Original Article

Potential antioxidant and antiradical agents from *Allium ascalonicum*: Superoxide dismutase and density functional theory *in silico* studies

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J. Adv. Pharm. Technol. Res.

Abstract

Antioxidants are compounds that can inhibit excessive free radical reactions in the body. Excessive free radicals can cause system imbalances in the body which can trigger oxidative stress and cause serious illness. The limitations of antioxidants in the body can be overcome by consuming safe natural additional antioxidants that can be obtained from natural products. Isolating compounds of *Allium ascalonicum* leaves as antioxidant and antiradical agents in inhibiting excessive free radicals by *in vitro* and *in silico*. The extracted compounds were purified by column chromatography. The compounds obtained were then characterized using ultraviolet, infrared, NMR, and mass spectrometry. Determination of antioxidant activity was carried out by *in vitro* using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the non-enzymatic superoxide dismutase (SOD) methods. The *in silico* study used the density functional theory (DFT) calculation method with global descriptive parameters (GDP), donor acceptor map (DAM), and frontier molecular orbitals (FMO) analysis. Three compounds have been isolated, of which compound **1** is a new compound. In the DPPH method, compound **1** has more strong antioxidant activity than others, as well as in the non-enzymatic SOD method. Whereas, in the DFT calculation shows that compound **1** has the best reactivity and stability between other compounds and was categorized as the best antiradical. Compound **1** has the highest antioxidant activity compared to the other compounds by *in vitro* both the DPPH and non-enzymatic SOD methods. *In silico,* compound **1** has the potential as the best antiradical.

Key words: *Allium ascalonicum,* antioxidant, density functional theory, oxidative stress, the nonenzymatic superoxide dismutase

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Submitted: 03-Dec-2023 **Revised:** 02-May-2024
Accepted: 06-May-2024 **Published:** 22-Jul-2024

Access this article online	
Quick Response Code:	Website: https://journals.lww.com/JAPTR
	DOI: 10.4103/japtr.japtr_525_23

INTRODUCTION

Shallot (*Allium ascalonicum* L.) is a plant which is widely cultivated and used as a spice and believed to be a traditional medicine.[1] According to previous studies, *A. ascalonicum* bulbs contain several important compounds that have potential as natural antioxidant compounds.[2]

How to cite this article: Ajiati D, Sumiarsa D, Amin MF, Kurnia D. Potential antioxidant and antiradical agents from *Allium ascalonicum*: Superoxide dismutase and density functional theory *in silico* studies. J Adv Pharm Technol Res 2024;15:171-6.

Antioxidants are compounds that can inhibit the occurrence of free radical reactions in the body.[3] It is important to protect the body from free radicals that can harm and prevent the body from oxidative stress that causes several serious diseases such as premature aging and cancer.[4] In addition, it is also caused by excessive production of reactive oxygen species.[5] Natural antioxidants can be obtained from fruits and vegetables because they contain important compounds which have an antioxidant activity.[6]

The antioxidant activity of a compound is influenced by its structure. The more polar the compound, the greater its ability is as an antioxidant. The structure which contains many hydroxyl groups bound to aromatic rings or conjugated carbon double bonds that can easily donate hydrogen or electrons to stabilize radical atoms because it has good resonance ability.[7] In other studies, antioxidant activity assay is mostly conducted using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, which is considered more effective, easy, and cheap.[8] DPPH is a stable radical compound which has reactive nitrogen species.[9] Another method used in this study is non-enzymatic superoxide dismutase (SOD) or known as mimic SOD because it mimics the workings of the antioxidant testing method using the SOD enzyme. In this method, the source of superoxide anion comes from riboflavin photoreduction and the role of SOD enzyme is replaced by secondary metabolite compounds.[10]

In recent years, many studies on antioxidants have used computational studies or better known as *in silico* studies to predict the antioxidant ability of a molecule because it is considered more effective and certainly more affordable. One method that is widely used to predict antioxidant reactivity and activity is density functional theory (DFT).[11,12] This method involves electron transfer ability and electron affinity in determining antioxidant ability.[13] This study uses quantitative and qualitative approaches involving laboratory research. In addition to a new compound, the use of mimic SOD (mSOD) method *in vitro* and DFT *in silico* is a novelty of this study.

