Trop J Nat Prod Res, March 2025; 9(3): 1097 - 1104 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

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

Original Research Article

Insight into the Biological Activity of Zingiber officinale var. Amarum Extracts as Antioxidant and SARS-CoV-2 Inhibitor *In-Silico*

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ARTICLE INFO

Article history:
Received 10 Decemebr 2024
Revised 13 Decemebr 2024
Accepted 10 February 2025
Published online 01 April 2025

ABSTRACT

Zingiber officinale var. Amarum (Ginger) is a medicinal spice of the Zingiberaceae family. Its medicinal value can be attributed to its secondary metabolites, such as flavonoids, terpenoids, gingerols, and shogaols, which posses potent antioxidant properties. This study aimed to evaluate the effect of extraction solvent on the total flavonoid content, and antioxidant activity of emprit ginger, and to determine the anti-SARS-CoV-2 activity of major compounds of ginger *in silico*. Emprit ginger was extracted by refluxing seperately with ethanol, ethyl acetate, and n-hexane. The extracts were tested for the presence of flavonoid, and the total flavonoid content was determined by the aluminium chloride colorimetric method. The antioxidant activity of the extracts was assessed using the 2,2'-azino-bis (3ethylbenzotiazolin)-6-sulfonic acid (ABTS) radical scavenging assay. Anti-SARS-CoV-2 activity of major phenolic and flavonoid compounds of ginger including catechin, epicatechin, rutin, naringenin, quercetin, gingerol, and zingiberol was determined *in silico* by molecular docking with SARS-CoV-2 main protease (Mpro). Emprit ginger extracts contained flavonoids with total flavonoid contents of 103.93 mgQE/g, 97.32 mgQE/g, and 81.5 mgQE/g for ethyl acetate, n-hexane, and ethanol extracts, respectively. The ethyl acetate extract had the highest antioxidant activity with IC_{50} value of 20.02 µg/mL. Emprit ginger ligands showed strong interactions with SARS-CoV-2 main protease (Mpro) with binding energies ranging from -4.80 to 9.90 kcal/mol. Gingerol and zingiberol exhibited the most promising anti-SARS-CoV-2 activity *in silico* with binding energies of -4.80 and -5.30 kcal/mol. The study revealed emprit ginger as a source of natural antioxidant with promising anti-SARS-Cov-2 activity.

Keywords: *Zingiber officinale* var. Amarum, Antioxidant, SARS-CoV-2, Secondary metabolites, *In silico.*

Introduction

Ginger contains several secondary metabolites including flavonoids, phenolics (such as gingerol, shogaol, zingerone), triterpenoid (sesquiterpene hydrocarbon), and alkaloid.[1,2]
Emprit or Java ginger is well known for its numerous pharmacological activity including antioxidant, hepatoprotection, antibacterial, analgesic, radio-protective, and anti-inflammatory activities.[3,4] The flavonoids in ginger are potent antioxidants, protecting cells from the damaging effect of free radicals.[1,5]
The extraction of flavonoid compounds in plants can be influenced by extraction method and solvent for extraction. The extraction solvent plays an important role in extracting active compounds because secondary metabolites have different solubility in various solvents. Extraction with water has been considered less effective, while organic solvents such as methanol, ethanol, ethyl acetate, n-hexane, chloroform, acetonitrile, acetone, and ether have been used widely in extraction because of their polarity index.

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Citation Vifta L R, Yanti S, Suyudi D S, Susmayanti W. Insight into the Biological Activity of Zingiber officinale var. Amarum Extracts as Antioxidant and SARS-CoV-2 Inhibitor In-Silico.Trop J Nat Prod Res. 2025; 9(3): 1097 – 1104 https://doi.org/10.26538/tjnpr/v9i3.26

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Ethyl acetate for example, is the preferred solvent for the extraction of highly alkylated aglycones, while some flavonoids were found to be preferencially extracted with acetonitrile and acetone.[6] The polarity of extraction solvent is usually considered before extraction and purification of metabolites from plants because the polarity of extraction solvent has been shown to not only affect the the nature of secondary metabolites extracted but also their biological activity, especially the antioxidant capacity of natural products.[4,7-9] The extraction method is just as important as the solvents. Despite being less environmentally friendly, conventional extraction methods are still employed primarily due to their usage of inexpensive equipment. Among them, maceration and soxhlet extraction are commonly employed because they enable the separation of flavonoids.[9,10]
Optimizing natural products as a therapeutic agent has been pursued through various studies. In addition to extraction and isolation, computational (*in silico*) studies of active compounds of natural products origin have been developed to determine the pharmacological potential of these compounds. *In silico* studies are relevant method to aid the discovery and of new drug candidate.[11-13]
There is limited studies on the potential of emprit ginger, also known as Java ginger as an antioxidant, including *in silico* studies. Therefore, the present study was designed to evaluate the total flavonoid content, and the antioxidant activity of different solvent extracts of emprit ginger, and also to investigate the potential SARS-CoV-2 inhibitory activity of major phenolic and flavonoid compounds of emprit ginger through an in silico molecular docking approach. The findings from the study will serve as a preliminary data for the antioxidant activity of

