



Biological Properties of Clove Oily Extracts and Therapeutic Application of Cloves Cream in Treatment of Inflammatory Pains Induced by Formalin in Mice Model

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Abstract

Introduction: This study aimed to investigate the phytochemical profile and biological properties of clove oil extracts (COE) as a preliminary step toward developing an anti-inflammatory clove cream.

Materials and Methods: Bioactive constituents of COE were identified using gas chromatography–mass spectrometry (GC-MS). Antioxidant activity was evaluated using the DPPH free-radical scavenging assay, total antioxidant capacity, ferric-reducing antioxidant power, and the β -carotene bleaching assay coupled with linoleic acid autoxidation. Antimicrobial activity was assessed using agar disc diffusion and microdilution methods. Anti-inflammatory activity was evaluated *in vitro* and *in vivo* using the bovine serum albumin denaturation inhibition assay and a formalin-induced inflammatory pain mouse model, respectively.

Results: GC-MS identified two major compounds, caryophyllene and humulene, together with other fatty acids. All extracts exhibited significant antioxidant activity, with DPPH free-radical inhibition exceeding 80% at 4 mg/ml. COE showed notable antimicrobial activity, with *Proteus mirabilis* being the most susceptible strain, showing inhibition zone diameters greater than 10 mm. Anti-inflammatory testing revealed significant effects for all extracts, with more than 80% inhibition of bovine serum albumin denaturation at 4 mg/ml. The cream formulated with COE1 (COEC1) reduced inflammation by 45.59%, compared with 33.04% for the standard (niflumic acid, FLUCIDAL® 3%). Skin toxicity evaluation yielded a Primary Irritation Index of zero.

Conclusions: These findings underscore the potential of the novel clove oil extracts obtained by vegetable oil maceration and the COE cream as new remedies warranting further investigation into their pharmacological applications.

Keywords: Clove Oil Extracts, Biological Properties, Clove Cream, Cutaneous Toxicity, Therapeutic Application, Inflammation

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Introduction

Natural remedies, particularly medicinal plants, have long served as the primary or even sole traditional treatment for various pathologies and as raw materials for modern medicine.¹ According to the World Health Organization (WHO), nearly 6,377 plant species are used in Africa, of which more than 4,000 are medicinal plants, accounting for 60% of traditional medicine.²

Phytotherapy is currently a highly studied discipline because plants contain secondary metabolites such as phenolic compounds (phenolic acids, flavonoids, and tannins), alkaloids (methylxanthines), terpenes (essential oils), and

nutrients (carbohydrates, proteins, and minerals), which constitute a source of bioactive molecules considered potent agents in the medical field. These bioactive substances are widely used in therapeutics as antibacterial, antiseptic, anti-inflammatory, and diuretic agents, as well as antioxidants that provide protection against oxidative stress.^{3,4}

Among this botanical diversity, the clove tree, scientifically known as *Syzygium aromaticum* and referred to as Qoronfol in the Arabic pharmacopoeia, occupies a special place because of its multiple therapeutic virtues. It belongs to the *Myrtaceae* family and originates from the Moluccas

Islands in Indonesia. It is a medicinal plant that has long been used in traditional medicine and especially in nutrition.⁵ Its richness in secondary metabolites accounts for several biological effects, including anti-inflammatory, antimicrobial, anticancer, and antioxidant activities.

Recent scientific research has highlighted that clove buds, which are rich in eugenol (up to 85–90% of their composition), possess powerful antioxidant properties capable of neutralizing free radicals involved in oxidative stress.⁶ Their anti-inflammatory action results from the inhibition of pro-inflammatory signaling pathways, including the suppression of cyclooxygenase-2 (COX-2) and inflammatory cytokines such as TNF- α .⁷ These pharmacological properties make clove a promising alternative to synthetic drugs, particularly in developing countries where access to modern treatments remains limited.

Indeed, non-steroidal anti-inflammatory drugs (NSAIDs) commonly used in the treatment of inflammatory pain and synthetic antibiotics widely used for various microbial infections have notable adverse effects, the most common being gastric ulcers and bacterial resistance, respectively, whereas clove extracts generally offer a better tolerance profile. Several pathogenic microbial strains have become increasingly resistant to antibiotics, making the treatment of microbial infections with these drugs ineffective, thus leading to persistent infection, worsening host health, and potentially death. In addition, the adverse effects induced by antibiotics and synthetic anti-inflammatory drugs used for microbial infections and inflammatory pathologies, respectively, are diverse and serious, which burdens patients and leads them to discontinue treatment.⁷

These concerns have motivated the present investigation to search for new and more effective anti-inflammatory and antimicrobial compounds in clove oil extracts (COE) as novel candidates for therapeutic development with fewer undesirable side effects. In addition, the use of cloves in topical formulations such as creams and oily macerates allows a therapeutic approach that is both traditionally and scientifically validated, thus meeting the needs of local populations while forming part of an integrative medicine approach.⁸ This is the first original study, conducted over three years, to elucidate the phytochemical profile of COE and to evaluate their anti-inflammatory, antioxidant, and antimicrobial properties. Cloves are known to be a valuable source of antiviral, antibacterial, and anticancer agents. These biological activities are attributed to the presence of bioactive molecules, mainly eugenol, which represents the major compound of the plant, along with eugenyl acetate and isoeugenyl acetate.⁸

Freitas et al.⁹ demonstrated the potent antibacterial effect of clove essential oil against various bacterial strains, with inhibition zone diameters reaching 28 mm. They determined the efficacy of clove against Gram-positive bacteria such as

Listeria monocytogenes and *Bacillus cereus*, as well as against Gram-negative multidrug-resistant bacteria such as *Salmonella typhimurium*. Research conducted by Lairungruang et al.¹⁰ demonstrated that clove inhibits the growth of *Candida albicans*, with a minimum inhibitory concentration (MIC) of 0.156 mg/ml. Clove essential oil has also demonstrated a broad spectrum of efficacy against fluconazole-resistant strains such as *Candida krusei* and *Candida glabrata*. It is also effective against dermatophytes, *Aspergillus* spp., and some *Candida* strains, such as *C. tropicalis* and *C. parapsilosis*.¹¹ Furthermore, clove eugenol has been shown to be effective against two types of herpes simplex virus (HSV): HSV-1 and HSV-2.¹²

Agbaje¹³ supported the traditional use of clove as an antiulcer agent based on an experiment conducted in mice with or without gastric ulcerative lesions, in which clove aqueous extract was shown to accelerate gastric emptying; eugenol in clove essential oil had no effect on acid production but protected the gastric mucosa by stimulating mucus secretion.