MATERIALS AND METHODS

Materials

The leaves of *A. ascalonicum* were obtained from Demak, Indonesia, which were then determined (No. 25/LBM/IT/12/2021) at the Taxonomy Laboratory, Padjadjaran University, Indonesia. Several organic solvents such as *n*-hexane, ethyl acetate (EtOAc), and methanol (MeOH) as well as several other chemicals, namely, Silica Gel 60 (0.2–0.5 mm) and (0.063–0.200 mm), Silica ODS RP-18 (0.040–0.063 mm), KLT Gel 60 F_{254} plate and ODS RP-18 F_{254} S plate were used for extraction, fractionation and isolation processes. In the process of identifying compound spots with KLT, 10% H_2SO_4 in ethanol (v/v) spotting solution was used, which was then monitored under UV light at λ 254 and 365 nm. *In vitro* research, antioxidant activity test with DPPH and non-enzymatic SOD methods using several chemicals such as DPPH, methanol, riboflavin, phosphate buffer pH 7.4, TEMED, distilled water, and NBT.[10,14] Materials for *in silico* study only require 3D-conformers of compounds **1–3** downloaded from PubChem in sdf format.

Extraction and isolation of *Allium ascalonicum* leaves

A. ascalonicum leaves were extracted using methanol. The methanol extract obtained was concentrated with a rotary evaporator at ± 50°C. The extract was partitioned using column chromatography (Silica G 60) with *n*-hexane, EtOAc, and MeOH to obtain fractions of each solvent. EtOAc and MeOH fractions were selected to be purified using Silica G 60 column chromatography (*n*-hexane-EtOAc) and ODS RP-18 (H_2O-MeOH) to obtain pure compounds **1** and **2**. Characterization of pure compounds was carried out using ^{1}H-NMR (JEOL, 500 MHz), ultraviolet-visible, infrared, and mass spectrometry.

Antioxidant activity assays using the 2,2-diphenyl-1-picrylhydrazyl method

This method was carried out twice, in the preliminary test and the pure compounds. Solvent used is methanol and DPPH as a radical with variation of sample concentration. The absorbance measurement was conducted using a microplate reader at 510 nm after being left in a dark room for ± 30 min. Percentage inhibition was calculated using the absorbance value of measurement result using $(A_{control} - A_{sample})/A_{control} \times 100\%$. Whereas, the IC_{50} value was calculated using Y = ax + b, which is the result of the regression equation.[15]

Antioxidant activity assays using the mSOD method

Initially, we prepared sample solutions of compounds with varying concentrations. The concentration variation was done by microdilution. Then, the sample solution, MeOH, working solution A (aquabides, phosphate buffer pH 7.4, NBT, TEMED, Riboflavin) and B (aquabides, phosphate buffer pH 7.4, NBT, TEMED) were put into 96-well and then irradiated for 10–15 min in a closed box. Absorbance measurement was performed using a microplate reader at λ 550 nm to obtain percentage inhibition and IC_{50} value.

Study of antioxidant activity with density functional theory calculation by *in silico*

In this antioxidant activity study by *in silico*, we used DFT calculation with basis sets B3 LYP/6-31G (2d, 2p) using MarvinSketch, Gaussian 09 W, and GaussView 5.0. The calculation was carried out under gas phase conditions.[16] We used three analyses consisting of global descriptive parameters (GDP), donor acceptor map (DAM), and frontier molecular orbitals (FMO). The three analyses involved calculations and DAM diagrams as follows [Figure 1].

RESULTS

Compounds **1** and **2** were obtained from the EtOAc fraction of the methanol extract of *A. ascalonicum* leaves. Meanwhile, compound **3** was obtained from the methanol fraction. Based on the characterization data, it shows that compound **1** belongs to the aromatic compound group and compounds **2–3** belongs to the steroid group.

