Research Article

Antioxidant activity of *Phyllanthus niruri* L. herbs: in vitro and in vivo models and isolation of active compound

Muhammad Da'i, Arifah Sri Wahyuni, Ika Trisharyanti DK, Tanti Azizah, Andi Suhendi, Azis Saifudin

Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, Indonesia..

Correspondence to: Muhammad Da'i, E-mail: Muhammad.Dai@ums.ac.id

Received October 5, 2015. Accepted October 15, 2015

Abstract

Background: *Phyllanthus niruri, Thymus vulgaris, Centella asiatica*, and *Apium graveolens* L. have been frequently used as medical herbs for various diseases. However, the use of the herbs is not based on any evidence of their contents. **Aims and Objective:** To examine their potential activity of becoming antioxidant, using in vitro and in vivo models and isolate active compound. **Materials and Methods:** The antioxidant activity of the samples was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as radical compounds. The decrease of DPPH radical was measured using spectrophotometer at 517 nm, after an incubation for 30 min. The highest antioxidant activity of the sample was then continued with in vivo model using Sprague Dawley (SD) rats. Paracetamol was used as an inducer to increase the malondialdehyde (MDA) levels in plasma, with doses of 2.5 g/kg BW. The rats were divided into four groups: negative control, dose of 50 mg/kg BW, dose of 100 mg/kg BW, and dose of 200 mg/kg BW. Isolation method was guided by antioxidant activity by using fractionation and column chromatography. The obtained compound was then characterized by 1H-nuclear magnetic resonance (NMR) and compared with the earlier study. **Result:** The IC_{50} value of these samples were 14.21 ± 0.73; 14.41 ± 1.13; 98.66 ± 9.59; and 237.33 ± 17.26 μg/mL, respectively, and the total phenolic contents were 81.59 ± 2.85; 154.09 ± 11.61; 6.36 ± 3.99; 2.03 ± 0.78 mg/g of sample extract, respectively. The in vivo study showed that *P. niruri* with dose of 100 mg/kg BW and 200 mg/kg BW were able to reduce MDA levels by 90.44% and 92.64%, respectively. Meanwhile, the proton and carbon NMR spectra showed that the isolate was quercetin. The IC_{50} value of the isolate, being 5.85 μg/mL, was lower than that of vitamin E, which was 6.85 μg/mL. **Conclusion:** The extract shows a potential effect with antioxidant activity, both in in vitro and in vivo models. Quercetin is the active compound, the antioxidant activity of which is higher than that of vitamin E.

KEY WORDS: Antioxidant; Phyllanthus niruri L.; Quercetin

Introduction

Free radicals have one or more electrons that are not paired in the outer orbitals[1] and produced under normal circumstances of metabolic processes.[2] Free radicals can damage proteins, lipids, carbohydrates, and nucleic acids.[3] Over longer terms, some damages can lead to degenerative diseases.[4] In diabetic patients, for example, hyperglycemia causes glucose auto oxidation, glycation of proteins, and activation of the polyol metabolic pathway that will accelerate the formation of reactive oxygen species (ROS).[5] This will result in an imbalance between antioxidants in the body and free radicals, which is the beginning of oxidative damage, known as oxidative stress. The risk of the disease can be minimized by the use of antioxidant compounds that can stabilize radicals.[6,7]

Obviously, the sources of antioxidants are natural and synthetic antioxidants. The Indonesian people have used a variety of medicinal plants to heal various diseases. A previous

Access this article online	
Website: http://www.njppp.com	Quick Response Code:
DOI: 10.5455/njppp.2015.5.0510201575	

study on 23 medicinal plants in Indonesia found four plants (*Phyllanthus niruri*, *Thymus vulgaris*, *Centella asiatica*, and *Apium graveolens*) that are potential as antioxidant agents, the activity of which is better or equal to that of vitamin E.[8] These plants contain alkaloids, flavonoids, lignin, tannins, coumarin, polyphenols, saponins, and terpenoids.[9] Active compounds that may work as antioxidants are tannins and[10] alkaloids[11] and phenolic components, such as phenolic diterpene, phenolic acids, and flavonoids.[12] Owing to reducing properties, the antioxidant activity of the phenolic content can stabilize singlet oxygen, capture free radicals, electron donors, and chelating metals.[13]

Therefore, this study was conducted to explore the potential of antioxidants from those four medicinal plants that have the best antioxidant activity based on in vitro and in vivo models, isolate the active compounds, and characterize the structure by proton and carbon nuclear magnetic resonance (NMR). The antioxidant activity is generally contributed by the phenolic content of the extract. The isolate was assessed through antioxidant activity and compared with vitamin E as an established antioxidant agent.

