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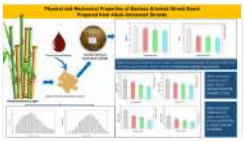

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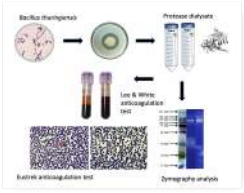

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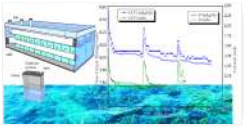

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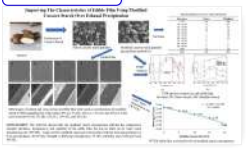

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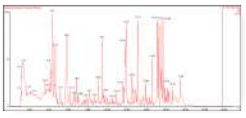

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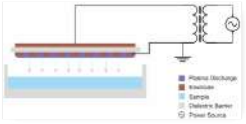

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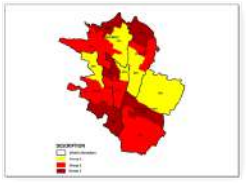

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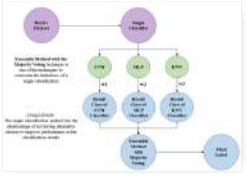

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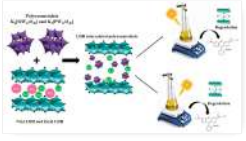

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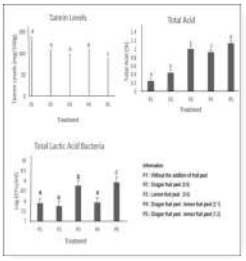

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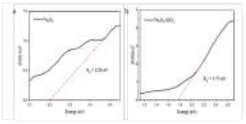

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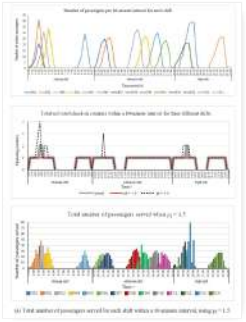

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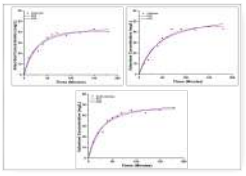

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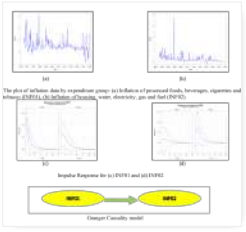

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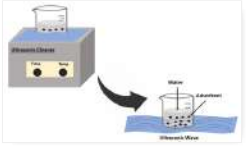

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Research Paper

Phytochemical, Metabolite Compound, and Antioxidant Activity of *Clinacanthus nutans* Leaf Extract from Indonesia

Nurlaili Susanti[1,2], Arifa Mustika[3*], Junaidi Khotib[4], Roihatul Muti'ah[5], Maftuchah Rochmanti[3]

[1]*Doctoral Program of Medical Science, Faculty of Medicine, Universitas Airlangga, Surabaya, 60132, Indonesia*
[2]*Faculty of Medicine and Health Science, Maulana Malik Ibrahim State Islamic University, Malang, 65144, Indonesia*
[3]*Department of Anatomy, Histology, and Pharmacology, Faculty of Medicine, Universitas Airlangga, Surabaya, 60132, Indonesia*
[4]*Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, 60132, Indonesia*
[5]*Department of Pharmacy, Faculty of Medical and Health Science, Maulana Malik Ibrahim State Islamic University, Malang, 65144, Indonesia*
*__Corresponding author__: arifa-m@fk.unair.ac.id

