Journal of
Food and Pharmaceutical Sciences

Original Article

Aerobe Fermentation Enhanced Antioxidant Activity Index of *Citrus limon* Leaves

Santi Herlina[1], Arif Setiawansyah[1,2]* and Nurul Hidayati[2]

[1]Faculty of Pharmacy, Universitas Kader Bangsa, Palembang, Indonesia
[2]Akademi Farmasi Cendikia Farma Husada, Bandar Lampung, Indonesia
*Corresponding author: Arif Setiawansyah | Email: arif12.setiawansyah@gmail.com

Received: 30 January 2024; Revised: 10 May 2024; Accepted: 04 July 2024; Published: 23 August 2024

Abstract: The chemical composition and antioxidant activity of a natural product are directly correlated with the preparation process which can alter certain enzymatic pathways, leading to the alteration of the production of secondary metabolites affecting its biological activity. This study aims to observe the effects of the pre-extraction process of *Citrus limon* on its TFC, TPC, and antioxidant activity. Samples were prepared by two different pre-extraction techniques (solar drying and aerobe fermentation) and extracted using ultrasound-assisted extraction with ethanol 96%. The TFC and TPC were measured by a colorimetric method using a spectrophotometer UV-Vis. The antioxidant activity was tested on DPPH and calculated as AAI with ascorbic acid as a standard. The result showed that solar dried sample exhibited higher TFC and TPC than the fermented sample with TFC and TPC values of 32.09 ± 0.45 mg QE/g and 335.80 ± 0.80 mg GAE/g extract. In contrast, the antioxidant activity assay revealed that fermented samples provided higher antioxidant activity than solar-dried samples and even better than ascorbic acid with an IC_{50} value of 2.23 ± 0.19 µg/mL. The pre-extraction process significantly influences the TFC, TPC, and antioxidant activity of *Citrus limon*.

Keywords: Pre-extraction process; *Citrus limon*; Flavonoid; Phenolic; Antioxidant activity index

1. INTRODUCTION

Free radicals are one of the main causes of various degenerative diseases, such as cancer [1], diabetes mellitus [2], and hypertension [3]. The emergence of various degenerative diseases is directly related to oxidative stress due to the presence of the reactive oxygen species (ROS) that oxidizes cells and tissues in the body [4]. The negative impact of ROS can be tackled by donating the electron to the empty orbital of the radicals, resulting in reducing the reactivity of ROS, leading to cell and tissue oxidation can be avoided [5].

Antioxidants are those molecules that can transfer their electron to neutralize the ROS in which various chemical compounds have been synthetically developed [6]. However, the use of synthetic antioxidants faces several adverse events emerging new health problems for the community. Natural products have been known as a source of numerous phytochemicals that are biologically and pharmacologically active, one of which is as antioxidants [7]. *Citrus limon* is one of the terrestrial plants that is known and scientifically proven to possess excellent antioxidant activity [8]. This antioxidant activity was reported in view of the fact that the presence of flavonoids and

phenolic compounds found in several parts of *Citrus limon* provide plentiful hydroxyl groups acting as the source of electrons to be donated to the free radicals [9].

Total flavonoid content (TFC) and total phenolic content (TPC) in a natural product are directly linked to their antioxidant capacity in which the higher the TFC and TPC, the greater the antioxidant activity [10]. However, the TFC and TPC of plants vary depending on numerous factors that can influence their antioxidant capacity. The pre-extraction process is pointed out to be one of the factors affecting the TFC and TPC of plants that has been widely reported in various scientific articles. Dried-powdered plants are reported to have higher TFC and TPC than the fresh samples [11]. In contrast, the fermented samples exhibited better antioxidant activity than dry samples [12]. This justified that the diversity of pre-extraction treatment of plants might give different results. However, until the present time, there have been no reports regarding the best pre-extraction process for Citrus limon to obtain high TFC, TPC, and antioxidant activity. Therefore, this work was undertaken to evaluate the influence of the pre-extraction process of *Citrus limon* leaves on its TFC, TPC, and antioxidant activity.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

All chemicals and reagents used in this work were analytical grade: Ethanol 96% (Bratachem, Indonesia), Ethanol absolute (Merck, Germany), Acetic acid (Merck, Germany), Sodium carbonate (Merck, Germany), $FeCl_3$ (Merck, Germany), $AlCl_3$ (Merck, Germany), Magnesium powder (Bratachem, Indonesia), DPPH (Sigma-Aldrich, Singapore), Quercetin (Sigma-Aldrich, Singapore), Gallic acid (Sigma-Aldrich, Singapore), Ascorbic acid (Merck, Germany).

