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Chemical Composition, Antioxidant, Cytotoxic and Anti-Inflammatory Activities of *Coleus ambionicus* Lour. Essential Oil against Antibiotic-Resistant *Helicobacter pylori*

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Abstract

The purpose of this study was to evaluate the efficacy of the *C. ambionicus* Lour. essential oil in the treatment of gastric ulcers and the eradication of drug-resistant *H. pylori* strains being the cause of gastric ulcers, which delay healing and increase the chance of gastric cancer. The essential oil was extracted from the fresh leaves of *C. amboinicus* Lour. yielded 0.05 ± 0.0032%. The main compounds of essential oil were thymol (52.79%) and caryophyllene (9.47%) as they exhibited inhibitory activity against amoxicillin -resistant *H. pylori* ATCC 43579 and metronidazole-resistant *H. pylori* ATCC 43504 with inhibition zones of 29.5 ± 0.71 and 25.5 ± 0.71 mm, respectively. The MIC values of essential oil for *H. pylori* ATCC 43579 and *H. pylori* ATCC 43504 were of 25 and 50 µg/mL, respectively. The antioxidant activity exhibited an IC_{50} of 4,126 ± 0.03 µg/mL in the DPPH radical scavenging assay which yielded a value of 142.93 ± 2.86 mg TE/g of essential oil extract in the FRAP assay. The essential oil concentrations of 5, 10 and 25 µg/mL exhibit anti-inflammatory effects by inhibiting NO production at values of 15.62 ± 7.29%, 19.67 ± 6.34% and 22.17 ± 5.03%, respectively. The anti-inflammatory effect significantly inhibited NO production in Lipopolysaccharides (LPS)-stimulated RAW 264.7 cells at no lower concentration than 5 µg/mL. The essential oil extract at concentrations were not higher than 250 µg/mL exhibited no cytotoxic effects on RAW 264.7 macrophage cell line. The findings suggest that the potential of essential oil from *C. amboinicus* Lour. shows antibiotic-resistant activity against *H. pylori* strains, exhibits antioxidant and anti-inflammatory properties, and is non-toxicity to cells. Consequently, it potentially serves as an alternative for developing drugs for the treatment of gastric ulcers and preventing recurrence, as it can be used alone or in combination with present drugs.

Keywords: Drug-resistant *Helicobacter pylori* strains, *Coleus amboinicus* Lour., Essential oil, Gastric ulcer

Introduction

The number of new cancer cases worldwide is rising annually. The 2020 global cancer patient survey conducted by The International Agency for Research on Cancer (IARC) reported approximately 20 million new cancer cases and 9.7 million fatalities. Gastric cancer or stomach cancer, ranks as the fifth most common cancer globally in terms of new cases, following lung cancer, female breast cancer, colorectal cancer, and prostate cancer, which occupy the top 4 positions [1]. Gastritis, peptic and duodenal ulcers and gastric cancer or mucosa-associated lymphoid tissue (MALT) lymphoma are primarily attributable to *Helicobacter pylori*

infection. Chronic infection with *H. pylori* leads to chronic gastritis and presents a lifetime risk of gastric cancer between 1.5% to 2%. For this reason, *H. pylori* remains the predominant bacterial infection of the human gastric mucosa, affecting around 50 percent of the global population [2-4]. This infection may be related to several types of cancer i.e. gastrointestinal cancer, including esophageal cancer, liver cancer, pancreatic cancer, gallbladder cancer, and colorectal cancer [4]. The mechanism of *H. pylori* in the causes of gastric and duodenal ulcers stimulates the immune system, where white blood cells releases proinflammatory mediators (nitric oxide and prostaglandin E2 (PGE2)) and proinflammatory cytokines (tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)), resulting in cellular inflammation. Moreover, this interaction with gastric acid induces ulcers in the stomach and duodenum, led in atrophy and modifications of the gastric mucosa which eventually develop to stomach cancer [5]. *H. pylori* exhibits multidrug resistant to antibiotics such as amoxicillin, metronidazole, clarithromycin, tetracycline, levofloxacin, metronidazole, and fluoroquinolone [6-10]. The evolution of resistance in *H. pylori* has increased significantly due to the utilization of non-recommended formulations, national antibiotic consumption, patient compliance, host factors, strain virulence, migration, and the overuse of azithromycin during the COVID-19 pandemic, which may influence resistance development [10]. A global monitoring of *H. pylori* resistance from 2013 to 2023, utilizing frequently administered medications across 36 countries, revealed a concerning rise in *H. pylori* resistance to antibiotics [11]. Currently, the *H. pylori* vaccination has failed to achieve success, as it stimulates only a moderate immune response in patients. Moreover, the majority of trial vaccination remains in preliminary phases, exhibiting inconsistency and demonstrating various results. The only oral vaccination comprising recombinant urease B has been randomized in phase 3 trials, demonstrating safety and efficacy in children [12].