2-((2-hydroxyphenanthrene-1-yl) oxy)-2-oxoacetic acid (**1**) is a white crystal-shaped compound and has the molecular formula of $C_{16}H_{10}O_5$; IR, ν_{max} 1094 (C-O), 1494 (C = C), 1683, 1701 (C = O) and 3453 cm^{-1} (OH); MS, m/z 282 (M − H)$^+$; It has the carbon *sp*^{2} at the shift δ_C 167.7 ppm which is a characteristic of the ester or carboxylic acid group. Then, there are several characteristics of alkene and aromatic carbon at δ_C 121.6–135.6 ppm.[17] According to the UV data of compound **1** at λ_{max} 255 nm is the absorption band of the benzenoid indicating that compound **1** has a benzene ring. Based on the shift δ_C, compound **1** has eight methine (CH) and eight *sp*^{2} quaternary (Cq) carbons.[18] β-sitosterol (**2**), the white crystal-shaped compound, has the molecular formula of $C_{29}H_{50}O$; IR, ν_{max} 1050 (C-O), 1381 (*gem*-dimethyl), 1465 (C = C), 2937 (C-H) and 3429 cm^{-1} (OH); MS, m/z 414 (M + H)$^+$; These data provide information that compound **2** has six methyl, eleven methylene, nine methine and three quaternary (Cq) carbons.[19] Sitosterol-3-*O*-glucoside (**3**) is a white powder-shaped compound with the molecular formula of $C_{35}H_{60}O_6$; IR, ν_{max} 1021 (C-O), 1367 (*gem*-dimethyl), 1639 (C = C), 2932 (C-H) and 3400 (OH); MS, m/z 576 (M − H)$^+$.[20]

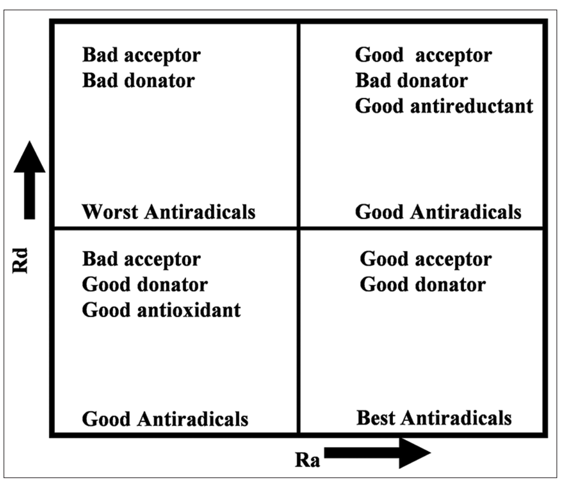

Figure 1: Donor acceptor map analysis diagram used to determine antioxidant activity *in silico*

Based on ^{1}H and ^{13}C-NMR analysis, compounds **2** and **3** have almost the same chemical shift. However, compound **3** has chemical shift that was the characteristics of glucoside at δ_C 61.5 (C-26), 73.9 (C-28), 77.2 (C-29, C-29a), 77.3 (C-30), and 101.2 ppm (C-31).[21] According to ^{13}C-NMR, compound **1** has carboxyl group at δ_C 167.7 ppm (C-12, C-12a).[22] Structures of compounds **1-3** are shown in Figure 2.

DISCUSSION

Antioxidant activity assays of *Allium ascalonicum* leave compounds by *in vitro*

Table 1 shows that compound **1** has the best antioxidant activity among the other compounds in DPPH and non-enzymatic SOD methods with IC_{50} of 76 and 40 μg/mL, respectively. The IC_{50} value of compound **1** indicates that the compound has strong antioxidant activity. Meanwhile, compounds **2–3** showed weak antioxidant activity in both methods. In this study, we used quercetin as a positive control in both methods because quercetin is known to have a strong antioxidant ability to inhibit free radicals.[14]

Based on Table 1, compound **1** has good activity because it is influenced by its structure. There are several influential groups from compound **1** that can affect antioxidant activity, namely the aromatic benzene ring, carbonyl groups, and phenol hydroxyl groups attached to it.[23] Compound **1** could donate its electrons to stabilize radical atoms to become more stable.[24] Meanwhile, compounds **2–3** do not have an influential group to donate its electrons. Although compound **3** has some hydroxyl groups on the

Table 1: Data of antioxidant activity of compounds[1-3]

Compounds	IC_{50} (μg/mL)	
	DPPH	SOD nonenzymatic
1	76	40
2	3273	3837
3	2500	10,000
Quercetin	3.8	5

DPPH: 2-diphenyl-1-picrylhydrazyl, SOD: Superoxide dismutase, IC: Inhibitory concentration