2.2 Sample collection and identification

Fresh leaves of Citrus limon were collected in Karang Endah, Muara Enim, South Sumatera-Indonesia in April 2023. The samples were then identified by the staff of Dr. Angga Dwihartama, School of Life Sciences and Technology, Bandung Institute of Technology.

2.3 Sample preparation and extraction

Fresh leaves of *Citrus limon* were prepared using two different techniques including solar drying and aerobe fermentation. The leaves were solar-dried using a direct solar drying method for three days. On the other side, some leaves were aerobe fermented in a dark and isolated chamber until wilted. The samples were then grounded, and 100 g of each sample were ultrasound-assisted extracted using 1 L of ethanol 96% for 30 mins at room temperature. The filtration using a Wathman filter paper was applied to separate the liquid extract from the residues. The residues were then re-extracted as much as two times using the same method as mentioned. The liquid extracts were collected and evaporated using a vacuum rotary evaporator (Buchi, Germany) at 70 rpm and 45°C. The extract yield was calculated using the formula described by Setiawansyah et al. (2018) [13]:

$$Yield\ (\%) = \frac{Weigh\ of\ crude\ extract\ (g)}{Weigh\ of\ dry\ powder\ sample\ (g)} x100$$

2.4 Total falvonoid content analysis

TFC was measured using a colorimetric method in a spectrophotometer UV-Vis (Shimadzu, Japan) as described by Nurlinda et al. (2021) [14] with minor modification. Approximately 15 mg of *Citrus limon* leaf extracts were dissolved in 15 mL ethanol and transferred 1 mL of extract solution to the test tube, mixing with 1 mL of $AlCl_3$ 5% and 1 mL of 120 mM acetic acid. The mixture was then measured at 370.5 nm after 30 mins incubation. The experiment was carried out in triplicate and TFC was calculated as quercetin equivalent using the following formula:

$$TFC = \frac{c\ x\ V\ x\ f}{m}$$

Where

TFC : Total flavonoid content (mg QE/g)
c : Quercetin equivalence (μg/mL)
V : Total volume of extract (mL)
f : Dilution factor
m : Extract mass (g)

2.5 Total phenolic content analysis

TPC determination was carried out using a method described by Sumaiyah et al. (2018) [15] with slight change by dissolving 10 mg of *Citrus limon* leaf extracts in 10 mL of ethanol. A 0.1 mL extract solution was then taken and mixed with 7.9 mL of distilled water and 0.5 mL of $FeCl_3$ 5%, then vortex for 1 min. The mixture was measured in a spectrophotometer UV-Vis (Shimadzu, Japan) at wavelength 745 nm after 30 mins incubation. The experiment was done in triplicate and TPC was calculated as gallic acid equivalent using the equation below:

$$TPC = \frac{c\ x\ V\ x\ f}{m}$$

Where

TPC : Total phenolic content (mg GAE/g)
c : Gallic acid equivalence (μg/mL)
V : Total volume of extract (mL)
f : Dilution factor
m : Extract mass (g)

2.6 Antioxidant activity assay

Antioxidant activity of *Citrus limon* leaf extract was determined using DPPH as explained by Setiawansyah et al. (2023) [16] with modifications. A stock solution of 50 μg/mL of DPPH was reacted with six different concentrations of *Citrus limon* leaves extract solution (1:1). The mixture was incubated in a dark room at 27°C for 30 mins and then measured in a spectrophotometer UV-Vis (Shimadzu, Japan) at the wavelength 517 nm. The experiment was run in three independent replications using ascorbic acid as standard. The antioxidant activity was calculated using the following formula:

$$Inhibition\ (\%) = \frac{Abs\ control - Abs\ sample}{Abs\ control} x\ 100$$

The IC_{50} calculation used the equation of linear regression obtained from the inhibition versus concentration and expressed as antioxidant activity index (AAI) using the following equation:

$$AAI = \frac{Final\ concentration\ of\ DPPH}{IC50}$$

2.7 Data analysis

The effect of the pre-extraction process of *Citrus limon* leaves extract on its TFC, TPC, and antioxidant activity index was analyzed statistically using One Way ANOVA followed by a Tukey's test in a GraphPad Prism 10 version.