Coleus amboinicus Lour. (synonym: *Plectranthus amboinicus* (Lour.) Spreng and *Coleus aromaticus* (Benth.) is a medicinal herb belonging to the Lamiaceae family. It occurs naturally in the tropical and warm climates of Africa, Asia, and Australia. It is grown in many countries and serves as a natural, cost-effective, and safer bioactive compound. This plant exhibits interesting pharmacological activities, including antibacterial, antifungal, antiviral, antiprotozoal, anthelmintic, embryotoxic, anti-inflammatory, antioxidant, antitumor, antiepileptic, analgesic, antidiabetic, wound healing, lactogenic stimulation, and pain relief, etc. [13-17]. Both the extract and essential oil of *C. aromaticus* show inhibitory effects on various types of microorganisms, particularly the essential oil. Previous research reports were found that the essential oil of *C. aromaticus* has the activity against gram-negative bacteria such as *Escherichia coli* [13,18-22], *Klebsiella pneumoniae* [19,20], *Pseudomonas aeruginosa* [18,20], *Proteus vulgaris* [18,23], *Salmonella* Typhi [13,19], *Salmonella* Typhimurium [24] and *Xanthomonas campestris* [18]; activity against gram-positive bacteria such as *Bacillus subtilis* [13,18,21], *Bacillus megaterium* [18], *Enterococcus faecalis* [19], *Lactobacillus acidophilus* [25], *Serratia marcescens* [23], *Staphylococcus aureus* [18-22], Methicillin resistant *Staphylococcus aureus* [23], *Staphylococcus epidermidis* [23], *Streptococcus mutans* [25]; and activity against fungi such as *Aspergillus niger* [18,26], *Aspergillus ochraceus* CFR 221 [27], *Aspergillus parasiticus* [18], *Alternaria brassicicola* [18], *Candida albicans* [13,18,22,23], *Candida tropicalis* [23], *Colletotrichum musae* [18], *Fusarium solani* [18], *Rhizopus oryzae* [18], *Rhizoctonia oryzae-sativae* [18]. The essential oil extracted from the dried leaves of *C. aromaticus* revealed greater inhibition against gram-positive bacteria than gram-negative bacteria [28]. The essential oil of *C. aromaticus* extracted from the leaves has more inhibitory effect against the same bacterial strain compared to the whole plant, aerial parts and stems [13,18-20,23]. *C. aromaticus* Lour. essential oil contains key compounds such as carvacrol and thymol, which possess bacteria killing mechanisms by disrupting bacterial membranes, binding to transmembrane and intracellular molecules, altering membrane fluidity, potassium ion permeability, and ATP contamination in microorganisms [29]. The mechanism of *S. aureus* killing involves carvacrol and thymol, which inhibit the activity of coagulase and lipase enzymes, leading to salt tolerance reduction and the suppression of the synthesis of staphylococcal enterotoxins [30]. The metronidazole-resistant strain *H.*

pylori ATCC 43504 was most effectively inhibited by essential oils derived from Lamiaceae family plants, specifically *Thymus capitatus* (L.), followed by *Thymus caramanicus* Jalas, *Thymus vulgaris* L., *Mentha piperita*, *Melissa* spp., *Ocimum basilicum* L., *Origanum vulgare* L., *Rosmarinus officinalis* Lindl., *Salvia hispanica* L., *Origanum majorana* L., *Mentha piperita* L., and *Hyssopus spp.* L. [29,31-33]. The major compounds identified in the Lamiaceae family exhibiting antibacterial potential against drug-resistant strains of *H. pylori* are thymol, piperitenoneoxide, menthol, carvone and carvacrol [29,31-33]. *C. amboinicus* Lour. is a medicinal herb utilised as food, with biological activity and therapeutic characteristics. Previous research indicates that both crude extracts and essential oils from leaves exhibit high anti-inflammatory and antioxidant activities [34,35]. Moreover, the aqueous extract of this plant is non-toxic to cells [36] which is highlighted by its antibacterial properties in the intestines and its balancing intestinal microbiota role [15,37]. This plant may be utilized in the treatment of gastric ulcers caused by *H. pylori* infection to reduce antibiotic usage contributing to drug resistance and lower the risk of gastric cancer. Nonetheless, there is an absence of research reported about the inhibition of *H. pylori* by *C. amboinicus* Lour., a member of the Lamiaceae family. The advancement of alternative pharmaceuticals that are health-safe and economically viable. Not only, the benefits of herbal medications include reduced side effects, a diminished recurrence rate, and suitability for usage either independently or alongside a conventional pharmaceutical, but also used as an alternative treatment of certain gastric ulcers and aid in preventing recurrence [38].

This study aimed to evaluate the efficacy of *C. amboinicus* Lour. essential oil extracts in suppressing drug-resistant *H. pylori* strains that cause stomach inflammation. The anti-inflammatory activities of essential oil were determined by the inhibition of nitric oxide (NO) production, antioxidant activity tests, and the measurement of cytotoxic doses. The antioxidant activity can inhibit the expression of pro-inflammatory genes and reduce the release of arachidonic acid, prostaglandins, and leukotrienes [39]. The chemical composition of these essential oils was studied as well. This essential oil may contribute to the development of a drug for the treatment of gastric ulcers in the future. Pharmaceuticals used for treating inflammation, including steroids and nonsteroidal anti-inflammatory medications (NSAIDs), may induce side effects such as gastrointestinal distress, suppression of platelet aggregation, and hepatotoxicity and nephrotoxicity [40].

Materials and methods

Plant material

The plant specimen utilised in this research was identified as *Coleus amboinicus* Lour. (herbarium number A 17847 (BCU)) by the Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. Fresh leaves of *C. amboinicus* Lour. were harvested from a pesticide-free garden in Nakhon Nayok Province, Thailand, during June to July 2024. The plants were collected during midday under sunshine. Fresh leaves were washed and diced into small bits. If these leaves were extracted, they were kept at −20 °C for subsequent extraction, as shown in **Figure 1**.

Figure 1 Characteristics of *C. amboinicus* Lour. plant utilized for oil extraction.

Essential oil extraction by hydro distillation

One hundred g of fresh leaf pieces were combined with 200 mL of distilled water in a 1 L round-bottom flask and subjected to hydro-distillation using a Clevenger-type apparatus for 3 - 4 h. After extracting the essential oil, it was dehydrated using anhydrous sodium sulfate, weighed and subsequently stored in amber bottles at 4 °C. The percentage of essential oil yield is determined using Eq. (1) [26].

Essential oil yield (% w/w) = (Essential oil mass/Mass of plant material)×100 (1)

Determination of chemical composition of essential oil extracted from *C. amboinicus* Lour. by gas chromatography mass spectrometry (GC-MS)

Chemical composition of *C. amboinicus* Lour. essential oil was evaluated via GC-MS utilizing Agilent technologies model 7890 B equipped with the triple quad mass selective detector model 7000 D under the specified conditions. The stationary phase utilized was HP -5MS capillary column of 30 m×0.25 mm with film thickness of 0.25 µm sourced from Santa Clara, CA, USA. The initial temperature of the injection section was established at 4 °C/min, then increasing to a range of 50 to 230 °C. The helium inlet pressure was established at 10.0 psi, with a constant flow rate of 1.2 mL/min, and an average velocity of 40 cm/s. The detector employed in this work was MSD, operating electron impact mode at 70 eV, with a scan range of 40 - 600 amu. The chemical profiles were identified using the Wiley 11th NIST 2014 + 2017 library.

