Trop J Nat Prod Res, December 2023; 7(12):5429-5435

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Antioxidant Activity and Anti-inflammatory Effect of Indian Borage Against Lipopolysaccharide-Induced Inflammation in Murine Macrophage (RAW 264.7) Cell Line

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ARTICLE INFO

Article history:
Received 09 August 2023
Revised 12 October 2023
Accepted 19 October 2023
Published online 01 January 2024

ABSTRACT

Indian borage (*Plectranthus amboinicus*) is a herb that has been reported to have numerous pharmacological activities including anti-inflammatory and antioxidant activities. The murine macrophage RAW 264.7 cell line is commonly utilized for anti-inflammatory drug screening. However, the anti-inflammatory effect of *Plectranthus ambonicus* ethanol extract (PaE) on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cell line has not been studied. This study is aimed at evaluating the antioxidant activity and anti-inflammatory effect of PaE in murine macrophage model. The anti-inflammatory activity was assessed via the inhibitory effect against NO production in RAW 264.7 cell line following LPS stimulation. The antioxidant activity of PaE was evaluated using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. The total flavonoid and phenolic contents were also evaluated using standard procedures. The antioxidant and anti-inflammatory activities of compounds in *Plectranthus amboinicus* were also predicted *in silico*. Results from the study has shown that PaE reduced nitric oxide production in LPS-stimulated RAW 264.7 cells. PaE also inhibited DPPH free radical and had a total flavonoid and phenolic contents of 2.04 ± 0.23 mgQE/g and 4.76 ± 0.03 mgGAE/g, respectively. From the *in silico* study, fifteen compounds from *Plectranthus amboinicus* were predicted to have strong antioxidant and anti-inflammatory activities. Thus, the findings from the present study has shown Indian borge as a plant with anti-inflammatory and antioxidant potentials. However, further investigation of the mechanism of action and the identification of its bioactive components is needed.

Keywords: Alternative drug, Anti-inflammatory, *Plectranthus amboinicus*, RAW 264.7 cells

Introduction

Free radicals are a byproduct of regular cellular energy production and functional activities, including cell signalling, gene expression, and ion transport.[1] One of such free radicals is nitric oxide (NO), which is involved in several physiological processes in the human body. However, excessive NO production causes inflammation-related tissue damage, and has been linked to increased risks of several inflammatory diseases, such as cancer, diabetes, atherosclerosis, and premature aging.[2,3] Therefore, the level of free radicals in the body must be minimized to reduce their harmful effects. In response to an irritant, such as injury or infection, the body elicits an inflammatory response, which is a normal process.[4] However, inflammation can become chronic, resulting in several illnesses, such as type 2 diabetes, autoimmune disorders, cancer, cardiovascular disorders, metabolic syndrome, and cardiovascular diseases.[5] An essential component in the pathophysiology of inflammation is the signalling molecule nitric oxide.[6]

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Citation: Puspitarini S, Dwijayanti DR, Wicaksono ST, Lestari ND, Rahayu RP, Nashi Widodo N. Antioxidant Activity and Anti-inflammatory Effect of Indian Borage Against Lipopolysaccharide-Induced Inflammation in Murine Macrophage (RAW 264.7) Cell Line. Trop J Nat Prod Res. 2023; 7(12):5429-5436. http://www.doi.org/10.26538/tjnpr/v7i12.10

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

A novel approach to inflammation treatment could be discovered via therapeutic strategy that inhibit inducible NO synthase.
In the mammalian immune system, macrophages play a crucial role by acting as an immediate line of defence against invaders before leukocytes migration and production of a variety of pro-inflammatory mediators, such as the transient free radical NO.[7] Lipopolysaccharides (LPS) of bacteria are one of the most potent macrophage activators that cause the production of cytokines that promote inflammation.[6] The RAW 264.7 cells, like macrophages, were created from a BALB/c mouse cell line transformed by the Abelson leukaemia virus. These cells produce higher nitric oxide levels after being stimulated with LPS. Therefore, measuring the reduction of NO synthesis after RAW 264.7 cells are stimulated with LPS can be used as a measure of the anti-inflammatory activity of bioactive compounds.[4,8,9]
Indian borage or *Plectranthus amboinicus* Lour. (*Coleus amboinicus* (Lour.) Spreng) is a herb that originated from Indonesia. This herb has been used as an ornamental plant and folk medicine.[10] The herb is used to treat various diseases in India, including malaria fever, cough, chronic asthma, bronchitis, and inflammation.[11] Phytochemical studies of *Plectranthus amboinicus* revealed that flavonoids, terpenoids, saponins, tannins, and volatile oils are constituents of this plant.[10,12,13] Flavonoids and other phenolic compounds of plant origin have been reported to act as free radical scavengers.[14,15] Although previous studies have reported several pharmacological activities of *Plectranthus amboinicus*,[2–6] but no study has reported on the effect of the ethanol extract of *Plectranthus amboinicus* (PaE) on NO synthesis in RAW 264.7 cells stimulated with LPS. Therefore, The objective of the study was to evaluate the antioxidant and anti-inflammatory activity of PaE via NO reduction in LPS-stimulated RAW 264.7 cells.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Materials and Methods

