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Research Article

Anti-oxidant and Aldose Reductase Inhibitory Activity of *Piper betle* Extracts

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Abstract: *Piper betle*, known as daun sirih, is one of popular jamu ingredients which could be consumed freshly from the natural product resources by Indonesian people as traditional medicine. The present study is the primary article on human aldose reductase inhibition and also antioxidant activity of *P. betle* leaves extracts. The ethanol extract of *P. betle* exhibited the most inhibitory activity of the aldose reductase enzyme among extracts. It was discovered to present an IC50 value of 18.8 µg/mL for in vitro human aldose reductase and showed antioxidant activity by ORAC assay with value of 3861.2 ± 451.0 µmol Trolox Equivalent/g extract. Further investigation on the chemical components of the ethanol extract showed a total of 14 compounds by GC-MS analysis. The major compounds were bisphenol A (13) (34.4%) isoxylic acid (3) (13.8%), *trans*-phytol (11) (6.6%) and octadecyl aldehyde (14) (6.4%). These results implied that *P. betle* leaves should be prospective as an aldose reductase inhibitor.

Keywords: *Piper betle,* Jamu, Natural products, Aldose reductase, Antioxidant.

1. INTRODUCTION

Aldose reductase (alditol: $NAD(P)^+$ 1-oxidoreductase) is recently known to work as a key player in the polyol signalling pathway. The enzyme converted the reaction of glucose to sorbitol, while sorbitol leads to the development of long term diabetic complications [1]. To overcome this phenomenon, several potential aldose reductase inhibitors have been practiced both from natural [2, 3] and synthetic one [4, 5].

The use of Indonesian traditional medicines has been expanded recently. Some of our papers related with biological activities of some Indonesian traditional medicines have been reported [6, 7, 8]. In some region in Indonesia, people have used *P. betle* as a health supplement for avoiding from obesity, ulcer, toothache, as well as dental healthy [9]. In addition, the leaf of *P. betle*, also known for having a strong pungent aromatic flavour, is the best traditional medicines for female health and vitality [10].

P. betle leaves are credited with many properties. In the past few years, *P. betle* was also reported for its biological activity such as antigiardial [11], antibacterial [12, 13, 14], antifungal [15, 16], cytotoxic [17], antifertility [18], antibiofilm [19], anti-atherogenic [20], anti-inflammatory [21], also antidiabetes [22, 23], and antioxidant [24, 25, 26]. The phenolic compounds, for instance allyl pyrocatechol, from the leaves prevented halitosis activity [27]. *P. betle* ethanol extract decreased both histamine and GM-CSF by a hypersensitive response significantly. Besides, the ethanol extract inhibited secretion activity by a TNF-α and IL-4-induced allergic reaction [28]. *P. betle* leaves also demonstrated the effect hepato-protective significantly and upgraded the tissue antioxidant activity by rising the non-enzymatic antioxidants levels. In addition, free radical-detoxifying enzymes activity of ethanol-treated rats in liver was also increased [29]. The other report presented

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that both hot water and cold ethanol extracts of leaves of *P. betle* reduced the blood glucose level significantly by oral administration of diabetic rats [23]. Furthermore, Siddiqui, *et al*., (2012) reported that *P. betle* extracts could be a potential agent of membrane bio-fouling aspect [30].

In the few past decades, there were studies about antioxidant and anti-diabetes also. However, this study reported from a different new approach. The previous study reported that *P. betle* has a good antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [14, 16, 24, 25, 26] together with the other reactive radicals such as thiobarbituric acid reactive substances (TBARS), nitric oxide (NO), hydroxyl or superoxide radicals. However, there is no report about free radical scavenging activity by ORAC yet. In addition, there is a little report about anti-diabetes of *P. betle*. The previous report showed that *P. betle* aqueous and ethanol extracts have an inhibitory activity on streptozotocin (STZ)-induced diabetic rats [22, 23]. From these report, *P. betle* is a potent to do further investigations regarding its biological activity as an aldose reductase inhibitor. In this study, aldose reductase inhibition of *P. betle* leaves extracts together with free radical scavenging activity of these extracts had been reported.