3. RESULTS AND DISCUSSION

3.1. Effect of pre-extraction process on extract yield

The samples were ultrasound-assisted extracted and provided a significantly different yield of extract. Figure 1 depicts the effect of the pre-extraction process on the extract yield of *Citrus limon* leaf extract. It shows that different sample preparations influenced the extract yield in which the solar-dried *Citrus limon* gives the higher yield (17.3 gram) compared to the aerobe-fermented samples (14.3 gram).

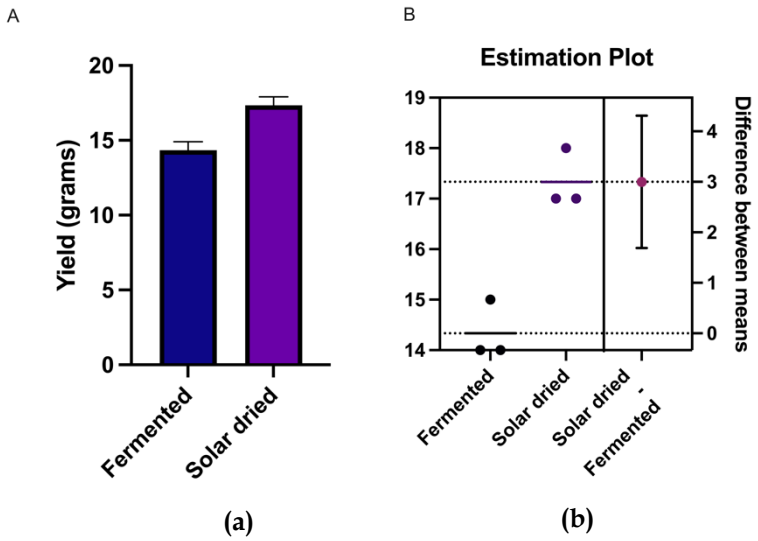

Figure 1. Effects of pre-extraction process on extract yield of *Citrus limon*. (**a**) Extract yield and (**b**) Unpaired independent t-Test result

The effect of the pre-extraction treatment was observed on the extract yield showing a significant difference between the fermented and solar-dried samples of *Citrus limon* leaves in which fermentation decreases the extract yield of *Citrus limon* leaves. This is not in line with previous work that reported natural fermentation increases the nutritional content of plant products, leading to an elevation of its extract yield [17]. However, this result is higher than other fermented plants including *A. artilis* (1.62-7.75%) [18]. The yield of the extract is directly correlated with the chemical constituent being extracted. It seems that fermentation alters the production of certain secondary metabolites production of *Citrus limon* leaves by enzymatic reaction being elevated which leads to the decomposition of the products. In contrast, solar drying decreases the water content of *Citrus limon*

leaves which causes degradation of enzymes responsible either for oxidizing or hydrolysis of constituents of *Citrus limon* leaves.

3.2 Effect of pre-extraction process on TFC and TPC

The flavonoids and phenolic content of *Citrus limon* were qualitatively analyzed prior to the quantitative determination. Table 1 describes the presence of flavonoids and phenols in *Citrus limon* leaves extract determined by colorimetric reaction using Mg^{2+} for flavonoids and $FeCl_3$ for phenols.

Table 1. Qualitative screening of flavonoids and phenols of *Citrus limon*

Secondary Metabolites	Samples	
	Fermented	**Solar dried**
Flavonoids	+	+
Phenols	+	+

The concept of flavonoid identification using magnesium (Mg^{2+}) is by harnessing the chemical properties of Mg^{2+} that can change the electron resonance of the gamma-benzopyrone within the C ring of the flavonoid. Changing the electron resonance causes the change of its structure to become a cyanidin structure that provides yellow, orange or reddish color [19] as illustrated in Figure 2b. Not all flavonoids can be identified by this reagent, but only applies to certain flavonoids that have gamma-benzopyrone structure including flavones, flavonols, and isoflavones.

