Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 5, 2014

Original Article

XANTHINE OXIDASE INHIBITORY AND IMMUNOMODULATORY ACTIVITIES OF FIFTEEN GRADES INDONESIA ORTHODOX BLACK TEA

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Received: 17 July 2014 Revised and Accepted: 14 Aug 2014

ABSTRACT

Objective: to study the effect of fifteen grades Indonesian orthodox black tea as xanthine oxidase (XO) inhibitor and immunomodulator.

Methods: Effect on XO activity was studied *in vitro* by using xanthine as a substrate. The degree of inhibition was determined by measuring the absorbance at 295 nm associated with uric acid formation. Effect of immune system was studied *in vivo* through carbon clearance test by determination of phagocytic index of reticulum endothelium system.

Results: among the fifteen grades, Broken Orange Pekoe (BOP), Pekoe Fanning (PF), and Broken Pekoe (BP) have inhibition effect on XO activity with an inhibition percentage more than 50%. The highest XO inhibitory effect was showed by BOP with inhibition percentage of 61.58%. Study on immune system showed that BOP, PF, Dust, and BP grades had immunostimulating, effect with the highest value was shown by PF with a phagocytic index of 1.41.

Conclusion: among the fifteen grades of orthodox black tea: BOP, PF, and BP have XO inhibitory and immunomodulatory activities.

Keywords: Xanthine oxidase inhibitory, Immunomodulatory activity, 15 grades of orthodox black tea.

INTRODUCTION

Tea, *Camellia sinensis* (Linnaeus) Kuntze, is one of the most frequently consumed beverages in the world. Black tea represents approximately 78% of total consumed tea, whereas green tea accounts for approximately 20% of tea consumed[1]. Tea contains number of bioactive chemicals like caffeine and different polyphenolic compounds. During manufacturing of black tea, green tea catechins undergo oxidation by polyphenol oxidase to form the complex condensation products, like theaflavins (TFs) and thearubigins (TRs). This process is known as fermentation[2]. TFs and TRs are the most exclusive polyphenols from black tea[3]. The contents of TFs and TRs were varied depend on the species, agronomic variables of tea and the process of fermentation[2].

Black tea and polyphenolic compounds were reported as antioxidants[4], anti-inflammatory[5], antidiarhoeal[6], and antimicrobial activities[7]. Tea also prevents cancer[8] and cellular DNA damage[9]. Tea can lower the risk of type 2 diabetes[10] and possesses neuroprotective properties[11]. The regular consumption of black and green tea may provide some protection against hypertension[12].

Black tea was reported to reduce uric acid level[13]. Furthermore it was reported that black tea decoction show immunomodulatory properties on experimental animal and human peripheral mononuclear cells[14]. However, investigation on XO inhibition and immunomodulatory activities by grade of orthodox black tea is limited. The aim of this study were to evaluate XO inhibitory and immunomodulatory activities of fifteen grades Indonesian orthodox black tea.

MATERIALS AND METHODS

Black Tea Processing

Young tea shoots, comprising an apical bud and tree expanded leaves of the clone Gambung 7 harvested from experimental farm Research Institute for Tea and Cinchona (RITC), Bandung Indonesia were used in this experiment. Freshly plucked tea shoots were loaded to the withering trough at the rate of 10 kg/m^2. Ambient air was passed for 18 hours to evaporate adequate physical and chemical withering. The withered leaves were passed through a mini orthodox machine and move to open top roller to get adequate maceration. The macerated leaves were fermented for 2 hours and dried in mini drier to a final moisture content which was 3%. The black tea samples were sorted using sieve shaker to get the 15 grades.

Analysis of catechin content

Orthodox black teas (25 g) was extracted with 250 mL of fresh boiling water and shaken for 10 minutes. Filtrate centrifuged to obtain clear filtrate to be further extracted with chloroform 1:1 (v/v). Low layer was removed and the top layer was further extracted with the same solvent for three times. The top layer was further extracted with ethyl acetate, 1:1 (v/v). The top layer was ethyl acetate fraction and the low removed. The ethyl acetate layer evaporated by rotary evaporator and dried by freeze drier to obtain crude catechins. Fifteen mg of crude catechin is solved into 25 mL of solvent (acetonitrile, ethyl acetate, phosphoric acid; 12:2:86), shaked to homogenize and filtered out through Whatman 0,45 µm. The filtrate was then analyzed by HPLC[15].

Preparation of extract

Each of orthodox black tea grades were grinded to reduce the size in order to get particle size with a mesh 20 then extracted for 6 minutes using fresh boiling water with a ratio tea : water, 1:50, w/v. The water extract was separated and make it dry using freeze dryer.

