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Research Article

Isolation of xanthine oxidase inhibitor compounds of pekoe fanning black tea and their activity on interferon-γ production *in vivo*

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ABSTRACT

Isolation of bioactive compounds on pekoe fanning black tea was done. The isolation was guided by xanthine oxidase (XO) inhibitory activity test. On the fractionation and separation steps, the ethyl acetate fraction and subfraction (SB)-3 had greater on XO activity with percentage of inhibition 71.38% and 74.49% respectively. The purification steps obtained four compounds and they were identified by spectral data of UV-Vis, ^{1}H-NMR, MS, and ^{13}C-NMR. From spectral data the compounds were known as kaempferol, quercetin, luteolin and myricetin. All isolates were assayed by XO inhibitory and their activity on interferon-γ (IFN-γ) production *in vivo*. Luteolin demonstrated the greatest of XO inhibitory activity with IC_{50} 5.06 µg/ml. On IFN-γ production indicated that quercetin demonstrated the greatest ability with IFN- γ production 14.45 pg/ml.

Keywords: Flavonoids; IFN-γ; Isolation; Pekoe Fanning; Xanthine Oxidase

INTRODUCTION

Xanthine Oxidase (XO) is a member of the xanthine oxidoreductase (XOR) group, found in mammals at highest concentration within the liver and intestine (Hearse *et al*., 1986). Under normal condition, the predominant mammalian XOR is xanthine dehydorogenase (XDH), but around 10% of the group is found as XO. However, under ischemic conditions, where oxygen is limited, XDH is converted to XO via limited proteolysis. Both XDH and XO convert hypoxanthine to uric acid via xanthine (Lin *et al*., 2000).

Plants were used by people for the treatment of gout, or diseases with associated symptomtps such as rheumatism or arthritis. XO inhibitors were found in a wide variety of plants used in traditional herbal medicines for the treatment of gout and rheumatism (Owen and Johns, 1999). One of the most plant documented well as XO inhibitor is tea, *Camellia sinensis* (L.) O. Kuntze. Bahorun *et al*., (2009) was reported that black tea successfully reduces uric acid in humans susceptible to cardiovascular diseases. Among types of tea, black tea was the most potent "tea" to inhibit XO (Dew *et al*., 2005). However, limited investigations grade of black tea on XO inhibitory activity were reported.

Our previous research was evaluated activities of 15 grades Indonesia black tea on XO inhibitory and immunostimulant effect through carbon clearance test to determine fagocyty index as selular natural immune system. Among 15 grades were evaluated, PF shown the best on both of activities (Rohdiana, et al., 2014). Furthermore PF will be evaluated its activity on humoral natural immune system. One of the most humoral natural immune system was documented well is interferon-γ (IFN-γ). Therefore the aim of this research was to isolate of XO inhibitor compounds of pekoe fanning black tea and its activity on IFN-γ production *in vivo*.

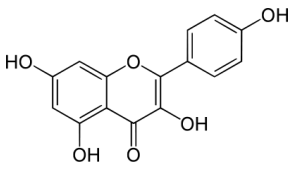

Figure 1: Kaempferol

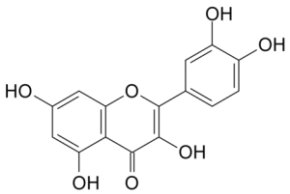

Figure 2: Quercetin

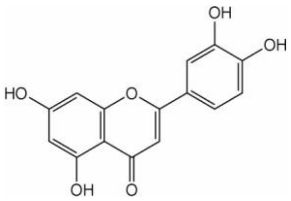

Figure 3: Luteolin

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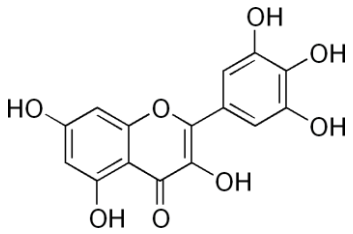

Figure 4: Myricetin

MATERIAL AND METHODS

Material

Pekoe Fanning (PF) was produced from Research Institute for Tea and Cinchona Gambung, Bandung, Indonesia in September 2012.

Methods

General Experiment Procedure

Melting points were determine with a Electrothermal Engineering LTD, Serial Number 9100 apparatus and are uncorrected. UV-Vis spectra were measured with a Hewlet Packard® 84524 UV/Vis Spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Agent 500 Mhz and 125 MHz respectively. Chemical shift were recorded in part per million (δ) in acetone-d6 with tetramethylsilane (TMS) as the internal reference. The ESI MS were obtained using a ESI-TOF Waters LCT Premier XE Mass Spectrometer.