Anti-*H. pylori* activity test

The anti-*H. pylori* activity test was performed utilizing the Disk diffusion method described by Korona-Glowniak [32]. The *H. pylori* strain cultures were cultivated in screw-cap tubes containing Muller-Hinton broth (MHB) supplemented with 7% sheep blood under microaerophilic conditions for a duration of 3 to 5 days. The culture turbidity was adjusted to 10^8 CFU/mL using a 0.5 McFarland standard. One hundred µL of the optimal cell concentration were then swabbed onto a MHA plate supplemented with 7% sheep blood using a 3-way streaking technique. Subsequently, 20 µL of the extracted essential oil at a concentration of 100 mg/mL, produced with 10% DMSO (dimethyl sulfoxide), was applied to a paper disk on a MHA plate supplemented with 7% sheep blood. The anti-*H. pylori* activity was assessed by measuring the diameter of the inhibitory zone.

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the essential oil were assessed using the resazurin microtitre assay (REMA) method, adapted from [32,41]. The MIC value was assessed using a 96-well plate. Fifty µL of MHB supplemented with 7% sheep blood were added to each well of the test row (wells 1 - 12). Then, 50 µL of 100 mg/mL essential oil was poured into well 1 and subjected to continuous 2-fold dilution through to well 12, resulting in concentrations of 50, 25, 12.5, 6.250, 3.125, 1.563, 0.781, 0.399, 0.195, 0.098, 0.049 and 0.021 mg/mL. Add 10 µL of 0.01% resazurin solution to wells 1 to 12. Ultimately, 10 µL of a concentrated culture solution of 5×10^6 colony forming unit (CFU)/mL was added in 12 wells to achieve a final concentration of 5×10^5 CFU/mL. In the negative control row, 50 µL of 10% DMSO solution was utilized in place of the essential oil. The plate was incubated at 37 °C under microaerobic conditions for 3 days. The minimum concentration at which the color of the resazurin solution transitions from purple to pink or colorless was designated as the MIC value. The MBC was determined by transferring 5 µL of bacterial culture from the final positive well and inoculating it into a MHA plate supplemented with 7% sheep blood, followed by incubation under microaerophilic conditions at 37 °C for 72 h.

Antioxidant activity of *C. amboinicus* Lour. leaf essential oil extract

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant activity of *C. amboinicus* Lour. essential oil was assessed using the DPPH radical scavenging assay, as modified by Itsarasook *et al.* [42]. This method uses the free radical scavenger DPPH (1,1-diphenyl-2-picrylhydrazyl) to interact with the antioxidant present in the sample, leading to a color change of the solution from purple to yellow and a related decrease in absorbance at 515 nm. The essential oil extract solutions were prepared in absolute ethanol at

concentrations of 10,000, 5,000, 2,500, 1,250, 625, 312.25, 156.25, and 78.13 µg/mL. The test reaction started with the addition of 75 µL of sample solution and 150 µL of DPPH solution to a 96-well plate (Perkin Elmer, Inc., Massachusetts, USA). The plate was incubated in the dark for 30 min, and the absorbance was measured at 515 nm. This test reaction was performed in triplicate. A standard solution of 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, Aldrich) was prepared in absolute ethanol at a concentration of 10,000 µg/mL for use as a positive control. The percentage of free radical scavenging activity was calculated using the following Eq. (2):

$$\% \text{ Free radical scavenging activity} = [(A_{blank} - A_{sample})/A_{blank}] \times 100 \quad (2)$$

Where A_{sample} indicates the absorbance measured for the mixture of DPPH solution and the sample and A_{blank} indicates the absorbance measured for the DPPH solution and absolute ethanol. The inhibitory concentration (IC_{50}) of the samples was calculated from the linear equation between different sample concentrations and percentage of free radical scavenging activity.

Ferric reducing antioxidant power (FRAP) assay

The antioxidant capacity was determined using the FRAP method, modified from the method of Tananuwong and Tewaruth [43]. This technique is employed to assess the ferric reduction capacity of the analyzed sample extracts. Prepare a 10:1:1 mixed solution comprising 300 mM acetate buffer solution (pH 3.6), 20 mM ferric chloride hexahydrate solution, and 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution (dissolved in 40 mM HCl). A mixed solution of 190 µL was combined with 10 µL of essential oil extract (5,000 µg/mL) in a 96-well plate and incubated at 37 °C for 30 min. The absorbance at 593 nm was quantified using trolox as a reference standard, reported as mg trolox equivalent (TE) /g extract.

Cytotoxicity test of *C. amboinicus* Lour. essential oil on RAW 264.7 tissue culture cells by MTT assay

RAW 264.7 macrophage cells were evaluated for the cytotoxicity of essential oil extracts via the 3- (4, 5-dimethylthiazol-2-yl) -2, 5-disphenyltetrazolium bromide (MTT) assay [5,44-45]. RAW 264.7 macrophages were cultivated in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% v/v fetal bovine serum and 1% penicillin/streptomycin, followed by incubation at 37 °C in a 5% CO_2 atmosphere for 24 h. The 5×10^4 cells were inoculated into each well of a 96-well plate. The cells were cultured with 1,000 µL of essential oil concentrations of 5, 10, 25, 50, 100 and 250 µg/mL, formulated with 0.0025% Tween 20, at 37 °C in a 5% carbon dioxide atmosphere for 24 h. The old cell culture media was removed, and the cells were washed with phosphate-buffered saline (pH 7.4). One thousand µL of mixture solution comprising 200 µL of 0.5% MTT solution mixed with 800 µL of cells culture medium was added to each well, followed by incubation at 37 °C for 2 h. After 2 h, the culture broth medium was removed. Prior to measuring the absorbance at 570 nm using a microplate reader, 500 µL of DMSO was added to each well to solubilize the blue formazan crystal product. Tween 20 (0.00025%) served as the control group (vehicle control). The amount of formazan is directly related to the count of active mitochondria in a living cell. The percentage of cell viability was subsequently estimated in relation to the control group (untreated cells) using Eq. (3).

$$\text{Cell viability (\%)} = (\text{Absorbance of treated well/Absorbance of control well}) \times 100 \quad (3)$$

Anti-inflammatory activity test of *C. amboinicus* Lour. essential oil on nitric oxide (NO) production

