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Terpenoid-lupeol of red dragon fruit (*Hylocereus polyrhizus*) and its immunomodulatory activity

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Abstract: Red dragon fruit (*Hylocereus polyrhizus,* (F.A.C. Weber) Britton and Rose) has been reported to have various biological activities such as antimicrobial, anti-hypercholesterolemia, anti-diabetes mellitus, cardiovascular risk reduction, health supplement, and melanoma cell inhibitory. The red thick peel of this fruit is just practically a waste that is possibly utilized to maintain health, therefore this research aimed to isolate and identify active compounds of *H. Polyrhizus* peels which can improve the immune system of body. In order to simplify methanol extract was partition and fractionation. The active compounds of petroleum ether fraction were separated and purified using preparative thin layer chromatography. The identification of the compounds structure was conducted through spectroscopic techniques, including UV, FT-IR, ^{13}CNMR and ^{1}HNMR spectroscopy. The data of spectra revealed that the isolate is lupeol. The statistical analysis of macrophage activity showed that the isolate with concentrations of 100, 50, 25, 12.5 and 6.25µg/mL could activate the macrophages higher than control negative. Terpenoid generated from the isolation of *Hylocereus polyrhizus* was identified as lupeol (1-isopropenyl-3a,5a,5b,8,8,11a-hexamethyl-eicosahydrocyclopenya [α] chrysen-9ol. *In vitro* test shows that the isolated compound had an immunomodulatory activity by increases macrophage phagocytosis of latex beads.

Keywords: *H. polyrhizus*, petroleum ether fraction, terpenoids - lupeol, immunomodulator.

INTRODUCTION

Dragon fruit is cactus subspecies of the genus Hylocereus of the family Cactaceae. The genus has about 16 species; one of them is red dragon fruit. The peel of red dragon fruit is a rich source of natural compounds such as phenolics, flavonoids, carotenoids, anthocyanins and terpenoids. It also contains pectin, galacturonic acid, mannose, and galactose, xylose and rhamnose (Muhammad *et al*., 2014).

The immune system needs antioxidant nutrients for hematopoiesis, cell membrane protection against Reactive oxygen species (ROS) and elimination pathogenic microorganisms. The diseases caused by Reactive oxygen species (*ROS*) include rhematoid arthritis, hemorrhagic shock, cardiovascular, metabolic disorders, neurodegenerative diseases, gastrointestinal, AIDS, etc (Aher *et al.,* 2011).

Immunomodulator is a substance that affects the immune system. The immunity consists of innate-immune system and adaptive immune system with a very complex biochemical systems. Some compounds which can be immunodulator are polysaccharides, terpenoids, saponins, alkaloids, isoflavonoids, glucosides, tannins, fatty acids, steroids, triterpenes, and flavonoids (Wagner *et al*., 1991; Parmar *et al*., 1997; Roshan and Savitri, 2013).

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This research aimed to isolate and identify active compounds of *H. Polyrhizus* peels which can improve the immune system of body.

MATERIALS AND METHODS

Plant materials: The sample used in this research was the pericarp of red dragon fruit obtained from Bantul, Yogyakarta, Indonesia. The total amount of the fruit used was 30.29 kg. Of the amount, the peel generated was 6.79 kg or 23.683% of the total fruit weight. The obtained simplisia was 800 grams.

Extraction, fractionation, isolation

Maceration was performed by dissolving 800 grams of simplisia in methanol at room temperature. The generated methanol extracts were partitioned with petroleum ether (PE) solvent. The soluble fractions of petroleum ether were fractionated with vacuum liquid chromatography (VLC). About 2.0 grams of the fractions were fractionated. The static phase used was silica gel 60 for column chromatography with increasing polarity gradient of mobile phase. Petroleum ether: ethyl acetate [50 ml PE], [49:1], [47.5:2.5], [45:5], [42.5:7.5], [40:10], [37.5:12.5], [35:15], [30:20] and [25:25] were used as the mobile phase. The eluates of each solvent comparison were colected. It generated 10 fractions which were then analyzed by thin-layer chromatography with promoter petroleum ether: Ethyl acetate [10:1] and spayed with

cerium sulfate reagent. Isolated target compound was by preparative thin-layer chromatography with static phase, silica gel $PF_{254,}$ 0.5mm of thickness and mobile phase, petroleum ether: Ethyl acetate [10:1]. The isolates were filtered and evaporated, it resulted in dry isolates (Wahdaningsih *et al*., 2018).