Fractionation

Pekoe fanning dried extract (100 g) was dissolved by 300 ml fresh hot boiling water. Then it was fractionated by liquid-liquid extraction successively with 300 ml n-hexane and ethylacetate for three times. All fractions were evaporated, dried and evaluated their activity on XO inhibitory.

Separation

The active fraction was separated by vacuum liquid chromatography with n-hexane, ethylacetate and methanol to obtain sub fractions of pekoe fanning. All sub fraction was evaporated, dried and evaluated their activity on XO inhibitory.

Purification

The active sub fraction was purified by radial chromatography with n-hexane, ethyl acetate, and methanol to obtain pure compounds. The pure compounds was evaluated their activity on XO inhibitory and its activity on interferon-γ production *in vivo*.

Spectral Data

Compound 1

UV-Vis (MeOH) λ_{max} nm: 256, 372; NaOH 0': 281, 425; NaOH 5': 240, 425; $AlCl_3$: 260, 429; $AlCl_3$+HCl: 259, 430; NaOAc 0': 275, 398; NaOAc 5': 275, 332, NaOAc+H_3BO_3: 260, 374. ^{1}H NMR (500 MHz, Acetone): δ 6,25 d (1H, J=2 Hz, H-6), 6,50 d (1H, J=2 Hz, H-8), 6,99 d (2H, J=9 Hz, H-3'; H-5'), 8,13 d (2H, J=8,5 Hz, H-2'; 6'). ^{13}C NMR (125 MHz, Acetone): δ 146,9 (C-2); 136,5 (C-3); 176,5 (C-4); 163,2 (C-5); 99,1 (C-6); 164,9 (C-7); 94,4 (C-8); 157,7 (C-9); 104,1 (C-10); 123,2 (C-1'); 130,3 (C-2'); 116,2 (C-3'); 160,0 (C-4'); 116,2 (C-5') and 130,3 (C-6'). ESI-MS m/z 287 [M+1] formula $C_{15}H_{10}O_6$.

Compound 2

UV-Vis (MeOH) λ_{max} nm: 256, 371; NaOH 0': 282, 424; NaOH 5': 282, 424; $AlCl_3$: 269, 436; $AlCl_3$+HCl: 266, 421; NaOAc 0': 275, 390; NaOAc 5': 275, 398; NaOAc+H_3BO_3: 261, 391. ^{1}H NMR (500 MHz, Acetone): δ 6,25 d (1H, J = 2 Hz, H-6), 6,50 d (1H, J = 2 Hz, H-8), 6,98 d (1H, J = 8,5 Hz, H-5'), 7,67 dd (1H, J = 2,5 Hz, H-2'), 7,69 d (1H, J = 2 Hz, H-6'). ^{13}C NMR (125 MHz, Acetone): δ 146,9 (C-2); 145,8 (C-3); 176,5 (C-4); 162,2 (C-5); 99,1 (C-6); 165,0 (C-7); 94,4 (C-8); 148,3 (C-9); 104,1 (C-10); 121,4 (C-1'); 123,7 (C-2'); 116,1 (C-3'); 157,7 (C-4'); 157,7 (C-5'); 136,7 (C-6'). ESI-MS m/z [M+1] formula $C_{15}H_{10}O_7$.

Compound 3

UV-Vis (MeOH) λ_{max} nm: 253, 348: NaOH 0': 265, 402; NaOH 5': 265, 402; AlCl3: 274, 425; $AlCl_3$+HCl: 273, 388; NaOAc 0': 270, 374; NaOAc 5': 270, 374, NaOAc+H_3BO_3: 262,367. ^{1}H NMR (500 MHz, Acetone): δ 6,24 d (1H, J = 2,5 Hz, H-6), 6,51 d (1H, J = 4,5 Hz, H-8), 6,57 s (1H, H-3), 6,99 d (1H, J = 8,5 Hz, H-5'), 7,45 dd (1H, J = 2,5 Hz, H-2'), 7,49 dd (1H, J = 2,5 Hz, H-6'). ^{13}C NMR (125 MHz, Acetone): δ 150,1 (C-2); 146,4 (C-3); 183,0 (C-4); 164,8 (C-5); 99,7 (C-6); 165,1 (C-7); 94,6 (C-8); 158,7 (C-9); 104,2 (C-10); 116,6 (C-1'); 120,1 (C-2'); 114,1 (C-3'); 163,3 (C-4'); 105,3 (C-5'); 123,7 (C-6'). ESI-MS m/z [M+1] 287 formula $C_{15}H_{10}O_6$.