Clove possesses powerful antioxidant activity, mainly due to its high eugenol content (up to 90% of the essential oil). According to Prasad and Singh,¹⁴ clove extracts showed excellent free-radical scavenging activity (IC₅₀ = 2.1 μ g/ml in the DPPH assay, exceeding that of vitamin C used as the standard). Moreover, *in vivo* studies have demonstrated that clove powder (500 mg/kg/day) reduced oxidative stress markers in tissues by 40–60%.

Eugenol, owing to its antioxidant properties, plays an important role in the prevention of various cancers, including liver cancer. *Syzygium aromaticum* inhibits cell proliferation and reduces oxidative stress, exerting a protective effect against liver cirrhosis, which may progress to cancer.¹⁵ In addition, recent studies have demonstrated the anti-inflammatory potential of clove extracts. A study conducted by El-gharib et al.¹⁶ demonstrated that clove extracts significantly inhibit pro-inflammatory signaling pathways in cellular models.

The aim of this study was to extract bioactive compounds from cloves by maceration in vegetable oil used as a non-polar extraction solvent, to characterize the resulting oily extracts through physicochemical, qualitative, and quantitative analyses, to determine their biochemical profile using quantitative chromatographic analysis, and to evaluate their biological activities, namely antioxidant, anti-inflammatory, and antimicrobial activities. To promote bioactive substances with anti-inflammatory effects, this is the first study conducted on COE to demonstrate their effectiveness in the prevention and treatment of inflammatory diseases. In addition, this study aimed to demonstrate the efficacy of a topical formulation, an “anti-inflammatory clove cream,” based on these oily extracts in the treatment of acute inflammation using a formalin-induced inflammatory pain

model in animals.

Materials and Methods

Experimental Animals

The *in vivo* evaluation of the anti-inflammatory effects of COE and clove cream, as well as the primary skin irritation test, were performed on male Swiss mice weighing 25 ± 3 g, in accordance with OECD Test Guideline 404.¹⁷ The animals were supplied by the Animal Care Facility of the Faculty of Life and Natural Sciences, University of Mascara, Algeria. The animals were divided into the groups described in the Experimental Design section, maintained under standard environmental conditions (25 ± 2 °C, 12/12 h light/dark cycle), and provided with a standard rodent pellet diet and ad libitum access to water.

Plant Material

Cloves used in this study were obtained from a local market

in the Mascara region of Algeria. Before use, they were thoroughly rinsed and then ground into a fine powder using a spice grinder. This powder was subsequently used to prepare the various oily extracts.

Microbial Strains

Different clinical microbial strains were used to evaluate the antimicrobial effect of COE. The bacterial strains tested included Gram-positive bacteria: *Staphylococcus aureus* SA1, *S. aureus* SA2, and *Streptococcus* sp.; Gram-negative bacteria: *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*; and the pathogenic fungus *Candida albicans*.

All strains were isolated and identified in the Laboratory of Bioconversion, Microbiological Engineering and Health Safety of the University of Mascara, Algeria. The antibiotic resistance profile of each microbial strain is presented in Table 1.

Table 1. Antibiotic Resistance Profile of the Tested Microbial Strains. Values are expressed as mean $\pm$ SD (n = 3)

Antibiotic discs	SP	AMX	PT	NI	N	OX	CT	P	P-G	FCA
<i>S. aureus</i> SA1	R ⁰	R ¹² $\pm$ 0	R ⁰	I ¹⁸ $\pm$ 0	I ¹⁵ $\pm$ 0	R ⁰	R ¹² $\pm$ 0	R ⁰	R ¹⁰ $\pm$ 0	/
<i>S. aureus</i> SA2	R ⁰	R ¹² $\pm$ 0	R ⁰	R ¹⁰ $\pm$ 0	R ⁸ $\pm$ 0	R ⁰	R ¹² $\pm$ 0	R ⁰	R ⁹ $\pm$ 0	/
<i>S. pyogenes</i>	R ⁰	R ¹⁵ $\pm$ 0	R ⁰	I ²² $\pm$ 0	I ¹⁵ $\pm$ 0	R ⁰	R ¹² $\pm$ 0	R ⁰	R ⁰	/
<i>E. coli</i>	R ⁰	R ⁰	R ⁰	I ²⁰ $\pm$ 0	R ¹⁵ $\pm$ 0	R ⁰	R ¹¹ $\pm$ 0	R ⁰	R ⁰	/
<i>K. pneumoniae</i>	R ⁰	R ⁰	R ⁰	I	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	/
<i>P. mirabilis</i>	R ⁰	R ¹⁵ $\pm$ 0	R ⁰	I ¹⁴ $\pm$ 0	R ¹⁶ $\pm$ 0	R ⁰	R ⁰	R ⁰	R ⁰	/
<i>P. aeruginosa</i>	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	/
<i>C. albicans</i>	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰	R ⁰

SP: Spiramycin, AMX: Amoxycillin, PT: Pristinamycin, NI: Nitroxolin, N: Neomycin, OX: Oxacillin, CT: Colistin, P: Penicillin, P-G: Penicillin-clavulanic acid, FCA: Fluconazole.

Oily Extract Preparation

The different COE were prepared by maceration under various conditions using vegetable soybean oil (VSO) as a non-polar extraction solvent (Table 2). In the first trial, 20 g of clove powder was macerated in 50 ml of VSO; in the second trial, 20 g was macerated in 100 ml of VSO. Each mixture was macerated either at room temperature for 4 weeks or at 60 °C for 3 consecutive hours in an oven. After extraction of the bioactive components, the mixtures were filtered through clean, sterile tissue. The filtrates were then centrifuged using a centrifuge (Hettich EBA) at 5000 rpm for 5 min. Each COE was separated from the clove powder residues after centrifugation and stored in small amber glass vials at 4 °C until use. The extraction yields for each COE and their organoleptic characteristics (appearance, color, and odor) were determined.

