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RESEARCH ARTICLE

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

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ABSTRACT

Chemical investigation of *Garcinia eugenifolia* and *Calophyllum enervosum* yielded six compounds. One of these was found to be a novel compound named as enervosanone. Five known compound such as cambogin, epicatechin, osajaxanthone, rubraxanthone and isocowanol were also isolated. These compounds were tested for their bioactivity as antimicrobial, antioxidant and cytotoxicity. Antimicrobial assay was performed using disc diffusion method. The antioxidative activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method by electron spin resonance. The cytotoxicity was measured by the MTT assay against MCF7 cell line. Enervosanone and rubraxanthone were active against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* with MIC value of 26.82, 26.82, 26.82, 26.82 and 60.97, 30.48, 60.97, 60.97 μM, respectively. Rubraxanthone and epicatechin exhibited antioxidant activities with IC_{50} of 0.89 M and 2.6 μM, respectively. The cytotoxicity assay on MCF7 cell line showed that enervosanone was found to be active in inhibiting cell proliferation of MCF7 with IC_{50} of 1.07 μM.

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

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

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

According to Sultanbawa [3], and Bennet and Lee [44], the Guttiferae family contains over 1000 species, mainly restricted to the tropics except to the genus *Hypericum* which occured widely in temperate region. The majority of these plants are trees or shrubs and some of them yield useful timber. Some of these genera and species are found to be endemic to certain land masses, e.g. *Kielmeyera* is confined to the South Africa continent, *Symphonia* and *Pentadesma* are confined to Africa.

Many chemical constituents have been studied from Guttiferaceous plants. The compounds, especially xanthones are usually found in this family. These results are considerable chemotaxanomic interest. In the review by Sultanbawa [3], 95 xanthones from Guttiferae have been listed. Since then there has been a steady stream of reports in which more than 80 new xanthones which have been characterized and many known xanthones reisolated from ca. 60 species of Guttiferae [4].

In this paper, we wish to report the bioactivities of enervosanone (1) cambogin (2), epicatechin (3) and osajaxanthone (4) isolated from *G. eugenifolia* and rubraxanthone (5) and isocowanol (6) purified from *C. enervosum*. Their activities were tested for antimicrobial, antioxidant and cytotoxicity. Anti platelet activating factor activity of two xanthones is also reported.

MATERIAL AND METHODS

General experimental procedures

Melting points were determined by using a Leica Gallen III apparatus and were uncorrected. UV spectra were measured on UV-100PC Shimadzu using methanol solution. IR spectra were measured on a FT-IR Perkin–Elmer 1600 as KBr discs. Optical rotations were measured by a Polarimeter Type AA-5. ^{1}H NMR (400 MHz) and ^{13}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 μg/mL)						
	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 ($3.9x10^{-4}$)
P. aeruginosa	11 (0.0125)	8 (0.05)	-		8(0.025)	-	
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_{50} of the compounds analyzed by ESR spectrometry

Sample	IC_{50}	Percent inhibition at 500 μg/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 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} (μM) (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 ± SD of three independent experiments performed in triplicate; *p<0.05 as compared with cedrol (Student's test).

Varian Unity INOVA spectrophotometer, using TMS as internal standard. EIMS were recorded on a Varian mass spectrometer at 70 eV. CC: silica gel (Merck 70–230 mesh and 230–400 mesh). Spots were visualized by UV (254 and 365 nm), $FeCl_3$ and *p*-anisaldehyde spraying reagent. Streptomycin sulphate standard was purchased from Oxoid (Hampshire,UK).

Plant materials

Stem bark of *C. enervosum* and *G. eugenifolia* were collected from Tilatang Kamang, 6 km East of Bukittinggi, West Sumatra, Indonesia in September 1998. The voucher specimen, MT-03 and MT-04, respectively are deposited at the Herbarium of Universitas Andalas (ANDA), Padang, Indonesia.