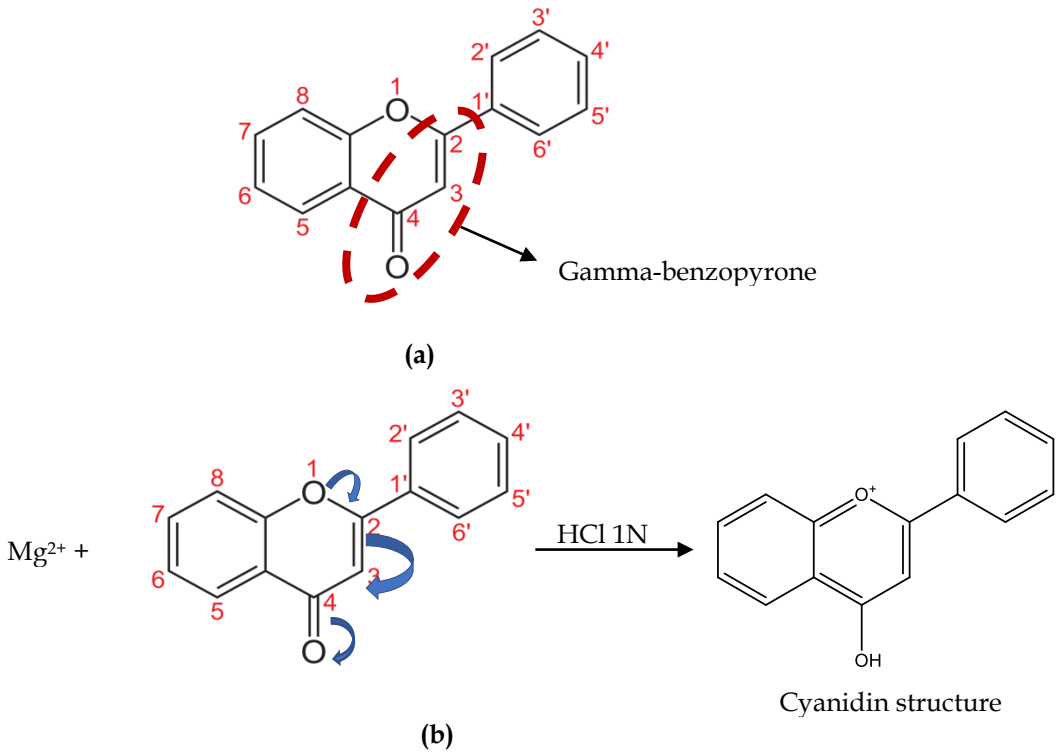

Figure 2. Schematic concept of flavonoid identification using Mg^{2+}. (A) a gamma-benzopyrone responsible for the reaction, and (B) the mechanism of reaction of a gamma-benzopyrone flavonoid with Mg^{2+}

While the concept of phenol identification using $FeCl_3$ is by harnessing the ability of the phenol compounds to form a phenol-iron complex with Fe^{3+} by direct reaction with the hydroxy (OH)

groups within the phenol structure (Figure 3) [19], [20]. This reagent is very non-specific because it can react with all compounds that have OH groups within their structure without any compromises.

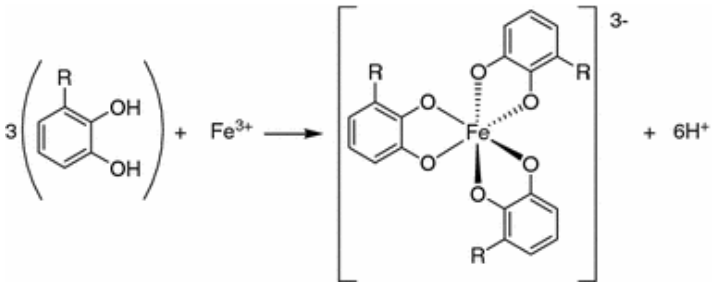

Figure 3. Polyphenol-iron complex resulted from reaction of $FeCl_3$ with the OH groups in phenol identification [20].

The existence of flavonoids and phenols in all samples was considered as a basic reference to be quantified in further experiments. The quantitative analysis of TFC and TPC of fermented and solar-dried *Citrus limon* leaves extract showed a diverse result in which solar dried sample provided a higher TPC (335.80 ± 0.80 mg GAE/g extract) (significantly different at $p<0.05$) than that of fermented sample (248.79 ± 0.25 mg GAE/g extract) as illustrated in Figure 4. However, the pre-extraction process did not significantly ($p>0.05$) affect the TFC of *Citrus limon* leaf extract from both aerobe-fermented and solar-dried samples with TFC values of 31.91 ± 0.19 mg QE/g extract and 32.09 ± 0.45 mg QE/g extract, respectively. *Citrus limon* provides higher phenolic compounds than flavonoids as reported by Makni et al. (2018) [21] which stated that *Citrus limon* contains phenolics and flavonoids content ranging from 105 – 204 mg GAE/g and 27 – 56 mg QE/g extract, respectively. However, our work is not in line in which the TFC and TPC were lower than other reports influenced by differences in the ecological environment affecting the production of secondary metabolites. The chemical composition of plants highly correlates with the ecological zone where the plants grow caused by whether the biotic or abiotic-related factors of the environment alters their production [22], [23].