Collection of Plant Sample and Extraction

Dried leaves of *Plectranthus amboinicus* were bought from UPT Materia Medica Batu, East Java, Indonesia in April, 2021 with Bets No. 190822.DJTH.6.R.004. The dried leaves were powdered and dissolved in ethanol (SmartLab) (1:10 w/v) and subjected to a microwave-assisted extraction. The extract was filtered using filter paper (MN 713) and then concentrated with the aid of a rotary evaporator (Heidolph), followed by freeze drying to obtain a dry extract.

Determination of Total Flavonoids Content

Total flavonoids of PaE were quantified using the aluminium chloride ($AlCl_3$) assay using quercetin (MarkHerb) as the standard. The extract or standard was mixed with $AlCl_3$ (Smartlab) (10% w/v), followed by 150 µL of ethanol (SmartLab). Ten microliter (10 µL) of 1 M Sodium acetate (CH_3COONa) (Sigma) was added and the solution was kept in the dark at room temperature for 40 min. The absorbance of the resulting solution was measured using an ELISA reader (BioTek ELx808) at a wavelength of 405 nm. The total flavonoids content of PaE was estimated from the quercetin calibration curve and expressed as milligram Quercetin Equivalent (QE) per gram of Extract.[4,6]

Determination of Total Phenolic Content

The total phenolic content of PaE was estimated using the Folin–Ciocalteu assay with Gallic acid (GA) (MarkHerb) as the standard. The extract or standard was mixed with the Folin–Ciocalteu solution (Merck) (10% w/v) and incubated for 5 min. Thereafter, sodium carbonate (Na_2CO_3) (Merck) (7.5% w/v) was added, then the mixture was kept in the dark at room temperature for 90 min. The absorbance of the mixture was determined using a spectrophotometer (Thermo spectronic) at a wavelength of 725 nm. The phenolic content was estimated from the gallic acid calibration curve and expressed as milligram Gallic Acid Equivalent (QE) per gram of Extract.[4,6]

Antioxidant Activity Assay

The antioxidant activity of *Plectranthus amboinicus* was determined using 2,2–Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. The extract was mixed with 0.4 mM DPPH (1:1 v/v). After incubation for 30 minutes in the dark, the absorbance was measured at 490 nm using an ELISA reader. The percentage scavenging activity was calculated using the formula below.[4,6]

% scavenging of DPPH radical = (Abs control- Abs extract)/(Abs control) x 100 (1)

Culture of murine macrophage RAW 264.7 cell line

LPS-induced RAW 264.7 cells were used for *in vitro* nitric oxide inhibitory activity assay. The cells were purchased from Elabscience (USA). They were maintained in Dulbecco's Modified Eagle's Medium-high glucose supplemented with 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and 10% fetal bovine serum in a 5% CO_2 incubator at 37°C).[7]

Nitric oxide inhibition assays

RAW 264.7 cells were grown in 24-well plates with a density of 1 x 10^5 cells/well for 24 hours in a 5% CO_2 incubator (temperature 37°C), and allowed to stabilize. Cells were then stimulated with LPS (4 µg/mL) and treated with PaE (50, 100, 200 µg/mL) for 24 hours. After 24 hours, the Griess reaction kit from Sigma was used to measure the concentration of nitrite in the culture medium as a sign of NO production. In a 96-well plate, 75 µL of cell culture supernatant was combined with 75 µL of Griess reagent and the absorbance was measured by an ELISA reader at wavelength of 571 nm. The concentration of nitrite in the extract was determined using the sodium nitrite ($NaNO_2$) standard curve.[7]