Physicochemical Characterization

The physicochemical characterization of the various COE was performed by determining selected parameters: pH,¹⁸ relative density at 20 °C,¹⁹ optical rotation,²⁰ refractive index,²¹ acid value,²² saponification and ester values,²³ and miscibility with ethanol.²⁴

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

The quantitative determination of the various bioactive compounds contained in the COE was performed using GC-MS according to the method described by Kaya et al.,²⁵ with slight modifications. A PerkinElmer Clarus 500 Autosystem GC (USA) was used for COE analysis. The COE were diluted with acetone (1:10) and injected into an HT-5 column (25 m $\times$ 0.22 mm, 0.1 μ m film thickness). The column temperature was programmed from 50 to 120 °C at 3 °C/min, then from 120 to 220 °C at 5 °C/min, with initial and final hold times of 0.64 min. Helium was used as the carrier gas at 5 psi inlet pressure. The injection and detection (FID) temperatures were set at 250 °C. Samples (1.0 μ l) were injected in split mode (50:1).

Extraction of Phenolic Compounds from Clove Oil Extracts

For isolation of polar fractions, each 6 g portion of COE was brought into contact with 50 ml of hexane in an ampoule. The mixtures were then shaken vigorously to completely dissolve the COE. Subsequently, 25 ml of 80% methanol was added in three successive extractions, with agitation to ensure efficient extraction of polar fractions (PF).

The mixtures were allowed to react for 15 min. Subsequently, the aqueous phases (polar fractions + methanol) were separated from the organic phases (non-polar fractions + hexane) and evaporated using a rotary evaporator (STUART type) to remove the solvent.

The concentrated polar fractions were collected in small amber tubes and then dried in an oven at 45 °C to obtain a powder, which was used for the subsequent analyses. Table

2 presents the codes of the different oily extracts used in this study. In addition, the extraction yields were determined for each polar fraction extract and calculated using the following formula:

$$EY (\%) = (W_f/W_i) \times 100$$

where W_f is the weight of the polar fraction extract in grams (g), and W_i is the weight of COE used for polar fraction isolation (g).

Table 2. Cloves Oily Extracts Codes

Code	Polar fractions of the oil extracts
COE1	Cloves oily extract obtained by maceration in 100 ml of VSO at room temperature for 4 weeks.
COE2	Cloves oily extract obtained by maceration in 50 ml of VSO at room temperature for 4 weeks.
COE3	Cloves oily extract obtained by maceration in 100 ml of VSO at 60 °C/3 hours.
COE4	Cloves oily extract obtained by maceration in 50 ml of VSO at 60 °C/3 hours.

Phytochemical Screening

Qualitative phytochemical analysis was performed using colorimetric methods to detect the presence of selected chemical groups of bioactive substances: phenolic compounds (PC), flavonoids (F), and tannins (T) in the polar fractions of clove oil extracts (PFCOE).^{26,27}

The detection of PC was performed using 10% ferric chloride ($FeCl_3$) as a colorimetric reagent. To 1 ml of each PFCOE, two drops of $FeCl_3$ were added; the appearance of an intense black-green precipitate indicated the presence of PC. Detection of F was carried out using a 2% aluminum trichloride ($AlCl_3$) solution. One milliliter of 2% $AlCl_3$ was added to 200 μ l of each PFCOE, and the appearance of a yellow color indicated the presence of flavonoids. For tannin detection, 1 ml of vanillin reagent was added to 200 μ l of each PFCOE. A positive reaction was indicated by the appearance of a red color in the solution.

Quantitative Determination of Polyphenols

Total phenolic compounds (TPC), total flavonoids (TFC), and total tannins (TTC) were determined according to the methods described by Boizot and Charpentier²⁸ using Folin–Ciocalteu reagent, Samatha et al.²⁹ using 2% aluminum trichloride ($AlCl_3$) solution, and Ba et al.³⁰ using vanillic acid reagent, respectively. The concentrations of each bioactive chemical group were calculated from absorbance measurements using a spectrophotometer (Jenway model 6400, UK). Gallic acid (GA) at concentrations of 0.05–0.2 mg/ml was used as the standard for TPC determination, and calculations were made using the following calibration curve:

$$Y = 0.751x + 0.0012.$$

Results were expressed as gallic acid equivalents (GAE) per gram of dry extract (mg GAE/g DE).

For TF determination, quercetin (Q) at concentrations of 0.05–0.25 mg/ml was used as the standard, and calculations were performed using the following calibration curve:

$$Y = 4.453x + 0.0115.$$

Results were expressed as mg quercetin equivalents per gram of dry extract (mg QE/g DE). Catechin was used as the standard for TT determination, and the following calibration curve was used to determine TT concentration:

$$Y = 0.1117x + 0.0014.$$

All determinations were performed in triplicate, and results were expressed as mean $\pm$ SD.

Antioxidant Activities Assessment

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Test

An aliquot of 250 μ l of each COE concentration (0.25–4 mg/ml) was added to 1 ml of freshly prepared ethanolic DPPH solution (0.1 mg/ml). A negative control was prepared using DPPH solution alone. The mixture was then incubated in the dark at 20 °C for 30 min. Absorbance was measured using a spectrophotometer (SHIMADZU UV mini-1240, Japan) at 517 nm.³¹ The anti-radical activity was evaluated using the following formula:

$$\text{Anti-radical activity (\%)} = [(A_1 - A_2) / A_1] \times 100,$$

where A_1 is the absorbance of the DPPH solution and A_2 is the absorbance of the DPPH solution after addition of the COE concentrations.

Results were expressed as percentages of DPPH free radical inhibition and as IC₁₀₀ values, which were established from the different calibration curves: ascorbic acid: $Y = 2.193x + 84.10$; $R^2 = 0.925$; COE1: $Y = 31.70x - 8.785$; $R^2 = 0.953$; COE2: $Y = 17.63x + 14.08$; $R^2 = 0.929$; COE3: $Y = 2.664x + 77.29$; $R^2 = 0.970$; COE4: $Y = 5.409x + 68.19$; $R^2 = 0.920$. IC₁₀₀ values were estimated by extrapolation from the linear regression curves. All assays were carried out in triplicate and expressed as mean $\pm$ SD.