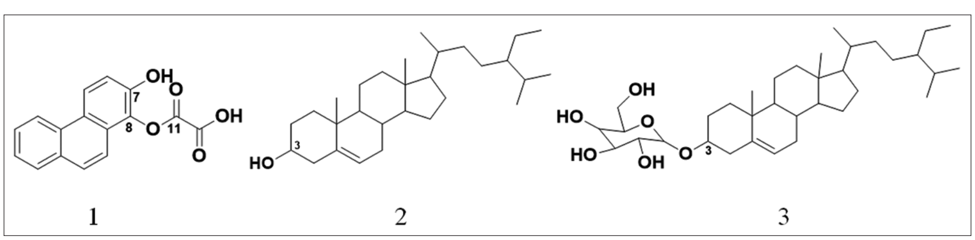

Figure 2: Compounds **1–3** were obtained from *Allium ascalonicum* leaves

glucoside substituents in its structure, it cannot conjugate and delocalize electrons when donating or transferring electrons.[25] Those functional groups have a relationship with the polarity of compounds and effect on antioxidant activity.[26] Figure 2 also shows that compound **1** is more polar than compounds **2** and **3**. The more polar the compound, the easier it is to break hydrogen bonds and donate electrons.[27]

Antioxidant activity assay of *Allium ascalonicum* leave compounds by in silico

In the DFT calculation in this study, we used three analysis methods to determine and validate the results of antioxidant activity *in vitro*, namely, GDP, DAM, and FMO.

Table 2: The result of global descriptive parameter calculation

Parameters (eV)	Compounds			
	1	2	3	Quercetin
I	10.85	7.62	7.51	9.43
A	1.12	−1.93	1.63	1.75
η	4.86	4.77	4.57	3.83
S	0.10	0.10	0.11	0.13
χ	5.99	2.84	2.94	5.59
μ	−5.99	−2.84	−2.94	−5.59
ω	17.96	4.05	4.32	15.64

Table 3: The calculation results of compounds 1–3 using donor acceptor map analysis

Compounds	Ω^- (eV)	ω^+ (eV)	Ra	Rd
1	7.29	1.30	0.62	2.08
2	2.86	0.02	0.01	0.82
3	2.98	0.04	0.02	0.85
Quercetin	7.35	1.75	0.84	2.10
Natrium	3.50	0.57	0.27	1.00
Flourin	10.68	2.09	1.00	3.05

Ra: Electron acceptor, Rd: Electron donor

Global descriptive parameters

GDP calculation was obtained from single point energy value of each charge (neutral, anion, and cation) using formulas that were mentioned above (method section). Those parameters are used to predict reactivities and stability of compounds.[28] They consist of ionization potential (I), electron affinity (A), hardness (η), softness (S), electronegativity (χ), chemical potential (μ), and electrophilicity index (ω).[29] Table 2 shows that compound **1** is more stable than compounds **2–3** as indicated by the higher hardness values than the others. The higher the hardness value, the more stable the compound. Stability can also be seen based on a more negative chemical potential value. Reactivity and stability greatly affect antioxidant activity as they are related to electron transfer in inhibiting radicals.[30]

Donor acceptor map

Determination of antioxidant activity based on DAM analysis is inseparable from the previous analysis, namely GDP. I and A values as in Table 2 were used to

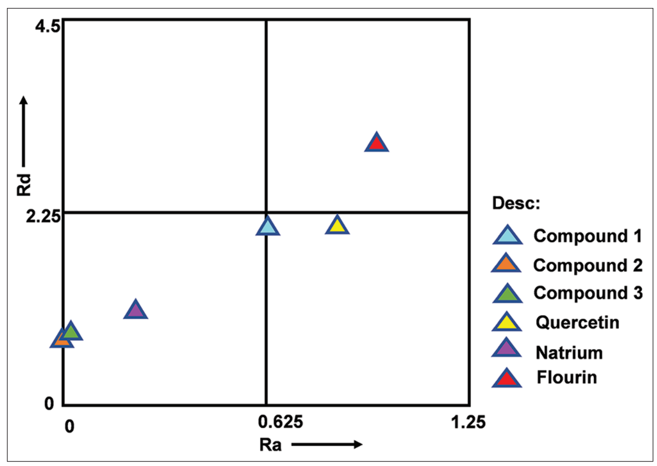

Figure 3: The results of compounds **1–3** were obtained using donor acceptor map diagram analysis

