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Isolation and Identification of Active Compound of n-hexane Fraction from Kasturi (*Mangifera casturi* Konsterm.) against Antioxidant and Immunomodulatory Activity

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Abstract: A research had been conducted to isolated, to identify and to test biological activity of active isolates from n-hexane extracts of casturi (*Mangifera casturi* Konsterm.) fruit. The research aims at isolating active isolates from n-hexane fractions and testing for antioxidant and immunomodulatory activities. n-hexane fractions were isolated using column chromatographic method by using n-hexane-ethylacetate as eluents with eluent gradient (20:1; 15:1; 10:1; 8:2; 7:3; 6:4 and 5:5) v/v. The isolated compounds (purified isolates) were white crystals identified as terpenoid compound. Identification of chemical structures found a compound, namely, 1-isopropenyl-3a, 5a, 5b, 8, 8, 11a-hexamethyl-eicosahydro-cyclopenta [α]chrysen-9-ol (lupeol). The isolated compound was tested for antioxidant activity using DPPH (1,1-diphenyl -2-picrylhydrazyl) method with an IC₅₀ value of 14384.71-lower than that of quercetin control (IC₅₀ = 2.962). The finding shows that the isolate is not a potent compound against DPPH free radical inhibition. *In vitro* immunomodulatory activity test was conducted for the isolated compound. The isolates were given at dosages of 6.25, 12.5, 25, 50 and 100 μ g. The test revealed increasing macrophage activity against latex bead phagocytosis. The highest phagocytosis activity and capacity were found at a dosage of 100 μ g, namely, latex-bead consuming macrophage by 26% and macrophage-eaten latex bead by 32% (significant, compared to the negative control, namely, latex-bead eating macrophage by 13% and macrophage-eaten latex bead by 15%).

Key words: *Mangifera casturi* Konsterm, n-hexane fraction, lupeol, antioxidant, immunomodulatory

INTRODUCTION

No research had been conducted to isolate and to identify active compounds of *Mangifera casturi* Konsterm fruit, even though some reports suggested that casturi fruit juice had an antioxidant activity (Edyson and Yasmina, 2008). *M. Casturi* is a plant of genus *Mangifera*, Anacardiaceae family which has more than 51 species. Isolation and identification of compound in the genus *Mangifera* had been reported by Anjaneyulu *et al.* (1999), suggesting two terpenoid compounds isolated from the neutral fractions of *Mangifera indica* L. bark, namely 25(R)-3-oxo-24-methylene cycloarthan-26-ol and taraxastanonol. Compounds of terpenoid group had also been found in some high-grade plants and found to have biological activities. Some terpenoid compound in the plant serves as a basic shaper of antiseptic, expectorant, gastrointestinal agent, pain reliever, immunostimulant and antioxidant. The study aims at isolating, identifying and

characterizing compound isolated from *M. casturi* fruit. n-hexane fraction from methanolic extract of *M. Casturi* was isolated using column chromatograph at solvent gradients of: n-hexane-ethylacetate (20:1; 15:1; 10:1; 8:2; 7:3; 6:4 and 5:5) v/v. The isolated compound was white crystals which were purified with recrystallization method. The compound identification was conducted using FT-IR, ¹HNMR, ¹³CNMR, COSY, HMBC, HMQC and LCMS spectroscopy. Based on the analysis, it can be concluded that the isolated compound was 1-isopropenyl-3a, 5a, 5b, 8, 8, 11a-hexamethyl-eicosahydro-cyclopenta[α]chrysen-9-ol. The isolated compound tested with DPPH method was found to have a very low antioxidant activity (IC₅₀ = 14384, 71). *In vitro* immunomodulatory activity test showed that at dosages of 6.25, 12.5, 25, 50 and 100 μ g, the compound could increase % average of macrophage phagocytosis of latex beads by 21, 21, 22, 23 and 26%, respectively and % average latex beads phagocytized by macrophage of 25, 25, 27, 28 and 32%, respectively.

MATERIALS AND METHODS

Plant materials: *M. casturi* fruits were collected from Banjar District, Southern Borneo, Indonesia. The sample of ripe fruits of *M. casturi* was collected from December 2010-January 2011. Plant identification was conducted at Pharmaceutical Biology Laboratory of Gadjah Mada University, Yogyakarta. Parts of the fruit used in the research were the fruit flesh and fruit bark (isolated from the seed), desiccated under 50°C for 3 days, made into powder and then weighed and extracted.

Extraction, fractionation and isolation: One kilogram of sample powder of *M. casturi* fruit was extracted in maceration using methanol pa. as solvent. Fractionation was conducted with liquid-liquid extraction method by weighing 100 g of thick methanolic extracts and then extracting the powder in 150 mL of n-hexane solvent. The extraction was repeated seven times and the n-hexane soluble compound was collected, then evaporated to get thick extract as n-hexane fraction. The resulting n-hexane fraction was 5, 3 g, dark green in color and stored in a refrigerator (10-15°C).