Total Antioxidant Capacity (TAC)

An aliquot of 100 μ l from each COE concentration (0.25–4 mg/ml) was mixed with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and then incubated at 95 °C for 90

min. After cooling, the absorbance of the solutions was measured at 695 nm against a blank containing 1 ml of reagent solution and 100 µl of methanol.³² All assays were performed in triplicate ($n = 3, \pm \text{SD}$). Ascorbic acid was prepared as a standard antioxidant at concentrations ranging from 0.25 to 4 mg/ml and analyzed under the same operating conditions for calibration curve preparation: $Y = 0.663x + 0.478$; $R^2 = 0.934$, from which the total antioxidant capacity calculations were made and expressed in milligrams of ascorbic acid equivalent per gram of COE (mg EAA/g COE).

Ferric Reducing Antioxidant Power (FRAP) Assay

The protocol described by Oyaizu³³ was used. Into test tubes containing 200 µl of each COE concentration (0.25–4 mg/ml), 500 µl of phosphate buffer (0.2 M, pH 6.6) was added, followed by 500 µl of 1% potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$]. The mixtures were then heated in a water bath at 50 °C for 20 min. Subsequently, 500 µl of 10% trichloroacetic acid was added to each tube, and the mixtures were centrifuged at 3000 rpm for 10 min.

Aliquots of 500 µl of the supernatant were transferred to other tubes, to which 500 µl of distilled water and 100 µl of 0.1% ferric chloride (FeCl_3) freshly prepared in distilled water were added. A blank was prepared under the same conditions using methanol. The absorbance of the reaction medium was read using a UV-Vis spectrophotometer (SHIMADZU UV mini-1240, Japan) at 700 nm against the blank. A positive control was also prepared using a standard solution of ascorbic acid, whose absorbance was measured under the same conditions as the COE samples. All tests were performed in triplicate ($n = 3; \pm \text{SD}$).

β-Carotene Bleaching Test Coupled with Linoleic Acid Auto-Oxidation

The inhibition of β-carotene bleaching by the different COE was evaluated using the method described by Marco³⁴ with some modifications. Briefly, 2 mg of β-carotene was dissolved in 10 ml of chloroform, and 5 ml of this solution was added to a vial containing 100 µl of linoleic acid and 1000 mg of Tween 20. After mixing both phases, the chloroform was evaporated using a rotary evaporator (STUART). Subsequently, 500 ml of diluted hydrogen peroxide was added to the above mixture with rigorous agitation, and 4 ml of the obtained emulsion was added to a series of tubes containing 200 µl of each COE at concentrations ranging from 0.25 to 4 mg/ml. The absorbance was measured immediately at 470 nm, corresponding to time zero ($t = 0$), against a blank containing the emulsion without β-carotene. The mixtures were then placed in a water bath at 50 °C for 120 min, and the absorbance of each solution was measured at 470 nm after 120 min. The negative control consisted of 200 µl of methanol instead of COE. All assays were

performed in triplicate ($n = 3; \pm \text{SD}$).

The β-carotene bleaching inhibition was calculated using the following formula:

$$\beta\text{-carotene bleaching inhibition (\%)} = (\text{Absorbance after 120 min} / \text{Absorbance at } t_0) \times 100$$

Antimicrobial Activity Assessment

The antimicrobial activity of COE was evaluated using two different methods: a qualitative analysis by the agar disc diffusion method and a quantitative analysis by the microdilution technique to determine antimicrobial parameters such as the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC).³⁵

Agar Disc Diffusion Method

A range of increasing COE concentrations was prepared and dissolved in 10% dimethyl sulfoxide. The concentrations used were: C1 = 1000 mg/ml, C2 = 500 mg/ml, C3 = 200 mg/ml, C4 = 100 mg/ml, C5 = 80 mg/ml, and C6 = 35 mg/ml, which were selected after several preliminary assays. Subsequently, 18-hour fresh cultures were prepared for each microbial strain, from which microbial suspensions were prepared in sterile physiological water (0.9% NaCl) with an optical density (OD) equivalent to 0.5 McFarland standard. The OD ranged from 0.08 to 0.13 at 625 nm for bacteria and was 0.5 at 540 nm for yeasts. This density corresponded to a final concentration of 10^6 to 10^8 CFU/ml for bacteria and 10^7 cells/ml for fungi.^{36,37} Thereafter, the different microbial strains were seeded by swabbing the surface of Mueller–Hinton (MH) agar (Sigma, USA) using sterile swabs. Sterile Whatman paper discs of 0.6 mm diameter were then impregnated with each COE concentration and aseptically deposited on the MH medium using sterile forceps. The plates were incubated at 4 °C to slow microbial growth and to promote good diffusion of the different COE on the MH medium around the discs. After incubation at 37 °C for 18–24 h, the microbial growth inhibition zone diameters around each disc were measured using a caliper,³⁸ and the effectiveness of COE was assessed according to the criterion of Poncé et al.³⁹ This test allowed the selection of the most active extracts for determination of the antimicrobial parameters. All assays were carried out in triplicate ($n = 3; \pm \text{SD}$).

Microdilution Method

The protocol described by Howaida et al.⁴⁰ was used with slight modifications. The quantitative evaluation of the antimicrobial effect of COE was performed using 96-well plates. The culture medium used was MH broth, which was introduced into each well at 50 µl per well. In the first well, 50 µl of the stock solution concentration prepared previously (2000 mg/ml) was added to obtain a concentration of 1000

mg/ml. A series of 1/2 dilutions was made until the 12th well, where the concentration was approximately 0.488 mg/ml (C12). Subsequently, 50 µl of the bacterial suspensions at 10⁸ CFU/ml concentration and *C. albicans* suspension at 10⁷ cells/ml were added to each plate well. The plates were then incubated under aseptic conditions at 37 °C for 72 h. Absorbance was read at time zero (t = 0) and after 4, 18, 24, and 72 hours to determine the microbial growth kinetics, from which the MIC values were selected for each microbial strain. The wells that did not show visible microbial growth were re-isolated on MH agar for MBC and MFC determination. The antibiotic potential of each strain was assessed by calculating the MBC/MIC and MFC/MIC ratios.

