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Original Research Article

In Vitro and *In Silico* Antioxidant Activity of Lemon-scented Gum (*Eucalyptus citriodora* Hook.) Cultivated in North Sumatra

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ABSTRACT

Eucalyptus citriodora Hook, also known as lemon-scented gum is a perennial plant that has been cultivated in North Sumatra for its essential oils and is being developed as a source medicinal compounds due to its bioactivities. While this plant has been extensively utilized for practical purposes in North Sumatra, empirical evidence regarding its antioxidant activity remains undisclosed, thereby piquing the interest of natural product chemists. To investigate the potential antioxidant activity of crude bioactive extracts from *E. citriodora* leaves, both *in vitro* and *in silico* studies were conducted. Crude extract was obtained by maceration of simplicia in methanol, followed by solvent-solvent extraction using hexane, and ethyl acetate. Antioxidant activity was determined using DPPH radical scavenging activity (*in vitro*). An *in silico* study was done using four protein targets namely; lipoxygenase, cytochrome P450 family 2 subfamily C member 9 (CYP2C9), NADPH-oxidase, and xanthine oxidase with curcumin and gallic acid as standard compounds. The highest yield was obtained from the hexane extract (54.3 g), followed by the methanol (42.5 g) and ethyl acetate extracts (11.6 g). The ethyl acetate extract of *E. citriodora* leaves showed the highest antioxidant activity (IC_{50} = 44.78 µg/mL), followed by the methanol (IC_{50} = 65.13 µg/mL), and hexane extracts (IC_{50} = 140.45 µg/mL). The best docking score expressed as high binding free energy was obtained on xanthine oxidase (ΔG = -6.7 kcal/mol) which was higher than the standard compound, gallic acid (ΔG = -6.5 kcal/mol). The high content of methyl gallate in the leaves of *E. citriodora* implies its prospect to be formulated into herbal drugs for the treatment hyperuricemia in the future.

Keywords: 2,2-Diphenyl-1-picrylhydrazyl, Antioxidant, *Eucalyptus citriodora*, Hyperuricemia, Xanthine oxidase.

Introduction

Medicinal plants have been used to treat a variety of disorders for ages.[1] There has been a surge of interest in discovering plant metabolites that have beneficial impacts on human health in recent decades.[2] Antioxidants, also known as free radical scavengers, have received a lot of interest because of their therapeutic potential.[3] Furthermore, the biological actions of plant phytochemicals may be useful in the pharmaceutical sector, as they reduce lipid peroxidative damage linked to pathological disorders including aging, coronary atherosclerosis, Alzheimer's disease, and carcinogenesis.[4,5] Natural products derived from plants as a source of antioxidant could be attributed to a number of factors that all contribute to a reduction in cellular oxidative stress. Some compounds may act as inducers of antioxidant enzymes, inducers of endogenous antioxidant compound biosynthesis, or inhibitors of enzymes which metabolic action generates reactive oxygen species (ROS) as a by-product.[6,7] Reactive oxygen species (ROS) play a physiological role in regular cell activity, but when their synthesis outnumbers their eradication, the body becomes overwhelmed with ROS, which can damage essential biomolecules, resulting in diseases.[8]

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In addition, antioxidant compounds function as molecules that can neutralize free radicals, thereby mitigating their harmful oxidative effects.[9–11] *Eucalyptus citriodora* Hook [syn. *Corymbia citriodora* (Hook.) K.D. Hill & L.A.S.Johnson] or lemon-scented gum is a perennial tree species commonly found in the eastern state of Australia also distributed in a number of tropical countries including Indonesia.[12] The plant is recognized for producing essential oils, primarily from the leaf portions, which are one of the richest and most cost-effective sources of citronellal, one of the key components that imparts the unique lemon odor. Oxygenated terpenes citronellal, citronellol, and isopulegol, as well as numerous other minor chemicals, are also detected in the extracts of this plant species.[13] The essential oils of *E. citriodora* exhibit biological activities such as antimicrobials,[14] insect repellent,[15] and antioxidant activities,[16] which has promoted its use as a medicinal plant to treat human disorders. There is a growing interest in commercial plantation of *E. citriodora* in Indonesia, especially North Sumatra because the majority of people still have limited information on its functionality as an essential oil-producing plant and on its medicinal properties.[17] Considering the limited information available on the phytochemical composition and medicinal properties of *E. citriodora* cultivated in Indonesia, particularly North Sumatra, this study aims to fill the knowledge gap by exploring its potential as a source of antioxidant compounds. The high content of phenolic compounds and the presence of methyl gallate in the leaf extract, as revealed by H/C-NMR spectroscopy analysis, suggest significant antioxidant activity.[18] By determining the antioxidant capacity of the crude plant extracts and evaluating the potential of methyl gallate as an antioxidant through *in silico* approaches, this study seeks to contribute to the understanding of *E. citriodora* therapeutic properties and its utility in the pharmaceutical sector. The results of this study could facilitate the development of natural antioxidant agents derived from *E.*

citriodora, thereby encouraging its commercial cultivation and medicinal applications.