Thin-Layer Chromatography (TLC) test was conducted on the n-hexane fraction using immobile phase of silica gel 60 F₂₅₄ and mobile phase of n-hexane-ethylacetate (8:2) v/v. The compound to isolate was monitored through a chromatogram sprayed with a specific reactant. The reactant used was 0.4 nM of DPPH to monitor antioxidant compounds and cerium sulfate for general compounds. Spots indicated as antioxidant were isolated with column chromatograph using a solvent gradient of n-hexane-ethylacetate (20:1; 15:1; 10:1; 8:2; 7:3; 6:4 and 5:5) v/v. Isolation using column chromatography yielded isolates, namely methanol and chloroform soluble white crystals. Crystals that adhered to the vial were purified by re-crystallization and by washing the crystals in n-hexane solvent.

Steroid compound test

Salkowski reaction: Crystals resulting from the isolation were dissolved in chloroform and some drops of thick sulfuric acid were added through the tube wall. Reddish steroid compounds would be seen on the upper chloroform layer.

Liebermann-burchardt reaction: Crystals resulting from isolation were dissolved in chloroform and some drops of thick sulfuric acid were added through the tube wall. The mixture was then added with 2-3 drops of anhydride acetate to change the color into purple-blue and finally into green (Harborne, 1998).

Structure analysis with spectroscopy: Structure analysis conducted on the isolated pure compounds using spectroscopic data Infrared spectroscopy (FTIR, Perkin Elmer 100), Liquid chromatography mass spectroscopy (LCMS, Mariner biospectrometry Hitachi L 6200), 1 D and 2 D nuclear magnetic resonantion (NMR, JEOL 500 MHZ): hydrogen nuclear magnetic resonantion spectroscopy (¹H-NMR), carbon nuclear magnetic resonantion spectroscopy (¹³C-NMR), Distortionless Enhancement by Polarization Transfer (DEPT), correlation spectroscopy (COSY), Heteronuclear Multiple Bond Correlation (HMBC) and Heteronuclear Multiple Bond Correlation (HMBC) from the Indonesian institute of Science (LIPI). Spectra were analyzed to determine the chemical structure of the isolated compounds.

Antioxidant activity test: Antioxidant activity test on the isolated compound was conducted with DPPH (1, 1-diphenyl-2-picrilhydrazyl) method. DPPH 0.4 mM solution in methanol pa used for the test must always be fresh. DPPH reactant was prepared by dissolving 15.7 mg in 100 mL of methanol pa. The isolated compound was dissolved in methanol pa, prepared at concentrations of (0.5; 1.0; 2.0; 4.0; 8.0; 16.0; 32) µg mL⁻¹ and then reacted in 1 mL of DPPH solution. Enough volume was obtained by adding up to 5 mL of methanol. The mixture was homogenated with vortex, let under room temperature for 20 min and calculated for absorbance with a spectrophotometer at a wavelength of 517 nm. Quercetin was used as a positive control. Antioxidant activity of the sample was presented in the reduction ratio of DPPH absorbance (%) that is calculated using the formula:

$$\text{Reduction ratio of DPPH absorbance} = \frac{\text{Abs. Blanco} - \text{Abs. sample}}{\text{Abs. Blanco}} \times 100$$

In vitro immunomodulatory activity test

Macrophage cell isolation: The test animals (mice) were anesthetized using chloroform before dissection. The mice were put in supine position, the abdominal skin were exposed and cleaned from peritoneal sheath using 70% alcohol. The mice were injected with 10 mL of cold RPMI down to the peritoneal cavity and let for 3 min while the mice were being rolled gradually. The peritoneal liquid was removed from the peritoneal cavity and aspired using an injection needle. The aspirates were put into an ice-filled Becker glass; the suspension was then put into a centrifugation tube. The aspirates were centrifuged at 1200 rpm under a temperature of 4°C for 10 min. The supernatant was removed and then 3 mL of complete medium was added into the resultant pellets. The number of cells was calculated using a hemocytometer, then

re-suspended in the complete medium to get cell suspension at a concentration of 2.5×10^6 mL⁻¹. The calculated cell suspension was cultured in a 24-well plate filled with round cover slips; each well contained 200 μ L (5×10^5 cells). The cells were incubated in 37°C, 5% of CO₂ for 30 min and then 1 mL well⁻¹ of complete medium was added and re-incubated for 2 h. The cells were washed twice using RPMI, then 1 mL well⁻¹ of complete medium was added and incubation continued up to 24 h.