Materials and Methods

The laboratory equipment used for this study was glassware (Pyrex), analytical balance (Ohaus), minispin (Eppendorf), centrifuge (Centrifuge PLC Series), Spectrophotometer uv-vis (Shimadzu 1140), ultrasound (Branson), and NMR (JEOL).

The materials used in this study consisted of *P. niruri* L., *T. vulgaris*, *C. asiatica*, and *A. graveolens* (local market), rats Sprague Dawley (SD) strain, paracetamol 2.5 g/kg BW (Pharmaceutical grade, Brataco Chemica), TMP-X (Sigma Aldrich), TCA 20% (Merck), and TBA 0.67% (Sigma Aldrich, T5500), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Merck), ethanol (Merck), gallic acid (Merck), Follin–Ciocalteu (Merck), Na_2CO_3 (Merck), Vitamin E (Sigma Aldrich), and plate sillica gel GF_{254}. This study was approved by Health Research Ethics Committee of Faculty of Medicine, Universitas Muhammadiyah Surakarta.

Extraction

The extraction process was performed by maceration using ethanol 96% as a solvent with a 1:7 ratio. Liquid extract was evaporated using a rotary evaporator and then heated in the water bath until a thick extract was formed.

Determination of Antioxidant in In Vitro Model

Seven hundred μL of DPPH solution (400mM) plus 1.5 mL of sample solution of various concentrations were then left in the dark for 30 min and then read at a wavelength of 517 nm. The blank used was 1.5 mL sample solution added by ethanol up to 5.0 mL, while the control used was 700 μL DPPH solution added by ethanol up to 5.0 mL. Inhibition concentration (IC_{50}) was the concentration of extract or fraction that gave antioxidant activity of 50%.

Determination of total phenolic contents

The total phenolic content was determined using the Folin–Ciocalteu reagent according to the method of Waterhouse.[14] The reaction mixture consisted of 100 μL of extract and 100 μL Folin–Ciocalteu reagent. The mixture was kept in darkness for 5 min, then combined with 75 μL of sodium carbonate and made up to 5.0 mL of aquadest. In order to complete the reaction, the mixture was kept in darkness for 45 min at room temperature. The absorbance at 742.5 nm was measured using a UV-Vis spectrophotometer (Shimadzu Corp.). Gallic acid was used as a standard, and the result was expressed as gallic acid equivalent (GAE)/g extract.

Determination of Antioxidant In Vivo Model[15]

The hepatoprotective activity of *P. niruri* extract was determined with hepatotoxic rat model using paracetamol as the inducer. After 7 days of acclimatization, the rats were classified into four groups of five. The treatments were done for 8 days. Group I served as the normal control and received only aquabidest (0.4 mL/kg BW) for 8 days. The group II served as the toxic control and was administered with paracetamol suspension (2.5 g/kg BW, po) once in every 72 h. Groups III until V were administered with extract (50; 100; and 200 mg/kg BW, po) daily and paracetamol suspension (2.5 g/kg BW, po) once in 5 days. About 1 mL of blood was collected from vena lateralis of rats on days 0, 5, and 8. The blood samples were centrifuged at 3,000 rpm at room temperature to separate serum and used to estimate the MDA levels.

Determination of malondialdehyde (MDA)[16] was used to evaluate lipid peroxidation in tissue samples. A volume of the serum (0.10 mL) was transferred to a vial and mixed with 2.45 mL of 20% (w/v) TCA solution and 2.45 L of a 0.67% (w/v) solution of TBA, and the final volume was adjusted to 5.0 ml with distilled water. Each vial was tightly capped and heated in a boiling water bath for 20 min. The vials were then cooled to ambient temperature. The absorbance of supernatant was measured at 532 nm (Shimadzu, UV Mini). The calibration curve used TMP-X as the standard, and the result was converted to MDA. The series of standard were 0.16; 0.33; 0.66; 1.32; and 2.64 μg/mL of TMP-X.