Extraction and isolation

Extraction of the dried and powdered stem bark of *C. enervosum* and *G. eugenifolia* were extracted using soxhlet extractor. *C. enervosum* was extracted as previously reported by Taher, et al. [5]. *G. eugenifolia* (900 g) was extracted by soxhlet apparatus for 18 hours with *n*-hexane (2.5 L), ethyl acetate (2.5 L) and acetone (2.5 L) successively. Each solvent was removed *in vacuo* by rotary evaporator. The *n*-hexane afforded the extract as brown sticky liquid (6 g, 0.66%) and the EtOAc extract gave a brown gummy (36.1 g, 4.01%).

The EtOAc extract (25 g) was subjected to column chromatography (60 cm length, 6 cm diameter) on silica gel 70-230 mesh (200 g) and eluted with the gradient solvent system of *n*-hexane-EtOAc (3:1, 2:1, 1:1, and 1:2) and EtOAc to give four fractions. The *n*-hexane-EtOAc (2:1) (9.2 g) soluble fraction was purified by column chromatography (42 cm length, 3.5 cm diameter) over silica gel 70-230 mesh (90 g) and eluted with *n*-hexane-EtOAc (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), (3:7), (2:8), and EtOAc. The combined 1-20 results were crystallized with PE and EtOAc to give rubraxanthone (5.78 g, 0.6425%) as yellow needles which exhibited positive test with $FeCl_3$ reagent; mp. 206-208 °C, lit. 205-206 °C [6].

The *n*-hexane-EtOAc (1:1) soluble fraction (3.7 g) from the EtOAc extract was purified by column chromatography (35 cm length, 2.5 cm diameter) on silica gel 230-400 mesh (60 g) using PE-EtOAc (9:1), (8:2), (7:3), (6:4), (5:5), (4:6), (3:7), (2:8) as eluent gave fractions 1-6 and 7-26. Purification of the combined fractions 7-26 was followed by column chromatography on sephadex LH-20 and eluted with MeOH afforded isocowanol (160 mg, 0.017 %) as yellow needles which reacted positively with $FeCl_3$ reagent; mp. 89-93°; TLC (silica gel): R_f 0.56 in EtOAc-PE (7:3).

Antimicrobial assay (disc diffusion method)

The isolated compounds from the stem bark of *G. eugenifolia* and *C. enervosum* were tested against *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus*. The antimicrobial assay was carried out using disc diffusion method [7]. The filter-paper discs were impregnated with 10 μL of methanol solution of each sample (1 mg/mL) and allowed to evaporate at room temperature. Streptomycin sulfate (30 μg/disc) was used as positive control. The discs were then incubated on the plate aerobically at 37 °C for 18 h. The diameter of inhibition zone around each disc was measured and recorded at the end of the incubation period.

Antioxidant assay (electron spin resonance (ESR) spectroscopy method)

DPPH radical scavenging test using electron spin resonance was carried out according to the method described by Ohtani et al. [8] with a slightly modification. The ethanolic solution of the test sample 100 μL (1 mg/mL) was added to 100 μL 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. The condition of ESR spectrometer were set at room temperature, power 1 mW, magnetic field 336.000 ± 5 mT, field modulation width 0.5 mT, sweep time 30 sec and time constant 0.03 s. The scavenging effect of DPPH was calculated by following formula:

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

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

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

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

Where:

Tc and Ts = total binding of control and sample, respectively

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

Cytotoxicity (MTT assay)

Cytotoxicity assay was carried out using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Mosmann [9]. MCF7 cell line was cultured in DMEM/F-12 with 15 mM HEPES buffer, L-glutamine and pyridoxine hydrochloride supplemented with 10% fetal bovine serum and 1% penicillin- streptomycin in 96-well plate at density of $6x10^4$ cells/mL. After reaching confluent ($2x10^5$ cells/ mL), the cells were treated with the sample. The sample was dissolved with dimethylsulfoxide (DMSO) at the final concentration of DMSO was 0.1% (v/v). 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 this period, 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 µl 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 µL) 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. The 50% reduction in cell number relative to the control or IC_{50} was estimate visually.