Macrophage phagocytosis test with latex beads: Latex beads with a diameter of 3 μ m (Sigma chem.Co.) were suspended in BPS at a concentration of 2.2×10^7 mL⁻¹. The isolates were suspended using 400 μ L of RPMI as a medium at some concentrations: 6.25, 12.5, 25.0, 50.0 and 100 μ g mL⁻¹. A solvent medium of DMSO 0.0025% was used as a negative control. Peritoneal macrophages that were cultured one day before were washed twice with RPMI, then added with the sample in RPMI medium 400 μ L well⁻¹, incubated for 60 minutes under a temperature of 37°C, 5% of CO₂. The RPMI suspension was cleaned, added with latex suspensions 200 μ L well⁻¹ and incubated for 240 min under a temperature of 37°C, 5% of CO₂. Then, the cells were washed 3 times with PBS to remove unphagocytized particles and then dried under room temperature before fixation with absolute methanol. When dried, the cover slips were polished with Giemsa 20% b/v for 30 min, washed with aquadest and removed from the culture wells and dried under a room temperature. Percent cells that phagocytized the latex beads were calculated from 100 cells examined under a light microscope. The treatment was repeated 4 times (Leijh *et al.*, 1986).

Statistical analysis: Determining IC₅₀ value through antioxidant test was conducted with linear regression, followed by one-way analysis of variance (ANOVA) version 16.0 and tukey analysis at a Confidence Interval (CI) of 95% ($p < 0.05$) to determine the significance between the test sample and positive control.

Data of immunomodulatory potential were analyzed based on the increase in cellular immune response, characterized by the increasing macrophage phagocytosis of the cells, compared to the control solvent. Data of the experiment were analyzed using one-way analysis of variance (ANOVA) versi 16.0 and tukey analysis at a Confidence Interval of 95% ($p < 0.05$).

RESULTS AND DISCUSSION

Phytochemical identification test on the isolated compound showed that n-hexane fraction of *M. casturi*

fruit had a compound of steroid group. The resultant isolates were white crystals which are characterized for chemical structure using FTIR, ¹HNMR, ¹³CNMR, DEPT, COSY, HMQC, HMBC and LCMS spectroscopy, with the following results.

FTIR (KBr): Vmax absorption 3338 cm⁻¹ indicated a characteristic hydroxyl group (-OH). Absorbance of 2921 and 2853 cm⁻¹ indicated aliphatic C-H and vibration of methyl group, an absorbance of 1639 cm⁻¹ indicated isolated C = C group (weak), an absorbance of 1379 cm⁻¹ indicated a dimethyl group (Silverstein and Webster, 2000).

¹HNMR (CD₃OD, 500 MHZ): δ 0.69 (1H, d, H-5), δ 0.76 (1H, s, H-24), δ 0.83 (1H, s, H-28), δ 0.86 (1H, s, H-25), δ 0.96 (1H, m, H-1b), δ 1.01 (1H, m, H-15b), 0.96 (1H, s, H-27), δ 0.98 (1H, s, H-23), δ 1.07 (1H, s, H-26), δ 1.20 (1H, m, H-12b), δ 1.43 (1H, m, H-22), δ 1.28 (1H, m, H-21), δ 1.31 (1H, m, H-11b), δ 1.34 (1H, m, H-9), δ 0.96 (1H, m, H-27), δ 1.42 (1H, m, H-6b), δ 1.43 (1H, m, H-18), δ 1.47 (1H, m, H-11a), δ 1.51 (1H, m, H-16), δ 1.56 (1H, m, H-6a), δ 1.60 (1H, m, H-15a), δ 1.63 (1H, m, H-2), δ 1.68 (1H, m, H-13), δ 1.70 (1H, m, H-1a), δ 1.72 (1H, m, H-12a), δ 1.69 (1H, s, H-30), δ 2.40 (1H, m, H-19), δ 3.14 (1H, dd, J=11,0; 5,3 Hz, H-3), δ 4.56 (1H, s, H-29b), δ 4.68 (1H, s, H-29a).

¹³CNMR (CD₃OD, 500 MHZ): δ 15.10 (CH₃, C-27), δ 16.22 (CH₃, C-24), δ 16.66 (CH₃, C-26), δ 16.81 (CH₃, C-25), δ 18.51 (CH₃, C-28), δ 19.52 (CH₂, C-6), δ 19.65 (CH₃, C30), δ 22.12 (CH₂, C-11), δ 26.53 (CH₂, C-12), δ 28.09 (CH₂, C-15), δ 28.66 (CH₂, C-2), δ 28.69 (CH₃, C-23), δ 30.84 (CH₂, C-21), δ 35.57 (CH₂, C-7), δ 35.57 (CH₂, C-16), δ 38.34 (C, C-10), δ 39.56 (CH, C-13), δ 40.02 (C, C-4), δ 40.13 (CH₂, C-1), δ 41.11 (CH₂, C-22), δ 42.10 (C, C-8), δ 44.01 (C, C-14), δ 44.17 (C, C 17), δ 49.3 (CH, C-19), δ 49.5 (CH, C-18), 51.92 (CH, C-9), δ 56.87 (CH, C-5), δ 79.71 (CH, C-3), 110.24 (CH₂, C-29), δ 151.97 (C, C-20).