Cell viability assay

The cell culture medium that had been used for the NO inhibition assay in the previous experiment was removed and changed with 150 µL of culture medium and 7.5 µL of Water-Soluble Tetrazolium (WST-1) reagent (Sigma) in each well, then incubated for 30 minutes in a 5% CO_2 incubator at 37°C. After 30 minutes, 100 µL of media was moved to a new 96-well plate and the absorbance was read with an ELISA reader (BioTek ELx808) at 450 nm.[7]

In silico Antioxidant Activity of Bioactive Compounds from Plectranthus amboinicus

The bioactive compounds of PaE were retrieved from a journal article database. The 3D structures of the compounds were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The conformational forms of the compounds were prepared using SMILE for molecular docking. The antioxidant and anti-inflammatory activities predictions of the bioactive compounds were done using the PASS online molecular docking tool (http://www.pharmaexpert.ru/passonline/predict.php).

Statistical Analysis

All data were presented as mean ± standard deviation (SD) of at least three replicates.

Results and Discussion

Total flavonoids and phenolic contents of Plectranthus amboinicus Extract

The Total Flavonoid Content (TFC) in PaE was calculated from the regression equation of the quercetin standard curve (Figure 1A). Quercetin equivalents (QE) were used to express the total flavonoid content (milligrams) of dry extract (gram) (mg QE/g of dry extract). PaE had a flavonoid content of 2.04 ± 0.23 mg QE/g dry extract (Table 1).

The Total Phenolic Content (TPC) in PaE was calculated from the regression equation of the gallic acid standard curve (Figure 1B). Gallic acid equivalents were used to express the total phenolic content as milligrams per gram of dry extract (mg GAE/g). The phenolic content of PaE was 4.76 ± 0.03 mg GAE/g (Table 1).

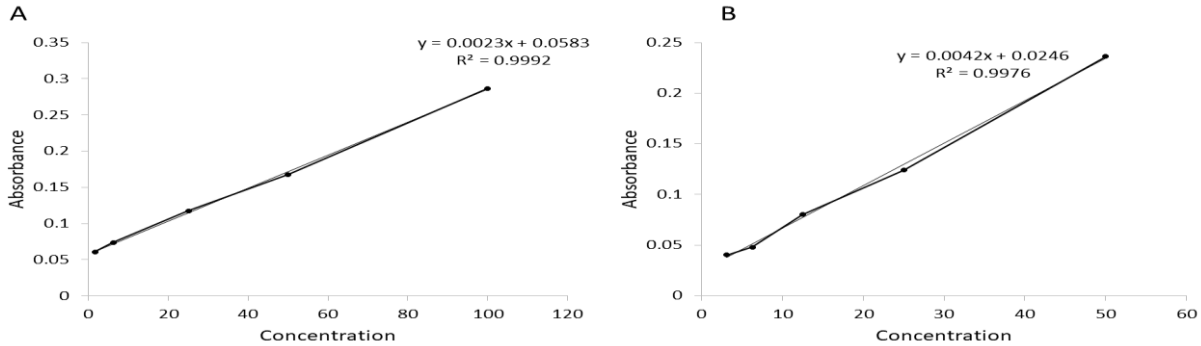

Figure 1: Standard curves for total phenols and total flavonoids. A: Quercetin calibration curve; B: Gallic acid calibration curve

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The phytochemical study of *Plectranthus amboinicus* found out that the plant contained flavonoids, terpenoids, saponins, tannins, and volatile oil.[10-13] Phenolic hydroxyl group serve as hydrogen atom donor that scavenge free radicals.[13,16] Furthermore, phenols, polyphenols, and tannins in plants have been found to inhibit nitric oxide production.[4,6,7] In this study, phenolic and flavonoid contents in PaE deviated slightly from those reported in the literature. For example, the works of Nguyen *et al*., obtained phenolic and flavonoids contents of *Plectranthus amboinicus* leaves to be 26.84 ± 0.91 µg GAE/mg and 12.14 ± 0.42 µg QE/mg, respectively.[13] This variation may be due to the different geographical location of the plant or the extraction methods, which may alter the amount of phenolics and flavonoids in the plant.