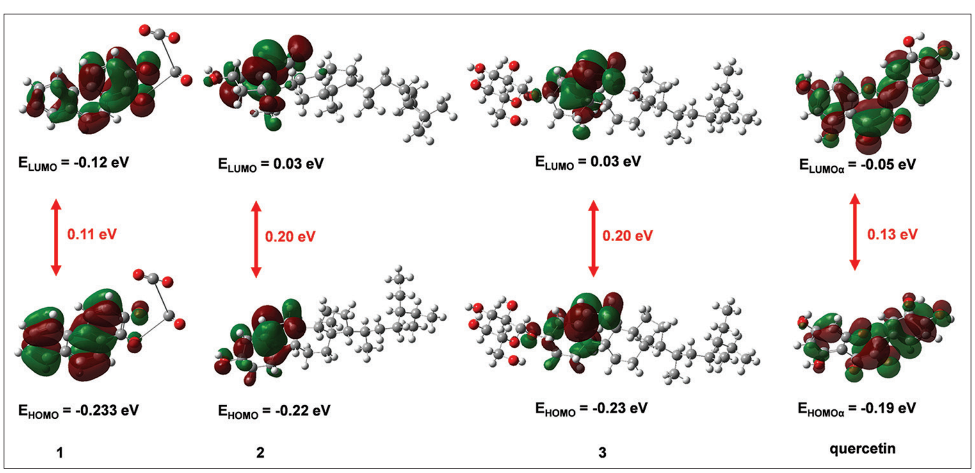

Figure 4: Frontier molecular orbitals analysis of compounds **1–3** involved highest occupied molecular orbital-lowest unfilled molecular orbital energy. HOMO: Highest occupied molecular orbital, LUMO: Lowest unfilled molecular orbital

calculate electron donor (ω^-) and electron acceptor (ω+) to obtain electron donor (Rd) and electron acceptor (Ra) index.[29,31] We determined these values using the formulas mentioned above. In this analysis, florin and sodium were also involved to obtain the Ra and Rd values. Florin (F) represents an electron acceptor and sodium (Na) a good electron donor.[31] The Ra and Rd values that have been obtained as in Table 3, then plotted on the DAM diagram. Figure 3 shows that compound **1** is categorized as the best antiradical that acts as a good acceptor and donor based on the DAM diagram [Figure 1]. Meanwhile, compounds **2–3** were categorized as a good antiradical with poor acceptor, good donor, and good antioxidant. When a molecule acts as a good electron donor and acceptor, it has high reactivity, so that hydrogen bonds can be broken more easily and donate electrons or hydrogen.[32]

Frontier molecular orbital

The third analysis is FMO which involves the highest occupied molecular orbital energy (HOMO)–the lowest unfilled molecular orbital energy (LUMO). In this analysis, the reactivity of the compound can be seen from the energy gap between the HOMO-LUMO energies of the molecule. The lower the energy gap, the more reactive the molecule is.[32] HOMO-LUMO visualization can also shed light on orbital distribution and electron density. In addition, this analysis can show the active sites or radical attack sites in the molecule, qualitatively.[33] As shown in Figure 4, of the three compounds, compound **1** has a lower energy gap than the other two compounds and even lower than the positive control. This shows that compound **1** has the highest reactivity with an energy gap of 0.11 eV.[13] Figure 2 shows that radicals can attack compound **1** at the aromatic ring and phenol hydroxyl group. Meanwhile, in compounds **2** and **3**, radicals can attack the cyclohexanol site. This can be seen from the orbital distribution in the HOMO-LUMO analysis as shown in Figure 4.

CONCLUSION

In this study, we have successfully isolated three compounds and one of them is a new compound. Based on the *in vitro* antioxidant activity test, compound **1** has strong antioxidant activity on DPPH and non-enzymatic SOD method. Based on *in silico* study, in GDP analysis, compound **1** has the highest reactivity and stability compared to other compounds. This is also reinforced by the results of FMO analysis which can be seen from the energy gap value. In DAM analysis, compound **1** is categorized as the best antiradical among the other. Thus, the antioxidant activity of compounds using *in vitro* methods does not correlate to certain analyses in *in silico* studies.

Acknowledgement

The authors are grateful to the Academic Leadership Grant (ALG) Dikdik Kurnia, Indonesia (1439/UN6.3.1/PT.00/2024, March 18, 2024) and also grateful to Universitas Padjadjaran for supporting all research facilities.

Financial support and sponsorship

The Academic Leadership Grant (ALG) Dikdik Kurnia, Indonesia (1439/UN6.3.1/PT.00/2024, March 18, 2024), Universitas Padjadjaran, Indonesia.

Conflicts of interest

There are no conflicts of interest.

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