The results of ¹HNMR analysis displayed two characteristic signals on the lupeol compound, namely, singlet olefinic protons at δ_H 4.56 and δ_H 4.68 (Fig. 1). The ¹³CNMR spectrum displayed 30 carbon atoms and DEPT analysis showed 6 quaternary carbon atoms (C), 6 methin atoms (CH), 11 methylene atoms (CH₂) and 7 methyl (CH₃). There were two signals at δ_C 110,24 and 151.97 ppm, showing 2 olefinic carbons at C-29 and C-20, in which the signals were deshielded at δ_C 151.97 ppm-a quaternary olefinic carbon at C-20. The signals were deshielded at δ_C 79.71 ppm, showing that there was a hydroxyl group at C-3.

The values of correlation between ¹H-¹H NMR (COSY), ¹H-¹³C NMR (HMQC) and ¹H-¹³C NMR (HMBC)

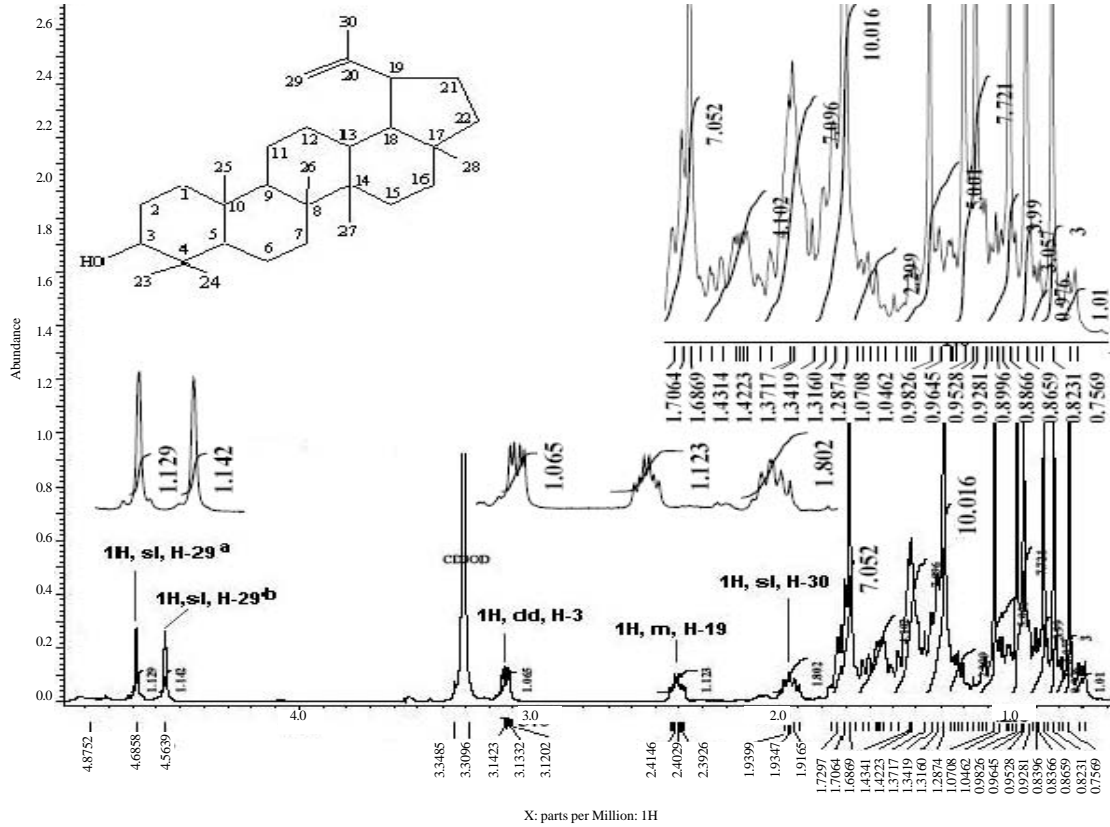


Fig. 1: ¹H-NMR spectrum isolate compound of n-hexane fraction from *M. casturi*

are displayed in Fig. 2-5. HMBC spectral analysis showed a correlation between hydrogen atom and carbon atom. Hydrogen atom at δ_H 1.07 (H-26) was correlated with carbon atom signals at δ_C 35.57 (C-7), δ_C 51.92 (C-9), δ_C 44.01 (C-14) and δ_C 28.66 (C-15). Figure 5 also shows a correlation between hydrogen atom at δ_H 4.68 (H-29a,b) and carbon atom signals at δ_C 19.65 (C-30) and δ_C 49.30 (C-19). Hydrogen atom at δ_H 1.69 (H-30) was correlated with carbon atom signals at δ_C 151.97 (C-20) and δ_C 110.24 (C-29). Hydrogen atom at 0.83 (H-28) was correlated with carbon atom signals at δ_C 36.76 (C-16), δ_C 44.17 (C-17), δ_C 49.50 (C-18) and δ_C 41.11 (C-22). Hydrogen atom at δ_H 0.98 (H-23) was correlated with carbon atom signals at δ_C 40.02 (C-4), δ_C 56.87 (C-5) and δ_C 16.22 (C-24). Hydrogen atom at δ_H 0.75 (H-24) was correlated with carbon atom signals at δ_C 40.02 (C-4), δ_C 56.87 (C-5) and δ_C 28.69 (C-23).