Antioxidant Activity of Plectranthus amboinicus

This study evaluated the potency of PaE as a natural antioxidant through the DPPH scavenging activity. The DPPH assay is an assessment of the antioxidant properties of a compound, an extract, or other biological samples that is frequently used. As a free radical, DPPH requires an electron from a hydrogen radical to transform it from a free radical to a stable molecule (DPPH-H). Due to its reduced state, DPPH-H has lower absorbance than DPPH, and this is evidenced from a colour change from purple to yellow. The DPPH scavenging activity of PaE is shown in Figure 2; as PaE concentration increased, the antioxidant activity also increased. The secondary metabolites, including phenolic and flavonoid groups are predicted to be responsible for the antioxidant activity of *Plectranthus amboinicus*. Plant phenolic and flavonoids compounds have hydroxyl (OH) groups that might offer hydrogen to scavenge free radicals. The potency of flavonoids and phenolics as antioxidants depends on the number and position of free OH groups. The ability of an antioxidants to donate hydrogen is crucial in their physiological function as antioxidants.[14,16] The findings from the present study agrees with the results obtained from previous studies on *Plectranthus amboinicus* which showed that *Plectranthus amboinicus* is a potent DPPH radical scavenger and its antioxidant activity could be dependent on the total phenolic and flavonoids contents.[10–13,17–19]

Plectranthus amboinicus reduces nitric oxide production in LPS-induced RAW 264.7 cells

The RAW 264.7 cells line is commonly utilized in anti-inflammatory drug screening. This study evaluated the effect of PaE on NO production after RAW 264.7 cells were stimulated with LPS. The LPS stimulation resulted in an increase in nitrite formation in the culture medium. These cell line were pre-treated with 0 to 200 µg/mL of PaE for 24 hours. The amount of nitrite in the culture medium was used to measure the NO production. Figure 3 shows that PaE reduced the NO production in a concentration-dependent manner. The IC_{50} value of PaE was 106.3 ± 10.65 µg/mL.

In a previous study, *Plectranthus amboinicus* aqueous extract showed the ability to reduce NO accumulation after RAW 264.7 cells were stimulated with LPS.[16] However, no study has reported the effect of *Plectranthus amboinicus* ethanol extract. Thus, this study is the first report on PaE effect on NO production after LPS stimulation of RAW 264.7 cells. The observed NO inhibitory activity of PaE in this study could be attributed to the phenolic and flavonoids compounds in the extract as well as its antioxidant effect. Plants with polyphenol, flavonoids, and tannins contents and robust antioxidant properties have been found to have NO generation inhibitory activity.[20,21]

Table 1: Total flavonoid and Total phenolic content of *Plectranthus amboinicus* ethanol extract

Sample	TFC (mgQE/g)	TPC (mgGAE/g)
PaE1	2.08	4.74
PaE2	2.25	4.74
PaE3	1.79	4.80
Mean	2.04 ± 0.23	4.76 ± 0.03

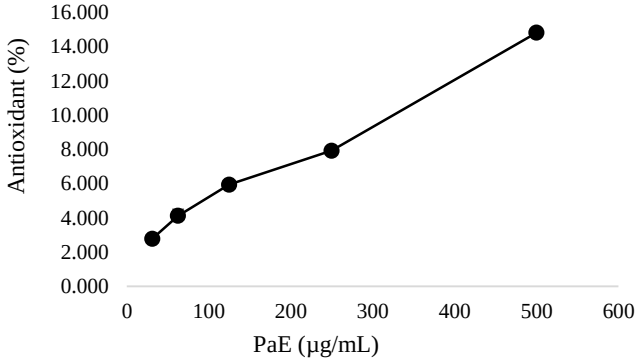
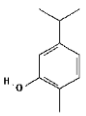
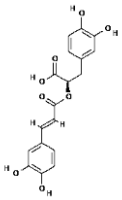


Figure 2: DPPH radical scavenging activity of Plectranthus amboinicus ethanol extract

Table 2: Secondary metabolites of Plectranthus amboinicus

PubChem ID	Bioactive compound	2D Structure	Reference
CID 10364	Carvacrol		(5)
CID 5281792	Rosmarinic acid		(2,8)