2. MATERIALS AND METHODS

2.1 Sample and Chemicals

P. betle leaves were purchased from Pasar Genteng, one of traditional markets in Surabaya, Indonesia. The dried powder leaves of *P. betle* were extracted for 24 hours using *n*-hexane, dichloromethane, methanol, and ethanol at 25 °C. The dissolved solvent was evaporated by rotary vacuum evaporator to afford each extracts. β-NADPH was purchased from Oriental Yeast Co., Ltd. DL-glyceraldehyde was obtained from Wako Pure Chemical Industries, Ltd. Human recombinant aldose-reductase (HRAR) was purchased from AT Gen Co., Ltd. All other chemicals are analytical grade or high purity commercially obtainable.

2.2 The Extraction with Solid Phase Micro (SPME)

We purchased polydimethylsiloxane fibers that have been coating with length of 1 cm and 100 **µm** film thicknesses from Supelco (Bellefonte, PA, USA). At 250 **°C the** fibers were prepared for 1 hour in the gas chromatograph inlet before use. By using a manual SPME container, the fibers were positioned for ready to use. By injecting the SPME penetrating needle across the foil and subjecting the headspace of fibers over the sample, the adsorption of the chemical components was obtained for 30 minutes. Once sampling was done, for desorption and analysis process, the fibers were directly transported to gas chromatograph's port of inlet.

2.3 Chemical Constituents Identification

The identification of chemical constituents was accomplished by means of Shimadzu QP-5050 gas chromatograph/mass spectrometer (GC/MS) from Kyoto, Japan. We used DB-5 with a fused and attached silica capillary column, with film length, 30 m; thickness, 0.25 µm; i.d., 0.25 mm; which produced by Agilent. Helium was used as carrier gas, with a 100 kPa column head pressure. The chemical constituents were desorbed on SPME in a split-less injector at 250 °C. The program of oven temperature was initiated for 5 min at 40 °C and enlarged with a slope of 3 °C/min until 300 °C continue by 300 °C for 10 min. Finally, The MS data were linked and comparing with the NIST62 MS library to identify the chemical constituents.

2.4 Aldose Reductase Assay

The activity of Human Recombinant Aldose Reductase (HRAR) was examined on a UV/VIS spectrophotometer, JASCO V-530 - Japan. The HRAR activities were determined conferring to our previous method [3]. The percentage of inhibitory activity (%) was calculated as this equation: [1-(Δ*A* sample/min - Δ*A* control/min)]×100. Δ*A* sample/min exposed a diminution of absorbance with a sample for 1 min and Δ*A* control/min with dimethyl sulfoxide (DMSO) instead of a sample. The determination of reaction were started with mixture of 10mM dl-glyceraldehyde, 0.15mM β-NADPH, 100 µl of tested sample solution on DMSO and 5 µl of HRAR, 100mM sodium phosphate buffer (pH 6.2) in a total volume 1.0 ml. Afterward the reaction mixes, the incubation at 25 ºC were performed for 5 min, then the reaction was initiated by adding HRAR, and later the reduction of absorbance at λ

340 nm was examined using a JASCO V-530 UV/VIS spectrophotometer for 10 min. Each plant extract was liquefied in DMSO at less than a 1% concentration which have no enzyme activity.

2.5 Oxygen radical absorbance capacity (ORAC) Assay

The ORAC assay was conducted based on previously described procedures [31, 32] but with slight modifications. *P. betle* extracts were pre-treated with DMSO with concentration of less than 0.1 % then dissolved in 75 µM phosphate buffer in pH of 7.4. After that, 20 µL of sample, buffer, and trolox solutions were added into tube wells, respectively. Next, 200 µL of fluorescein solution was added. After 10 min incubation at 37 °C, 75 µL of 2,2'-azobis(2-amidino-propane) di-hydrochloride (AAPH) working solution was also injected. Finally, fluorescence degradation was measured over 90 minutes. Every 30 second interval was measured by using Molecular Devices Flex Station 3 microplate reader. The excited and emission wavelengths were 485 nm and 535 nm, respectively. The result data were managed by Soft Max Pro 5.4.1. The minimum and maximum concentrations of extracts in buffer were 6.25 and 50 µg/mL, respectively. In our assay system, trolox solutions with concentration of 6.25, 12.5, 25, and 50 µM were used to make the standard curve.

3. RESULTS

The extracts were prepared from the dried of *P. betle* leaves with maceration process for 24 h to yield *n*-hexane, dichloromethane, methanol and ethanol extract. The inhibitory activity of HRAR of each extracts is shown in Fig.1.