Liquid chromatography mass spectra of isolate was obtained from electron spray ionization (LC MS-Esi pos ion, Mariner biospectrometry Hitachi L 6200). Its fragment ion were m/z: 426 360, 338, 309, 296, 274, 269, 230, 206, 187, 162. The molecular ion was found at m/z 426 indicating its molecular weight. Therefore, the molecular formula of isolat was $C_{30}H_{50}O$ calculated for 426.

Some lupeol activities had been reported, for instance, as a dead cell stimulant programmed on human leukemic cells (HL-60) (Aratanechemuge *et al.*, 2004). Lupeol had been known for its anti-inflammatory and anti-arthritis activities (Agarwal and Rangari, 2003; Gallo and Sarachine, 2009), for its activity as a very aggressive inhibitor of human metastatic melanoma cell growth (Saleem *et al.*, 2008), for angiogenic activity (You *et al.*, 2003) and for antioxaluric and anticalsiuric activities (Anand *et al.*, 1995). Lupeol derivatives were reported to have antimalarial activity (Fotie *et al.*, 2006; Kumar *et al.*, 2008) and anti-microbial activity (Ragasa *et al.*, 2005; Gallo and Sarachine, 2009).

Antioxidant activity test for the isolated compound was conducted with DPPH method. DPPH is the most commonly used method in antioxidant activity test. Advantages of the method include simplicity, quickness and sensitivity to test the antioxidant activity of certain compounds or plant extracts (Koleva *et al.*, 2002; Prakash *et al.*, 2010). The mechanism is based upon the reaction that involves DPPH as an electron scavenger or as a hydrogen radical scavenger by producing a stable diamagnetic compound to neutralize

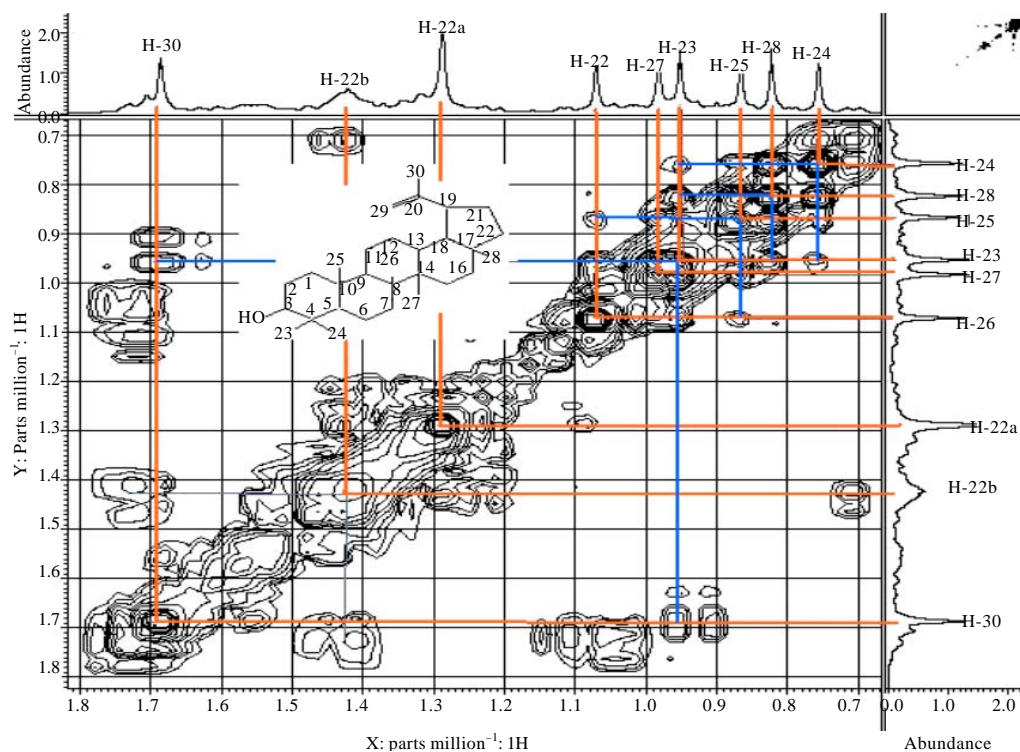


Fig. 2: $^1\text{H}, ^1\text{H}$ -COSY spectrum correlations of isolated compounds