Materials and Methods

Collection and Authentication of Eucalyptus citriodora Leaves
The plant material, *Eucalyptus citriodora* Hook was collected in April 2019 from an area of plantation owned by PT. Toba Pulp Lestari Tbk located in Dolok Nauli Distict, Toba Samosir Regency, North Sumatra Province, Indonesia. The specimen was authenticated by plant taxonomists in Herbarium MEDA/ Medanense, Universitas Sumatera Utara, Medan, Indonesia (No. 189/MEDA/2019).

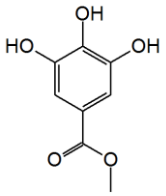
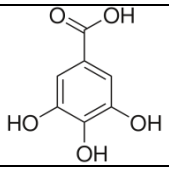
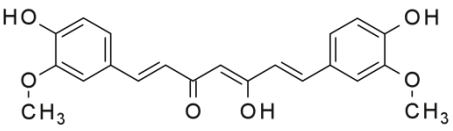
Extraction of Eucalyptus citriodora Leaves
The air-dried leaves of *E. citriodora* were pulverized into powder and macerated in methanol (MeOH pro analysis, Merck, Germany) for 48 h with three repetitions to yield a total of 153.6 g of crude extracts concentrated *in vacuo* using a rotary evaporator. The crude extracts were further fractionated using two different solvents namely; hexane (C6 pro analysis, Merck, Germany) and ethyl acetate (EtOAc pro analysis, Merck, Germany), concentrated *in vacuo*, weighed for its yield (g) and stored in dark vials prior to to use.

Antioxidant assay using DPPH
The antioxidant capacity of the extracts was determined through an *in vitro* radical scavenging assay. 2,2-diphenyl-1-picryl-hydrezyl (DPPH) Merck, Germany) was used as free radical. Various concentrations (100, 75, 50, 25, 10 µg/mL) of crude extracts (0.1 mL) and ascorbic acid as a control were added to methanol solution (0.004%) of DPPH. After 30 min of incubation at room temperature, the absorbance of the solution was read at 517 nm using UV-Vis spectrophotometer 1800 (Shimadzu, Japan). Decrease of absorbance indicated the radical scavenging activity of the solutions as determined using the following formula:[19]

$$DPPH\ Radical\ scavenging\ activity(\%) = \frac{(Absorbance_{control} - Absorbance_{sample})}{Absorbance_{control}} x100 \quad \ldots (1)$$

In Silico analysis of antioxidant compounds
Crystalographic or 3D protein structure of target proteins involved in inflammation and oxidative stress were retrieved from the RSCB Protein Data Bank through https://www.rcsb.org/. The target proteins were Lipoxygenase (PDB: 1N8Q), Cytochrome P450 family 2 subfamily C member 9/ CYP2C9 (PDB: 1OG5), NADPH-oxidase (PDB: 2CDU) and Xanthine oxidase (PDB: 3NRZ). Protein structures were prepared by removing the intact ligands, metal atoms, water molecules and other non-covalent bonds and saved into pdbqt format using AutoDock Tools. Ligand structures used in this study were retrieved from PubChem through https://pubchem.ncbi.nlm.nih.gov/ with a dataset as shown in Table 1. The 3D structures were optimized using Open Babel featured in PyRx. Molecular docking was performed for protein structures with grid box centre coordinates and size from previous study[20] and not exceeding 2 Å threshold using AutoDock Vina. The results from molecular docking analysis were expressed as ΔG binding energy values (kcal/mol). The 2D/3D interactions between proteins and ligands were visualized using BIOVIA Discovery Studio (Dassault Systems, San Diego, CA, USA).