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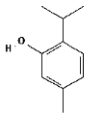
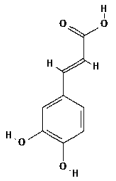
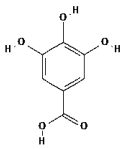
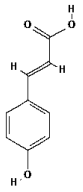
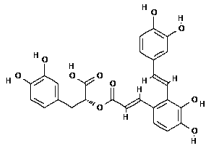
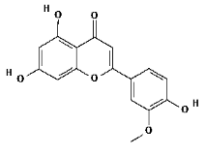
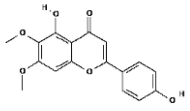
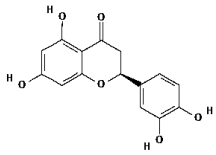
CID 6989	Thymol	(9) 
CID 689043	Caffeic acid	(2,8) 
CID 370	Gallic acid	(2) 
CID 637542	p-Coumaric acid	(2) 
CID 5281793	Salvianolic acid A	(10) 
CID 5280666	Chrysoeriol	(5) 
CID 188323	Cirsimaritin	(5) 
CID 440735	Eriodyctiol	(8) 

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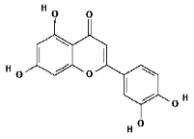
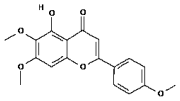
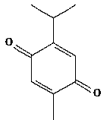
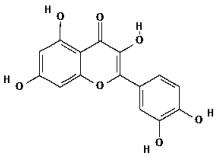
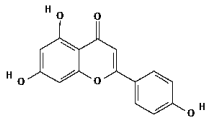
CID 5280445	Luteolin	(8) 
CID 161271	Salvigenin	(5) 
CID 10281	Thymoquinone	(10) 
CID 5280343	Quercetin	(2) 
CID 5280443	4′,5,7-Trihydroxyflavone (apigenin)	(8) 

Effect of Plectranthus amboinicus extract on RAW 264.7 cell viability
The effect of PaE on LPS-stimulated murine macrophage (RAW 264.7 cells) viability was evaluated at 0 to 200 g/mL concentrations. WST-1 assay was applied to measure cell viability. The result showed a cell viability of >99% (Figure 4). This result indicates that the range of PaE doses used is not toxic to the cells (Figure 4). *In vivo* studies gave a similar result which showed that hematological and histological parameters were not altered by *Plectranthus amboinicus* treatment. *Plectranthus amboinicus* extract could be regarded as safe because no toxicity or adverse effects was observed *in vitro* and *in vivo*.

In silico Antioxidants and Anti-inflammatory Activities of Plectranthus amboinicus Secondary Metabolites
Molecular docking of bioactive compounds of *Plectranthus amboinicus* was conducted by PASS online molecular docking tool using SMILE structures of the secondary metabolite through bioinformatic study. This study was conducted to predict the activity of *Plectranthus amboinicus* compounds as antioxidant and anti-inflammatory agents. The result was obtained as Probability of Activity (Pa) Score, with a range of 0-1. This score describes how effect is the predicted pharmacological activity of secondary metabolites. A score of close to 1 indicates that the secondary metabolite is very effective in terms of the predicted pharmacological activity. Figure 5 shows the mean values of Pa score for fifteen secondary metabolites of *Plectranthus amboinicus* (Table 2) as antioxidant and anti-inflammatory agents. Future studies will focus on the isolation and the pharmacological activities of bioactive compounds from *Plectranthus amboinicus*.

Plants have the potential as alternative medicines because of their active compounds.[22] *Plectranthus amboinicus* has several active compounds, and the present study has shown the antioxidant and anti-inflammatory activities of these compounds *in silico* from their ability to act as free radical scavenger, Nitric oxide scavenger, and nitric oxide antagonist (Figure 5). Antioxidants are chemicals that protect cells from the damaging effect of free radicals which is significant in the prevention and treatment of several diseases, including inflammatory diseases.[23-25] The association between the antioxidant activity, total phenolics, and flavonoids contents in PaE suggests that PaE has the potential as a source of natural antioxidant and anti-inflammatory agents through DPPH scavenging and nitric oxide inhibition with little or no cytotoxic effect.