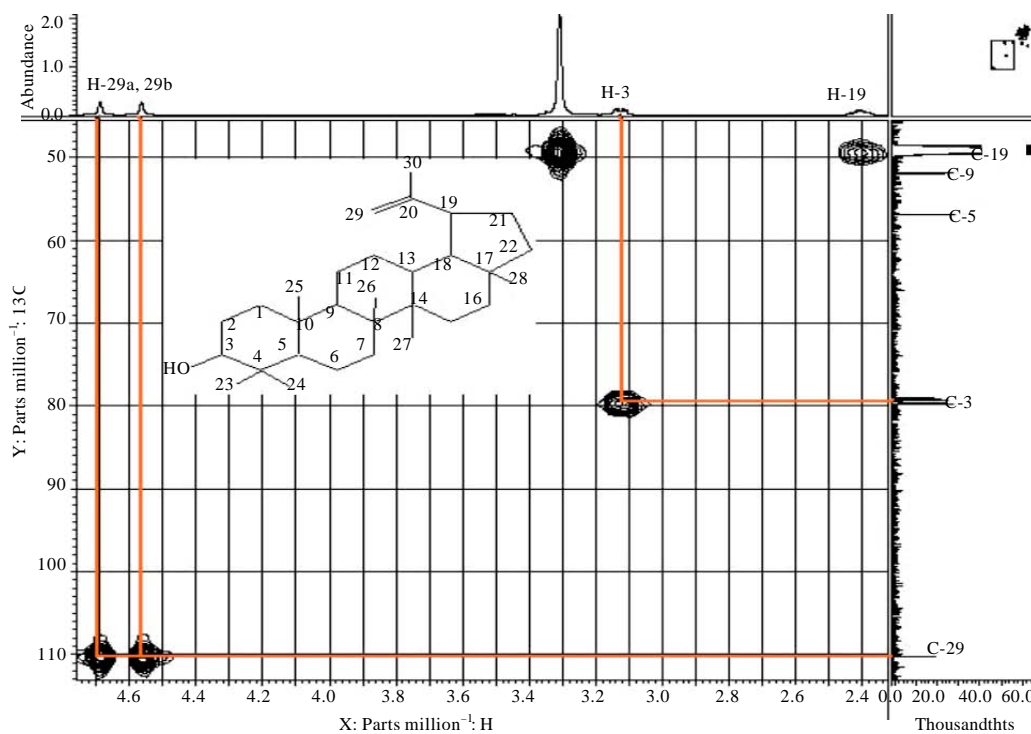


Fig. 3: $^1\text{H}, ^{13}\text{C}$ -HMQC spectrum correlations of isolated compounds