Table 1: Ligands used in molecular docking simulation against protein targets

Compound(s)	PubChem ID	Molecular weight (g/mol)	Formula	2D Structure
Methyl gallate	7428	184.15	$C_8H_8O_5$	
Gallic acid (Control)	370	170.12	$C_7H_6O_5$	
Curcumin (Control)	969516	368.4	$C_{21}H_{20}O_6$	

Statistical analysis
All numerical data were presented as the mean of three replicates, except for yield and binding affinities, which were analyzed descriptively. The inhibitory concentration required by an antioxidant to achieve a 50% reduction of DPPH radicals (IC_{50} in µg/mL) was determined using linear regression analysis, by plotting the concentration of samples against DPPH scavenging activity, performed with Microsoft Excel version 2019.

Results and Discussion

The data in Table 2 showed that dried leaves of *E. citridora* yielded a high amount of hexane extract (54.3 g), followed by methanol extract (42.5 g) and ethyl acetate extract (11.6 g). The result of phytochemical screening is also presented in Table 2. It is expected that the highest yielding extract or hexane extract contained a high portion of terpenoids as indicated from the screening while other major groups such as saponins were only detected from the MeOH extract and phenolics detected in both EtOAc and MeOH extracts. It was later shown that, in addition to steam distillation for essential oils, solvent extraction was also effective in extracting this group of compounds. However we did not conduct any further phytochemical identification from the hexane extract to validate the presence of citronellal, citronellol, and eucalyptol as the dominant compounds of the species. Various extraction techniques and solvent selections significantly alter the yield, composition, and bioactivity of *Eucalyptus* extracts. Although essential