emprit ginger, and the potential anti-SARS-CoV-2 activity of major phenolic and flavonoid compounds of emprit ginger.

Materials and Methods

Chemicals and reagents

Ethanol (96%), methanol, n-hexane, ethyl acetate, potassium persulfate ($K_2S_2O_8$), vitamin C, glacial acetic acid, aluminium chloride ($AlCl_3$), and quercetin were products of Merck®. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) was product of Sigma-Aldrich®, while distilled water was from CV Bratacho.

Molecular docking software

The following tools were used for the *in silico* molecular docking study; PubChem library database, AutoDock Vina 1.5.6, MGLTools-1.5.6, Pyrx, PyMOL, BIOVIA Discovery Studio 2021.

Collection and identification of plant material

Emprit ginger rhizomes were collected from Kemetul (7°23'43.0"S 110°33'00.0"E), Semarang Regency, Central Java, Indonesia in October 2023. The plant material was identified and authenticated at the Laboratory of Ecology and Biosystematics, Diponegoro University, Indonesia.

Preparation of ginger extracts

Dried powdered ginger (100 g) was extracted by refluxing seperately with 300 mL each of ethanol, ethyl acetate, and n-hexane. The refluxing was done at the boiling points of the respective solvents for 4 h. The extracts were filtered through a Whatmann filter paper, and the filtrates were evaporated using a rotary evaporator until a thick extract was obtained. The percentage yeilds of the extracts were calculated based on the weight of the dried powdered ginger used.

Qualitative test for flavonoids

Qualitative test for flavonoids in the ginger extracts was carried out according to method previously described.[14] A few quantity of magnesium powder was added to solution (I mL) of the extracts, followed by the addition of a few drops of concentrated hydrochloric acid, then the mixture was shaken. The appearance of a brownish red colour indicates the presence of flavonoids.

Determination of total flavonoid content (TFC)

The TFC of the ginger extracts was determined according to the method described by Wardana *et al*. (2024).[15] A stock solution of ginger extract (500 ppm) was prepared, and 1 mL of this solution was transferred into a 10 mL volumetric flask, followed by the addition of 1 mL of 10% $AlCl_3$ and 1 mL of sodium acetate (5%). The mixture was vortexed, and then incubated at room temperature for 20 minutes. The absorbance of the reaction mixture was measured at 415 nm using a UV-Visible spectrophotometer. Quercetin was used to prepare a calibration curve. The total flavonoid content was determined from the quercetin calibration curve, and expressed as milligram quercetin equivalent per gram of extract (mg QE/g extract). The experiment was performed in triplicate.

Determination of antioxidant activity

The antioxidant activity of ginger extracts was evaluated using ABTS (2,2'-azino-bis (3-ethylbenzothiazolin)-6-sulfonic acid) radical scavenging assay. The reaction scheme of the ABTS assay is shown in Figure 2. A stock solution (100 ppm) of each extract was prepared in methanol. A series of dilutions (10, 20, 30, 40, 50, and 60 ppm) were prepared from the standard solutions. To 3 mL each of the dilutions was added 2 mL of ABTS solution, and the mixture was incubated at room teperature for 16 minutes. Thereafter, the absorbance of the solutions was read at 739 nm using a UV-Vis Spectrophotometer (Simadzu UV-1800). The percentage of radical inhibition was evaluated using the formula shown in equation 1 below.[16]

$$\%inhibition = \frac{A_{control} - A_{sample}}{A_{sampel}} \times 100\%$$

IC_{50} value was calculated from the plot of concentration of ginger extracts versus percentage inhibition of ABTS radical.