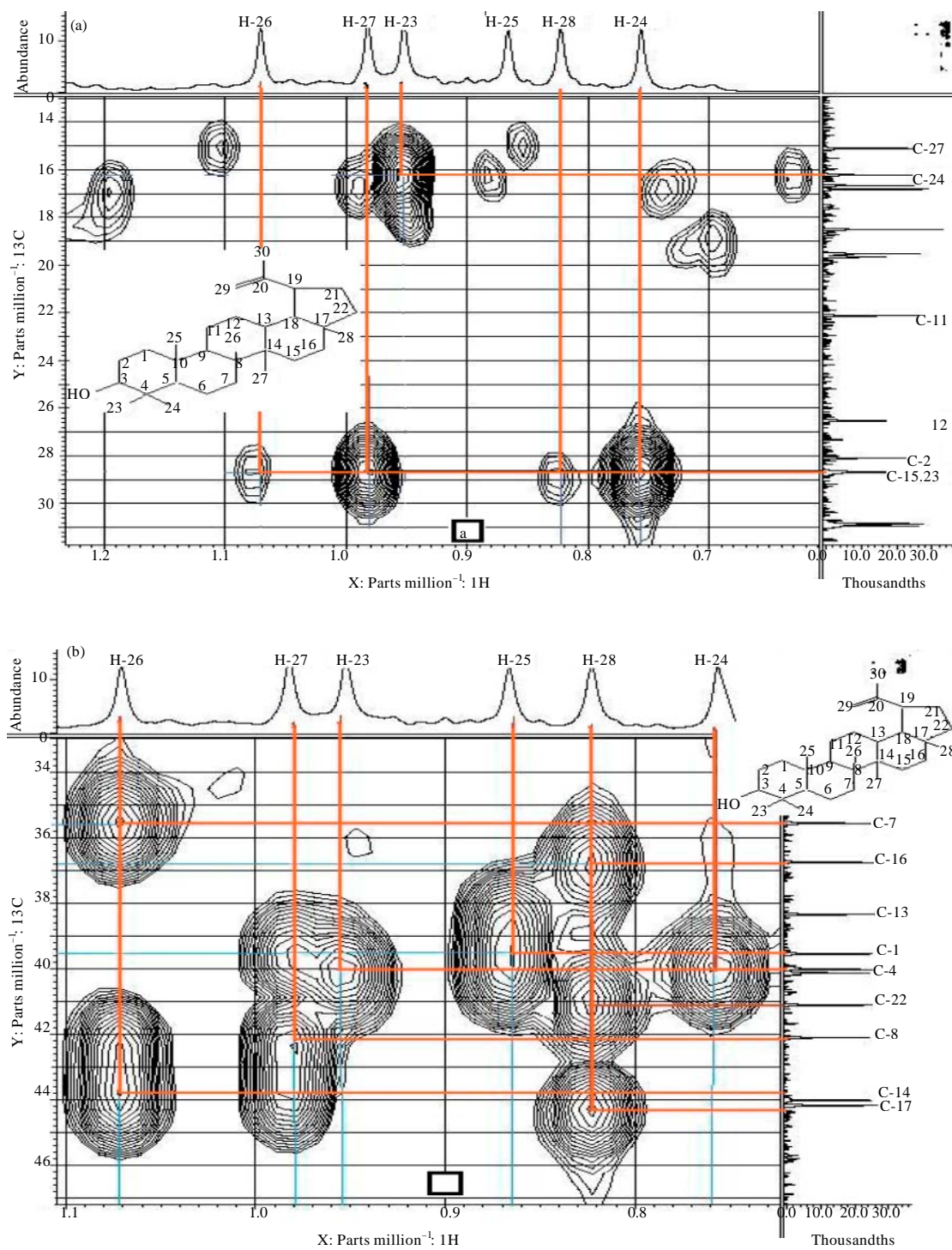


Fig. 4(a-b): ^1H , ^{13}C -HMBC spectrum (a and b) correlation of symmetrical proton-carbon in isolated compounds