Isolation and Characterization

Isolation of active compound of *P. niruri* was conducted by guiding antioxidant in in vitro models. The extract of *P. niruri* was fractionated to obtain *n*-hexene fraction, ethyl acetate fraction, and residues fraction. All fractions obtained were then assessed with antioxidant activity. Then, ethyl acetate fraction, which has a potential activity, was fractionated with vacuum column chromatography. Stationary and mobile phases used were silica gel (Merck 7734) and a mixture of $CHCl_3$:*n*-hexene (8:2), and $CHCl_3$:methanol (9:1, 8:2, 7:3, 6:4, and 5:5). Elution was performed three times with volume of each mobile phase of 350 mL. The volume of the collected fraction was 130 mL. Altogether, 58 fractions were obtained. On the basis of the thin

Table 1: Value of IC_{50} and total phenolic content (n = 3)

Samples	IC_{50} (μg/mL)	Classification[17]	Total phenolic content GAE (mg/g extract)
P. niruri	14.21 ± 0.73	Active	81.59 ± 2.85
T. vulgaris	14.41 ± 1.13	Active	154.09 ± 11.61
C. asiatica	98.66 ± 9.59	Medium	6.36 ± 3.99
A. graveolens	237.33 ± 17.26	Not active	2.03 ± 0.78
Vitamin E	12.55 ± 2.519	Active	–

layer chromatography (TLC) examination, the same profiles of fractions were then combined.

After TLC examination, nine subfractions were collected, and, then, the antioxidant activity was determined. The highest antioxidant activity of subfraction, then fractionated by flash chromatography, silica G 60 (Cat Number 7731) as stationary phase and mixtures of *n*-hexene:ethyl acetate (4:1; 3:2; 2:3; and 1:4), $CHCl_3$:ethyl acetate (4:1; 3:2; 2:3; and 1:4), and $CHCl_3$: methanol (7:3) as the mobile phase. Elution was performed three times, each using 200 mL. Fraction was collected; the separation profiles were then evaluated by TLC. The same profiles combined resulted in four subfractions, and, then, the antioxidant activity was assessed. Owing to the impurity content of subfraction, the purification was performed by preparative RP-TLC (Silica-C18, as a stationary phase and mixtures of acetonitrile:methanol:water = 1:1:1 as mobile phase). Separated subfraction was then cleaned up using flash chromatography [methanol:$CHCl_3$ = 3:7, as mobile phase, and silica G 60 (Cat Number 7731), as stationary phase]. The isolate characterized by proton NMR (JEOL, 500 MHZ), $CDCl_3$ was used as a solvent. Antioxidant activity of isolate was assessed and compared with vitamin E.

Result

Screening Antioxidant Activity

Potential antioxidant activity was classified based on the IC_{50} value.[17] The result showed that the antioxidant activity of *P. niruri* and *T. vulgaris* showed similar values, around 14 μg/mL, which were close to that of the vitamin E [Table 1]. The other extracts are not active as an antioxidant.

Antioxidant of In Vivo Models

The objective of in vivo models was to optimize the induction time of paracetamol as hepatotoxic inducer, which is administered per oral. Induction of hepatic may increase MDA levels in serum. As shown in Table 2, the optimum time to measure the MDA levels is 72 h after induction. The increase of MDA is almost double of the first time, about 62.88 ± 12.38 μg/mL.

Paracetamol metabolite, N-acetyl-p-benzoquinone imine (NAPQI) is a reactive electrophilic that induces tissues impairment,[18] indicated with the increase of MDA levels in the serum.[19]

Isolation and Characterization of Active Compound

The result shows that the rendemen of nonpolar fraction is the lowest, while residue is the highest [Figure 1]. This indicates that the chemical components of the extract are mainly polar and semipolar compounds.

Characterization of Isolate 4 by proton NMR

The spectra of proton MNR show five peaks [Figure 3A]. These peaks at 7.728 ppm (d,J = 2.6 Hz) represent proton position at 2'; 6.879 ppm (d,J = 10.60 Hz) represent proton at 5'; 7.617 ppm (d,J = 10.65 Hz, 2.65 Hz) represent proton at 6'; at 6.384 ppm (d,J = 2.5 Hz) represent proton at 6'; and 6.177 ppm (d,J = 2.5 Hz) represent proton position at 8' [Figure 2].

