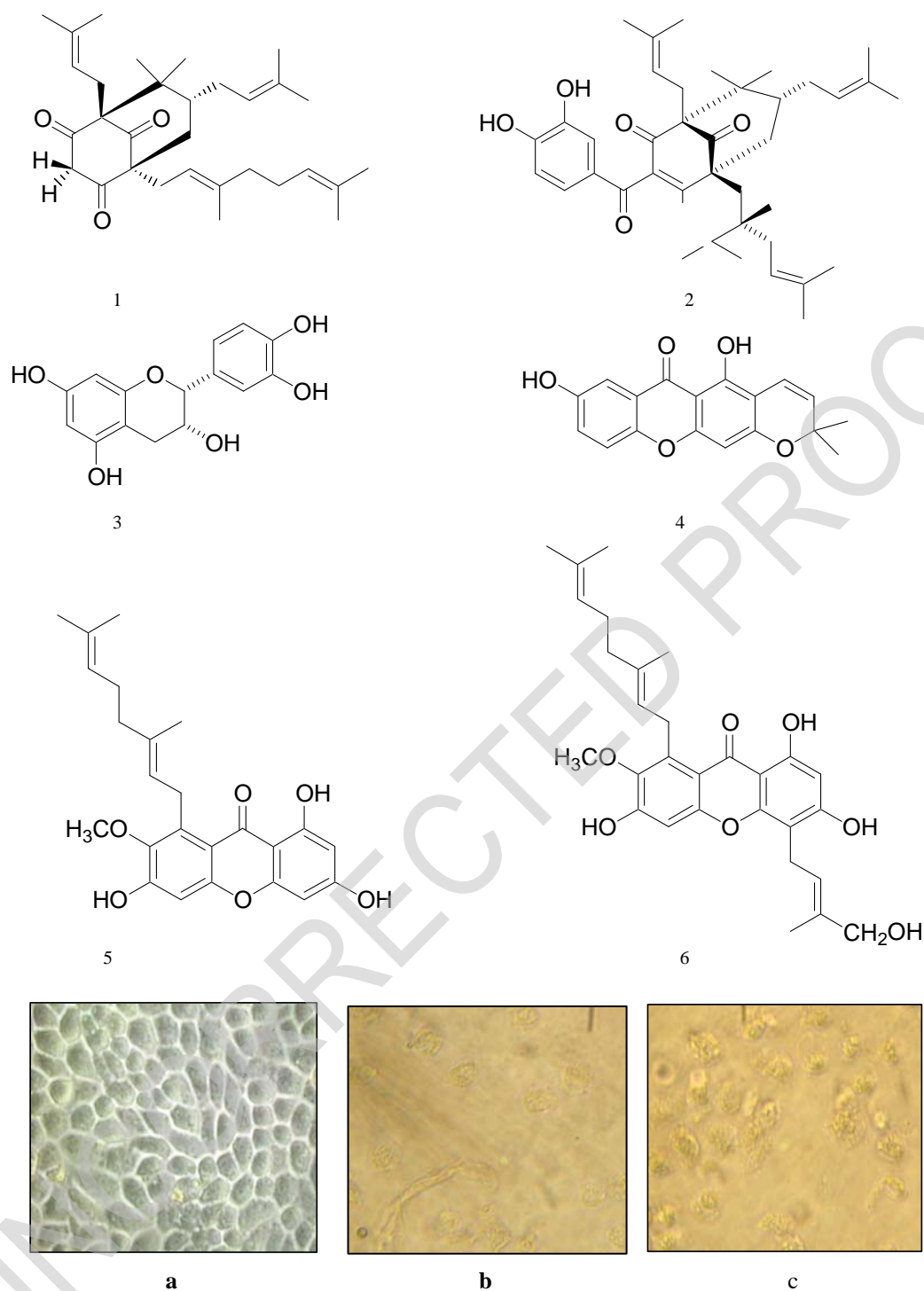


Fig. 1. Morphology of human breast cancer (MCF7). (a) Confluent cells (untreated), (b) After 48 hour treatment with enervosanone ( $IC_{50}$  1.07  $\mu$ M), (c) After 48 h treatment with  $\mu$ M tamoxifen ( $IC_{50}$  0.76  $\mu$ M). Different concentrations of the sample were made with serial dilution.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