Structure analysis with spectroscopy

The structure analysis was conducted on the isolated pure compounds using spectroscopic data UV, Infrared spectroscopy (FTIR. Perkins Elmer 100) and 1 D nuclear magnetic resonantion (NMR, JEOL 500 MHZ).

In vitro test of immunomodulatory activity

Macrophage cell isolation

Mice, were anesthetized with chloroform and dissected. The mouse was put on dissection tray. Its frontal belly was cleaned with 70% alcohol and the peritoneal cavity were dissected. RPMI solvent (10mL) was injected into the peritoneal cavity, left for 3 minutes and rolled slowly. Peritoneal liquid was secreted from the cavity by squeezing the inner organ with two fingers; the non-fatty tissues, which were far from intestine, were aspirated using a hypodermic needle. The needle was then put into an icy beaker glass. The suspension was put into centrifuge tube; it was centrifuged at 1200 rpm at 40C for 10 minutes. Supernatant was removed and 3mL of complete RPMI medium was added to the obtained pellet. The obtained cell pellet was calculated using hemocytometer and was suspended over in complete medium to get cell suspension at 2.5X106/mL of cell density. The calculated cell suspensions were cultured on 24-well plates completed with rounded coverslips; each well was 200µl ($5x10^5$). Incubation was done in incubator with 5% CO2 at 37°C for 30 minutes. Each well was added with 1mL of complete medium and was re-incubated for 2 hours. The cells were washed twice with RPMI; 1mL of complete medium was added to each well. The incubation continued until 24 hours

Test of macrophage phagocytic activity with latex beads

Non specific *in vitro* test of phagocytic capacity was performed with latex beads (3µm). The latex beads were suspended in PBS to get concentration of 2.2 x 10^7 /mL. Isolate was added into RPMI media (400µL) with some concentrations using solvent control media DMSO of 0.0025%. The macrophages cultured a day before were washed and incubated for 60 minutes at 37°C, CO_2 (5%). The cells were then washed three times with PBS to remove particles which were not phagocytosed; they were dried at room temperature and fixated with absolute methanol. After dried, the coverslips were stained with Giemsa 20% b/v for 30 minutes and washed with aquades. The cell was then gently lifted from culture well and dried at room temperature. The percentage of cell phagocytosing latex particles was counted from 100 cells observed under light microscope at 400x magnification. The treatments were performed three times (Leijh *et al*., 1986).

Ethical approval

Please insert here ethical approval statement

STATISTICAL ANALYSIS

The homogeneity and distribution of the phagocytic data result were analyzed using Shapiro-Wilk test. If the data were normally distributed and homogeneous, the analysis would be continued using one-way analysis of variance (ANOVA) and LSD analysis at the 95% level of confidence to see the differences among the treatments.

RESULTS

Identification of isolates

UV-Vis

Ultraviolet-visible spectroscopy of the isolates wasdone by dissolving isolate in chloroform and run in ultraviolet–visible spectroscopy showed the max absorption at (λ_{maks}) 292.2 nm. The spectra UV-Vis were run at range λ 200-400 nm.

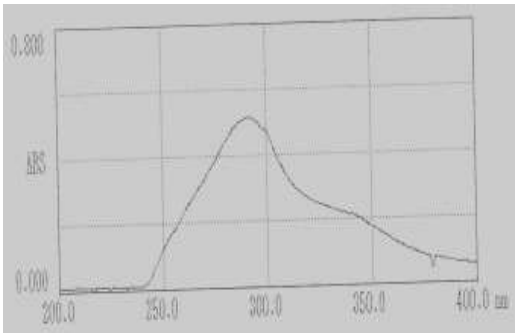

Fig. 1: Spectrum UV isolate (Shimadzu UV-2900)

FT-IR

The spectra FT-IR of isolates revealed a unique absorbance of some functional groups, such as stretching absorbance absorption at 3339.4 cm^{-1} indicating hydroxyl group (-OH). The strong ribbon at 2944.0 cm^{-1} indicated aliphatic hydrocarbons (C-H). The weak absorbance at 1639.5 cm^{-1} showed isolated double C=C bond, gem-dimethyl (ν_{maks} 1456.1 cm^{-1} and 1380.2 cm^{-1}) and ether (ν_{maks} 1040.2 cm^{-1}) (Silvester and Webster, 1998; Pavia *et al.,* 1998).