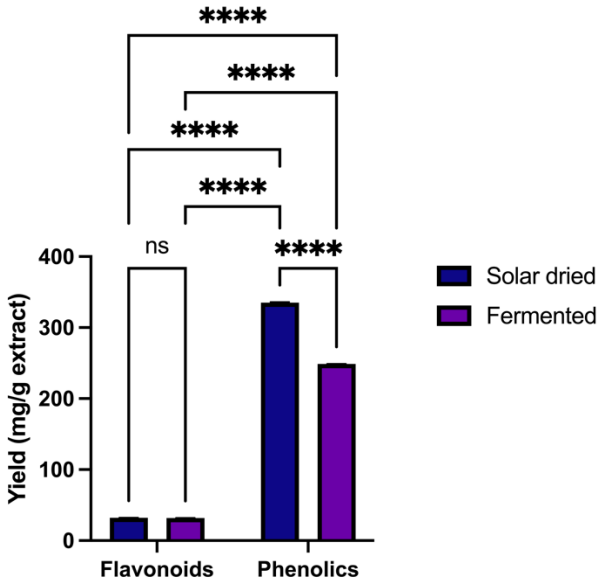

Figure 4. TFC and TPC of solar dried and fermented *Citrus limon* leaves extract

As depicted in Figure 4, it seems that fermentation diminishes the production of phenolic compounds in *Citrus limon* leaves. In this work, aerobe fermentation was used which is known to

retain the water content in the samples which causes the enzyme responsible for catalytic phenols to remain active. Additionally, high water content is the major contributor to bacterial growth which alters the production of phenol components. Certain bacterium encoded several genes coding for enzymes like phenyl phosphate synthase and phenyl phospate carboxylase involved in phenol degradation through phosphorylation and carboxylation [24]. The solar drying reduces the water content in the samples inhibit the bacterial growth and inactivates the enzymes, leading to the degradation of phenolic compound being avoided.

3.3 Effect of pre-extraction process on antioxidant activity index

The antioxidant activity of *Citrus limon* leaf extract was tested on DPPH using an ascorbic acid as a standard. All samples both fermented and solar-dried showed a significant free radical scavenging activity by reducing the absorbance of the test solution. IC_{50} calculation revealed that fermented *Citrus limon* leaf extract provides stronger antioxidant activity than solar-dried samples, even better than ascorbic acid as summarized in Table 2. In this work, we also utilized the antioxidant activity index (AAI) to observe the capacity of *Citrus limon* to scavenge the radical from DPPH. The AAI was used to avoid different results when tested on diverse DPPH concentrations. Statistical analysis indicated that the pre-extraction treatment significantly influences the antioxidant activity of *Citrus limon* leaf extract.

Table 2. Antioxidant activity of *Citrus limon* leaves extracts

Samples	**IC_{50} (µg/mL) ± SD**	**AAI**
Solar dried	35.11 ± 1.23	1.42 ± 0.05
Fermentation	2.23 ± 0.19	21.91 ± 1.90
Ascorbic acid	4.13 ± 0.25	12.10 ± 0.72

Apart from being a quite plentiful source of phenolic compounds, *Citrus limon* leaves are reported to contain ascorbic acid ranging from 317 – 580 mg per 100 grams leaves [25]. Fermented *Citrus limon* leaves provide high antioxidant activity due to the presence of high content of ascorbic acid. Anyiam et al. (2023) [26] reported that fermentation elevates the production of ascorbic acid in plants by approximately 125%. Additionally, the fermentation process results in the production of new bioactive compounds that might increase the antioxidant activity [27].

4. CONCLUSION

The pre-extraction process highly influenced the TPC and antioxidant activity of *Citrus limon* leaves but did not affect the TFC. Fermentation significantly decreases the TPC, while increasing the antioxidant activity. This indicates that fermentation can be applied to enhance the biological activity related to the antioxidants of *Citrus limon* leaves. However, further research is needed to prove this hypothesis.

Conflicts of interest: The authors declare no conflict of interest.