Conclusion

This study showed that the bioactive compounds of *Plectranthus amboinicus* are mainly of the flavonoids and phenolic groups. These compounds may be associated with the antioxidant and anti-inflammatory activities of the plant through their ability to act as DPPH radical scavengers and nitric oxide inhibitors in LPS-stimulated RAW 264.7 cells. Although, the present study has shown the potential of *Plectranthus amboinicus* ethanol extract as a natural antioxidant source, further studies will be needed for detailed biological activity, mechanisms of action, and the isolation and characterization of the bioactive compounds.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

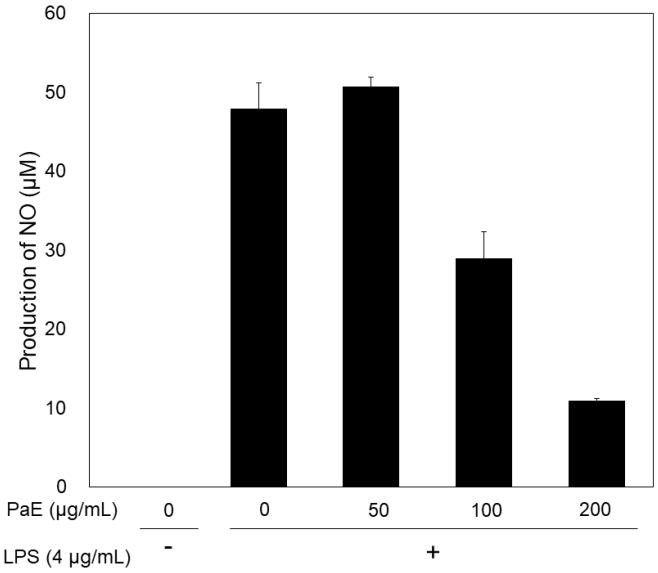


Figure 3: Inhibitory effects of *Plectranthus amboinicus* ethanol extract on NO production in LPS-induced RAW 264.7 cells

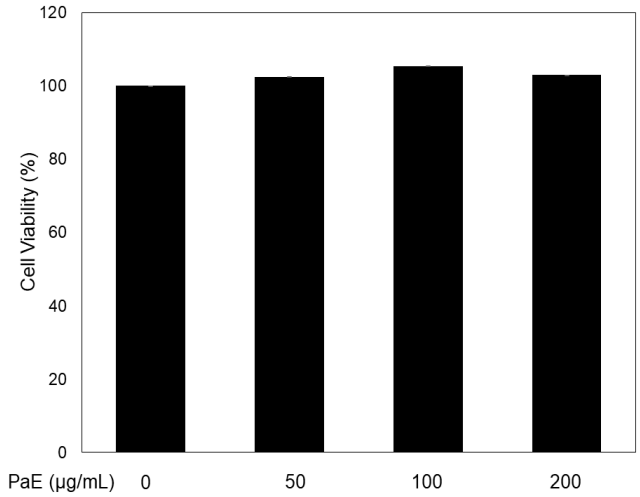


Figure 4: WST-1 analysis of RAW 264.7 cell viability after treatment with *Plectranthus amboinicus* ethanol extract

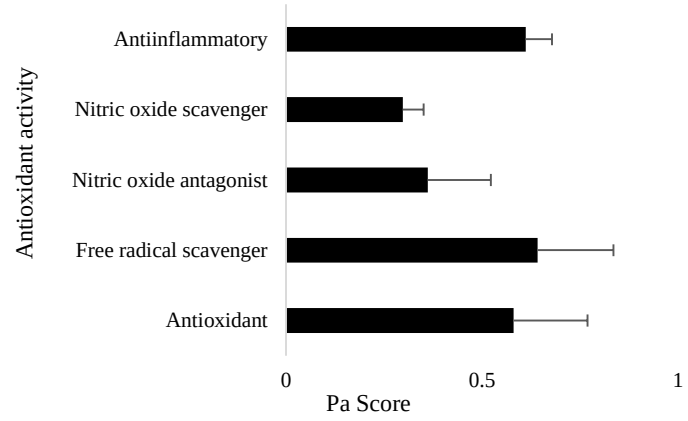


Figure 5: Pharmacological Activity Prediction of *Plectranthus amboinicus* Bioactive Compounds

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors would like to acknowledge the Universitas Brawijaya for providing the facilities for this study under the scheme of Hibah Guru Besar.

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