Abstract
The antioxidant properties from medicinal plants have protective effects against oxidative stress-linked diseases. Their antioxidant capacities are related to phenolic compounds, including phenols and flavonoids. *Clinacanthus nutans*, a native to Asia, including Malaysia, Thailand, and Indonesia, are used for treating diabetes and other diseases, but the exploration of the plant in Indonesia is still limited. Therefore, this study aimed to reveal the phytochemical constituents, metabolic compounds, and antioxidant activity of *C. nutans* extracts. Dried leaf powder was extracted with 70% ethanol and aqueous, each extract was subjected to qualitative phytochemical screening. Subsequently, total phenolic and flavonoid content was calculated using Folin-Ciocalteu and AlCl3 colorimetric assay. The antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. The profiles of phenolic and flavonoid compounds of 70% ethanolic extract were investigated by LC-MS/MS. Phytochemical screening showed that the extracts contain phenolic, flavonoid, tannin, and saponin. The total phenolic content of the 70% ethanol and aqueous extract was 4.14±1.9% mgGAE/g and 3.89±3.2% mgGAE/g, respectively. Meanwhile, the total flavonoid content obtained was 0.19±1.0% mgQE/g and 0.03±0.5% mgQE/g for 70% ethanol and aqueous extracts, respectively. Both extracts showed high antioxidant activity, with DPPH IC50 30.57 μg/mL for 70% ethanol and 37.24 μg/mL for aqueous extract. LC-MS/MS analysis of 70% ethanol extract revealed 5 phenolic and 3 flavonoid compounds, where DL-glutamic monohydrate and Boesenbergin B were the most dominant based on the percentage of area under the curve. *C. nutans* originating from Indonesia can be a good source of natural antioxidants because 70% ethanol and aqueous extract show high phytochemical content and antioxidant activity.

Keywords
Clinacanthus nutans, DPPH, LC-MS/MS, Total Flavonoid Content, Total Phenolic Content

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1. INTRODUCTION

Oxidative stress is related to the progressivity of various diseases, including diabetes, cardiovascular diseases, malignancy, and neurodegenerative disease (Tan et al., 2018). This condition is triggered by a disproportion in the generation of reactive free radicals and cellular antioxidant defense mechanisms, which causes oxidative stress and damage to cell macromolecular components such as proteins, lipids, and nucleic acids (Adwas et al., 2019). Although free radicals can arise from external sources including air pollution, radiation, smoking, alcohol, heavy metals, and some medicines (Valko et al., 2007), the body system is the most significant producer. Free radicals are also stimulated to produce in cells due to the respiratory chain, the cytochrome P450 system, oxidative processes in peroxisomes, and oxidase in phagocytic cells (Santo et al., 2016).

The body uses antioxidants as a defense against oxidative stress (Bhat et al., 2015). Recently, natural antioxidants have drawn interest of many people due to their potential health benefits as a safer alternative than synthetic antioxidants, which are labile and cause undesirable effects (Mahmoud et al., 2021). Numerous studies have revealed how natural antioxidants work, such as acting as hydrogen donors, interacting with certain proteins in the intracellular signaling pathway, affecting epigenetic processes, and modifying gut flora (Hrelia and Angeloni, 2020). Phytochemicals such as phenolics and flavonoids contribute to the antioxidant ability of plants. The presence of at least one phenol ring is required for the action, with the hydrogen

replaced by a hydroxyl, methyl, or acetyl group (Forni et al., 2019). The antioxidant potential of the plant is associated with the solvent selected, which determines the extraction yield (Hossain et al., 2021).

Clinacanthus nutans is a plant from Southeast Asia that belongs to the family of *Acanthaceae*. This plant is familiar with the local name "dandang gendis" in Indonesia, "belalai gajah" in Malaysia, and "payayor" in Thailand. Traditionally, it is utilized to treat skin infections, venomous bites, cancer, and diabetes mellitus (Kamarudin et al., 2017). Previous reports showed that *C. nutans* has analgetic, antioxidant, antiinflammation, immunomodulator, antimicrobial, anticancer, and antidiabetic activities (Khoo et al., 2018), but the exploration of this plant in Indonesia is still limited. Therefore, this study aimed to reveal the phytochemical constituents, metabolic compounds, and antioxidant activity of *C. nutans* leaf extracts.

2. EXPERIMENTAL SECTION

2.1 Materials

The plants were obtained from UPT Laboratorium Herbal Materia Medika Batu, East Java, Indonesia. The material used was identified to be *Clinacanthus nutans* leaves. The plant was thoroughly washed under running water then dried in 50°C oven for 48 hours. The dried leaves were powdered with an electric mill and sifted with a sieve of 90 mesh. Powder moisture content was calculated to be 9.77% with a moisture analyzer (HC103) and stored under dry conditions for extraction. Subsequently, the analytical grade chemicals used in this study were bought from Sigma-Aldrich.