$$\text{Cells number after treatment} = \frac{Abs.\ sample}{Abs.\ DMSO} \times 2x10^5 \text{ cells/mL}$$

Statistical analysis

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

RESULTS

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

Extraction of the air dried and powdered of the stem bark of *Garcinia eugenifolia* by soxhlet apparatus with *n*-hexane, ethyl acetate, and acetone successively, afforded the *n*-hexane extract as a brown sticky liquid and the ethyl acetate extract as a brown gummy. The ethyl acetate extract of the stem bark of *G. eugenifolia* was submitted to chromatographic separation to afford rubraxanthone (5) and isocowanol (6).

Several bioactivities were tested to these isolated compounds such antimicrobial, antioxidant, anti platelet activating factor and cytotoxicity. Antimicrobial assay was tested against two strain bacteria, gram-positive (*Bacillus subtilis* and *Escherecia coli*) and gram-negative (*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 spin resonance spectroscopy. Their activities are given in 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) were identified as reported in Taher et al., [5]. Rubraxanthone (5) and isocowanol (6) were elucidated by comparing data with literature [6].

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

In the cytotoxicity assay, enervosanone showed a significant cytotoxic effect against MCF7 by affecting cell proliferation and changing the cell morphology (Fig. 1). Enervosanone was found to be active in inhibiting cell proliferation of MCF7 with IC_{50} of 1.07 µM. However, IC_{50} value of evervosanone was greater than the positive control, tamoxifen which had the IC_{50} 0.76