In silico study against SARS-CoV-2 main protease

Molecular docking simulation of selected ligands, including catechin (290.27 g/mol), epicatechin (290.27 g/mol), rutin (610.5 g/mol), naringenin (272.25 g/mol), quercetin (302.23 g/mol), gingerol (294.4 g/mol) and zingiberol (222.37 g/mol) with SARS-CoV-2 main protease (Mpro) apo (code 7cam protein) was done using AutoDoc Vina and MGL tools. The selected ligands were flavonoids and phenolic compounds previously isolated from ginger. The 3D structure of SARS-CoV-2 main protease (Mpro) apo (7cam protein) with molecular weight 67.77 kDa was downloaded from the protein data bank (*https://www.rcsb.org*), and saved in "pdb" format. Structures of the ligands in 3D format were downloaded from the Pubchem data base (*https://pubchem.ncbi.nlm.nih.gov*), and saved in "sdf" format, which was later converted to "pdb" using Pymol. The binding sites of the protein with the ligands was obtained by setting the grid box at center x = -1.739; center y = 3.049; center z = -16.315. The interactions between the selected ligands and the target protein were measured in terms of bining affinities. The 3D interaction of the ligands with the target protein was visualized using the BIOVIA Discovery Studio.[17,18]

Statistical analysis

Statistical analysis was done using SPSS 26 software. Data were subjected to one-way analysis of variance (ANOVA), followed by LSD post hoc test.

Results and Discussion

Extraction yields

Emprit ginger was extracted using the reflux method with different solvents. The extraction of active compounds from plants can be influence by both method and solvent used. Extraction conditions such as temperature and extraction time also affect the solubility of active compounds in a given solvent. For example, high temperature simultaneously increase the solubility and mass transfer of active substances, and also decrease the viscosity and surface tension of the solvent, contributing to higher extraction yields.[9,19]

Table 1: Percent yields and moisture contents of *Zingiber officinalle* var. Amarum extracts

Extraction solvent	Extract weight (g)	Moisture content (%)	Yield (%w/w)
n-hexane	3.126	3.12	3.126
Ethyl acetate	6.425	4.21	6.425
Ethanol 96%	8.183	6.80	8.183

Table 2: Total flavonoid content (TFC) of *Zingiber officinalle* var. Amarum extracts

Ginger extract	TFC (mgQE/g)
n-hexane	103.9 ± 2.4
Ethyl acetate	97.3 ± 1.9
Ethanol 96%	81.5 ± 1.4

Table 1 shows the extraction yields of the different solvent extracts of emprit ginger. The results showed that ethanol produced the highest yield of 8.183% compared to ethyl acetate and n-hexane. The differences in yield could be attributed to the differences in the polarity of the solvents used for the extraction. Solvent polarity have been found to affect the extraction yield of active compounds, and probably their pharmacological activity.[20] Extracts from non-polar solvents usually produce low yields and pharmacological activity, while polar solvents produce higher yields, and better pharmacological activity. Polar solvents such as ethanol effectively extract flavonoids and their glycosides, phenolics, and tannins.[1,21] Total flavonoids, total

phenols, and antioxidant activity are usually higher in ethanol extract compared to other less polar solvent extracts.

Flavonoid content of ginger extract

Determination of flavonoids in emprit ginger was carried out both qualitatively and quantitatively. The qualitative analysis was aimed at determining the presence of flavonoids in emprit ginger extract by observing the colour change on reaction with magnesium powder. The results of the qualitative test showed that the n-hexane, ethyl acetate, and ethanol extracts of emprit ginger contained flavonoids. This was consistent with the results obtained from previous studies which have shown the presence of flavonoids, and other secondary metabolites including phenolics in emprit ginger extracts.[2,13,22]

The determination of the total flavonoids content of emprit ginger extracts was based on the aluminium chloride colorimetric method. The method is based on the principle of the formation of a stable complex of $AlCl_3$ with the C-4 keto group and the C-3 or C-5 hydroxyl groups of flavones and flavonols.[15] The $AlCl_3$-flavonoid complex is shown in Figure 1. The formation of this complex is indicated by the appearance

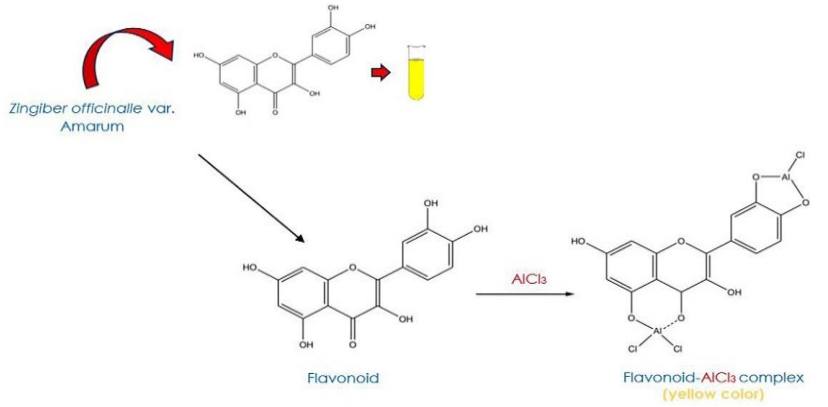

Figure 1: Complexation of Flavonoid compound with aluminium chloride (AlC_3)