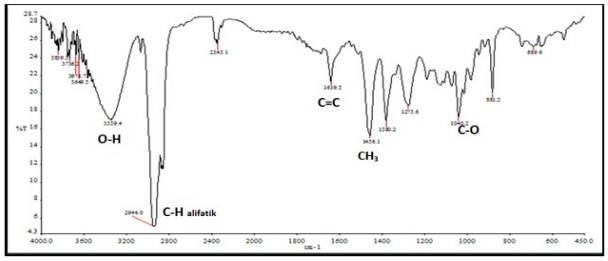

Fig. 2 : Spectrum FT-IR isolate (in pellets KBR)

[13]C-NMR

The spectra ^{13}C-NMR revealed the existence of 30 carbon atoms in a compound. There were two carbon atoms which were *downfield* at δ 150.1 ppm dan 109.4 ppm; this was led by the appearance of double bond groups (C=C). At δ 79.1 ppm dan δ 55.5 ppm, carbon methylenes (C-H) was found (table 1).

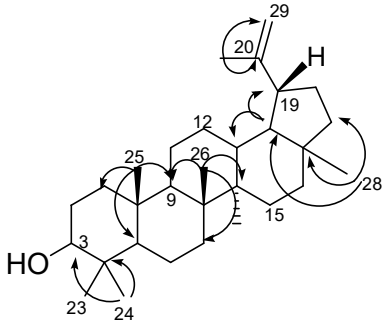

Fig. 3: Chemical Structure of Lupeol (isolate)

[1]H-NMR

The spectra ^{1}H-NMR showed seven methyl tertiers at δ_H 0.78; 0.81; 0.82; 0.98; 0.98; 1.01; and 1.68 (singlet, 3H), oxidized methine protons at δ_H 3.17 (1H, dd *J*=6.5; 11.5 Hz), two olefinic protons at δ_H 4.55 (H-29a) and 4,67 (H-29b) and specific protons of H_{19} at δ_H 2.36 (1H, dt, *J*=6; 10.0 Hz) (Silverstein and Webster, 1998; William and Fleming, 1998) (table 2).

DISCUSSION

Based on those analysis of spectra UV-Vis, FT- IR, LC-MS, 13 C-NMR and 1H-NMR, isolate 1 is similar to lupeol ($C_{30}H_{50}O$). fig. 3 shows its chemical structure.

Lupeol has some synonyms including Fagarsterol, Lupenol, β-Viscol, Cautchicol, Xanthosterin and (3β)-Lup-20(29)-en-3-ol. Lupeol has been isolated from some plants; it is just isolated from red dragon fruit peels. Some

Table 1: Spectra ^{13}C NMR

Position	^{13}C NMR δc (isolate)	Comparisons
	^{13}C NMR δc	^{13}C NMR δc (a)
1	39.7	38.7
2	28.1	27.5
3	79.1	79.3
4	38.8	39.8
5	55.5	55.5
6	21.0	19.0
7	34.4	34.2
8	42.9	41.1
9	47.7	50.9
10	38.9	37.2
11	26.1	21.2
12	28.2	25.3
13	38.9	38.5
14	43.1	42.8
15	31.3	27.2
16	34.8	35.9
17	47.3	43.2
18	48.4	48.5
19	48.1	47.8
20	150.1	151.2
21	29.8	30.1
22	40.9	40.3
23	28.2	28.4
24	16.2	15.6
25	16.1	16.2
26	15.6	16.1
27	15.4	14.8
28	14.6	18.1
29	109.4	109.5
30	18.5	19.8

Data of lupeol comparisons (Mouffok *et al*., 2012).