In vitro Anti-inflammatory Activity

Inhibition of Bovine Albumin Denaturation

The technique described by Kandikattu et al.⁴¹ was applied with slight modifications. Briefly, 1 ml of each COE concentration and the standard (diclofenac) at different concentrations (0.25–4 mg/ml) were mixed with 1 ml of 1% bovine serum albumin (BSA) solution. The mixtures were incubated at 37 °C for 20 min. Subsequently, the tubes were placed in a water bath at 60 °C for 3 min, and the absorbance was measured at 660 nm. Each assay was performed in triplicate (n = 3; ± SD). Control tubes were also prepared by mixing 1 ml of BSA solution with 1 ml of ethanol (100% albumin denaturation) or physiological saline. The bovine albumin denaturation percentages were determined according to the following formula:

$$\text{Bovine albumin denaturation (\%)} = [(Ac - As) / Ac] \times 100,$$

where Ac is the control absorbance at 660 nm (1% bovine albumin solution) and as is the sample absorbance at 660 nm.

The inhibition of bovine albumin denaturation was calculated using the following formula:

$$\text{Bovine albumin denaturation inhibition (\%)} = [(Ac - As) / Ac] \times 100,$$

where Ac is the absorbance of the positive control (100% denatured BSA) and as is the sample absorbance at 660 nm.

Antihemolytic Activity and Erythrocyte Membrane Stabilization

The assay was based on the effect of COE on erythrocyte stabilization following hemolysis induced by a hypotonic solution, according to the protocol of Gadamsetty et al.⁴² The antihemolytic activity was assessed using human blood collected from students of the Department of Biology, University of Mascara, Algeria. Blood was collected under aseptic conditions from the antecubital fossa after disinfection with alcohol and collected into heparinized tubes to prevent coagulation. An equal volume of Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride in 100 ml of distilled water) was added, and the mixture was centrifuged at 3000

rpm for 5 min to separate erythrocytes from other blood components. After removal of the plasma, the erythrocyte pellet was washed three successive times with physiological saline (0.9% NaCl) until a clear supernatant was obtained, with centrifugation at 3000 rpm for 5 min after each wash. The resulting pellet was diluted to 10% with sterile physiological saline. Aliquots of 0.5 ml of each COE, soybean oil, and diclofenac (used as the standard) at different concentrations were added to 1 ml of phosphate buffer (pH 7.4), 2 ml of hypotonic saline solution (0.36%), and 0.5 ml of the previously prepared erythrocyte suspension. After incubation at 37 °C for 30 min, the mixtures were centrifuged again at 3000 rpm for 20 min. The absorbance of the supernatant was measured at 560 nm using a spectrophotometer. All assays were performed in triplicate (n = 3; ± SD). Control tubes were prepared by mixing 0.5 ml of erythrocyte suspension with 0.5 ml of physiological saline or ethanol to verify the erythrocyte status or to induce 100% hemolysis, respectively. The hemolysis percentage was determined using the following formula:

$$\text{Hemolysis (\%)} = [(As - Apc) / As] \times 100,$$

where as is the sample absorbance at 560 nm and Apc is the positive control absorbance (100% hemolysis). Thus, the red blood cell protection percentage, or hemolysis inhibition, was calculated using the following formula:

$$\text{Hemolysis inhibition (\%)} = 100 - \{[(As - Apc) / As] \times 100\}.$$

Preparation of the Clove Oil Extract Cream (COEC)

A topical formulation refers to all processes that enable active ingredients to be transformed into a drug in a suitable form for administration.⁴³ The clove oil extract cream (COEC) was prepared using two different phases: an aqueous phase containing distilled water and moisturizing agents, and an oily phase containing clove oil extracts as the active ingredient, along with waxes and emulsifiers to moisturize and protect the skin.

The objectives were to ensure good skin penetration of the clove cream active ingredients, to ensure cream stability and effectiveness, and to provide a pleasant, easy-to-apply texture.

The COEC was prepared according to the following protocol: Two 100 ml beakers were placed in a water bath at 60 °C. The first beaker contained 50 g of distilled water with 3 g of starch, and the second beaker contained 4 g of Tween 80, 12 g of paraffin oil, 10 g of stearic acid, 10 g of beeswax, and 10 g of COE. Each beaker was covered with aluminum foil, and the mixtures were left to melt for 45 min. After dissolution of the ingredients, the oily phase was added to the aqueous phase and mixed immediately and vigorously using an electric mixer (Silverson type) until a smooth cream was obtained.

Finally, 1 g of sodium benzoate was added to COEC as a preservative. The final product was packaged in sterile amber

Table 3. Clove Cream Composition

	Ingredients	Roles	Amounts (%)
Oily phase	Paraffin oil	Mild laxative	12
	Stearic acid	Emulsifier	10
	Beeswax	Thickening agent	10
	COE	Active ingredient	10
Aqueous phase	Distilled water	Solvent	50
	Starch	Thickening agent	3
	Tween 80	Emulsifier	4
Preservative agents	Sodium benzoate	Preservative agent	1

glass containers and stored at 4 °C until use. The composition of COEC is presented in Table 3.