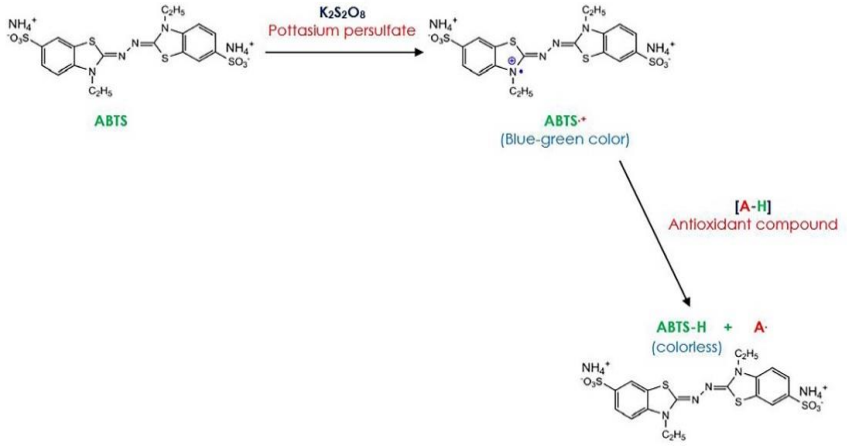

Figure 2: Interaction between ABTS radicals and antioxidant compound.[27]

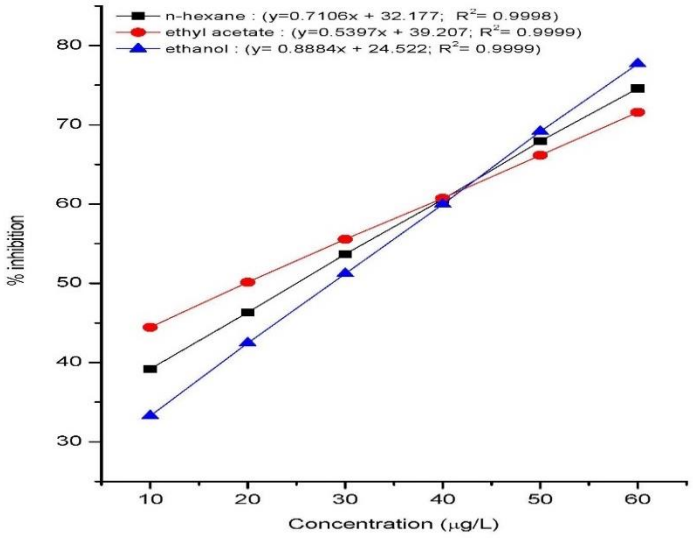

Figure 3: Plot of concentration of ginger extracts versus percentage inhibition of ABTS radical