Table 2: Spectra ^{1}H-NMR

Position	Isolate	Comparisons
	^{1}H NMR δ_H (Integral, mult, *J*=Hz)	^{1}H NMR δ_H (Integral, mult, *J*=Hz)
1	1,24 (1H, m)	1,25 (1H, m)
	1,33 (1H, m)	1,35 (1H, m)
2	1,50 (1H, m)	1,49 (1H, m)
	1,60 (1H, m)	1,63 (1H, m)
3	3,17 (1H, dd; 6,5; 11,5)	3,19 (1H, dd; 5,5; 9,8)
4	-	-
5	0,67 (1H, d; 9,5)	0,71 (1H, d; 6,5)
6	1,41 (2H, m)	1,41 (2H, m)
7	1,42 (2H, m)	1,43 (2H, m)
8	-	-
9	1,09 (1H, d; 13,5)	1,09 (1H, d; 7,2)
10	-	-
11	1,43 (2H, m)	1,42 (2H, m)
12	1,44 (2H, m)	1,43 (2H, m)
13	0,78 (1H, s)	0,77 (1H, s)
14	-	-
15	1,25 (2H, m)	1,25 (2H, m)
16	1,35 (2H, m)	1,35 (2H, m)
17	-	-
18	0,93 (1H, dd; 3; 8)	0,95 (1H, dd; 3,5; 10,5)
19	2,36 (1H, dt; 6; 11,5)	2,36 (1H, dt; 5,4; 10,0)
20	-	-
21	2,25 (1H, m)	2,15 (1H, m)
	2,29 (1H, m)	2,25 (1H, m)
22	1,98 (2H, m)	2,15 (2H, m)
23	0,98 (3H, s)	0,96 (3H, s)
24	0,82 (3H, s)	0,75 (3H, s)
25	0,81 (3H, s)	0,82 (3H, s)
26	1,01 (3H, s)	1,03 (3H, s)
27	0,98 (3H, s)	0,94 (3H, s)
28	0,78 (3H, s)	0,78 (3H, s)
29	4,55 (1H, s)	4,58 (1H, s)
	4,67 (1H, s)	4,61 (2H, s)
30	1,68 (3H, s)	1,59 (3H, s)

Data of lupeol comparisons (Harison, 2014).

plants, such as *Mangifera casturi*, are reported to contain lupeol (Sutomo, 2013), *Diospyros kaki* L.F (Tjitaresmi, 2002) and *Strobilanthus callosus* Nees (Sarpate, 2012). Lupeol has low antioxidant activities to catch free radicals DPPH. The compound has some health benefits; it can be used for antiinflammatory, antiarthritic, antigeogenic, antioxaluric and anticalciuric, and metastatic melanoma inhibitory for human (Agarwal, 2003; You *et al.,* 2003; Anand *et al*., 1995; Saleem *et al*., 2008).

The *in vitro* immunomodulatory activity assay of the isolates was conducted according to Leijh *et al*. (1986). The test used live macrophages isolated from mice. The macrophages were the main phagocytic cells which can counter pathogens through phagocytosis mechanism; it is important for of both innate immune response and adaptive immune response (Sanchez *et al.,* 2008). *In vitro* test of macrophage phagocytic activity was performed. The active macrophages will express major histocompatibility complex (MHC) class II and molecules of co stimulatory such as CD80, CD86 and CD40 able to induce cell T effectively (Tripathi *et al*., 2008). In this research, isolation of macrophage cells from mice's peritoneal cavity. The coloring method was Giemsa 20% application. It resulted in dark purple of the nucleus and red and blue of the cytoplasm. The cells were observed under light microscope at 400x magnification (Akrom *et al*., 2015; Woronzoff-Dashkoff, 2002).

Phagocytic index refers to the ability of active macrophage cells to phagocytose latex. The phagocytic capacity of macrophages indicated that the immune system improved to destroyed or killed pathogens. The increasing number of macrophage and its latex

Table 3: Percentage of macrophage activity

Concentrations µg/mL	N	Phagocytic capacity (means ± SD)	Phagocytic index (means ± SD)
100.00	3	21.45±1.35	1.61±0.18
50.00	3	20.67±0.67	1.79±0.12
25.00	3	17.78±0.51	1.52±0.06
12.50	3	20.33±1.21	1.77±0.14
6.25	3	17.21±1.17	1.58±0.18
Control positive	3	32.56±0.83	1.52±0.04
Control negative	3	5.67±2.08	1.32±0.11

phagocytosis showed positive activity, compared to the control negative. This revealed that the isolates were potential for immunodulatory effects.

The statistical analysis showed that the isolate with concentrations of 100, 50, 25, 12.5 and 6.25µg/mL could activate the macrophages higher than control negative.

But no better than the positive control. The results of ANOVA and LSD analysis showed differences between the treatment group and the control negative and control positive with confidence level 95% ($p<0.05$).

Percentage of macrophage phagocytic activity significantly different with control and significantly different with IM 100 IM 50 dan IM 12,5. Meanwhile, percentage of phagocytic index significantly different with control and significantly different with IM 100 dan IM 50.

CONCLUSION

Terpenoid generated from the isolation of *Hylocereus polyrhizus* was identified as lupeol (1-isopropenyl-3a,5a,5b,8,8,11a-hexamethyl-eicosahydrocyclopenya[α] chrysen-9ol. *In vitro* test shows that the isolated compound had an immunomodulatory activity by increases macrophage phagocytosis of latex beads.

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