Ethanol extract of *P. betle* showed the highest inhibition among extracts at concentrations of 100 µg/mL. The methanol extract exposed some inhibition, but it was fewer than that of the ethanol extract. The dichloromethane was discovered to be somewhat more effective than that of the *n*-hexane extract. In the present study, quercetin was used as a positive control which is known as a naturally occurring HRAR inhibitor, and in our assay system exhibited an IC_{50} of 2.9 µg/mL. We determined the inhibitory activity of HRAR of the ethanol extract of *P. betle* (Fig. 2) and it displayed the dose dependently (IC_{50} = 18.8 µg/mL) inhibitory activity. These results indicated that ethanol extract of *P. betle* can constrain the progression of *in vitro* HRAR.

The results of ORAC assays of *P. betle* extracts are shown in Table 1. ORAC values (µmol TE/g

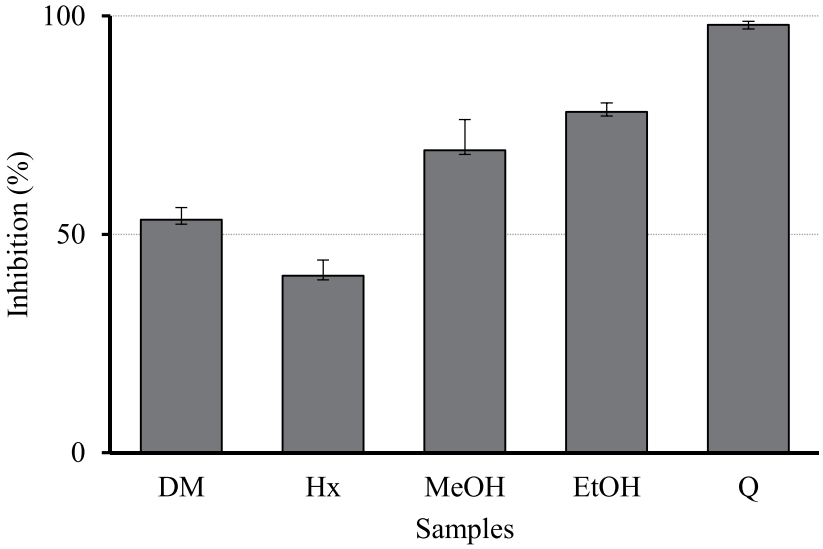

Fig. 1. Aldose reductase inhibitory activity of extracts of *P. betle* at a concentration of 100 µg/mL. DM is dichloromethane extract; Hx is *n*-hexane extract; MeOH is methanol extract; EtOH is ethanol extract; and Q is quercetin (positive control). Each column represents the mean ± SD, n = 3.

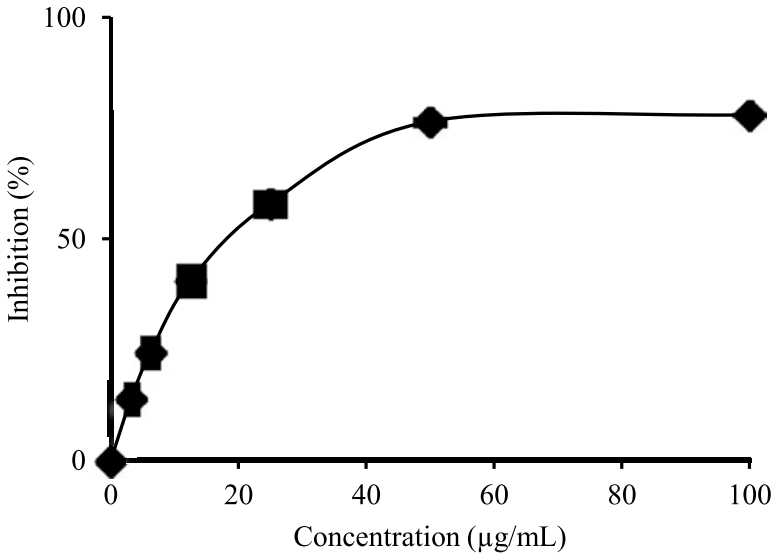

Fig. 1. The effect of *P. betle* ethanol extract on aldose reductase.

Table 1. ORAC values of *P. betle* extracts.

Samples	ORAC Values (µmol TE/g extract)
n-Hexane extract	832.4 ± 244.1
Dichloromethane extract	2343.2 ± 421.4
Ethanol extract	3861.2 ± 451.0
Methanol extract	4107.3 ± 487.6

Table 2. Chemical composition of *P. betle* ethanol extracts with SPME.