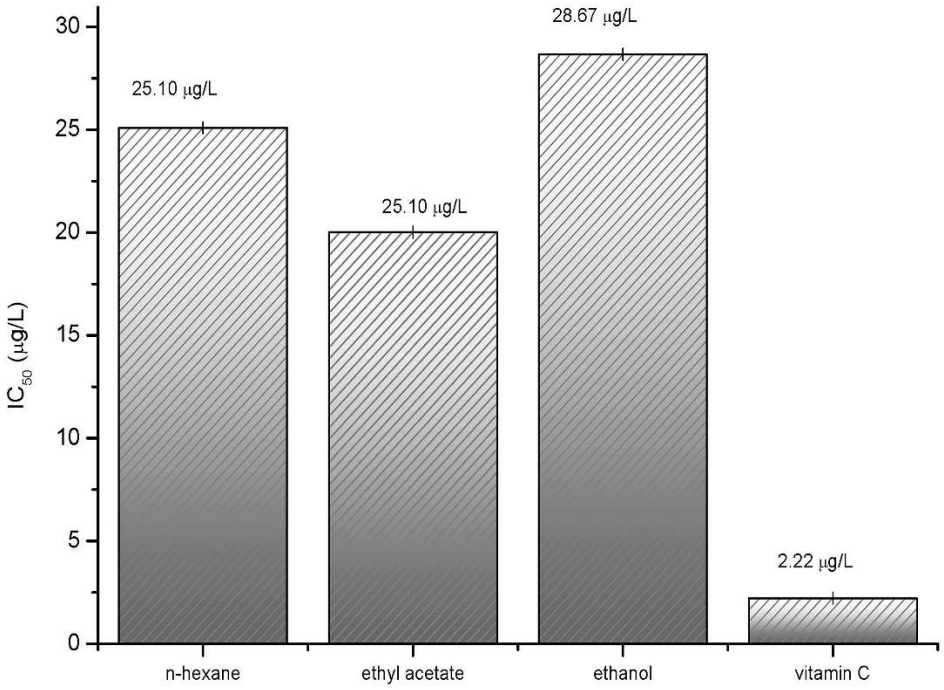

Figure 4: Antioxidant activity (IC_{50}) of *ginger* extracts and vitamin C

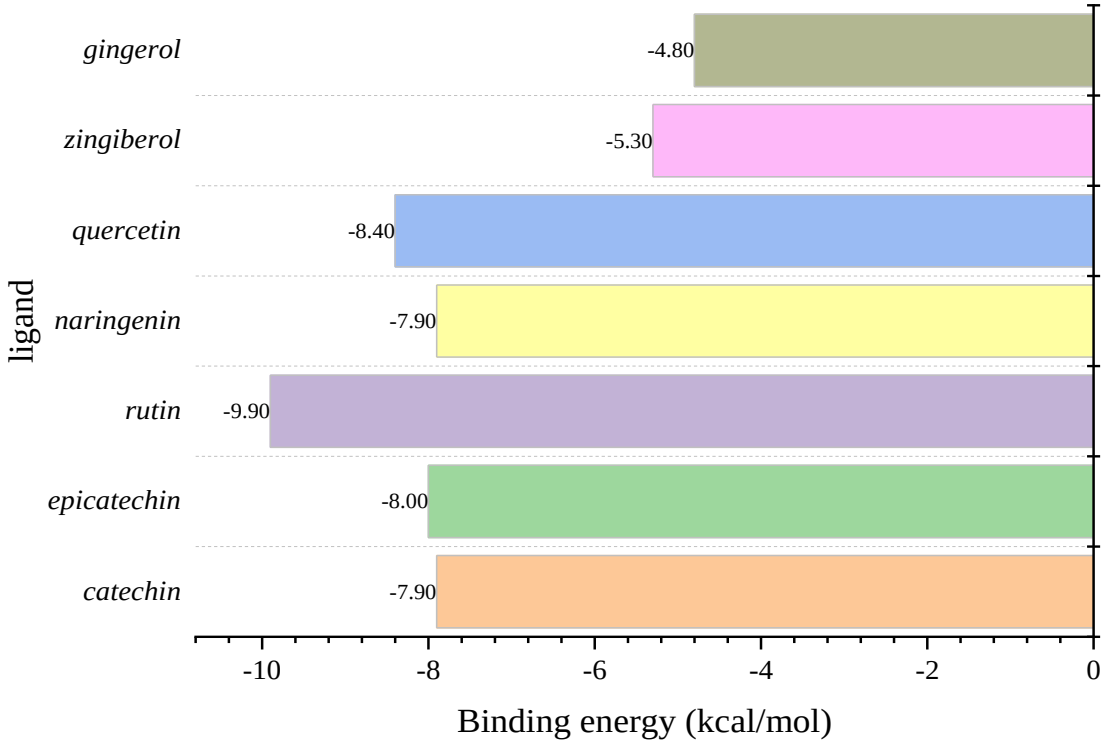

Figure 5: Binding energy of ligand interaction with SARS-CoV-2 main protease

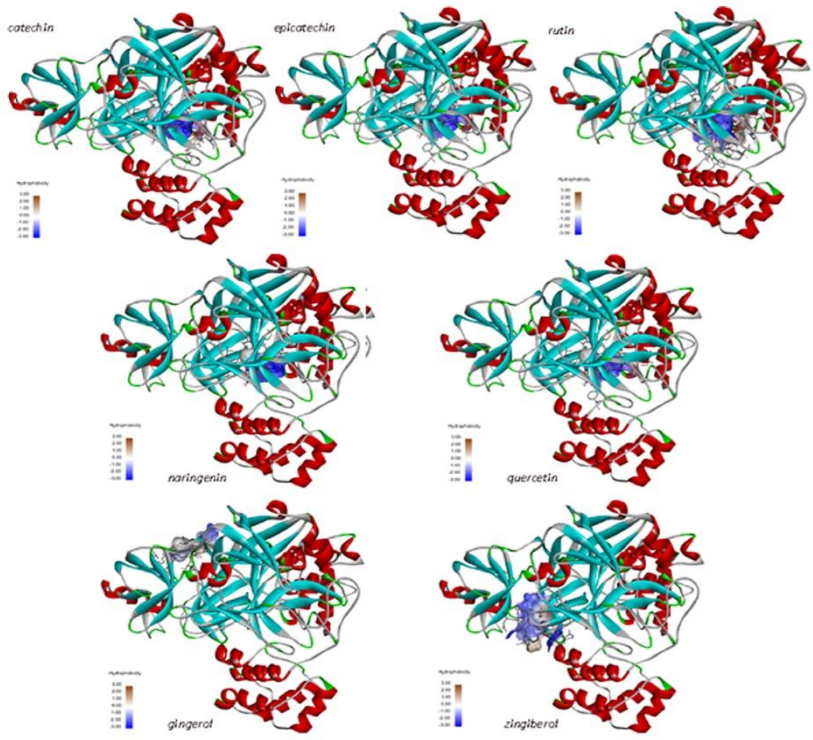

Figure 6: Visualization of ligand interaction with SARS CoV-2 main protease

of intense yellow colour. Sodium acetate is usually added to keep the wavelength of maximum absorption in the visible region.[23]

Based on the results as shown in Table 2, the ethyl acetate extract of emprit ginger had the highest total flavonoid content of 103.93 mg QE/g, which was significantly ($P < 0.05$) higher than that of the n-hexane and ethanol extracts with total flavonoid contents of 97.32 mg QE/g and 81.50 mg QE/g, respectively. Semipolar solvents like ethyl acetate have the ability to extract both polar and non-polar

compounds.[8,20] therefore, there is a greater possibility of the extraction of active compounds in emprit ginger by ethyl acetate.
It is important to state that flavonoid compounds in ginger, such as quercetin, catechin, and epicatechin which are from the flavonol group, are less polar or semipolar in nature, hence they are more commonly found in semipolar than in polar solvents.[4,24] In addition, flavonoid glycosides such as rutin, which are more polar than quercetin, can still be extracted by semipolar solvents like ethyl acetate, thus making the total flavonoid content in ethyl acetate to be higher than in other solvents.[4,20]

Antioxidant activity of Emprit ginger extracts

The antioxidant activity of emprit ginger extract was evaluated using the ABTS radical scavenging method. The method is based on the oxidation reaction of potassium persulfate with ABTS diammonium salt, where ABTS is oxidized by potassium persulfate or manganese dioxide to form ABTS radicals ($ABTS^{\bullet+}$).[15] $ABTS^{\bullet+}$ absorbs light in the visible region, producing a bluish-green colour by losing electron from the ABTS nitrogen atom. The intensity of the bluish-green colour is measured at a wavelength of 739 nm. The loss of the blue colour is an indication of the reduction of ABTS radical by an antioxidant.