Compound 4

UV-Vis (MeOH) λ_{max} nm: 256, 372; NaOH 0': 281, 423; NaOH 5': 280, 423; $AlCl_3$: 270, 439; $AlCl_3$+HCl: 266, 420: NaOAc 0': 272, 425; NaOAc 5': 272, 438; NaOAc+H_3BO_3: 266, 390. ^{1}H NMR (500 MHz, Acetone): δ 6,25 d (1H, J = 2, H-6), 6,49 d (1H, J = 1,5 Hz, H-8), 7,41 s (2H, H-2', 6'). ^{13}C NMR (125 MHz, Acetone): δ 146,4 (C-2); 146,4 (C-3); 176,5 (C-4); 162,3 (C-5); 99,1 (C-6); 165,0 (C-7); 94,4 (C-8); 146,9 (C-9); 104,1 (C-10); 122,7 (C-1'); 136,4 (C-2'); 108,3 (C-3'); 157,7 (C-4'); 108,3 (C-5') dan 136,9 (C-6'). ESI-MS m/z [M+1] 319 $C_{15}H_{10}O_8$.

Assay of XO inhibitory activity

Mixture consisted of 1 ml tea extract solution (100 µg/ml), 2.9 mL 50 mM potassium phosphate buffer (pH 7.5 at 25°C) that were initiated by adding to 2 ml of the substrate solution (xanthine 0.15 mM). Xanthine 0.15 mM was prepared by dissolving it in 100 µl NaOH and the pH was adjusted to 7.5. The mixture was incubated at 25°C for 15 min. After preincubation, the reaction was initiated by the addition of 0.1 ml (0.1 units/ml in phosphate buffer, pH 7.5 at 25°C) xanthine oxidase enzyme (from bovine milk, Sigma X1875). Xanthine

oxidase was prepared in cold potassium phosphate buffer immediately before used. The mixture was incu---bated at 25°C for 30 min, for stopping reaction 1 ml HCl 1 N was added. The absorbance was recorded at 295 nm using Ultra Violet (UV) spectrophotometer. Allopu---rinol (100µg/ml) was used as positive control (Berg---meyer *et al*., 1974; Kong *et al*., 2000; Owen and Johns, 1999; Yumita *et al*., 2013). One unit will convert 1.0 µmol of xanthine to uric acid per minute. XO activity was expressed as the percentage inhibition of XO, which was calculated as:

$$Inhibition\,(\%) = \left(\frac{x-y}{x}\right)100\%$$

Where: x is the activity of the enzyme without black tea extract (Δabs. with enzyme --- Δabs. without en---zyme), and y is the activity of the enzyme with black tea extract (Δabs. with enzyme -Δabs. without en---zyme).

Assay of IFN-γ Production

Analysis of production of IFN-γ was conducted trough administration of isolates as drug 12 days. On third day, induction of Sheep Red Blood Cell (SRBC) for im-munization was started. On the seventh day primary anti body titer and second of SRBC induction was con---ducted. On tenth day the second antibody was tittered. On the twelfth day of coating plate was conducted. While the mice on thirteenth day were sacrifice. The determination of production IFN-γ on mice *in vivo* by ELISA*(Enzyme Linked Immunosorbent Assay)* on the fourteenth day was analyzed.

RESULT AND DISCUSSION

Table 1 summarizes among three fractions were moni---tored, ethylacetate fraction had the greatest activity on XO inhibitory with percentage of inhibition 71.38% followed water and n-hexane fractions each 27.21% and 12.51% respectively. These results are consistent with studies have been conducted by Yeragamreddy *et al* (2013), that ethylacetate fraction more stronger than other fractions on XO inhibitory. Furthermore Yeragamreddy *et al* (2013) stating that the main chem-ical constituent on ethylacetate fractions is flavonoids. While Nagao *et al*., (1999) stating that flavonoids have been found to show an inhibitory activity on XO which produces hydrogen peroxide and superoxide anion during the oxidation of hypoxanthine to xanthine and then to uric acid.