Assay of xanthine oxidase inhibitory activity

Mixture consisted of 1 mL tea extract solution (100 µg/mL), 2.9 mL 50 mM potassium phosphate buffer (pH 7.5) and add 2 mL of the substrate solution (xanthine 0.15 mM) are incubated at 25^{o}C for 15 minutes. Xanthine 0.15 mM was prepared by dissolved 2.2816 mg of xanthine in a minimal volume of NaOH. Add approximately 90 ml of deionized water. Adjust to pH 7.5 at 25°C with either 1 M NaOH or 1 M HCl. Dilute to a final volume of 100 mL. 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^{o}C) XO enzyme (from bovine milk, Sigma X1875). XO was prepared in cold potassium phosphate buffer immediately before used. The mixture was then incubated at 25^{o}C for 30 minutes and 1 mL HCl 1 N was added to stop the reaction. The absorbance was recorded at 295 nm using Ultra Violet (UV) spectrophotometer. Allopurinol (100 µg/mL) was used as positive

control[16-19]. One unit of XO will convert 1.0 µmol of xanthine to uric acid per minute. XO activity was expressed as the inhibition percentage of XO, which was calculated using formula:

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

where: x is the activity of the enzyme without test extract (Δabs. with enzyme - Δabs. without enzyme), and y is the activity of the enzyme with test extract (Δabs. with enzyme -Δabs. without enzyme).

Carbon clearance test

The immunomodulatory effect of the fifteen grades of Indonesia orthodox tea was studied through carbon clearance test to determine the phagocytic activity of reticulum endothelium system. The test was done in Swiss Webster mice. Randomly the mice were grouping into seventeen groups (five mouse/group). Each of tea grade extract was administered orally once a day for 7 consecutive days. Zymosan A was used as standard. On day 8th, colloid carbon suspension was injected intravenously 0.1 mL per 10 g of mouse[20].

Blood samples were collected before 0 and at 5, 10, and 15 min after carbon administration. Amount of 20 µL of blood sample was suspended in 4 mL 1% acetic acid solution and the transmittance was measure at 650 nm. The graph for absorbance versus time was plotted for each animal in respective test groups and phagocytic index (PI) was calculated using formula:

$$\text{Phagocytic Index (PI)}: \frac{\text{Ksample}}{\text{Kcontrol}}$$

Where K_{Sample} represent the slope of absorbance versus time curve for extract-treated sample and $K_{Control}$ represent the slope of absorbance versus time curve for control[21].

RESULT AND DISCUSSION

In the tea market, black teas were often graded by the size and quality of the leaf. According to Indonesian National Standard (SNI) of tea, the orthodox black teas were classified into three classes, *i.e.*, class I, II, and III. Those three classes of these orthodox black teas are further grading into 15 grades based on its particle size and appearance as can be seen in Table 1.

Class I of orthodox black tea usually consist of young leave, while orthodox black tea class II consist mostly medium and class III was old leaves. Catechin content was higher in young leaves compare to medium and old leaves. Antioxidant activity of young leaves also higher than those of medium and old leaves[22].

Catechin content of its grade of orthodox black tea and its effect on XO inhibitory activity determined in this research were in Table 2 below. Based on data shown in Table 2, generally class I have highest catechin content compared to class II and III. Data in Table 2 shown that its XO inhibitory activity is not linier to its catechin content. BOP and BP (class I) showed the similar and highest effect on XO activity indicated from both inhibition activity (61.58±2.12 % and 60.43±1.55 %). While PF and DUST (Class I), PF II and BP II (class II), DUST III (class III), showed medium effect with inhibition percentage around 45 - 57 %.

Table 1: Characteristic of fifteen Indonesia orthodox black tea grade (SNI)

Class	Grade	Mesh		Appearance
		Pass	Un-pass	
	BOP (Broken Orange Pekoe)	10	12	Blackish, well-made, neat leaf of medium size
	BOPF (Broken Orange Pekoe Fanning)	12	16	Blackish, neat leaf, fairly clean, but smaller than the BOP grade
	PF (Pekoe Fanning)	16	24	Blackish, neat leaf, fairly clean, but smaller than the BOPF grade
I	Dust	24	30	Blackish, neat leaf, fairly clean, but smaller than the PF grade
	BP (Broken Pekoe)	10	12	Blackish, choppy, hard, produces from young stems
	BT (Broken Tea)	12	16	Blackish, flaky
	PF II (Pekoe Fanning II)	16	24	Brownish than PF, a few fiber and stalk
II	Dust II	24	30	Brownish than Dust, a few fiber and stalk
	BP II (Broken Pekoe II)	10	12	Brownish than BP, a few fiber and stalk
	BT II (Broken Tea II)	12	16	Brownish than BT, a few fiber and stalk
	PF III (Pekoe Fanning III)	16	24	Reddish than PF II, more fiber and stalk
III	Dust III	24	30	Reddish than Dust II, more fiber and stalk
	BM (Broken Mix)	30	60	Mixed flaky leaf tea, more fiber and stalk
	BTL (Bohea Tulang)	US	US	Unsorted stalk
	BBL (Bohea Bulu)	US	US	Unsorted fiber

Table 2: Catechin content and XO inhibitory activity of fifteen grades of Indonesia orthodox black tea