Discussion

The antioxidant activity of *P. niruri* and *T. vulgaris* is caused by phenolic contents. The activity of *T. vulgaris* and *P. niruri* is almost similar [Table 1], although the phenolic content of *T. vulgaris* is almost double that of *P. niruri*. The correlation of antioxidant activity and phenolic contents is not high, with the coefficient constant of only 0.61. It can be concluded that the antioxidant activity is caused not only by phenolic contents but also by other compounds such as polyhydroxy flavonoids, cumarin, ferulic acid, carotenoid, quinone, saponin, alkaloid,

Table 2: MDA content after induction of paracetamol (2.5 g/kg BW)

Groups	MDA content (μg/mL) postinduction			
	0 h	24 h	48 h	72 h
Normal control	34.52, (n = 2)	27.29 ± 18.88, (n = 3)	16.11 ± 2.05, (n = 3)	27 ± 6.70, (n = 4)
Negative control	0, (n = 5)	23.66 ± 4.12(n = 5)	26.63 ± 3.51, (n = 4)	62.88 ± 12.38, (n = 4)

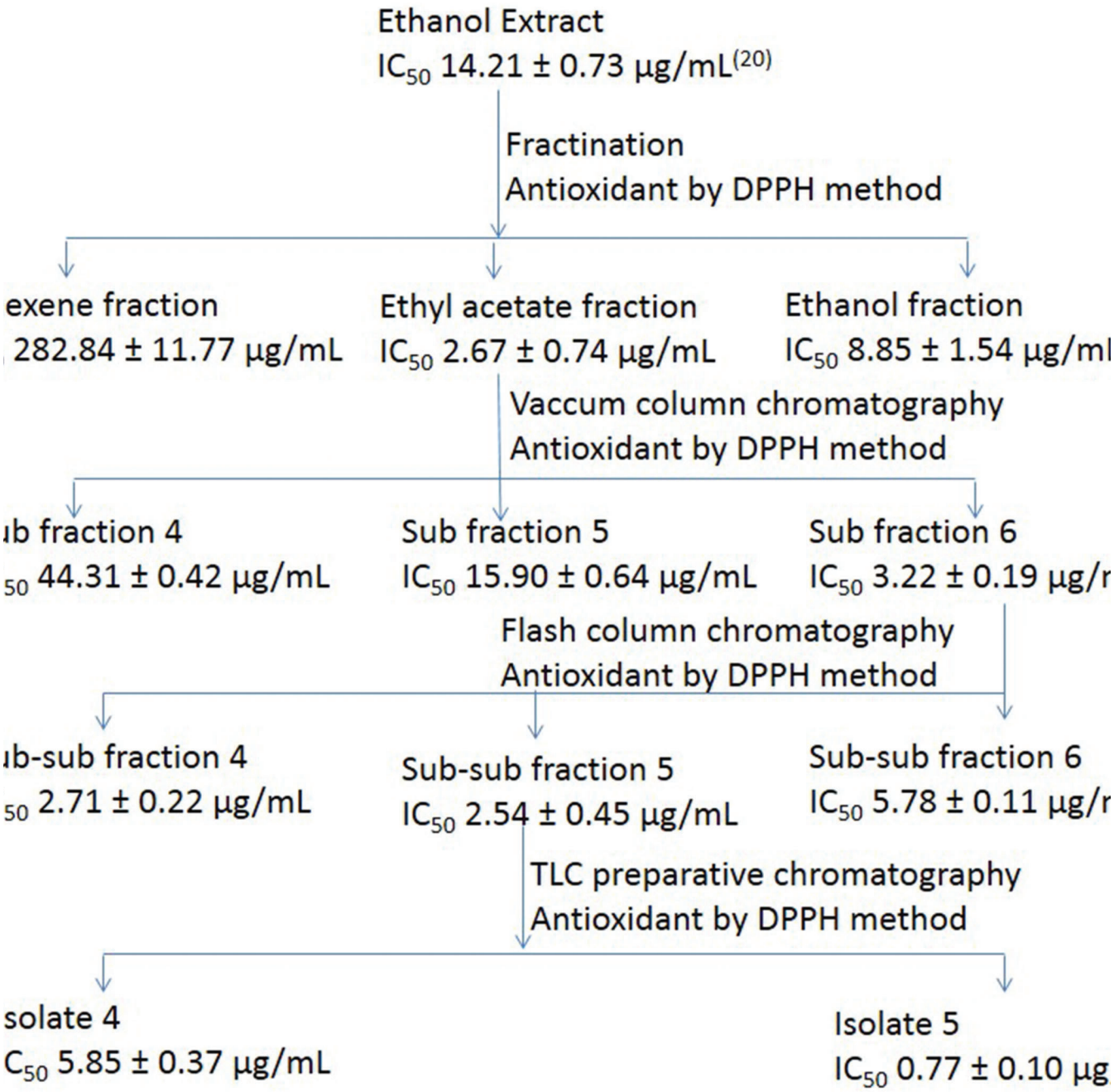

Figure 1: Scheme for isolation of antioxidant compound from extract *P. niruri*.

and tanins. The differences of antioxidant activity can be the influence of several factors, such as the solvent of extraction, chemical composition of samples, and determination methods.[21]

Evaluation of antioxidant of in vivo models was conducted by referring to the activity of previous study on antioxidant and antidiabetic effects of *P. niruri*.[22] The data [Table 3] shows that *P. niruri* presents an antioxidant activity that is characterized by the decrease of MDA levels in the serum. The decrease of *P. niruri* dose of 100 and 200 mg/kg BW was almost close. This study also shows a similar result[23] that *P. niruri* presents an antioxidant