[23] Antioxidant compounds and $ABTS^{\bullet+}$ react in two stages. In the first stage, one molecule of $ABTS^{\bullet+}$ removes an electron or hydrogen atom from the antioxidant compound and generates a semiquinone radical. In the second stage, the semiquinone radical combines with another $ABTS^{\bullet+}$molecule to produce $ABTS^{\bullet+}$/antioxidant adduct. In summary, antioxidant compounds donate hydrogen atom to $ABTS^{\bullet+}$, resulting in
decolorization of the solution, due to the reducing concentration of $ABTS^{\bullet+}$ and eventual loss of the blue colour (Figure 2).[25,26]
The result of the antioxidant activity of ginger extracts, as well as vitamin C is presented in Figures 3 and 4. Figure 3 shows the relationship between the concentration of ginger extracts and the percentage inhibition of ABTS radical. The result showed a concentration-dependent increase in the ABTS radical scavenging activity of the extracts. In Figure 4, the antioxidant activity of ginger extracts was expressed in terms of IC_{50} value, which is the antioxidant concentration that reduces 50% of the ABTS free radical. The higher the IC_{50} value, the weaker the antioxidant activity, and the lower the IC_{50} value obtained, the higher the antioxidant activity.[25,26] The result showed that the ethyl acetate extract had the highest antioxidant activity with IC_{50} value of 20.02 µg/mL. The IC_{50} value of the n-hexane and ethanol extracts were 25.10 µg/mL and 28.67 µg/mL, respectively. Vitamin C (positive control) produced the lowest IC_{50} value of 2.22 µg/mL, which is an indication of its potent antioxidant activity.[27]
The ethyl acetate extract of ginger had the most potent and significantly higher antioxidant activity compared to the other two extracts ($P < 0.05$), and this correlated with the flavonoid content of the extracts. Bioactive compounds such as flavonoids and phenolics have been shown to contribute significantly to the antioxidant activity of plant extracts, including ginger extracts.[8,28-30]