oil yields are typically higher in fresh leaves, dried leaves of Brazilian cultivars produced higher yields, with citronellal and neo-isopulegol being the most dominant components.[21] Six *Eucalyptus* species were optimized through extraction techniques to achieve the highest yield, with methanol proving to be the most effective solvent.[22] In *Eucalyptus camaldulensis*, hexane extraction at 65°C gave the highest yeild.[23] Another study using methanol extracts of *Eucalyptus globulus* demonstrated the highest yield and bioactivity, with 1,8-cineole identified as the dominant component.[24] The antioxidant capacity was determined to evaluate the potential of phytochemicals present in each extract of *E. citridora*. Results of DPPH radical scavenging activity are presented in Figure 1. All tested extracts were effective in a concentration-dependant manner. The highest DPPH radical scavenging activity of 97% was achieved at a concentration of 100 µg/mL for EtOAc, followed by MeOH (69%), and hexane (38%) at the same concentration. The formula generated from a linear regression analysis between DPPH radical scavenging activity (%) and sample concentration (µg/mL) of each extract ands is presented in Figure 2. The EtOAc extract exhibited significant DPPH radical scavenging capacity with IC_{50} value of 44.78 µg/mL, which indicated its prominent antioxidant capacity. In contrary, the highest yield extract (hexane) failed to display a higher antioxidant activity than the other samples. The antioxidant properties of ethanol extract of *E. citriodora* from Taiwan has been reported with IC_{50} value of 5.11 µg/mL which was better than findings from this study.[25] In addition, a study led by Singh *et al*[26] reported an IC_{50} value of 425.4 µg/mL from essential oils (citronellal, *β*-citronellol, isopulegol) of *E. citriodora* cultivated in India. Other study reported that the highest antioxidant capacity when tested using ABTS assay was obtained in the essential oil of *E. citriodora* than its relatives such as *Eucalyptus dives, Eucalyptus globulus, Eucalyptus delegatensis, Eucalyptus pauciflora, Eucalyptus radiata, Eucalyptus smithii, Eucalyptus urophylla* and *Eucalyptus viminalis*.[14] As a result, the antioxidant capacity of *E. citriodora* may vary depending on its cultivation and growth area, extraction method, and assay. Lenny *et al*[18] has reported for the first time, the methyl gallate as a dominant phenolic compound found in the leaves part of *E. citriodora* grown in North Sumatra. Methyl gallate is an abundant phenolic acid in plants, and its use in herbal remedies may be due to its notable biological benefits, including antioxidant, anticancer, and antibacterial properties.[27] The antioxidant activity of methyl gallate and its original compound, gallic acid were subjected to a molecular docking study for its potential in free radical scavenging by interact directly with specific proteins in the human body. The reducing group, such as hydroxyl and thiol, are common features in phenolic compounds including methyl gallate and gallic acid.[28] Curcumin, a polyphenol compound commonly aids in the management of oxidative and inflammatory conditions, was used in this study as a gold standard to compare the potential of methyl gallate as antioxidant compound.[29] Antioxidants can function either as single chemical entities or synergistically in diverse groups commonly found in herbal mixtures. This can be advantageous in certain aspects; however, purity remains essential for advancement to the pharmaceutical industry.[30,31] An *in silico* investigation was then conducted to elucidate the potential mechanism of methyl gallate from *E. citriodora* as an inhibitor of hyperuricemia (Table 3). Xanthine oxidase was the most vulnerable protein target during the simulated interaction with methyl gallate, gallic acid and curcumin. However, curcumin still bonded more effective than methyl gallate and gallic acid to xanthine oxidase as indicated from its more negative ΔG value (-8.6 kcal/mol). Here, we obtained a higher binding activity of methyl gallate than gallic acid for three protein targets (CYP2C9, NADPH-oxidase, xanthine oxidase) which is quite interesting. A similar study has been conducted by Sutomo and Pratama[32] to test the antioxidant potential of methyl gallate and gallic acid with xanthine oxidase (3NRZ). They also reported a higher ΔG value for methyl gallate (-7.45 kcal/mol) than gallic acid (-5.93 kcal/mol) producing a series of conserved interaction to amino acid residues such as 802-Glu, 880-Arg, 914-Phe and 1010-Thr. Negative ΔG scores indicate that the interaction between all receptors and curcumin, methyl gallate, and gallic acid will occur spontaneously. Three out of four receptors (except 1N8Q) exhibit stronger affinity for methyl gallate than for gallic acid. This is noteworthy because methyl gallate is known to have antioxidant activity through a direct scavenging mechanism that is weaker than that of gallic acid.[33,34] Nonetheless, both ligands demonstrate inferior activity compared to curcumin. The molecular interaction between methyl gallate and gallic acid to xanthine oxidase is presented in Figure 3. Methyl gallate bonded with the same amino acid residue by gallic acid namely 262-Thr and 347-Ser (2 H-bonds) which also explained its relative position to the protein. In this study, the compound was failed to interact with the active sides which was located further from 648-Leu to 1076-Pro as compared to the affinity by co-crystalized ligand or febuxostat which may due to the different ligand and protein preparation technique. Febuxostat is a non-purine XO inhibitor that effectively reduces uric acid production by forming a stable complex with both reduced and oxidized forms of the enzyme. It also reduces reactive oxygen species (ROS) formation, potentially mitigating vascular inflammation and oxidative stress.[35] Recent research shows that febuxostat suppresses NLRP3 inflammasome-mediated IL-1β secretion and cell death through both mitochondrial ROS-dependent and independent mechanisms, including increased intracellular ATP and improved mitochondrial energetics via activation of the purine salvage pathway.[36] The enzyme, xanthine oxidase (XO) catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid, which is eliminated by the kidneys. Hyperuricemia is caused by excessive uric acid synthesis and/or insufficient uric acid removal. Xanthine oxidase has been identified as a highly effective therapeutic target for the treatment and control of pathological diseases caused by excessive blood uric acid levels. Selective and direct inhibition to XO could be a superior therapy strategy for diseases induced by XO, such as gout, inflammatory disease, oxidative damage, and cancer. Based on our findings, it is implied that the use of *E. citriodora* leaves may be potential to treat hyperuricemia as indicated from its prominent antioxidant capacity (IC_{50}) and evidence through *in silico* analysis between methyl gallate and xanthine oxidase.

Table 2: Phytochemical constituents and the yeilds of the crude extracts of *Eucalyptus citriodora*

Extract(s)	Alkaloids	Phenolics	Saponins	Steroids	Terpenoids	Yield (g)
Hexane (C6)	–	–	–	–	+	54.3
Ethyl acetate (EtOAc)	–	+	–	–	–	11.6
Methanol (MeOH)	–	+	+	–	–	42.5

Colorimetric detection [(+) = Presence, (–) = Absence]

Table 3: The results of molecular docking simulation against protein targets

Ligand(s)	Binding Free Energy ΔG (kcal/mol)			
	1N8Q	1OG5	2CDU	3NRZ
Methyl gallate	-6.1	-6.2	-6.6	-6.7
Gallic acid (Control)	-6.7	-6.0	-6.4	-6.5
Curcumin (Control)	-7.2	-8.3	-8.3	-8.6

1N8Q = Lipoxygenase, 1OG5 = CYP2C9, 2CDU = NADPH-oxidase, 3NRZ = Xanthine oxidase