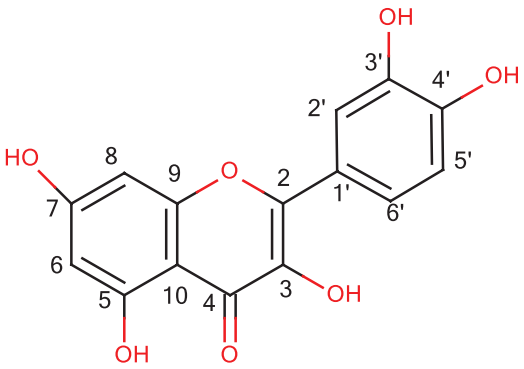

Figure 2: Structure of quercetin.

activity using in vivo model. Chemical composition of *P. niruri*, such as transiphytol, 4-t-butylpyrocatechol, phyllanthin, and hypophyllantin can protect cytotoxicity hepatocyte culture.[24,25]

Isolation is guided by the antioxidant activity, initiated by redissolving the extract by using *n*-hexene (as nonpolar solvent), ethyl acetate (as semipolar solvent), and residue (as polar fraction).On the basis of the evaluation of the antioxidant, the candidate to be isolated was the ethyl acetate fraction. In order to obtain simple components in the fractions, gradient polarity mobile phase is used in the vacuum column chromatography. The fraction obtained classified based on the TLC profiles became nine subfractions. The assessment of antioxidant activity shows that group VI shows the highest activity [Figure 1]. On the basis of the results in Fig 1, the antioxidant activity of isolated compound are higher than vitamin E (IC_{50} = 6.85 μg/mL) which is known as an established antioxidant compound. Isolate 4 can be elucidated by using ^{1}H-MR and compared with the reference. The spectra of isolate [Figure 3A] are quite similar to the result of an earlier study (Fig. 3B).[26] Therefore, the isolate is quercetin.

Conclusion

P. niruri L. herb is a promising antioxidant herb that has the best DPPH radical scavenging activity when compared with *T. vulgaris, C. asiatica*, and *Apium graveolens* L.; meanwhile, the extract at dose 200 mg/kg BW could decrease MDA levels around 92.64%. Isolation guided by the antioxidant activity of *P. niruri* as a promising antioxidant herb showed the fractions