2.2 Methods

2.2.1 Extraction Procedure

A 200 g of dried powder of *C. nutans* leaves was split into 2 portions for aqueous and ethanolic 70% solvent, where 100 g of the powder was dissolved in 1,000 mL of solvent (1:10) (Khoo et al., 2015). The extraction was performed by sonication method (SONICA 2400EP S3) 3 times for 10 minutes at room temperature. The filtrate was evaporated using a Rotary Evaporator (Heidolph Hei-VAP ML Adv/Pre) at 50°C, 70 rpm, and Cyler temperature 10°C. Subsequently, the obtained concentrated extract was dried in oven at 40°C. The dry extract was calculated with its yield value as follows:

$$\text{Yield value}(\%) = \frac{\text{extract weight}}{\text{powder weight}} \times 100\% \quad (1)$$

2.2.2 Screening for Phytochemical

A qualitative phytochemical test was conducted using standard phytochemical screening (Harborne, 1998). This test identified a group of metabolite compounds, including phenol (ferric chloride), flavonoid (lead acetate), tannin (ferric chloride), saponin (froth), Alkaloid (Wagner's), triterpenoid and steroid (Salkowski test). The total phenolic content (TPC) was performed using the Folin-Ciocalteu method as previously reported by Guchu et al. (2020). Test tubes with aliquots of 1 mg/mL were filled with 0.5 mL of Folin Ciocalteu reagent and mixed for 10 minutes. This solution then added by 10% sodium carbonate and incubated for 10 minutes. The absorbance was detected with a UV-Vis spectrophotometer at 765 nm, where gallic acid was provided as standard and a reagent in solvent as a blank. The total flavonoid content (TFC) was calculated with an AlCl3 colorimetric assay adapted from Kamtekar et al. (2014). During the process, test tubes with aliquots of 1 mL were filled with 4 mL of aquadest, 5% sodium nitrite solution, and 10% aluminum chloride. Subsequently, 5 minutes later, 2 mL of 1M sodium hydroxide and 10 mL aquadest were put in solution and mixed. A UV-vis spectrophotometer at 425 nm was used to calculate the absorbance of the solution, with distilled water as the blank and quercetin as the standard.

2.2.3 Antioxidant Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical inhibition method was used to test the in-vitro antioxidant activity (El Sayed et al., 2015). A total of 5 concentrations of extracts of 5, 10, 15, 20, and 25 μg/mL were prepared in ethanol. Test tubes with 3 mL of each extract and 1 mL of 0.2 mM DPPH diluted in solvent and mixed. Subsequently, the solution was incubated in light free conditions for 30 minutes. In this study, 4 mL of 0.2 mM DPPH solution in ethanol was used as a blank, and L-ascorbic acid was used as a control. Finally, the absorbance of the solution was detected by UV-Vis spectrophotometer at 517 nm. This test was conducted in triplicates. The inhibition activity was determined as follows:

$$\text{Inhibition activity}(\%) = \frac{[Ac - As]}{Ac} \times 100\% \quad (2)$$

As and *Ac* denote sample and control absorbance, respectively. The IC50 value was calculated from sample concentration and percentage of inhibition activity using linear regression.

2.2.4 LC-MS/MS Analysis

Aliquots of 10 mg was diluted in solvent and filtered with a 0.45-micron millipore membrane. Subsequently, 5 μl solution was injected using a micro syringe in the UPLC-QTOF-MS system. Chromatography was carried out using a gradient elution system. The velocity was adjusted at 0.2 mL/min and the mass spectrometry used was UPLC-MS/MS positive ionization mode with a QTOF analyzer. The gas temperature, the desolvation temperature, and the desolvation gas were set at 100°C, 350°C, and 796°C, respectively (Yildirim et al., 2022).

2.2.5 Statistical Analysis

The data were presented as mean ± standard deviation. The SPSS ver. 26 software was utilized for statistical computations, and differences were examined using an independent t-test, with $P<0.05$ determined significant.

3. RESULT AND DISCUSSION

3.1 Percentage Yield of Extracts

According to this study, the 70% ethanol and aqueous extract produced yield percentage of 13.74% and 13.40%. The yield of *C. nutans* leaf extract varies in previous studies, which include 15.29% (Yang and Song, 2021), 4.5% (Ismail et al., 2017), and 6.85% (Ismail et al., 2020), based on the raw materials used, the extraction process, and the choice of solvent. The yield of extract measures the proportion of raw materials that are usable from all raw materials. Meanwhile, a higher yield percentage indicates that the raw material has a high possibility of being used (Solihah et al., 2018). It was also discovered that the sonication method can improve extraction yield because the cavitation effect reduced material and solvent waste (Dzah et al., 2020).