References

[1] N. Gupta, K. Verma, S. Nalla, A. Kulshreshtha, R. Lall, and S. Prasad, "Free radicals as a double-edged sword: the cancer preventive and therapeutic roles of curcumin," *Molecules*, vol. 25, no. 22. MDPI, Nov. 01, 2020. doi: 10.3390/MOLECULES25225390.

[2] M. Hayakawa and F. Kuzuya, "Free radicals and diabetes mellitus," in *Japanese Journal of Geriatrics*, 1990, pp. 149–154. doi: 10.47760/ijpsm.2023.v08i03.001.

[3] R. Loperena and D. G. Harrison, "Oxidative stress and hypertensive diseases," *Medical Clinics of North America*, vol. 101, no. 1. W.B. Saunders, pp. 169–193, Jan. 01, 2017. doi: 10.1016/j.mcna.2016.08.004.

[4] E. O. Olufunmilayo, M. B. Gerke-Duncan, and R. M. D. Holsinger, "Oxidative stress and antioxidants in neurodegenerative disorders," *Antioxidants*, vol. 12, no. 2. MDPI, Feb. 01, 2023. doi: 10.3390/antiox12020517.

[5] L. He, T. He, S. Farrar, L. Ji, T. Liu, and X. Ma, "Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species," *Cellular Physiology and Biochemistry*, vol. 44, no. 2. S. Karger AG, pp. 532–553, Dec. 01, 2017. doi: 10.1159/000485089.

[6] K. Neha, M. R. Haider, A. Pathak, and M. S. Yar, "Medicinal prospects of antioxidants: A review," *Eur J Med Chem*, vol. 178, pp. 687–704, 2019, doi: https://doi.org/10.1016/j.ejmech.2019.06.010.

[7] S. C. Lourenço, M. Moldão-Martins, and V. D. Alves, "Antioxidants of natural plant origins: From sources to food industry applications," *Molecules*, vol. 24, no. 22. MDPI AG, 2019. doi: 10.3390/molecules24224132.

[8] D. Rizaldy *et al.*, "Lemon (*Citrus limon* L.): Antioxidative activity and its marker compound," *Biointerface Res Appl Chem*, vol. 13, no. 1, Feb. 2023, doi: 10.33263/BRIAC131.021.

[9] M. Klimek-szczykutowicz, A. Szopa, and H. Ekiert, "*Citrus limon* (Lemon) phenomenon—a review of the chemistry, pharmacological properties, applications in the modern pharmaceutical, food, and cosmetics industries, and biotechnological studies," *Plants*, vol. 9, no. 1. MDPI AG, Jan. 01, 2020. doi: 10.3390/plants9010119.

[10] E. S. Prasedya *et al.*, "Effect of particle size on phytochemical composition and antioxidant properties of *Sargassum cristaefolium* ethanol extract," *Sci Rep*, vol. 11, no. 1, Dec. 2021, doi: 10.1038/s41598-021-95769-y.

[11] M. H. Rahmah, N. Nurfila, and A. P. Sari, "Total phenol and total flavonoid of graded fractination fresh and dried *Muntingia calabura* extract: a sustainable immunomodulator bioagent for functional health drink," *Jurnal Pembelajaran Dan Biologi Nukleus*, vol. 8, no. 3, pp. 767–780, Nov. 2022, doi: 10.36987/jpbn.v8i3.3375.

[12] S. Luliana, N. U. Purwanti, and K. N. Manihuruk, "Pengaruh cara pengeringan simplisia daun senggani (*Melastoma malabathricum* L.) terhadap aktivitas antioksidan menggunakan metode DPPH (2,2-difenil-1-pikrilhidrazil)". *Pharmaceutical Sciences and Research*, vol. 3, no. 3, pp. 120-129, Dec. 2016.

[13] A. Setiawansyah, A. Hakim, and D. G. Wirasisya, "Evaluasi dan identifikasi golongan senyawa potensial antibakteri pada daun dan kulit batang mimba (*Azhadirachta indica* A. Juss) terhadap *Escherichia coli*," *Jurnal Tumbuhan Obat Indonesia*, vol. 11, no. 2, pp. 40–48, 2019, doi: 10.22435/jtoi.v11i2.1003.

[14] N. Nurlinda, V. Handayani, and F. A. Rasyid, "Spectrophotometric determination of total flavonoid content in biancaea sappan (*Caesalpinia sappan* L.) leaves," *Jurnal Fitofarmaka Indonesia*, vol. 8, no. 3, pp. 1–4, Mar. 2021, doi: 10.33096/jffi.v8i3.712.