Quality Control of Clove Cream

Macroscopic and Organoleptic Characteristics

A small amount of COEC was spread on a clean surface (glass plate) using a sterile spatula. The color, appearance, homogeneity, and odor were then observed. These observations made it possible to detect formulation defects such as phase separation, granulation, or product alteration.⁴⁴

pH Measurement

The pH of the COEC was measured using a previously calibrated pH meter. The ideal value should range between 4.5 and 6.5 to respect the skin pH. A result outside this range may indicate a risk of product irritation or instability.⁴⁵

Microbiological Quality Control

Prior to application, microbiological evaluation of pharmaceutical creams intended as anti-inflammatory products for therapeutic applications is crucial to ensure their quality and safety. According to the European and American Pharmacopoeias, the main objectives were to measure the total aerobic germ count (TAGC), total yeast and mold count (TYMC), and to detect the presence of pathogenic bacteria that may contaminate this type of product. Briefly, 1 g of COEC was aseptically added to 9 ml of sterile physiological saline to obtain a stock solution. The solution was then homogenized using a vortex mixer for 30 s, and a series of decimal dilutions was prepared to a final dilution of 10⁻⁴. For total aerobic germ (TAGC) enumeration, 0.1 ml of the 10⁻² and 10⁻³ dilutions were inoculated on Plate Count Agar (PCA) medium (Sigma, USA). The same protocol was applied for yeast and mold (TYMC) enumeration using Sabouraud agar medium (Sigma, USA). The detection of pathogenic bacteria was therefore carried out by inoculating the stock solution on selective agar media, specifically Chapman agar and Cetrimide agar (Sigma, USA) for the detection of *S. aureus* and *P. aeruginosa*, respectively.⁴⁶ The Petri dishes were then incubated at 37 °C for 24 h, except those for yeasts and molds, which were incubated at 25 °C for 3–5 days.

The TAGC was determined using the following formula:

$$N \left(\frac{CFU}{g} \right) = \sum \frac{Colonies}{D} \cdot V \cdot (n1 + 0.1n2)$$

According to European and American Pharmacopoeia, the TAGC must be ≤ 102 UFC/g, while the TYMC must be <101 UFC/g.

In vitro Anti-inflammatory Effect of Clove Cream

The anti-inflammatory effect of the COEC1 was evaluated *in vitro* using the BSA denaturation inhibition method to confirm the *in vitro* efficacy of the topical formulation as a potent anti-inflammatory product. This cream was selected regarding its potential *in vitro* antioxidant and antimicrobial effects. Briefly, 0.5 g of COEC1 was mixed with 10 ml of sterile physiological water (0.9%) to prepare a stock solution at a concentration of 50 mg/ml, from which serial dilutions were made to obtain a series of concentrations ranging from 0.25 to 4 mg/ml. Diclofenac sodium 1% cream was used as the standard. Then, 1 ml of each COEC concentration and the standard were mixed with 1 ml of 1% BSA solution. The mixtures were incubated at 37 °C for 20 min, and the tubes were then placed in a water bath at 60 °C for 3 min. After cooling, the absorbance was measured using a spectrophotometer at 660 nm. All assays were performed in triplicate (n = 3; ± SD).

Primary Skin Irritation Test

The skin irritation index (SII) is a quantitative method used in this study to assess the severity of skin reactions after application of the COEC to mouse skin. It was used in the present toxicological study to determine the irritant potential of the COEC.⁴⁷

The study was conducted on male mice weighing 25 g each, maintained under standardized conditions. After shaving a 2 cm² area of skin, the animals in the first control group received physiological saline (0.9% NaCl) via cutaneous application.

Animals in the second test group received clove oily extract labeled COE1; animals in the third test group received clove oily extract labeled COE2; mice in the fourth test group received clove oily extract labeled COE3; and animals in the fifth test group received clove oily extract labeled COE4. Animals in the 6th and 7th test groups received

the COEC1 and COEC4 respectively, by cutaneous application to the specific skin area previously prepared, which was then covered with a moistened sterile compress and held with a hypoallergenic dressing. The products remained in contact with the skin for 4 hours, and irritation

signs such as erythema and edema were examined at 24, 48, and 72 hours according to standardized scales (Table 4).⁴⁸ Results in the test groups were compared with those of the control groups to determine the irritant potential of the formulation.⁴⁹

Table 4. Erythema and Edema Numerical Scale

Scale	Erythema (Redness)	Edema (swelling)
0	None	None
1	slight redness	Slight swelling
2	Moderate redness	Moderate swelling
3	Severe redness	Severe swelling
4	Extreme redness/crusting	Extreme swelling/necrosis

The PII was calculated by averaging the erythema and edema scores for each mouse and then calculating the average for the entire group using the following formula:

$$PII = (\Sigma \text{erythema score} + \text{edema score}) / N,$$

where N is the number of animals.

The irritant potential of the COEC was determined using the following criteria: $SII < 0.5$: non-irritating substance; $0.5 \leq SII < 2$: mildly irritating substance; $2 \leq SII < 5$: moderately irritating substance; $SII \geq 5$: severely irritating or corrosive substance.

In vivo Anti-inflammatory Activity

To evaluate the *in vivo* anti-inflammatory effect, seven groups of five mice each were used. The mice were fasted for 17 hours before testing. The first group served as the positive control and received an injection of 1% formalin (25 ml/kg b.w.) into the right paw arch (Batch 1). Mice in the second group (negative control) were injected with physiological saline used as a placebo (Batch 2). Cutaneous applications of COE1 extract were performed on the paws of animals in the third test group (Batch 3), and COE4 was applied to animals in the fourth test group (Batch 4). In addition, COEC1 was applied to mice in the fifth test group (Batch 5), and COEC4 was applied to animals in the sixth test group (Batch 6). The seventh test group (Batch 7) received a therapeutic application of FLUCIDAL® 3% niflumic acid ointment, used as the standard treatment. Formalin (1%) was then injected into all animals of the different test groups 30 min after the application of COE and COEC. Assays were carried out in replicate ($n = 5$; mean $\pm$ SD).

Edema evolution was measured in both mouse paws (treated and untreated) at different time points ($t = 0, 30, 60, 120, 180, 210, 240, 270, 300, 330$, and 360 min) after the 1% formalin injection. The anti-inflammatory activity of the tested products and its progression were estimated by determining the average percentage of edema inhibition, calculated according to the following formula:

$$\text{Inflammation inhibition (\%)} = \left[\frac{((V_c - V_0)_{\text{control}} - (V_c - V_0)_{\text{treated}})}{(V_c - V_0)_{\text{control}}} \right] \times 100$$

where V_0 is the mouse paw volume at $t = 0$ (before formalin injection), and V_c is the mouse paw volume at the different time points ($t \neq 0$).