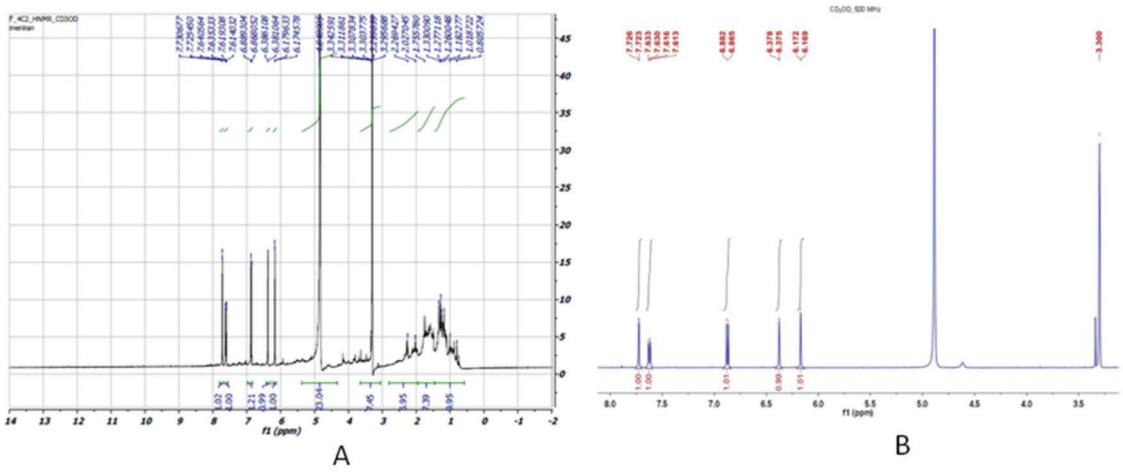

Figure 3: Proton NMR spectra of isolate from *P. niruri* (A) and quercetin (B)[26].

Table 3: MDA levels of groups treatments

Groups	MDA levels (μg/mL) days			Reduction MDA levels (%)
	0	5	8	
Negative control	ND, (n = 5)	36.34 ± 6.59, (n = 5)	81.65 ± 11.09, (n = 5)	–
P. niruri, 50 mg/kg BW	10.60 ± 1.50,(n = 3)	70.52, (n = 2)	32.27 ± 14.08*, (n = 3)	60.47
P. niruri, 100 mg/kg BW	ND, (n = 5)	1.58, (n = 2)	6.99*, (n = 1)	91.44
P. niruri, 200 mg/kg BW	ND, (n = 5)	22.55 ± 2.73, (n = 5)	6.01*, (n = 1)	92.64

ND = not detected.
*$p < 0.05$, compared with negative control group (one-way ANOVA, followed by a *t*-test)

of n-hexane, ethyl acetate, and ethanol, which showed values of IC_{50} such as 14.21 ± 0.73, 282.84 ± 11.77, 2.67 ± 0.74, and 8.85 ± 1.54 μg/mL, respectively. Quercetin was successfully isolated from the extract and showed a higher antioxidant activity than vitamin E.