The nitrite concentration is commonly measured as a key indicator of iNOS activity because iNOS catalyzes the production of nitric oxide (NO), which is rapidly converted to nitrite and nitrate in aqueous solutions. Thus, nitrite levels reflect NO generation and serve as a reliable marker of iNOS activity. The concentration of nitrite in the cell culture medium serves as an indicator of iNOS enzyme activity. The nitrite concentration was analyzed using the Griess reaction as per the methodology of Srisook *et al.* [5] and Chiu *et al.* [36]. Macrophages (RAW 264.7) were activated by culturing them in DME medium supplemented with 10% (v/v) fetal bovine serum and 1%

penicillin/streptomycin until reaching 80% cell density. Subsequently, 5×10^4 cells were inoculated into each well of a 96-well cell culture plate for the duration of 24 h. Then, 100 µL various quantities of essential oils, 50 µM indomethacin and 50 µg/mL nitro-L-arginine methyl ester (L-NAME) as a positive control, together with 0.00025% Tween20 being a vehicle control, were added into each well and incubated for 30 min. Cells were stimulated to provoke inflammation with the addition of 1 µg/mL LPS to each well and incubated in a CO_2 incubator for 24 h. The amount of NO produced by the cells was subsequently quantified with a Griess reagent assay. One hundred microliters of the examined cell culture supernatant were combined with Griess reagent, consisting of 50 mL each of 0.1% (w/v) naphthyl-ethylene-diamine dihydrochloride in water and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid, and allowing to incubate at room temperature for 20 min. The interaction between cell culture supernatant and Griess reagent was quantified for NO at 540 nm utilizing a microplate reader (CLARIO star, BMG Labtech, DKSH Technology Limited, Thailand). The percentage of NO was calculated by comparing the measured absorbance values with the control group values induced only by LPS. The experiments were performed a minimum of 3 times, with 3 replications each experimental condition.

Statistical Analysis

The data performed one-way analysis of variance (ANOVA) utilizing SPSS Statistics 17.0 (SPSS, USA). The different treatments were analyzed using Duncan's multiple range test at a 95% confidence level.

Results and discussion

Extraction and composition of the essential oil from *C. amboinicus* Lour.

Fresh leaves of *C. amboinicus* Lour. were harvested during June - July 2024 from a pesticide-free garden in Nakhon Nayok Province, Thailand, and were subsequently extracted via hydro distillation. Hydro distillation extraction utilizes a solvent that is free from hazardous residues and non-toxic to cells [36], exhibiting significant anti-inflammatory properties [34]. The yield of essential oil was 0.05 ± 0.0032% as shown in **Table 1**. The essential oil extract of *C. amboinicus* Lour. exhibited a golden yellow color and a distinctive aroma, as illustrated in **Table 2** and **Figure 2**. Mangathayaru *et al*. [46] reported that steam distillation of fresh leaves for 3 h yielded 0.24% volatile oil, characterized by a golden yellow viscosity, a nice thymol aroma and a harsh pungent flavor, was highly soluble in 80% ethanol and diethyl ether. The findings indicated that the yield of essential oil from the leaves of *C. aromaticus* Lour. was measured at 0.05 ± 0.0032%. This finding yield of *C. amboinicus* Lour. essential oil aligns closely with those from India (0.04% to 0.077%) [47] and Venezuela (0.05%) [19], also closely with *P. amboinicus* from southern Ecuador (0.025% - 0.049%) [48]. Additionally, the extraction of essential oil results from fresh leaves varies in various regions i.e. Thailand (0.08% - 1.0%) [24,49], India (0.13% - 0.6%) [50-52], Malaysia (0.54%) [26], and Venezuela (0.05%) [19]. The findings by Arumugam *et al.* [15] indicated that *in vitro* root cultivation yielded 0.29% essential oil concentration where thymol levels were 3 times exceeding values after 4 weeks of growth. Mallavarapu *et al.* [53] reported that the quality of essential oil was the best quality of essential oil when harvested during September, while plants harvested in May yielded higher oil quantities than those harvested in September. The yield and main chemical components of essential oils are influenced by various environmental conditions and seasonal variations.

Table 1 Extraction yield and physicochemical characteristics of essential oil from *C. amboinicus* Lour. leaves.

Plant	Physical properties		Yield (%)
	Color	Odour	
Fresh leaves of *C. amboinicus* Lour.	Golden yellow	Aromatic fragrant	0.05 ± 0.0032

Figure 2 Characteristics of essential oils extracted from the leaves of *C. amboinicus* Lour.

C. amboinicus Lour. essential oil is pale yellow, sticky substance with a pronounced better aroma. The chemical composition and essential oil content were evaluated using GC-MS. The testing results identified 16 compounds rich in monoterpenes (thymol, γ-terpinene and p-cymene) and sesquiterpenes (caryophyllene, α-bergamotene, α-humulene and δ-cadinene). The predominant chemical was thymol (52.79%), followed by caryophyllene (9.47%), γ-terpinene (7.93%), p-cymene (5.11%), α-bergamotene (5.31%), α-humulene (3.63%) and δ-cadinine (2.17%), all of which were present in moderate concentrations (**Table 2** and **Figure 3**). Thymol (2-isopropyl-5-methylphenol) is an isomer of carvacrol and a derivative of p-cymene [54]. The noteworthy effects of thymol are anti-inflammatory, antioxidant, antibacterial, antihyperlipidemic, antispasmodic, immunostimulatory, and anticancer properties [55], where The United States Food and Drug Administration has recognized thymol as a safe food additive, indicating its minimal toxicity for consumers [55]. The results of this study correlate with prior research, indicating that the predominant compounds in *C. aromaticus* Lour. essential oil include thymol, sourced from plants in Thailand, India and Malaysia [26,50-52,56-58], followed by carvacrol from plants in Thailand, India, Egypt and Venezuela [19,24,28,46,59] and 3-Carene (20.78%) from plants in Malaysia [60]. The soils containing iron-manganese ores could result in differences in the oil composition [60]. Consequently, this data indicates that the chemical composition of the components fluctuates based on the plant growing region [23]. The age of the plant influenced the yield of thymol and carvacrol, with younger *Lippia berlandieri* Schauer plant (30 days) exhibiting greater quantities of thymol compared to mature plants [14,61]. Factors influencing the yield and main chemical compounds of essential oils depend on geographical area, plant variety, plant age, harvesting time, plant location, and extraction and drying techniques [14,15,23,27].