Statistical Analysis

Replicates were prepared for all *in vitro* and *in vivo* experiments ($n = 3$ and $n = 5$, respectively), and results are presented as means $\pm$ standard deviations (mean $\pm$ SD). Statistical analyses were performed using SPSS software to compare means by one-way and multivariate analysis of variance (ANOVA). Significant differences were considered at $p < 0.05$.

Results and Discussion

Clove Oil Extract Yield and Organoleptic Characteristics

The extraction yields, appearance, odor, and color of the different COE are presented in Table 5. The COE prepared in this study exhibited an oily appearance, brown to yellowish-brown coloration, and a strong aromatic odor characteristic of pure clove oil volatile compounds (Table 5). The extract labeled COE2 was the only one with a different color; it was dark brown with a very strong clove aromatic odor, which may explain its richness in bioactive and volatile clove substances (Table 5). The color intensity and odor of the extracts reflected the concentration of bioactive molecules in the cloves used in this study.

The homogeneous and oily consistency confirmed the adequate quality of the different COE extracts. Furthermore, chromatographic analyses were performed to demonstrate differences in the chemical composition of each COE obtained by the different extraction techniques.

Several studies have reported that clove oil has a light-yellow color and often appears as a colorless volatile product of eugenol, the major compound found in clove oils in most research, with a strong odor and intense flavor.⁵⁰⁻⁵² Ayoola et al.⁵³ also reported a yellowish color for clove essential oils.

The COE labeled COE1 and COE2, extracted by hot maceration at 60°C for 3 h, showed the highest yields ($59.6 \pm 1.03\%$ and $23.16 \pm 1\%$, respectively) compared with COE extracted by maceration at room temperature for 4 weeks

(Table 5). The highest yields were obtained for the extracts using 100 ml of VSO. Thus, the effects of the extraction technique and the extraction solvent volume on the yield of plant secondary metabolites were clearly observed.

To the best of our knowledge, no studies have been conducted on COE prepared by maceration in vegetable oil

as a non-polar extraction solvent. However, many recent studies have reported on different clove phenolic extracts and essential oils. This is the first study on COE in which we demonstrate a design for new therapeutic clove extracts that could be used in the preparation of novel topical formulations exhibiting interesting therapeutic efficacy.

Table 5. Extraction Yields and Organoleptic Characteristics of Cloves Oily Extracts. Values are given as mean $\pm$ SD ($n = 3$), $p < 0.05$

	Yield (%)	Appearance	Color	Odor
COE1	55.72 $\pm$ 1.93	Oily and homogeneous	Brown	Pure clove oil strong odor
COE2	20.64 $\pm$ 0.89		Dark brown	Very strong cloves aromatic odor
COE3	59.6 $\pm$ 1.03		Yellow to yellow brown	Light aromatic odor, refer to cloves aroma
COE4	23.16 $\pm$ 1		Brown	

In a study conducted by Kara,⁵⁴ the clove EO yield was approximately 8.389%. Sokomate et al.⁵⁵ reported similar results, recording yields of 7% and 7.6%, respectively, for clove EO extracted by hydrodistillation. However, Guan et al.⁵⁶ reported higher yields of 11.5%, 19.6%, and 10.1% for essential oils obtained by hydrodistillation, supercritical fluid extraction, and steam distillation, respectively. In addition, Selles et al.⁵⁷ found an EO yield of approximately 11.6%, which was in agreement with another study conducted by Nana et al.,⁵⁸ who obtained yields of approximately 10.54%. The extraction yields of clove EO remained lower than those of the COE prepared in this study using both maceration at room temperature and at 60 °C, with extraction yields exceeding 50% and 20%, respectively, for the two methods (Table 5). The extraction yield was mainly influenced by the extraction method used and, in particular, by the type of extracting solvent (solvent polarity). Furthermore, it is necessary to evaporate chemical solvents after the extraction process, which concentrates the final extract and therefore reduces the percentage yield. In contrast, a different solvent type was used in this study, VSO, which has a completely different structure and

composition; it is not suitable for evaporation, and as a result, a considerable extract volume was preserved. To the best of our knowledge, this study is among the first to evaluate the effectiveness of COE as potential antioxidant and anti-inflammatory products and to assess the antimicrobial potential of COE. In addition, this study reports original research highlighting the anti-inflammatory potential of a clove cream prepared herein for the treatment of inflammation induced in a mouse model.

Physicochemical Characterization of Clove Oil Extracts

The results of the physicochemical characterization of COE are presented in Table 6. According to the obtained results, the COE extracts exhibited an acidic pH (pH 5) due to their chemical composition (Table 6). These values were lower than those obtained by Muhammad et al.,⁵⁹ who reported pH values of clove methanolic extracts ranging from 5.20 to 6.70. Regarding the relative density of the different COE, a value of 0.934 was recorded for the extracts labeled COE1 and COE2, 0.893 for the COE3 extract, and 0.884 for the COE4 extract, while a value of 0.917 was recorded for the VSO used (Table 6).

Table 6. Physicochemical Characteristics of Clove Oil Extracts

Physicochemical parameters	COE1	COE2	COE3	COE4	VSO
PH	5	5	5	5	4.33
Relative density at 20 °C	0.934	0.934	0.893	0.884	0.917
Rotation angle (α)	0.02	0.04	0.03	0.05	0.01
Refractive index	1.475	1.478	1.476	1.478	1.474
Rotating power	5	10	7.5	12.5	2.5
Acid index	1.68	1.12	0.56	0.76	0.54
Saponification index	151.4	148.7	140.3	136.7	190.5
Ester index	149.7	147.5	139.7	136.6	190.4
Iodine index	31.72	63.45	158.6	126.9	129.2
peroxide index	5	7.5	10	12.5	11.5
Miscibility to ethanol 70%	Immiscible				

A clear difference in density was observed between the different COE and the VSO used as the non-polar extraction solvent. The COE prepared by maceration at room temperature for 4 weeks showed higher density values than those prepared by maceration at 60 °C for 3 h (Table 6).