[15] Sumaiyah, Masfria, and A. Dalimunthe, "Determination of total phenolic content, total flavonoid content, and antimutagenic activity of ethanol extract nanoparticles of *Rhaphidophora pinnata* (L.F) Schott leaves," *Rasayan Journal of Chemistry*, vol. 11, no. 2, pp. 505–510, 2018, doi: 10.31788/rjc.2018.1122068.

[16] A. Setiawansyah, M. I. Arsul, S. Sukrasno, S. Damayanti, M. Insanu, and I. Fidrianny, "Anti-hyperuricemic potential of caryophyllene from *Syzygium aromaticum* essential oil: SiO_2-$AgNO_3$-based column chromatography purification, antioxidant, and xanthine oxidase inhibitory activities," *Advances in Traditional Medicine*, 2023, doi: 10.1007/s13596-023-00710-5.

[17] A. B. Hassan *et al.*, "Effect of natural fermentation on the chemical composition, mineral content, phytochemical compounds, and antioxidant activity of *Ziziphus spina-christi* (L.) 'nabag' seeds," *Processes*, vol. 9, no. 7, Jul. 2021, doi: 10.3390/pr9071228.

[18] H. Riasari, S. Fitriansyah, and I. Hoeriah, "Perbandingan metode fermentasi, ekstraksi, dan kepolaran pelarut terhadap kadar total flavonoid dan steroid pada daun sukun (*Artocarpus altilis* (Parkinson) Fosberg)," *Jurnal Sains dan Teknologi Farmasi Indonesia*, vol. 11, no. 1, pp. 1–17, 2022.

[19] N. R. Farnsworth, "Screening plants for new medicine," in *Biodiversity*, S. Staff and E. Wilson, Eds., Washington DC: National Academy of Sciences Press, 1988, pp. 83–97.

[20] N. R. Perron and J. L. Brumaghim, "A review of the antioxidant mechanisms of polyphenol compounds related to iron binding," *Cell Biochem Biophys*, vol. 53, no. 2, pp. 75–100, Mar. 2009, doi: 10.1007/s12013-009-9043-x.

[21] M. Makni, R. Jemai, W. Kriaa, Y. Chtourou, and H. Fetoui, "*Citrus limon* from Tunisia: Phytochemical and Physicochemical Properties and Biological Activities," *Biomed Res Int*, vol. 2018, 2018, doi: 10.1155/2018/6251546.

[22] N. Utami, S. Susianti, S. Bakri, B. Kurniawan, and A. Setiawansyah, "Cytotoxic activity of *Cyperus rotundus* L. rhizome collected from three ecological zones in Lampung-Indonesia against HeLa cervical cancer cell," *J Appl Pharm Sci*, 2023, doi: 10.7324/japs.2023.113764.

[23] C. S. F. Boaro, M. A. R. Vieira, F. G. Campos, G. Ferreira, I. De-la-Cruz-Chacón, and M. O. M. Marques, "Factors influencing the production and chemical composition of essential oils in aromatic plants from brazil," in *Essential Oil Research: Trends in Biosynthesis, Analytics, Industrial Applications and Biotechnological Production*, S. Malik, Ed., Cham: Springer International Publishing, 2019, pp. 19–47. doi: 10.1007/978-3-030-16546-8_2.

[24] X. Xie and N. Müller, "Enzymes involved in the anaerobic degradation of phenol by the sulfate-reducing bacterium *Desulfatiglans anilini*," *BMC Microbiol*, vol. 18, no. 1, Aug. 2018, doi: 10.1186/s12866-018-1238-0.

[25] I. Eaks, "Ascorbic acid content of citrus during growth and development," *Botanical Gazette*, vol. 125, no. 3, pp. 186–191, 1964.

[26] P. N. Anyiam *et al.*, "Effect of fermentation time on nutritional, antinutritional factors and in-vitro protein digestibility of *Macrotermes nigeriensis*-cassava mahewu," *Measurement: Food*, vol. 11, Sep. 2023, doi: 10.1016/j.meafoo.2023.100096.

[27] Y. S. Zhao *et al.*, "Fermentation affects the antioxidant activity of plant-based food material through the release and production of bioactive components," *Antioxidants*, vol. 10, no. 12. MDPI, Dec. 01, 2021. doi: 10.3390/antiox10122004.