Table 2 Types and quantities of chemical compositions in the essential oil from the leaves of *C. amboinicus* Lour.

RT	Component name	Area (%)
5.06	α-thujene	0.38
7.36	β-pinene	0.47
8.13	α-terpinene	1.74
8.40	p-cymene	5.11
9.47	γ- terpinene	7.93
13.49	terpinene-4-ol	1.11
18.06	thymol	52.79
19.93	α-copaene	0.98

RT	Component name	Area (%)
21.33	caryophyllene	9.47
21.82	α-bergamotene	5.31
22.39	α-humulene	3.63
23.23	germacrene D	1.70
24.50	δ-cadinene	2.17
26.29	caryophyllene oxide	1.68
28.38	α-cadinol	1.42
29.37	shyobunol	1.12

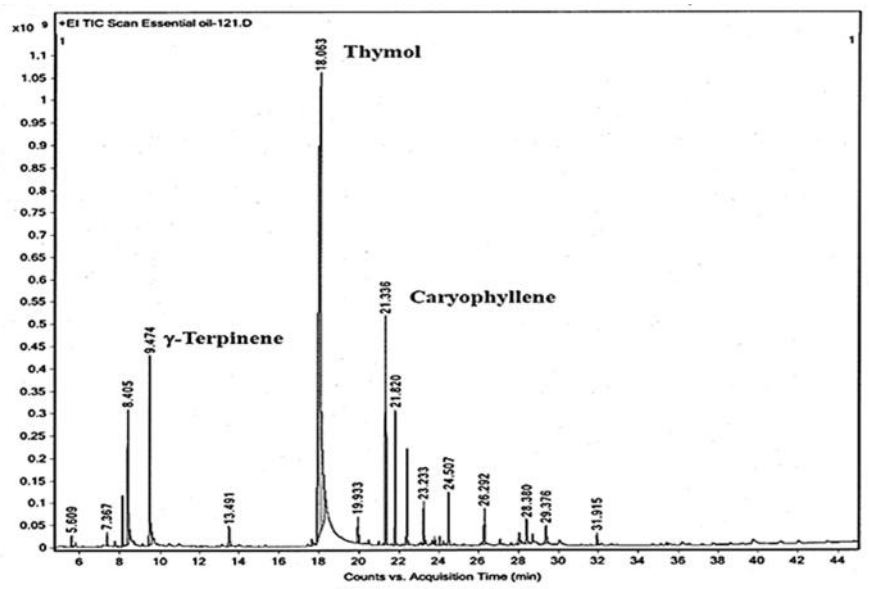

Figure 3 The retention time of chemical components in the essential oil of *C. amboinicus* Lour. was investigated using GC-MS.

Effect of *C. amboinicus* Lour. essential oil on anti-drug-resistant *H. pylori* activity

C. amboinicus Lour. essential oil at a concentration of 100 mg/mL was utilized to evaluate the inhibitory zone against *H. pylori* ATCC 43579 (Amoxicillin-resistant strain) and *H. pylori* ATCC 43504 (Metronidazole-resistant strain). The essential oils exhibited inhibitory activity against both *H. pylori* ATCC 43579 and *H. pylori* ATCC 43504, with inhibition zones of 29.5 ± 0.71 and 25.5 ± 0.71 mm, respectively (**Table 3** and **Figure 4**).

Table 3 Inhibition zone of *C. amboinicus* Lour. essential oil effective against drug-resistant *H. pylori* strains.

Plant	Inhibition zones (mm)	
	***H. pylori* ATCC 43579**	***H. pylori* ATCC 43504**
Leaf of *C. amboinicus* Lour.		
Pure	32 ± 0.00	30 ± 0.00
100 mg/mL	29.5 ± 0.71	25.5 ± 0.71
Erythromycin (15 µg)	30 ± 0.00	28 ± 0.00

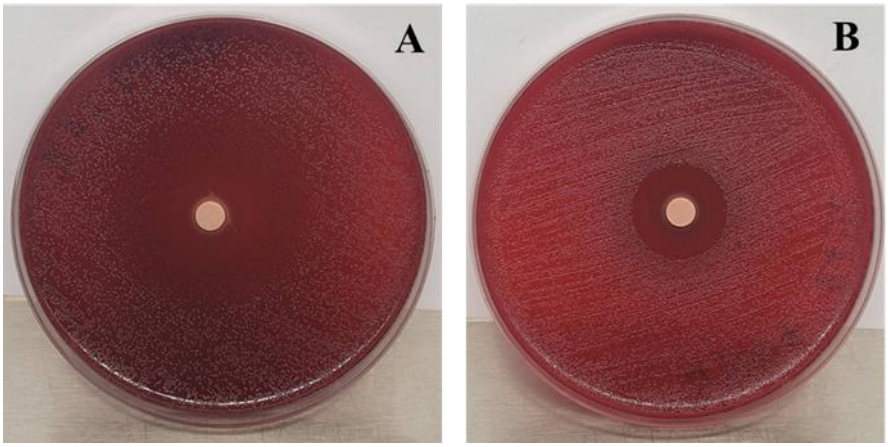

Figure 4 Inhibitory zone of *C. amboinicus* Lour. essential oil at a concentration of 100 mg/mL against *H. pylori* ATCC 43579 (A) and *H. pylori* ATCC 43504 (B).

The susceptibility test results against *H. pylori* ATCC 43579 and *H. pylori* ATCC 43504 showed MIC values of 25 and 50 µg/mL, respectively (**Table 4**). Previous research reports, it was found that essential oil from *C. amboinicus* Lour. has the ability to inhibit many types of pathogens, such as having the ability to inhibit negative bacteria such as *Enterobacter* sp., diarrhea caused by *Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* Typhi, *Salmonella* Typhimurium *and Xanthomonas campestris* [13,18-20,22-24] and having the ability to inhibit gram-positive bacteria such as *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Serratia marcescens*, Methicillin resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans* and *Streptococcus pyogenes* [13,18-20,22-23,25]. It also has antifungal activity against *Aspergillus niger*, *Aspergillus ochraceus* CFR 221, *Aspergillus parasiticus*, *Alternaria brassicicola*, *Candida albicans*, *Candida tropicalis*, *Colletotrichum musae*, *Fusarium solani*, *Rhizopus oryzae* and *Rhizoctonia oryzae-sativae* [13,18,22,23,26,27]. However, there is no research report on the inhibition drug-resistant strains of *H. pylori* by essential oils extracted *C. amboinicus* Lour., which is in the Lamiaceae family.