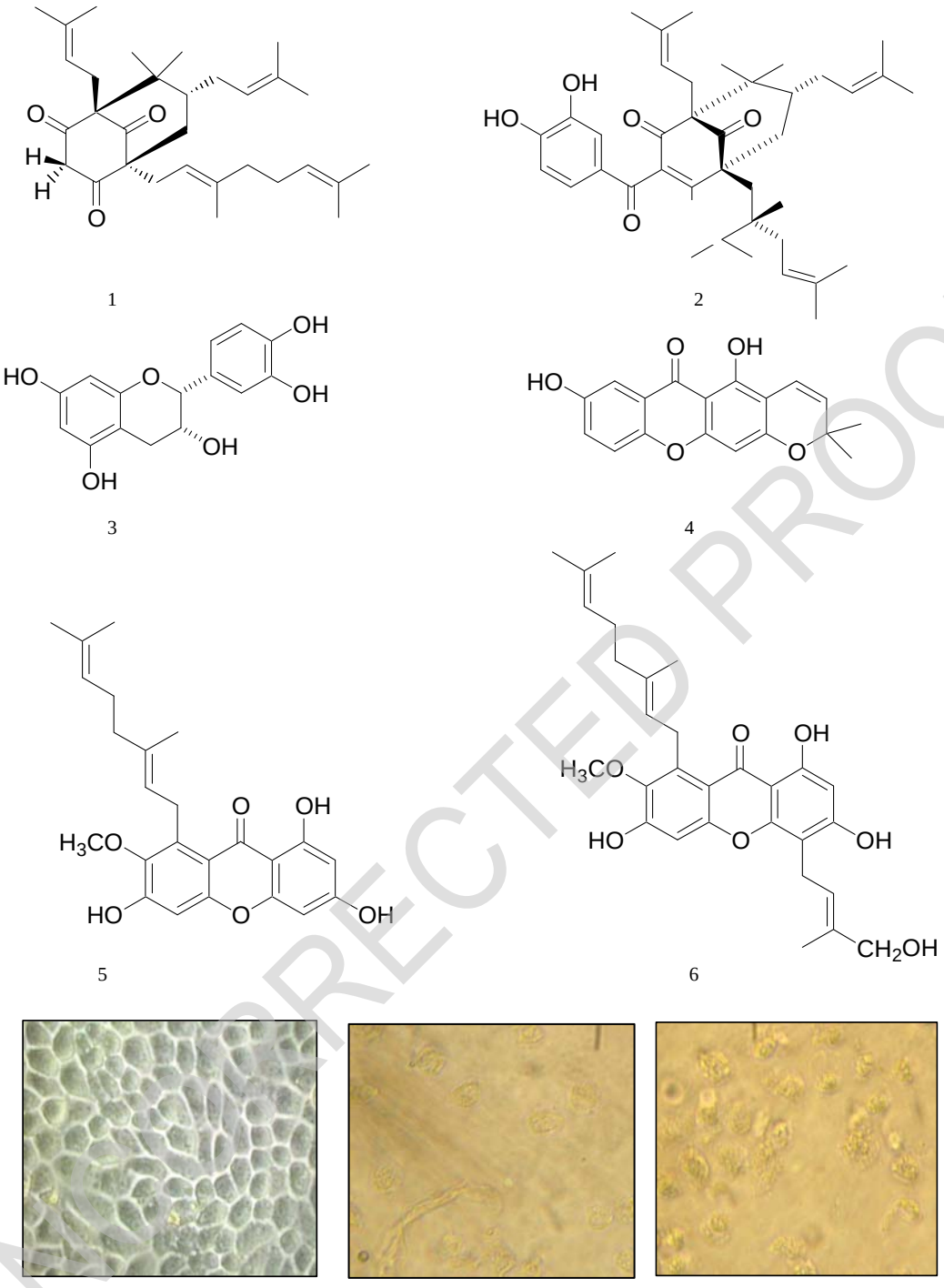

Fig. 1. Morphology of human breast cancer (MCF7). (a) Confluent cells (untreated), (b) After 48 hour treatment with enervosanone (IC_{50} 1.07 µM), (c) After 48 h treatment with µM tamoxifen (IC_{50} 0.76 µM). Different concentrations of the sample were made with serial dilution. Tamoxifen was used as 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 µl 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 µL) 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.

µM (SD ± 0.005). Hence, it is indicated that enervosanone was less active than the control of tamoxifen.

Based on the cell morphology, it was proposed that enervosanone active against human breast cell cancer by inhibiting the cell proliferation. However, the mechanism of action of enervosanone is still not understood.

There is now increasing interest in the antioxidant activity of phytochemical present in the diet, in health food supplement (nutraceutical), and in topical preparations of cosmetic (cosmaceuticals) from environmental exposure. A simple and rapid estimation of hydroxyl and superoxide anion radical scavenging activities can