Components	R.T (min)	%*
Chavicol(1)	28.1	2.3
Isoeugenol(2)	34.2	2.0
Isoxylic acid (3)	38.9	13.8
α-curcumene(4)	39.2	3.5
1,1'-[1-(2,2-Dimethylbutyl)-1,3-propanediyl]biscyclohexane(5)	39.9	1.4
Cinnamyltiglate(6)	46.6	2.8
(3E,7E)-10-Isopropenyl-3,7-cyclodecadien-1-one (7)	47.8	1.3
Scobenol(8)	50.3	2.7
Hexadecanoic acid (9)	57.2	1.8
Ethyl pentadecanoate(10)	58.3	1.7
trans-Phytol(11)	62.1	6.6
13-Tetradecenal (12)	63.8	1.1
Bisphenol A (13)	63.9	34.4
Octadecyl aldehyde (14)	65.3	6.4

*area percentage

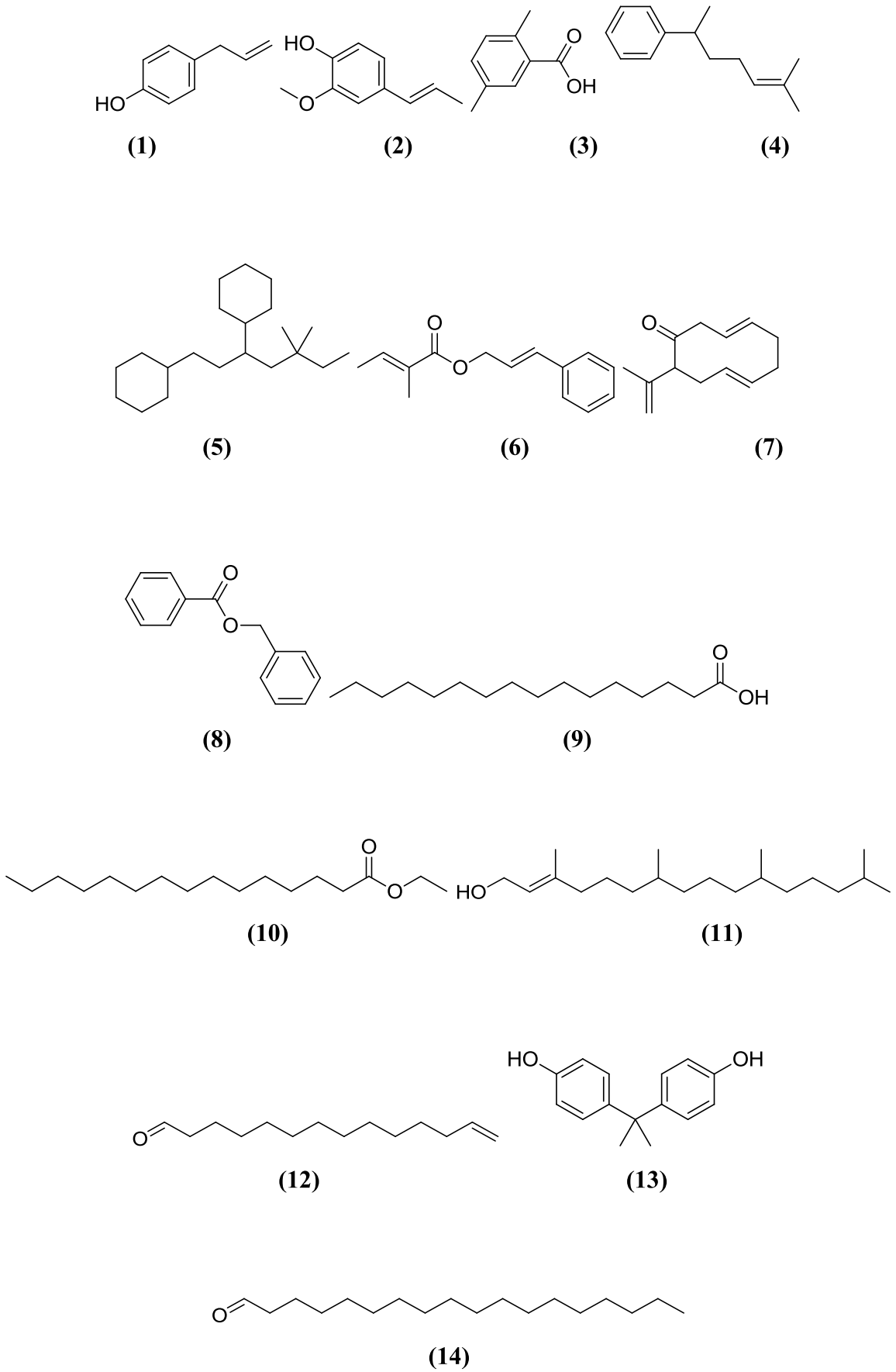

Fig. 3. GC-MS based chemical composition analysis of the *P. betle* ethanol extracts showed a total of 14 compounds