The MIC value for anti-*H. pylori* ATCC 43579 was correlated with *Allium sativum* L. (MIC = 10 - 25 µg/mL). The MIC value for commercial essential oils from some plants within the same family as *C. amboinicus* Lour. was 62.5 µg/mL against *H. pylori* ATCC 43504, which exceeded the MIC values of the essential oils examined in this study, specifically *Mentha piperita* L., *Hyssopus* spp. L., *Lavandula angustifolia* Mill., *Mentha piperita* L., *Origanum majorana* L., *Rosmarinus officinalis* Lindl., and *Salvia hispanica* L. [32]. Conversely, some plants exhibited lower MIC values than those observed in this study, including *Thymus vulgaris* (0.04 - 15.16 µg/mL), *Satureja montana* (0.04 µg/mL), *Ocimum basilicum* L. (15.6 µg/mL), *Melissa* spp. (15.6 - 31.1 µg/mL), and *Origanum vulgare* L. (31.1 - 40 µg/mL) [32,62]. Furthermore, essential oil from aerial parts of *Thymus capitatus* (L.) Hoffmanns. & Link (0.25 µg/mL) [31] and from the leaves of *Mentha piperita* (15.6 - 31.3 µg/mL) [33] gave lower MIC value against *H. pylori* ATCC 43504 than this study. The essential oils from other family plants, *Eugenia caryophyllus* (family Myrtaceae) and *Citrus paradisi* (family Rutaceae) gave higher MIC value against *H. pylori* ATCC 43504 in this study [62]. Nonetheless, certain plants exhibited lower MIC values in this study such as *Syzygium aromaticum* L. (family Myrtaceae), *Leptospermum scoparium* (family Myrtaceae), *Cymbopogon citratus* (family Poaceae) and *Lippia citriodora* (family Verbenaceae) [32,62].

Table 4 MIC and MBC values of *C. amboinicus* Lour. essential oil at a concentration of 100 mg/mL against drug-resistant *H. pylori* strains.

Plant	*Helicobacter pylori* ATCC 43579		*Helicobacter pylori* ATCC 43504	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
Essential oil from the leaf of *C. amboinicus* Lour.	25	50	50	100

The major compounds in essential oils of medicinal plants from the family Lamiaceae have exhibited antimicrobial potential against metronidazole-resistant *H. pylori* ATCC 43504 were thymol [31,32,62] and carvacrol [32,62,63]. The essential oil extracted from dried leaves of *C. aromaticus* Lour. revealed higher inhibition against gram-positive bacteria than gram-negative bacteria [28]. Thus, the major compound, thymol (52.79%), of the *C. amboinicus* Lour. essential oil extract in this study exhibited good activity against both amoxicillin-resistant *H. pylori* ATCC 43579 and metronidazole-resistant *H. pylori* ATCC 43504 strains. In the Lamiaceae family, the major compounds, thymol and carvacrol, demonstrated antibacterial activity against the metronidazole-resistant *H. pylori* ATCC 43504 strain. Thymol exhibited an MIC value of 15.6 µg/mL [32], while carvacrol showed MIC values ranging from 29 to 31.3 µg/mL [32,63]. Furthermore, essential oil extracted from *Thymus capitatus* (L.) Hoffmanns. & Link, which contains 47.2% thymol, displayed excellent inhibitory activity with an MIC of 0.5 µg/mL [31]. Furthermore, it was found that the commercial pure essential oils thymol and carvacrol from Merck (Germany) displayed MIC values of 100 and 40 µg/mL, respectively [63]. In addition, the commercial pure thymol from Sigma-Aldrich® exhibited antibacterial activity, giving an MIC value of 7.8 µg/mL [32]. Based on these reports indicated that the commercial essential oils extracted from medicinal plants in the Lamiaceae family exhibited activity against metronidazole-resistant *H. pylori* ATCC 43504 strain related to the commercial pure essential oils. Both thymol and carvacrol have bactericidal mechanisms by causing damage to the cell membrane, leading to inhibition of cell wall biosynthesis [64]. In addition, act as ergosterol synthesis inhibitor in the fungal cell membrane [64] and *Staphylococcus aureus* killer by inhibiting the activity of coagulase and lipase enzymes, resulting in reduced salt tolerance and inhibiting the production of staphylococcal enterotoxins [30]. Other compounds that have been shown active against metronidazole-resistant *H. pylori* ATCC 43504 strain include citronellal, menthol, piperitenoneoxide, linalool, menthone, bisabolol, eugenol [32,33,62]. Anti-metronidazole-resistant *H. pylori* ATCC 43504 activity was also found in 95% ethanolic extracts of *Boesenbergia rotunda* (MIC = 3.1 µg/mL) and *Curcuma longa* (MIC = 3.1 µg/mL) [65].

The effect of antioxidant activities extracted from *C. amboinicus* (Lour.) leaves essential oil

The antioxidant activity of *C. amboinicus* (Lour.) leaf essential oil extract using DPPH radical scavenging assay showed an IC_{50} of 4,126 ± 0.03 µg/mL, revealing lower antioxidant activity than trolox, the positive control (IC_{50} = 220 ± 0.00 µg/mL). The antioxidant capacity estimated value using FRAP is 142.93 ± 2.86 mg TE/g of essential oil extract, as presented in **Table 5**. The antioxidant activity observed in this study was notably stronger, as indicated by a much lower DPPH IC_{50} value compared to the 9,518 µg/mL previously reported by Thanaseelungkoon *et al.* [66] for *C. amboinicus* Lour. essential oil from Northern Thailand (Mae Hong Son and Chiang Mai Provinces). The essential oil extract of *C. amboinicus* Lour. in this research showed the greater amounts of thymol (52.79%), caryophyllene (9.47%), γ-terpinene (7.93%), p-cymene (5.11%), and α-bergamotene (5.31%). The results were similar to the antioxidant activity observed in the essential oil of *C. amboinicus* Lour., with carvacrol and thymol identified as the predominant components in high concentrations [51,66]. The previous research suggested that the essential oil extract from *C. amboinicus* Lour. shows the most significant DPPH radical scavenging activity, predominantly comprising the compound 2,3,5,6-tetramethylphenol, followed by carvacrol, γ-terpinene, and germacrene [35,48,67]. Furthermore, research on the antioxidant

activities of essential oil from *P. amboinicus*, performed both *in vitro* and *in vivo*, found the primary components as carvacrol, thymol, cis-caryophyllene, trans-caryophyllene, and p-cymene [51,68]. Generally, the primary antioxidant constituents in plants consist various phenolic compounds and terpenes [69], thus both thymol and carvacrol are classified as phenolic monoterpenoids, with carvacrol being an isomer of thymol [30,64].