Table 3: Amino acid residues of SARS CoV-2 main protease bound with the ligands

Ligand	Amino acid residue				Ligand	Amino acid residue			
	Hydrogen Bond (Å)	**Van der Waals**	**Pi interaction**	**Hydrophobic**		**Hydrogen Bond (Å)**	**Van der Waals**	**Pi interaction**	**Hydrophobic**
catechin	GLY A127 (2.68, 2.94) GLU B288 (2.35)	GLN B127 TYR B126 ARG A4 TYR A126 PHE B3 PHE B291 SER B284	LYS A5 ARG B4 LYS B5	LYS A5 LYS B5	naringenin	ARG B4 (3.25) TYR B126 (5.84)	PHE B3 GLN B127 ALA B7 VAL B125 VAL A125 ALA A7 TYR A126 GLN A127 GLU B288	LYS A5 LYS B5	LYS A5 LYS B5
epicatechin	GLY A127 (4.16, 3.96) GLU B288 (4.90)	ALA A7 VAL B126 ALA B7 GLN 127 TYR B126 TYR B127 SER B284	LYS A5 LYS B5	LYS A5 LYS B5	quercetin	PHE B3 (4.29) LYS B5 (3.38) GLN A127 (4.83) LYS A5 (2.84)	SER B284 GLU B288 TRP B207 PHE B291 ARG B4 TYR A126 ALA A7 VAL B125 TYR B126 ALA B7 GLN B127 MET A6 ARG A4	LYS A5 LYS B5	LYS A5 LYS B5
rutin	SER B284 (4.27) SER A284 (4.08) GLN A127 (4.97) VAL A125 (4.44) GLU A288 (5.74) LYS B5 (3.31)	LEU A282 LEU B282 GLY B283 PHE B291 GLY A283 GLU B288 LEU A286 LEU B286 ALA A285 ALA B285 TYR B126 ALA B7 ALA A7 MET B6 TYR A126 ARG A4 PHE B3	ARG B4 LYS A5	GLU A288 ARG B4 LYS A5 ARG A4 PHE B3 LYS B5	gingerol	ASP A155 (4.21, 5.14)	GLN A299 ARG A298 ASP A153 SER B123 PHE A8 PRO B122 LYS A12	TYR B118 LEU B141 PRO A9 ILE A152 TYR A154	ARG A298 SER B123 TYR B118 LEU B141
					zingiberol	PHE B103 (4.90)	GLU B178 TYR B37	TYR B101 LYS B102 PHE B103	TYR B101 LYS B102 PHE B103

Phenolic and flavonoid compounds in ginger with strong antioxidant activity include gingerol, shogaol, zingeberols, and zingerone.[30] Flavonoids donate hydrogen or electrons to free radicals to stabilize them, such that the higher the flavonoid content in an extract, the higher the antioxidant activity.[23]

In general, ginger is thought of as a powerful antioxidant. It scavenges harmful oxidants such as peroxides, super oxides, hydroxy radicals, and other reactive oxygen species (ROS).[1,5] The antioxidant activity of ginger may be impacted by the type of extraction solvent used. For

example, ethyl acetate and aqueous extracts of ginger have been reported to have more excellent antioxidant qualities than the ethanol, diethyl ether, and n-butanol extracts.[4]

In silico activity against SARS-CoV-2 main protease

Molecular docking simulation was carried out using flavonoids identified in ginger, namely; catechin, epicatechin, rutin, naringenin, quercetin, gingerol and zingiberol. The selected ligands were docked with SARS-CoV-2 main protease (Mpro) (code 7cam protein) which comprises two monomers code 6lu7. The active site of the monomer is in the N-terminal belonging to domain I residues (10-99) and domain II residues (100-182). The C-terminal of the protease belonging to residues 198–303 plays a role in dimer formation. Dimerization makes the active site of one monomer inactive and the other monomer active. Compounds that inhibit dimerization of the protease have the potential to be used as protease inhibitors.[17,18] Ligands from the flavonoids, phenols, and xanthones class have been shown to inhibit the SARS-CoV-2 main protease.[31]