3.2 Screening for Phytochemical

The qualitative phytochemical of *C. nutans* extracts confirmed positive for phenols, flavonoids, tannins, and saponins but lacked for alkaloids, terpenoids, and steroids as presented in Table 1. From Table 2, the quantitative phytochemical tests showed phenolic and flavonoid content in both extracts. Based on the results of statistical analysis, 70% ethanol and aqueous extract had no significant difference in TPC and TFC. Since the quality of plant extraction influenced by the solvent used, solvents with different polarities significantly affect the phytochemical constituents carried away during the extraction process (Ismail et al., 2020). Methanol and ethanol were selected because they can extract polyphenols. However, water was used since it is universally soluble in polar substances. Ethanol is safer than methanol for human consumption (Dai and Mumper, 2010).

The values of phytochemical screening in this study are slightly different compared to the previous literature. Thongrakard and Tencomnao (2010) extracted *C. nutans* leaves from Thailand (ethanol solvent 1:5) using maceration technique and reported a higher total phenolic content than in this study, at levels of 4.67 ± 3.60 mgGAE/g. Yang and Song (2021) revealed that the phytochemical screening of *C. nutans* from Malaysia extracted by maceration method with methanol solvent 1:4 contained phenolics, flavonoids, saponins, and phytosterols. Furthermore, the TPC obtained was 1.77 ± 0.008 mgGAE/g, which was lower than that found in this study, while the TFC with a value of 0.04 ± 0.001 mgQE/g was higher compared to the results of this study. Khoo et al. (2015) compared the TPC of each part of *C. nutans* from Malaysia with various extraction and drying methods. The leaf part and the combination of air/oven drying with the sonication method produced the highest phenolic content was 7.29 ± 0.11 mgGAE/g. The differences found in various studies can be influenced by geographical variations and extraction methods, which affected the number of phenolics and flavonoids (Ghasemzadeh et al., 2014; Ismail et al., 2017).

3.3 Antioxidant Activity of *C. nutans* Extracts

DPPH assay is commonly utilized to evaluate the antioxidant activity of phytoconstituents because of its low cost, simplicity in experimentation, repeatability, application at room temperature, and potential for automation (Munteanu and Apetrei, 2021). The antioxidant activity depends on its ability to donate electrons to DPPH radical. Furthermore, a colour change in the solution from purple to yellow can be detected using a UV-Vis spectrophotometer at 517 nm (Syarifah et al., 2021). The value of inhibitory concentration 50 (IC50), the concentration of antioxidants which eliminate 50% of free radicals, is the indicator used to demonstrate antioxidant activity (Foti, 2015). Furthermore, the power levels of antioxidants are determined based on the IC50 value, which is very strong if the value is <50 µg/mL and strong if the value is 50-100 μg/mL (Marjoni and Zulfisa, 2017).

This study discovered that both *C. nutans* extracts showed DPPH radical scavenging activity, as shown in Table 3. According to the grading, both extracts had very strong activity with IC50 values lower than 50 μg/mL. The 70% ethanol extract had a lower IC50 value than the aqueous extract but no significant difference based on statistical analysis. Antioxidant activity of *C. nutans* extracts from different countries showed varying results. Ghasemzadeh et al. (2014) examined the methanol extract of *C. nutans* cultivated in Malaysia and found that the IC50 for DPPH activity was at a higher concentration of 64.6 μg/mL. Similarly, IC50 for DPPH activity at higher concentrations was found in a study of *C. nutans* from Thailand extracted with 50% ethanol with a value of 110.4 ± 6.59 μg/mL (Pourreza, 2013). Among these results, *C. nutans* leaves from Indonesia have the highest antioxidant activity. However, the values obtained in this study must be confirmed with other antioxidant test methods other than DPPH.

Medicinal herbs are rich in phytochemicals with antioxidant properties (Altemimi et al., 2017). The presence of phenols, flavonoids, tannins, and saponins was discovered in a qualitative phytochemical examination of *C. nutans* extract. Quantitative phytochemical analysis of flavonoids and phenols showed considerable quantities. Since phenolic and flavonoid compounds have a high capacity to scavenge free radicals, the presence of these phytoconstituents increases the possibility that *C. nutans* is a potent antioxidant. Furthermore, the content of tannins and saponins can also impact the antioxidant power (Rodrigues et al., 2019).