The antioxidant activity of *C. amboinicus* Lour. (*Plectranthus amboinicus* Lour.) essential oil can considerably vary depending upon environmental and biological conditions. Key factors influencing this variability include geographical location, soil type [35], low temperatures [70], extended photoperiods [70], increased solar irradiation [71], harvesting time (morning) [67] and plant age (30 days) [61]. Therefore, the essential oil showed varying levels of antioxidant activity, with high levels observed in Vietnam (DPPH IC_{50} = 44.15 ± 0.15 µg/mL) [35] and India (DPPH IC_{50} = 68 µg/mL) [51], moderate levels were recorded in Brazil (DPPH IC_{50} = 500 µg/mL) [67], Poland (DPPH EC_{50} = 60.69 ± 1.3 µg/mL), and India (DPPH EC_{50} = 95.46 ± 1.2 - 152.8 ± 1.6 µg/mL) [70], while diminished levels were observed in southern Ecuador (DPPH IC_{50} = 816.57 ± 1.02 - 1,688.67 ± 1.03 µg/mL) [4].

Table 5 Antioxidant activities of *C. amboinicus* (Lour.) leaf essential oil extract.

Parameters	Essential oil extract
DPPH IC_{50} (µg/mL)	4,126 ± 0.03
Trolox IC_{50} (µg/mL)	220 ± 0.00
FRAP (mg TE/g extract)	142.93 ± 2.86

Effect of *Coleus amboinicus* essential oil on the viability of RAW 264.7 macrophages

The toxicity of the essential oil extract against RAW 264.7 macrophage tissue culture cells was evaluated using MTT assay, revealing that the cells were tested with concentrations ranging from 5 - 250 µg/mL, as illustrated in **Figure 5**. The survival assay of RAW 264.7 macrophages was performed by treating live cells with MTT to produce purple crystals (formazan) [72]. Incubation of cells with various concentrations of essential oil for 24 h revealed that a concentration of 5 - 10 µg/mL did not significantly reduce cell viability in comparison to the control group.

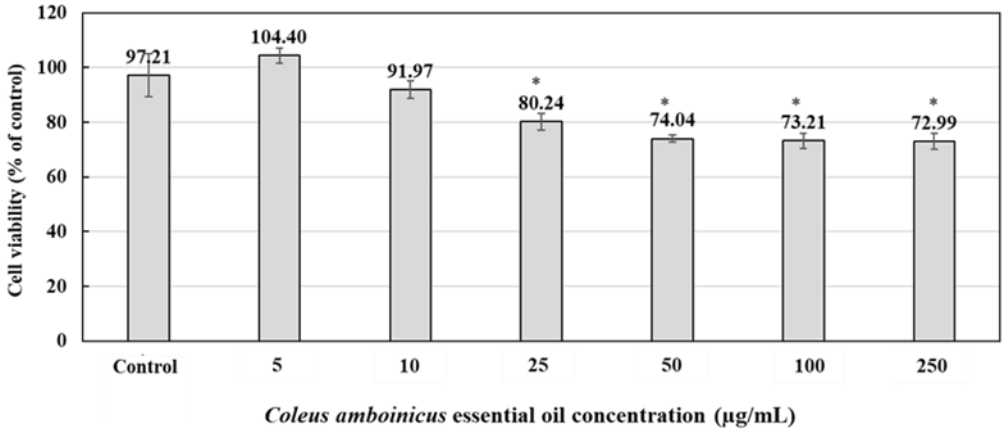

Figure 5 Effect of *C. amboinicus* Lour. essential oil on the survival of cultured RAW 264.7 macrophages. The data shown are the mean of 3 replicates ± S.D., with * denoting a statistically significant difference ($p < 0.05$) relative to the untested group.

Despite the significant difference in cell survival rates within the concentration range of 25 - 250 µg/mL compared to the control group, the survival percentages ranged from 72.99% to 91.97%, exceeding 70% of

RAW 264.7 macrophage cells, thus indicating that the tested sample concentrations were non-toxic to the cells [73]. When the concentration of essential oil exceeded 500 µg/mL, cell viability was markedly diminished (no presented data). Consequently, it may be concluded that essential oil at doses not greater than 250 µg/mL exhibited no toxicity to RAW 264.7 macrophage cells. The essential oil extracted from *C. amboinicus* Lour. in this study showed lowest cytotoxicity towards RAW 264.7 cells compared to the aqueous extract from *Plectranthus amboinicus* (Lour.), with over 80% cell survival at concentrations of 100 - 1,000 µg/mL [36]. Nevertheless, the essential oil extracted from *C. amboinicus* Lour. in this research exhibited lower cytotoxicity to cells compared to the dry leaf extracted *C. amboinicus* and ethanol extract of *C. amboinicus* leaves. Furthermore, *C. amboinicus* leaf extracts exhibited non-toxicity at lower concentrations compared to the essential oils in this study i.e. to normal lung cell line (VERO) (31.2 µg/mL), normal human lung fibroblast Wi38 cell line (10 - 30 µg/mL) and the human lung cancer cell line (A549) (10 - 31.2 µg/mL) [44,74,75].