The binding energy of the docking interaction of the ginger ligands with SARS-CoV-2 main protease is presented in Figure 5. The negative values for the binding energy means that the interaction took place spontaneously and exothermically. The phenolic compounds (zingiberol and gingerol) were found to have lower binding energy compared to the flavonoid compounds. The smaller the binding energy, the stronger the interaction.[32,33] The flavonoids had binding energies ranging from -7.9 kcal/mol to -9.90 kcal/mol. Based on their binding energy values, zingiberol and gingerol are considered to have great potential as inhibitors of SARS-CoV-2 main protease. Figure 6 shows that phenols and flavonoids of ginger have different binding regions. Flavonoids such as catechin, epicatechin, rutin, naringenin, and quercetin tend to bind at the same binding region. Meanwhile, zingiberol and gingerol have different binding regions.

SARS-CoV-2 main protease has a secondary structure of α-helix, *β*-sheet, random coil, and *β*-turn. A total of 7 α-helix, 13 *β*-sheets, and 18 *β*-turns have been reported to be the active site of SARS-CoV-2.[31] Changes in the secondary structure of a protein can affect its stability.[34,35] Visualization of the docking interaction of the SARS-CoV-2 main protease with ligands from ginger extract shows that the interactions were through hydrogen bonds, Van der Waals, and π-π orbital interactions (Figure 6). The interactions were dominated by Van der Waals interaction, and the amino acid residues involved are shown in Table 3.

The 7cam protein has been used to determine the binding site of SARS-CoV-2 Mpro in dimer form. Changes in residue stability in the dimer structure can inhibit the enzyme's catalytic activity. SARS-CoV-2 Mpro is a homodimer with a nearly perpendicular structure.[17] SARS-CoV-2 Mpro is one of the targets for COVID-19 drug discovery.[36]

SARS-CoV-2 causes oxidative stress (OS) and hyperinflammation, which result in pulmonary and extra-pulmonary symptoms.[37] Suppression of nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of antioxidative responses and cellular homeostasis, may cause OS and hyperinflammation in COVID-19.[4] The Nrf2 pathway prevents the development of cytokine storm and OS, and the expression of pro-inflammatory cytokines. The active compounds in ginger, including gingerol and zingiberol have demonstrated antioxidant action through the Nrf2 signaling pathway. Nrf2 activators may be crucial in lowering inflammation caused by SARS-CoV-2 infection by inhibiting the NLRP3 inflammasome in COVID-19.[37,38]

Naringenin in an *in silico* study shows binding to SARS-CoV-2 main protease by forming H-bond with an amino acid of the main protease active site.[12] In another *in silico* study of flavonoids with SARS-CoV-2, it was reported that quercetin, cathecin, and epigallocathecin are potential inhibitors of SARS-CoV-2 main protease.[32]

In a clinical trial, a combination of zingiber and echinacea were administered alongside standard treatment (hydroxychloroquine) to a group of SARS-CoV-2 infected patients, while another group of SARS-CoV-2 infected patients (control) received the standard treatment only. The result showed a significant ($P<0.05$) improvement in symptoms of COVID-19 including coughing, dyspnea, and muscle pain in the zingiber and echinacea treated group compared to the standard treatment (control) group.[3]

Conclusion

The results of the present study have shown that the total flavonoid content and antioxidant activity of Emprit ginger (*Zingiber officinalle* var Amarum) extract were greatly impacted by the extraction solvent. Ethyl acetate, a semipolar solvent was more effective in extracting flavonoids from ginger, and the ethyl acetate extract demonstrated a significantly higher antioxidant activity than the n-hexane and ethanol extracts. Major phenolic and flavonoid compounds identified in ginger including catechin, epicatechin, rutin, naringenin, quercetin, gingerol and zingiberol showed various interactions including hydrogen bond, Van der Waals, and π-π orbital interactions with SARS-CoV-2 main protease (Mpro) with binding energies ranging from -4.80 to 9.90 kcal/mol. The findings from the study have shown that ginger has potent antioxidant properties, and also showed promising potential as SARS-CoV-2 inhibitor.

Conflict of Interest

All the authors declare no conflicts 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

This research was supported by Regional Planning, Research, and Development Agency (BARENLITBANGDA) Semarang Regency, which assisted in exploring local herbs in Semarang Regency. The authors also thank to the Kemetul Village Government in Semarang Regency, Central Java, Indonesia, for the technical support.

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