253  $\mu$ 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

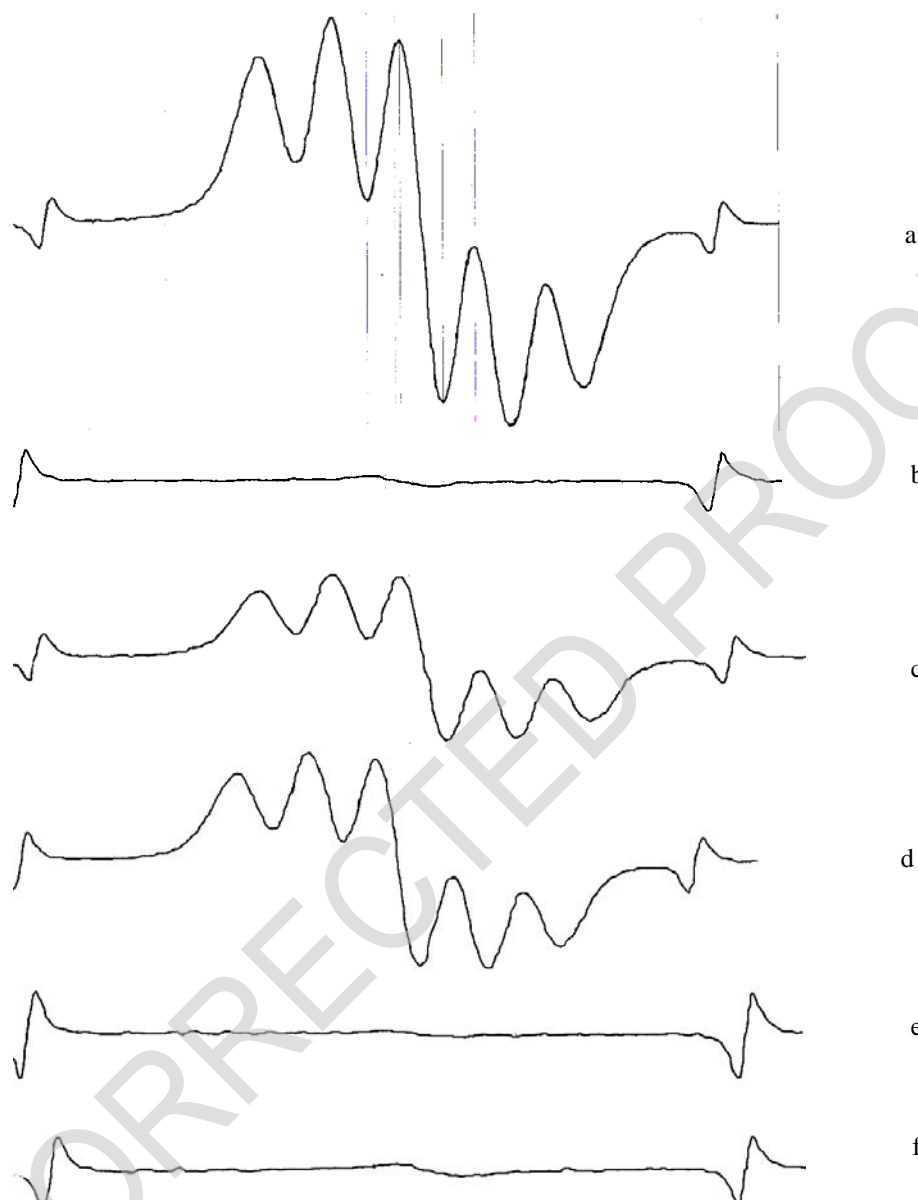


Fig. 2. Scavenging activity of, (b) epicatechin + DPPH 25.3 mM (percent inhibition: 96.5 %), (c) rubraxanthone + DPPH 25.3 mM (percent inhibition: 55.3%), (d) enervosanone + DPPH 25.3 mM (percent inhibition: 42.7 %), (e) Vitamin C + DPPH 25.3 mM (percent inhibition: 97.7%), (f) Vitamin E + DPPH 25.3 mM (percent inhibition 95.7%) against (a) DPPH (25.3 mM) free radical by ESR spectrometry. The ethanolic solution of the test sample 100  $\mu$ 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.

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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