Class	Grade	Catechin (%)	XO inhibitory (%)
I	BOP	8.60 ± 0.12^{c}	61.58 ± 2.12^{b}
	BOPF	10.13 ± 0.14^{a}	34.70 ± 3.15^{e}
	PF	10.18 ± 0.11^{a}	57.01 ± 1.31^{c}
	Dust	9.76 ± 0.12^{b}	45.79 ± 1.51^{d}
	BP	6.27 ± 0.09^{i}	60.43 ± 1.55^{b}
	BT	8.07 ± 0.10^{d}	23.28 ± 2.09^{f}
II	PF II	7.86 ± 0.09^{e}	49.66 ± 5.32^{d}
	Dust II	7.19 ± 0.10^{g}	46.98 ± 3.19^{d}
	BP II	6.94 ± 0.09^{h}	49.96 ± 3.41^{d}
	BT II	7.89 ± 0.08^{e}	35.90 ± 2.86^{e}
III	PF III	6.98 ± 0.10^{h}	12.31 ± 1.45^{g}
	Dust III	7.45 ± 0.09^{f}	19.75 ± 1.62^{f}
	BM	7.43 ± 0.11^{f}	32.11 ± 2.38^{e}
	BTL	6.81 ± 0.13^{h}	23.70 ± 3.42^{f}
	BBL	7.54 ± 0.11^{f}	11.25 ± 1.65^{g}
Allopurinol			92.76 ± 1.27^{a}

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

The other grades *i.e.*, BOPF and BT (Class I), BM and BT II (class II), PF III, DUST III, BTL, and BBL (Class III) have low activity with inhibited percentage < 45%. Allopurinol, which is used as a standard in this research inhibit XO activity by 92.76 ± 1.37%. Based on those

result, it can be concluded that there is no positive correlation between catechin content and XO inhibitory activity of those grades of orthodox black tea.

Beside having antioxidant activity, number of publications mention that tea can also promote health by induce immune system [23]. In order to determine the potency of those grades of orthodox black teas in immune system, carbon clearance test was carried out in Swiss Webster mice. Through this test, it can concluded that immune system can only stimulated by certain grades of this orthodox black tea. Phagocytic Index of reticulum endothelium system of mice received orthodox black tea are exposed in the Table 3.

As can be seen on Table 3, PF, BOP, BP and Dust (Class I) exhibited high PI *i.e.* 1.41, 1.38, 1.36 and 1.28, respectively. Based on it PI, this types of orthodox black tea shown moderate immunostimulating activity. While BT (Class I), PF II and BP II (Class II), Dust III, BTL and BBL (Class III) had no effect on immune system indicated by its PI < 1.00. These results showed that the class I type of orthodox black tea have higher activity on immune system compared to the other two classes. Our previous study of these fifteen grades of orthodox black teas on *1,1-diphenyl-2-picrylhydrazyl* (DPPH) scavenger activity showed similar result with the effect of orthodox black tea on immune system, *i.e*. the class I type of orthodox black tea gave highest scavenger activity. This result indicated that the catechin content of class I of orthodox black tea responsible for immunostimulating and DPPH scavenger activities. While for XO activity are suggest not because of catechin content, however base on other compound. Data on Table 2 exposed that BOPF and PF which is contained highest amount of catechin (10,13 and 10,18 %, respectively) shown XO inhibitory activity lower than BOP (8,6 %) and BP (6,27%) that contained lower amount of catechin. Beside that, BTL which is contained 7, 5% of catechin showed the lowest XO inhibitory activity (Inhibitory effect: 11,25%).

Based on those results, for XO activity only BOP, PF and BP (Class I) were potential to be develop further as an XO inhibitor, while for enhance immune system and as an antioxidant can be used orthodox black tea class I group *i.e*., BOP, PF, Dust, and BP.

Table 3: Phagocytic index (PI) of fifteen grades of Indonesia orthodox black tea

Class	Group	Grade	Phagocytic index
	1	BOP	**1.38**
I	2	BOPF	1.02
II	3	PF	**1.41**
III	4	Dust	**1.28**
	5	BP	**1.36**
	6	BT	0.99
	7	PF II	0.93
	8	Dust II	1.12
	9	BP II	0.13
	10	BT II	1.18
	11	PF III	1.16
	12	Dust III	0.87
	13	BM	0.40
	14	BTL	1.03
	15	BBL	0.95
	16	Zymosan A	1.41
	17	Methyl Prednison	0.26

CONCLUSION

Among the fifteen grades of Indonesia orthodox black tea, only BOP, PF, and BP (Class I) are potential to be further develop as medicine for enhance immune system and as a XO inhibitor.

ACKNOWLEDGEMENT

This study was supported by Beasiswa Pendidikan Pasca Sarjana (BPPS) Scholarship, Education Ministry of Indonesia Government. The authors would acknowledge to management of Research Institute for Tea and Cinchona, PT. Cahaya Karunia Persada, Pharmaceutical Biology and Pharmacology Research Groups, School of Pharmacy, Bandung Institute of Technology, for the facilities used.

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