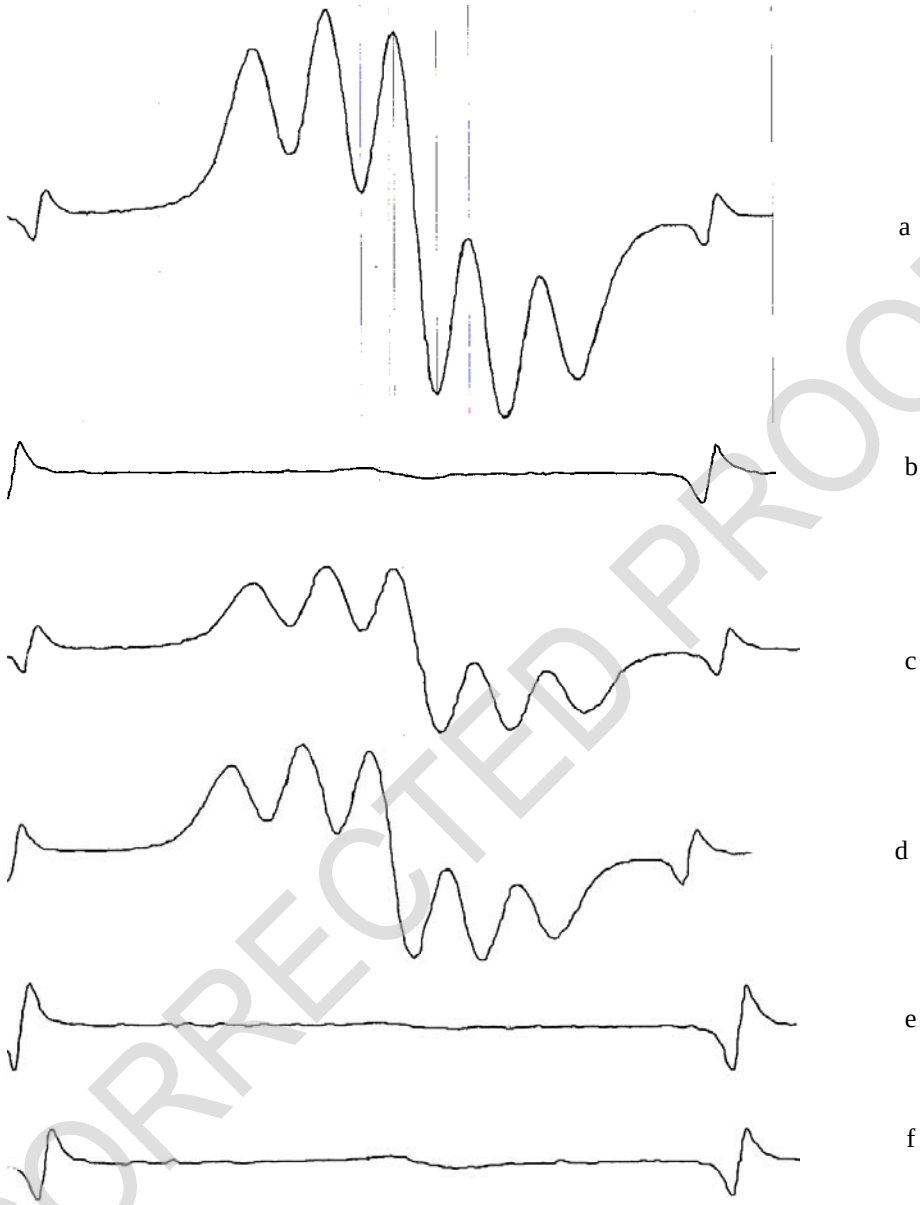

Fig. 2. Scavenging activity of, (b) epicathechin + DPPH 25.3 mM (percent inhibition: 96.5 %), (c) rubraxanthone + DPPH 25.3 mM (percent inhibition: 55.3%), (d) enervosanone + DPPH 25.3 mM (percent inhibition: 42.7 %), (e) Vitamin C + DPPH 25.3 mM (percent inhibition: 97.7%), (f) Vitamin E + DPPH 25.3 mM (percent inhibition 95.7%) against (a) DPPH (25.3 mM) free radical by ESR spectrometry. The ethanolic solution of the test sample 100 µL (1 mg/mL) was added to 100 µL 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 antioxidant activity of the compounds, Electron Spin Resonance method was applied. The results showed that epicatechin exhibited the best antioxidant property against DPPH radicals with IC_{50} of 2.60 µM and percent inhibition of 96.5 at concentration of 500 µg/mL (Table 2). Its activity was almost similar to the antioxidant activity of the standard, vitamin C and Vitamin D with IC_{50} of 8.01 and 210.20 µM, respectively (percent inhibition of 97.7 and 95.7 at 500 µg/mL, respectively). The others tested samples, enervosanone and rubraxanthone showed no significant antioxidant activity (Fig. 2).

Inhibitory effects of rubraxanthone and isocowanol 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 % inhibition increased. The results revealed the structure-activity relationship between rubraxanthone and isocowanol on the PAF receptor binding. Compound

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

As the conclusion, the different types of chemicals isolated from *Garcinia eugenifolia* and *Calophyllum enervosum* exhibited different activities. The most interesting compounds were enervosanone, rubraxanthone and epicatechin that exhibited the antimicrobial, inhibitory effects on platelet-activating factors binding and antioxidative activities, respectively.

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