3.4 Metabolite Profiling

The 70% ethanol extract of *C. nutans* leaves was further selected to detect for its phenolic and flavonoid content using LC-MS/MS. The chromatogram was processed using Masslynx 4.1 to determine the m/z spectrum. The prediction of the compounds interpreted by PubChem (https://pubchem.ncbi.nlm.nih.gov/). One molecule needs to be subtracted from the whole molecule when typing the molecular formula. It was necessary to deduct the entire m/z from the actual mass of the H atom, which was 1.0078 because the compound will acquire

Table 1. Phytochemical Screening of Leaf Extracts of *C. nutans*

Phytochemicals	70% Ethanol	Aqueous
Phenols	+	+
Flavonoids	+	+
Tannins	+	+
Saponins	+	+
Alkaloids	-	-
Terpenoids	-	-
Steroids	-	-

+, present; -, absent

Table 2. Phytochemical Quantitative of Leaf Extracts of *C. nutans*

Type of Solvent	TPC (mgGAE/g)	TFC (mgQE/g)
70% Ethanol	4.14 ± 1.9 [a]	0.19 ± 1.0 [a]
Aqueous	3.89 ± 3.2 [a]	0.03 ± 0.5 [a]

Data are shown as mean ± SD. Significant differences are indicated by different superscript letters in the same column ($p < 0.05$)

Table 3. DPPH Inhibition Activity of Leaf Extracts of *C. nutans*

Concentration (μg/mL)	% Inhibition 70% Ethanol	Aqueous	Ascorbic Acid
5	6.62 ± 0.26 [a]	7.00 ± 0.63 [a]	79.88 ± 0.64 [c]
10	15.33 ± 0.66 [a]	12.15 ± 0.63 [a]	93.88 ± 0.90 [c]
15	18.61 ± 0.19 [a]	15.49 ± 0.84 [b]	94.67 ± 1.78 [c]
20	26.56 ± 0.56 [a]	36.62 ± 0.07 [b]	94.87 ± 1.23 [c]
25	45.76 ± 1.73 [a]	32.50 ± 0.32 [b]	95.46 ± 0.91 [c]
IC50	30.57 μg/mL [a]	37.24 μg/mL [a]	1.70 μg/mL [c]

Data are shown as mean ± SD. Significant differences are indicated by different superscript letters in the same column ($p < 0.05$)

Table 4. Identification of Phenolic and Flavonoid Compound of 70% Ethanolic Extract of *C. nutans* Leaf

Retention Time	Area (%)	Measured Mass (m/z)	Formula	Compound	Group
1.344	19.51	118.0867	$C_5H_{11}NO_5$	DL-glutamic acid monohydrate	Phenol
3.629	3.25	162.0588	$C_6H_{11}NO_2S$	S-allyl-l-cysteine	Phenol
5.19	0.71	249.113	$C_{14}H_{16}O_4$	Alloevodionol	Phenol
6.885	2.17	440.2494	$C_{19}H_{37}NO_{10}$	5-[5-(2-Ethylbutoxy) -3,4-dihydroxy-6-(hydroxymethyl) -2-(methylamino) oxane-3,4-diol	Phenol
10.949	0.73	297.2069	$C_{17}H_{28}O_4$	Gingerdiol	Phenol
4.395	13.72	565.1572	$C_{26}H_{28}O_4$	Boesenbergin B	Flavonoid
6.596	0.77	262.1795	$C_{12}H_{19}N_7$	Phendimetrazine tartrate	Flavonoid
11.321	0.01	237.1857	$C_{15}H_{24}O_2$	Capsidiol	Flavonoid