extract) of ethanol and methanol extract were 3861.2 and 4107.3, respectively. Solid-phase micro extraction was used to identify chemical composition in ethanol extract of *P. betle*. GC-MS based chemical composition analysis of the *P. betle* ethanol extracts showed a total of 14 compounds presented in Figure 3 and summarized in Table 2. The major compounds were bisphenol A (13) (34.4%) isoxylic acid (3) (13.8%), *trans*-phytol (11) (6.6%) and octa-decylaldehyde (14) (6.4%).

4. DISCUSSION

This present report confirmed the HRAR inhibitory activity of *P. betle* extracts for the earliest time. *P. betle* is one of Piperaceae famili mostly consumed in Asia [33]. This plant, known as daun sirih, could be found in some Indonesia traditional markets. Related to this study, the four extracts of *P. betle* were founded for their inhibitory activity on HRAR at a minimum concentration of 100 µg/mL for each of the extracts. In our assay system, quercetin, as known as a potent aldose reductase inhibitor, was used as a standard. The results showed that the ethanol extract is the most effective to inhibit aldose reductase enzyme. As a *like dissolved like* concept, the ethanol has a polar side to extract the polar compounds from *P. betle*. They might be called as aldose reductase inhibitor. On the other hand, an ethanol extract of *P. betle* also reported significantly lowered the blood glucose level on STZ-induced diabetic rats [23, 34]. Further investigation of ethanol extract *P. betle* also was reported by ORAC values. The results presented the ethanol extract has a fine amount of µmol TE/g extract. It should be notable that both HRAR inhibition and the ORAC value of the ethanol extract of *P. betle* were almost the alike as that of the methanol extract. Furthermore, these results indicated that here gave a linear correlation among aldose reductase inhibitory activity and free radical scavenging.

Aldose reductase enzyme is frequently used to *in vitro* antidiabetic assay model [3, 6, 7]. This enzyme, as the first enzyme in polyol pathway, is catalysed glucose to sorbitol. For diabetic disorders, a hyperglycaemic condition will be activated the polyol pathway highly. These conditions made more sorbitol's produce. Unfortunately, a high accumulated sorbitol caused the diabetic complications [1] such as cataracts, neuropathy and nephropathy. Literally, sorbitol could be converted to fructose by sorbitol dehydrogenase then to be fructose-6-phosphate catalysed by hexokinase. Thus, fructose-6-phosphate could be used for further metabolism circle to produce an energy namely glycolysis. But these metabolism circles do not work as simple process as well, when a hyperglycaemic condition caused a high affinity of aldose reductase. However, a hyperglycaemic will inhibit the activity of sorbitol dehydrogenase, hexokinase as well as NADPH as the main body cofactor. Certainly, many aspects in the metabolism system concern for more investigation.

Based on aldose reductase inhibition and ORAC values results, further experiments were focused on chemical composition of ethanol extract. The chemical compositions were determined by SPME connected to GC-MS identification. SPME is a solid phase extraction method [35]. This is a recommended method for extraction because it is simple, fast and solvent less [31]. After extraction, the SPME fiber is transferred to the inlet port of GCMS instrument. In addition, the GCMS is a good choice instrument for the chemical identification of *P. betle* because most of chemical compositions of this plant are the volatile oil components. The major components of ethanol extract of *P. betle* typically have hydroxyl group and/or carboxyl group which are important group as aldose reductase inhibition [36].

5. CONCLUSIONS

In conclusion, this paper presents a primary study on *P. betle* leaves for the inhibition of HRAR and free radical scavenging by using ORAC assay. Among extracts, ethanol extract showed the uppermost inhibitory in contradiction of HRAR activity, and it was applicable in reducing free radical scavenging by the ORAC assay. Advance examinations will emphasis on the isolation of the bioactive constituents dependable for the HRAR inhibitory effects and antioxidant of *P. betle* ethanol extract.

6. ACKNOWLEDGEMENTS

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