Table 1: Activity of pekoe fanning fractions on xan---thine oxidase inhibitory

Fractions	Inhibitory activity (%)
n-hexane	12.51 ± 3.55 d
ethyl acetate	71.38 ± 5.39 b
water	27.21 ± 7.33 c
allopurinol	93.46 ± 1.61 a

Data is mean ± SD of three determinations; Value bear---ing different letters within columns are significantly different by Tukey's HSD (P≤0.05).

To the ethylacetate fraction the separation process was continued using vacuum liquid chromatography (VLC). The activity of all sub fractions on XO inhibitory was showed in Table 2.

Among 11 sub fractions that were monitored, SB-3 had the highest activity on XO inhibitory with percentage of inhibition 74.49% followed by SB-4 and SB-5 which were 63.68% and 62.36% respectively. Furthermore, SB-3 as the active sub fraction was purified by radial chromatography with n-hexane : ethylacetate (5:5), (3:7), (2:8), 100% ethylacetate, ethylacetate: methanol (9:1), (7:3), (5:5) and (3:7). SB-3 which was purified by n-hexane : ethyl acetate (5:5), (3:7), (2:8), 100% ethylacetate obtain the yellow crystal and named of compound 1 to 4. Furthermore all compounds was analyzed their chemical structure with activity on XO inhibition and production of IFN-γ *in vivo* as showed in Table 3 and 4.

Table 3 summarizes that the hydroxyl groups at C-5 and C-7 were essential for a high inhibitory activity on XO. There were concluded that flovone has stronger activity than flavonol on XO inhibition. The absence of

Table 2: Activity of acetate ethyl sub fractions on xanthine oxidase inhibitory

Sub Fractions	Inhibitory activity (%)
SB-1 ethyl acetate (7:3)	7.66 ± 3.54 g
SB-2 ethyl acetate (6:4)	10.37 ± 1.17 fg
SB-3 ethyl acetate (3:7)	74.49 ± 2.44 b
SB-4 ethyl acetate (3:7)	63.86 ± 2.69 c
SB-5 ethyl acetate (3:7)	62.36 ± 5.43 c
SB-6 ethyl acetate: methanol (9:1)	44.59 ± 3.66 d
SB-7 ethyl acetate: methanol (1:1)	31.89 ± 3.98 e
SB-8 ethyl acetate: methanol (1:1)	14.42 ± 2.55 f
SB-9 ethyl acetate: methanol (1:1)	32.52 ± 2.16 e
SB-10 ethyl acetate: methanol (3:7)	33.09 ± 5.51 e
SB-11 methanol	33.09 ± 5.51 e
Allopurinol	92.65 ± 0.49 a

Data is mean ± SD of three determinations; Value bearing different letters within columns are significantly different by Tukey's HSD (P≤0.05).

hydroxyl group at C-3 enhanced the inhibitory activity on XO. The present of hydroxyl groups at C-5', especially for flavonol were enhanced the inhibitory activity on XO. The other hand, the present of hydroxyl group at C-3' was reduce the inhibition. These results are consistent with studies have been conducted by (Cos *et al*., 1998).

Table 3: Production of IFN-γ by isolates

Compounds	IC50 (µl/mg)
Kaempferol	10.24
Quercetin	26.88
Luteolin	5.06
Myricetin	22.72
Allopurinol	2.75

Assay of IFN-γ Production

Table 4 summarizes that the presence of an OH group at position C-5 'will decrease the formation of IFN-γ. This can be seen by comparing kaempferol against myricetin.The absence of the OH group at position C-3 also lowers the activity of IFN-γ production. This can be seen by comparing quercetin with IFN-γ production value amounted to 14.45 pg / ml to luteolin that IFN-γ production value amounted to 10.36 its pg / ml. Effect of the absence of the OH group at position C-3 is not as big as the decline caused by the presence of an OH group at position C-5'.

Table 4: Production of IFN-γ by isolates

Compounds	Production of IFN-γ (pg/ml)
Kaempferol	13.61 ± 0.60
Quercetin	14.45 ± 0.46
Luteolin	10.36 ± 0.90
Myricetin	9.15 ± 0.34
Selenium	9.07 ± 0.23

Data is mean ± SD of three determinations

CONCLUSION

Four flavonoids isolated from pekoe fanning black tea showed strong activities on XO inhibitory and IFN-γ production *in vivo.* Luteolin has activity on XO inhibition similar with Allopurinol. Meanwhile on IFN-γ production in vivo all flavonoids was assayed had activity more stronger than selenium as control. Furthermore, flavonoids especially luteolin was recommended to develop as bioactive compound for anti gout and immunostimulant.

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