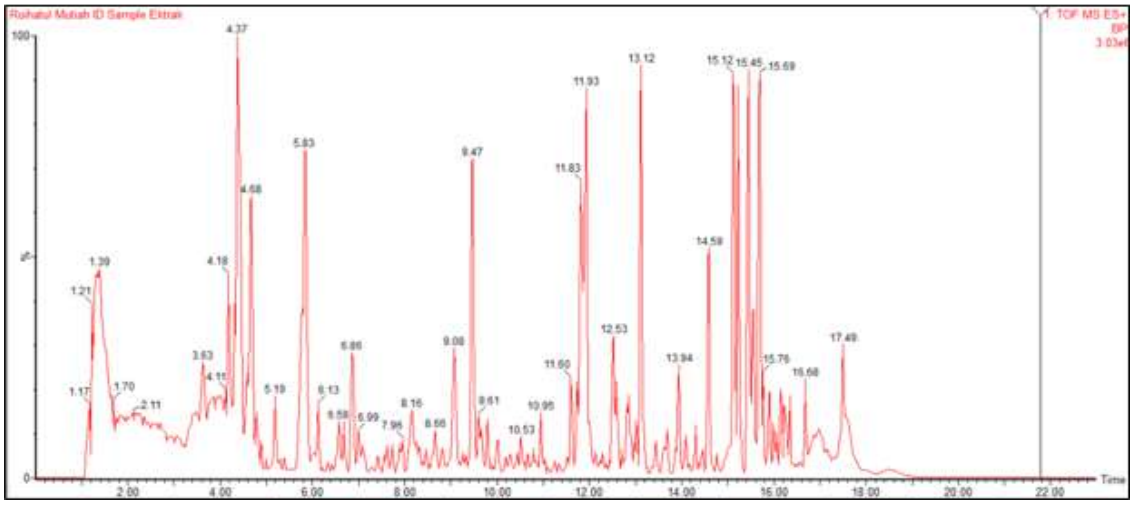

Figure 1. LC-MS/MS Chromatogram of 70% Ethanolic Extract of *C. nutans* Leaf

the H charge due to the positive ESI ion source. The observed and computed m/z were compared after learning the name of the chemical and its structure. When the difference was less than 0.0005, the expected compound was represented by the peak.

The chromatogram and results of data interpretation are shown in Figure 1 and Table 4. There were 5 phenolic compounds, which include DL-glutamic acid monohydrate, S-allyl-l-cysteine, Alloevodionol, 5-[5-(2-Ethylbutoxy)-3,4-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-6-(hydroxymethyl)-2-(methil amino)oxane-3,4-diol, and Gingerdiol. A total of 3 flavonoid compounds were also obtained, namely Boesenbergin B, Phendi metrazine tartrate, and Capsidiol. Several investigations stated that these compounds have antioxidant activity and other diverse pharmacological activities. S-allyl-L-cysteine extracted from *Allium sativum* had antioxidant activity at a concentration of 58.43 mg/L to scavenge DPPH and hydroxyl radicals (Sun and Wang, 2016). The antioxidant mechanism of S-allylcysteine is associated with its ability to scavenge free radicals and activate the transcription factor Nrf2 (Colín González et al., 2012). Alloevodionol contained in *Melicope sp.* also showed antioxidant, antiproliferative and anti-hepatitis C virus activity (Johnson et al., 2010; Widyawaruyanti et al., 2021; Le et al., 2021.

Boesenbergin is a compound from *Boesenbergia rotunda* with antioxidant and cytotoxic effects on various cancer cell lines and anti-inflammatory activity on RAW 264.7 (Isa et al., 2012). Meanwhile, 6-Gingerdiol, a Gingerol metabolite from Zingiberaceae, is reported to ameliorate cisplatin-induced oxidative stress by decreasing lipid peroxide markers and increasing antioxidant enzymes (Kuhad et al., 2006). Ginger supplementation in chemotherapy patients was also reported to reduce oxidative stress markers (Danwilai et al., 2017). Capsidiol isolated from *Capsicum annuum* exhibited immunomodulatory and antineuroinflammatory effects by decreasing levels of IL-6, IFNg, iNOS, and COX-2 in microglial cells (Yang and Song, 2021).

Phenolic compounds are a widely diverse group of phytoconstituents extensively distributed in plants that is an important part of food intake (Pourreza, 2013). Due to their antioxidant capacity, various phenolic compounds in daily diets can lower the risk of aging-related diseases (Shahidi and Ambigaipalan, 2015; Martins et al., 2016). Flavonoids are one of the phenolic compounds, where the hydroxyl atom in its structure is necessary for antioxidant activity and displays powerful scavenging of free radicals (Banjarnahor and Artanti, 2014). Furthermore, plants' antioxidant activity also increases endogenous antioxidants and triggers optimal homeostasis and appropriate enzymatic activity (Li et al., 2014; Shahidi and Ambigaipalan, 2015).

4. CONCLUSION

C. nutans is a source of medicinal plants with promising natural antioxidant activity. According to the results, it was discovered that 70% ethanol and aqueous extract had no significant difference in phytochemical content and antioxidant activity. Meanwhile, preparations based on these herbal raw materials require further investigation to prevent and treat the diseases associated with oxidative stress.

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