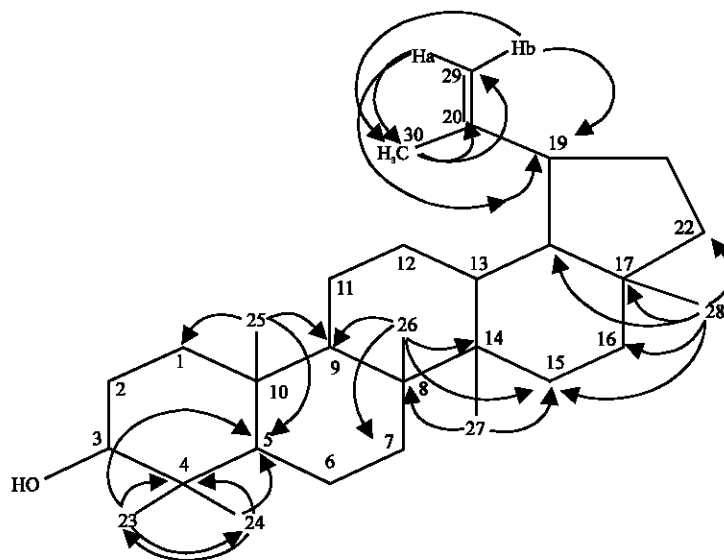


Fig. 5: HMBC correlations of symmetrical proton with carbon in isolate compound

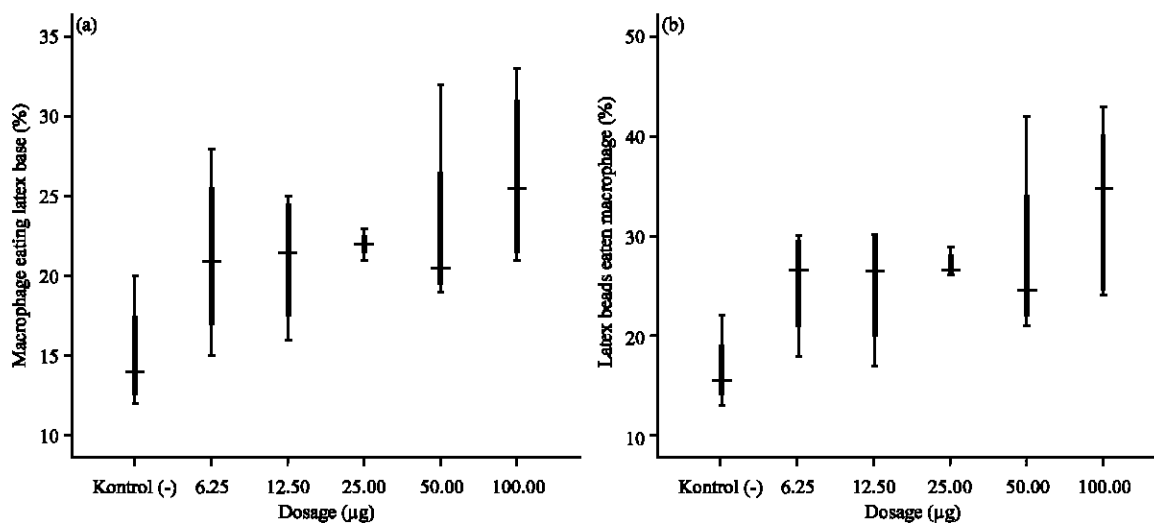


Fig. 6(a-b): Relationship between dosage and activity of macrophages (%) after administration of the isolate compounds

free radicals in the DPPH. The results of antioxidant activity test for the isolated compounds are presented in Table 1.

Antioxidant activity test for the isolated compounds with DPPH method yielded an IC_{50} value of $14.384, 71 \mu\text{g mL}^{-1}$, compared to $IC_{50} = 2.96 \mu\text{g mL}^{-1}$ for the positive control (quercetin). ANOVA analysis at a Confidence Interval (CI) of 95% ($p < 0.05$) showed that, within the same range of concentration, a significant difference were observed in the activity as a DPPH free radical scavenger between quercetin (positive control) and the isolated compound.

In vitro immunomodulatory activity test for the isolated compounds was conducted based on the method proposed by Leijh *et al.* (1986). The results of activity test for the isolates as an immunomodulator are presented in Table 2, meanwhile the correlation between dosage and % latex-eating macrophage cell activity is presented in Fig. 6.

The isolated compound gave a response to the increasing macrophage phagocytosis activity of latex beads. The test was repeated 4 times. Calculation for the number of cells per 100 latex-phagocytizing macrophages showed an increase in average percentage, compared to

Table 1: Antioxidant activities of isolat compound and quercetin (positive control) free radical scavenging assay

Sample	Concentrations ($\mu\text{g mL}^{-1}$)						IC ₅₀	
	0.5	1.0	2.0	4.0	8.0	16.0		32.0
Isolate compounds	-	0.20 ^a	0.88 ^a	1.47 ^a	1.77 ^a	2.46 ^a	4.81 ^a	14384.71 ^b
Quercetin	20.43 ^a	25.84 ^a	38.41 ^a	54.42 ^a	66.48 ^a	88.41 ^a	90.52 ^a	2.96 ^c

^aPercentage inhibition of DPPH radical, ^bInhibitory activity was expressed as the Mean of 50% inhibitory concentration

Table 2: Percentage of activated macrophages after administration of isolate±SE

Doses ($\mu\text{g mL}^{-1}$)	N	Σ Macrophage eating latex beads (%) (Mean±SE)	Σ Latex beads eaten macrophage (%) (Mean±SE)
6.25	4	21.25±2.78	25.25±2.75
12.50	4	21.00±2.12	25.00±3.13
25.00	4	22.00±0.41	27.00±0.71
50.00	4	23.00±3.02	28.00±4.77
100.00	4	26.25±2.87	32.25±4.64
Control (-)	4	15.00±1.78	16.50±1.93

the negative control (Table 2). ANOVA analysis at a Confidence Interval (CI) of 95% ($p < 0.05$) revealed that the treatment dosages did not significantly influence the % latex-phagocytizing macrophages. However, at a dosage of $100 \mu\text{g mL}^{-1}$, a significant impact was observed on the % macrophage-phagocytized latex beads.

CONCLUSION

n-hexane fractions of Casturi (*Mangifera casturi* Konsterm) fruits contained terpenoid compound, namely, 1-isopropenyl-3a,5a,5b,8,8,11a-hexamethyl-icosahydrocyclopenya[α] chrysen-9ol (lupeol) which is isolated for the first time for the plant. Antioxidant activity test with DPPH method for the isolates showed a very weak antioxidant activity (IC₅₀ 14.384,71 $\mu\text{g mL}^{-1}$), compared to the quercetin (IC₅₀ 2,96 $\mu\text{g mL}^{-1}$). *In vitro* test shows that the isolated compound had an immunomodulatory activity, namely, to increase macrophage phagocytosis of latex beads.

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