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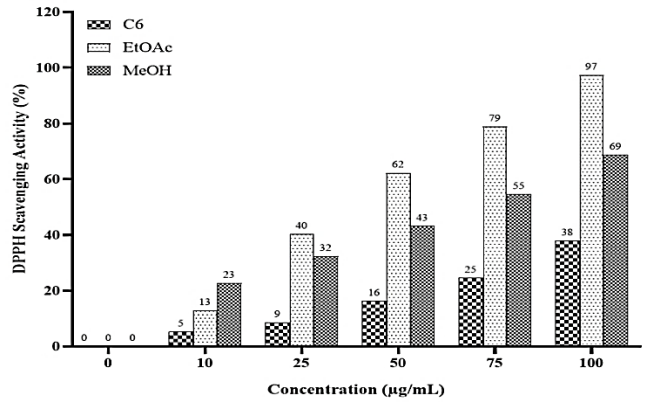

Figure 1: DPPH scavenging ability of different extracts of *Eucalyptus citriodora*. C6: Hexane, EtOAc: Ethyl acetate, MeOH: Methanol

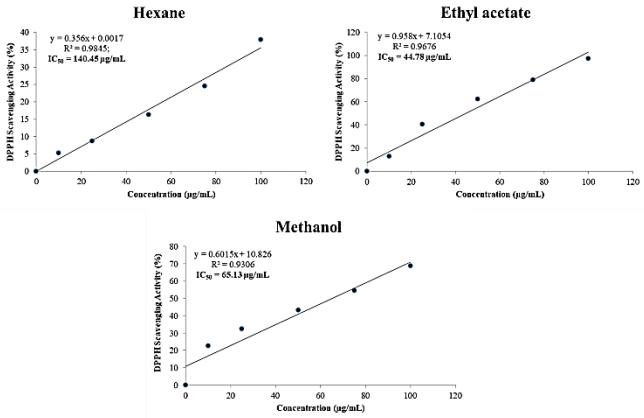

Figure 2: Half-maximal Inhibitory concentration (IC_{50}) value of tested extracts in DPPH assay

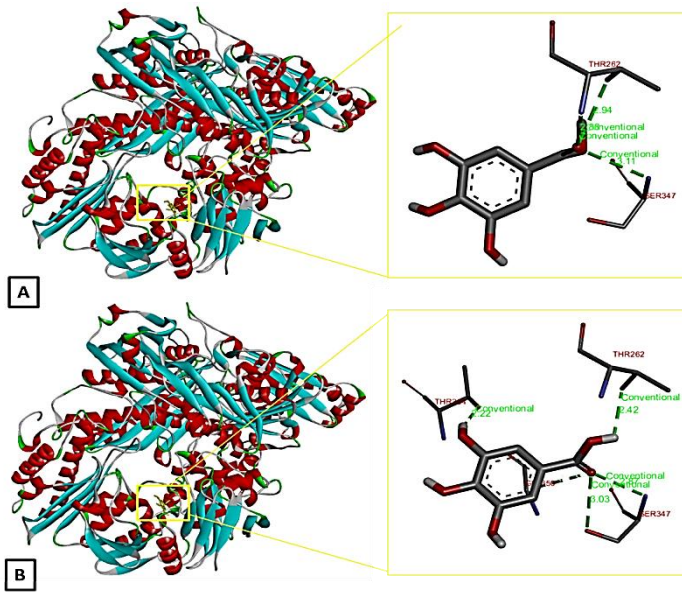

Figure 3: Comparison of the ligand interaction between methyl gallate (a) and gallic acid (b) on xanthine oxidase receptor and its interaction.

Conclusion

This study highlights the potential of *Eucalyptus citriodora* Hook. as a medicinal plant and a source of essential oils. The crude extract with high yields was obtained using solvent extraction, particularly hexane. The ethyl acetate extract showed the highest antioxidant activity, followed by methanol and hexane extracts. *In silico* studies revealed promising interactions with protein targets, particularly xanthine oxidase. The presence of methyl gallate suggests potential use as herbal remedy for the treatment of hyperuricemia. Furthermore, the results of this study can be utilized by plantations to initiate research and development related to biopharmaceuticals derived from *E. citriodora*, targeting methyl gallate as one of the dominant compounds. This collaboration can be extended to universities and pharmaceutical industries, broadening the application of *E. citriodora* beyond just the production of essential oils and timber.

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.

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