References

1. Zou Y, Lu Y, Wei D. Antioxidant activity of flavonoid-rich extract of *Hypericum perforatum* L. in vitro. J Agric Food Chem. 2004;52 (16):5032–9.
2. Hariyatmi. Kemampuan Vitamin E sebagai Antioksidan terhadap Radikal Bebas pada Lanjut Usia. *MIPA*, Jurusan Pendidikan Biologi FKIP UMS. Surakarta. 2004;14(1):52–60.
3. Suirta IWNM, Puspawati, Gumiati NK. Isolasi dan Identifikasi Senyawa Aktif Larvasida dari Biji Mimba *(Azadirachta indika A. Juss)* Terhadap Larva Nyamuk Demam Berdarah *(Aedes aegypti).* J Kimia. 2007;1(1):47–54.
4. Temple NJ. Antioxidants and disease: more questions than answers. Nutrition Research. 2000;20(3):449–59.
5. Ueno Y, Kizaki M, Nakagiri R, Kamiya T, Sumi H, Oswa T. Dietary glutathione protects rats from diabetic nephropathy and neuropathy. J. Nutr. 2000;132(5):897–900.
6. Amrun MH, Umiyah Evi UU. Uji Aktivitas Antioksidan Ekstrak Air dan Ekstrak Metanol beberapa Varian Buah Kenitu (*Chrysophyllum cainito* L.) dari Daerah Jember Berkala Penelelitian Hayati. 2007;13:45–50.
7. Rosen P, Tritschler HJ, Packer L. Vascular complications in diabetes: mechanisms and the influence of antioxidants In:(Eds.) *Handbook of Antioxidants*, 2nd edn. , New York: Marcel Dekker, 2000. pp. 511–24.
8. Da'i M, Wahyuni AS, Kusumowati ITD, Setiawan D, Dhi'fi HJ, Suhendi A, et al. *The Corrrelation of Phenolic Content with Antioxidant Activity Five Types Indonesian Herbs* International Seminar on Translational Research in Cancer Chemoprevention2011.
9. Paithankar VV, Raut KS, Charde RM, Vyas JV. *Phyllanthus niruri*: a magic herb. Res Pharm. 2011;1(4):1–9.
10. Zhang LL, Lin YM. Antioxidant tannins from *Syzygium cumini* fruit Afr J Biotechnol. 2009;8(10):2301–9.
11. Alfarabi M, Bintang M, Suryani, Safithri M. The Comparative ability of antioxidant activity of *Piper crocatum* in inhibiting fatty acid oxidation and free radical scavenging. Hayati J Biosci. 2010;17(4):201–4.
12. Javanmardi J, Stushnoff C, Locke E, Vivanco JM. Antioxidant activity and total phenolic content of Iranian *Ocimum* accessions. Food Chem. 2003;83(4):547–50.
13. Karadeniz F, Burdurlu HS, Koca N, Soyer Y. Antioxidant activity of selected fruits and vegetables grown in Turkey. Turk J Agric For. 2004;29:297–303.
14. Waterhouse AL. Current Protocols in Food Analytical Chemistry. United States: John Wiley and Sons, 2000. pp. 11–8.
15. Sriningsih. Efek protektif pemberian ekstrak etanol herba meniran (*Phyllantus niruri* L.) terhadap aktivitas dan kapasitas fagositosis makrofag peritoneum tikus. Artocarpus. 2006;6(2):91–6.
16. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95 (2):351–8.
17. Reynertson KA. *Phytochemical Analysis of Bioactive Constituents from Edible Myrtaceae Fruit*, Dissertation, The City University of New York2007. pp. 70–4.
18. Corcoran GB, Racz WJ, Smith CV, Mitchell JR. Effects of N-acetylcysteine on acetaminophen covalent binding and hepatic necrosis in mice. J Pharmacol Exp Ther. 1985;232(3):864–72.
19. Josephy PD. Molecular Toxicology. , New York: Oxford University Press, 2007. pp. 97–104.
20. Widhihastuti E. Pengukuran Aktivitas Antioksidan dengan Metode DPPH serta Korelasinya dengan Kadar Fenolik pada Lima Jenis Herba Bahan Obat Alam Indonesia. Skripsi. Fakultas Farmasi Universitas Muhammadiyah Surakarta. 2011.
21. Widyastuti N. Pengukuran Aktivitas Antioksidan dengan Metode CUPRAC, DPPH, dan FRAP serta Korelasinya dengan Fenol dan Flavonoid pada Enam Tanaman. Skripsi. Fakultas MIPA Institut Pertanian Bogor2010.
22. Da'i M, Wahyuni AS, Kusumowati ITD, Suhendi A, Azizah T. Pemanfaatan Antioksidan Alami Pada Upaya Pencegahan Komplikasi Akibat Stres Oksidatif Pada Tikus Yang Diinduksi Diabetes Melitus, Laporan Penelitian LPPM UMS2012.
23. Shivanandappa T, Harish R. Antioxidant activity and hepatoprotective potential of *Phyllanthus niruri*. Food Chem. 2006;95(2): 180–5.
24. Shamasundar KV, Singh B, Thakur RS, Husain A, Kiso Y, Hikino H. Antihepatotoxic principles of *Phyllanthus niruri* herbs. J Ethnopharmacol. 1985;14(1):41–4.
25. Makoshi MS, Adanyeguh IM, Nwatu LI. Hepatoprotective effect of *Phyllanthus niruri* aqueous extract in acetaminophen sub-acutec exposure rabbits. J Vet Med Anim Health. 2013;5(1):8–15.
26. Foo LY, Lu Y, Molan AL, Woodfield DR, McNabb WC. The phenols and prodelphinidins of white clover flowers. Phytochemistry. 2000;54(5):539–48.

How to cite this article: Da'i M, Wahyuni AS, DK IT, Azizah T, Suhendi A, Saifudin A. Antioxidant activity of *Phyllanthus niruri* L. herbs: in vitro and in vivo models and isolation of active compound. Natl J Physiol Pharm Pharmacol 2016;6:32-37.

Source of Support: Nil, **Conflict of Interest:** None declared.