Inhibitory effect of *C. amboinicus* Lour. essential oil on nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages

The majority of research on the anti-inflammatory properties of plant extracts has evaluated their capacity to decrease the production of NO, PGE2 and iNOS, including the expression of COX-2 in murine macrophage cells (RAW264.7) stimulated by bacterial LPS. Furthermore, they decrease the synthesis of pro-inflammatory cytokines, primarily TNF-α, IL-1β, and IL-6 [76]. This study evaluated the anti-inflammatory effect of essential oil extracted from *C. amboinicus* Lour. by assessing the inhibition of nitric oxide (NO) production in RAW264.7 cells stimulated with bacterial LPS. The results indicated that essential oil at concentrations of 5, 10, and 25 µg/mL yielded NO levels of 84.73 ± 7.29%, 80.66 ± 6.34%, and 78.15 ± 5.0379%, respectively, which were significantly different from the control group that received only LPS (100.41 ± 4.16%). The essential oil exhibited NO inhibitory capacity at concentrations of 5, 10, and 25 µg/mL, resulting in NO production inhibition of 15.62 ± 7.29%, 19.67 ± 6.34%, and 22.17 ± 5.03%, respectively, with no significant difference compared to the positive control group (**Table 6** and **Figure 6**). The findings of this study indicated that essential oil was more effective in inhibiting NO production than by aqueous extracts from *Plectranthus amboinicus* (Lour.), where at a concentration higher than 100 µg/mL, the nitrite levels were significantly different ($p < 0.05$) compared to those of the control group not exposed to LPS [77]. Furthermore, the essential oil of *C. amboinicus* Lour. in this study contained a high level of thymol, which was identical to thymoquinone from the hexane extract of *Plectranthus amboinicus*, has been shown to inhibit LPS-induced expression of tumor necrosis factor-alpha (TNF-α) [77]. The inflammatory was stimulated by the primary mediators prostaglandins (PG) and nitric oxide (NO), produced by the enzymes cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS), respectively. Thus, the inhibition of inflammation involves a decrease of prostaglandins and nitric oxide production [78]. Inflammation and cellular toxicity triggered cells to release pro-inflammatory mediators such as cytokines (tumor necrosis factor alpha [TNF-α], tumor necrosis factor gamma [TNF-γ], interleukin-**4** [IL-**4**)], and interleukin-**6**[IL-**6**]), eicosanoids and nitric oxide (NO) [77,79].

Table 6 The inhibitory effect of *C. amboinicus* Lour. essential oil on nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages.

Samples	NO production (% of control)	Inhibition of NO production (%)
Control	37.50 ± 1.56	
Control + LPS	100.41 ± 4.16	
25 µg/mL of CAEO	36.76 ± 2.18	
5 µg/mL of CAEO + LPS	84.73 ± 7.29	15.62 ± 7.29
10 µg/mL of CAEO + LPS	80.66 ± 6.34	19.67 ± 6.34

Samples	NO production (% of control)	Inhibition of NO production (%)
25 µg/mL of CAEO + LPS	78.15 ± 5.03	22.17 ± 5.03
50 µg/mL L-NAME + LPS (positive control)	67.18 ± 8.80	33.10 ± 8.80
50 µM indomethacin + LPS (positive control)	47.86 ± 0.94	52.34 ± 0.94
0.00025% Tween20 + LPS (vehicle control)	91.62 ± 5.18	
0.00025% Tween20 (vehicle control)	37.87 ± 2.53	

Note: Control = Medium, CAEO = *C. amboinicus* Lour. essential oil, LPS = Lipopolysacchari, L-NAME = nitro-L-arginine methyl ester

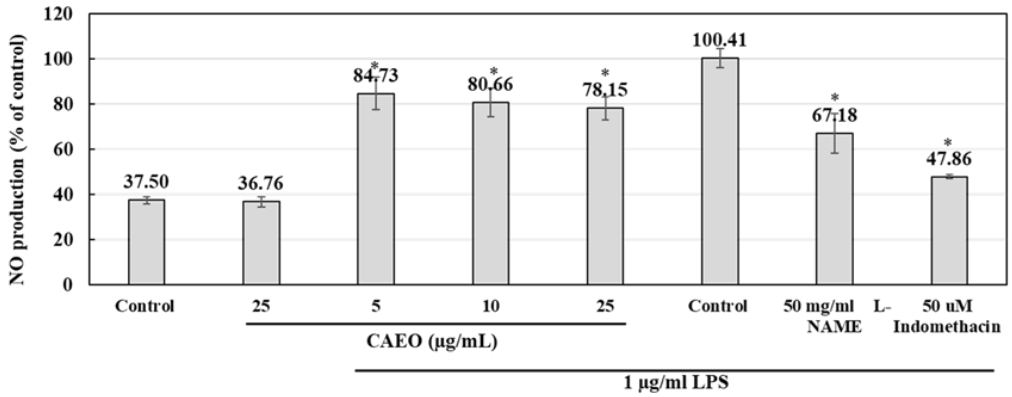

Figure 6 The inhibitory effect of *C. amboinicus* Lour. essential oil on NO production in LPS-stimulated RAW 264.7 macrophages. The presented data shows the mean of 3 replicates ± S.D. with * denoting a statistically significant difference ($p < 0.05$) relative to the untreated group. Control = Medium, CAEO = *C. amboinicus* Lour. essential oil, LPS = Lipopolysacchari, L-NAME = nitro-L-arginine methyl ester.

Conclusions

The *C. amboinicus* Lour. plant yielded the greatest concentration of 0.05 ± 0.0032%. The main compounds of *C. amboinicus* Lour. plant were thymol (52.79%) and caryophyllene (9.47%). The susceptibility test against *H. pylori* ATCC 43579 and *H. pylori* ATCC 43504 revealed MIC values of 25 and 50 µg/mL, respectively. The *in vitro* anti-inflammatory and toxicity studies indicated that the essential oil significantly inhibited pro-inflammatory mediators in LPS-stimulated RAW 264.7 cells at a dose of at least 5 µg/mL, with no cytotoxicity observed at a concentration not higher than 250 µg/mL. Furthermore, it exhibited antioxidant activity with an IC50 of 4,126 ± 0.03 µg/mL using the DPPH radical scavenging assay, while the FRAP assay gave a value of 142.93 ± 2.86 mg TE/g of essential oil extract. The results indicated that the essential oil exhibited antioxidant, non-cytotoxic and anti-inflammatory properties, including activity against antibiotic-resistant *H. pylori*, indicating its potential application in the treatment of *H. pylori*-related gastric ulcers.

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