Therefore, it was confirmed that the composition of VSO

was modified after the extraction process, during which new molecules were extracted; consequently, the initial chemical profile of the VSO was altered, yielding a completely different product (clove oil) containing new bioactive molecules and exhibiting variable density. This was demonstrated by our results, as each extraction technique

produced results different from those of the others. When compared with the clove essential oil density standards specified by the French national organization for standardization (AFNOR), which range between 1.030 and 1.063, the different COE could be qualified as less dense than clove essential oil. Various studies have shown that clove oil density is mainly related to the density of various compounds, such as eugenol (1.0651), eugenol acetate (1.0806), and β -cis-caryophyllene (0.9075).⁶⁰⁻⁶² The COE prepared by hot maceration (60 °C for 3 h) had the lowest density compared with those prepared by maceration at room temperature for 4 weeks, which could be due to changes in long carbon chains and the high-molecular-weight compounds contained in these oily extracts (Table 6). An optical rotation of 2.5 was recorded for the VSO used for COE preparation, whereas a higher value of 12.5 was recorded for the extract labeled COE4 (Table 6). These results were particularly noteworthy, reflecting the effectiveness of the extraction processes in obtaining a final product with a chemical composition of bioactive substances completely different from that of the initial oil, thereby increasing the optical activity of the final product, which was therefore characterized as pure clove oily extract.

Our results were higher than those obtained by Alfikri et al.,⁶³ who reported lower optical rotation values for clove essential oil. Essential oils and oily extracts are different products, and each therefore has its own physicochemical indices that characterize it, depending on the nature of the final product obtained and especially its chemical composition. The polarization angle increased in the different COE compared with the value obtained for VSO ($\alpha = 0.01$) (Table 6). This variation was mainly due to a decrease in viscosity of the final product. Clove oils are known to be volatile products containing several long carbon chains of high molecular weight, such as isolongifolene, β -cis-caryophyllene, α -humulene, eugenol acetate, and caryophyllene oxide, which affect viscosity and the polarization rotation angle.⁶⁴

Refractive indices of 1.475, 1.478, 1.476, and 1.478 were determined for the COE labeled COE1, COE2, COE3, and COE4, respectively (Table 6). These values were higher than that recorded for VSO (1.474). Our results were not consistent with those obtained by Ayoola et al.,⁶⁵ who determined a refractive index of 1.526, or with those of Alfikri et al.,⁶⁶ who reported values ranging from 1.534 to 1.536 and 1.608, respectively, for clove essential oil. The lower refractive index of COE compared with that of clove essential oils reported in various studies, as mentioned previously, was mainly related to the chemical compounds contained in these extracts, as well as to water content, which may influence the refractive index by decreasing it.⁶⁷ The reference value for this index for clove essential oil according to AFNOR is in the range of 1.528 to 1.573. The

difference in refractive index between clove essential oil and COE can be attributed to variations in chemical composition, purity, extraction process, and extract density. The peroxide value is a measure of oil oxidation, especially for vegetable oils, and is used to assess their freshness and quality. The extract labeled COE1 had a peroxide value of 5, indicating slight oil oxidation (Table 6). This suggested good freshness and relatively high quality of the clove oily extract. The extract labeled COE2 had a peroxide value of approximately 7.5, indicating a slight increase compared with COE1; however, oxidation remained moderate. This extract can still be considered fresh, but it may be slightly less stable than COE1. The extract labeled COE3 had a peroxide value of approximately 10, indicating moderate oil oxidation (Table 6).

In addition, the extract labeled COE4 had a peroxide value of 12.5; oxidation of this oil was more pronounced. This suggested lower quality of this extract compared with the others, and as a result, further deterioration could occur (Table 6). Comparing our results with those of Louni,⁶⁸ who found a peroxide value of 6.34 meq O₂/kg oil in clove essential oil, our results were lower for the extracts labeled COE2, COE3, and COE4 but higher than that obtained for the extract labeled COE1. This led us to conclude that clove essential oils have greater stability than oily extracts (Table 6). Higher peroxide values indicate greater oil oxidation, which can lead to loss of freshness and quality. Extracts with lower peroxide values (such as COE1) are generally considered to be of higher quality and more chemically stable.

Acidity characterizes oil purity and stability at room temperature and allows assessment of the degree of alteration by oil hydrolysis.⁶⁹ The results showed that the prepared COE had acceptable acidity compared with the value indicated by NF ISO 1242: 0.3 mg KOH/g MG. Studies on clove essential oil conducted by Louni⁷⁰ showed that the acid index was about 1.2. A value of less than 2 was attributed to optimal oil preservation. Conversely, acid index values greater than 2 may indicate oil degradation (ester hydrolysis) during storage.⁷¹ From these references, it can be concluded that all COE samples were of good quality in terms of acidity level. A high acid index was recorded for the different COE compared with that obtained for VSO (0.54) (Table 6). These results indicated that the prepared COE were rich in free fatty acids and even in esterified fatty acids. The saponification index (SI) indicates the quantity of free and bound fatty acids contained in 1 g of fat. Thus, the saponification index is the mass (in mg) of potassium hydroxide (KOH) required to saponify 1 g of fat. A higher saponification index indicates a lower fatty acid molar mass; therefore, our results indicated that the different COE had a low molar mass (Table 6). The results showed that VSO (extraction solvent) had a high iodine value (129.2), followed by the extracts labeled COE3 and COE4, which

had iodine values of 158.6 and 126.9, respectively, and then the extracts labeled COE1 and COE2, which had low values of 31.72 and 63.45, respectively (Table 6). Thus, the tested extraction solvent (VSO) had a value in accordance with CEVITAL unit standards (124–139 g I2/100 g oil). The degree of oil unsaturation was measured by the iodine value, which provided an overall indication of the unsaturation status. Its value increased with an increasing number of unsaturations in the oil.⁷² According to research conducted by Meng et al.,⁷³ a low iodine value can be considered an expected advantage, as it would help confirm the quality of an oil and improve shelf life by preventing oxidative degradation of edible oils. The COE were immiscible with 70% ethanol (Table 6). This test was important because it indicated which solvent type should be used to dissolve the COE for the various other experimental analyses; therefore, the choice of solubilizing solvent was essential. This analysis confirmed that the different COE were immiscible with 70% ethanol, a more polar solvent, whereas the COE are non-polar products containing some polar substances. Thus, to solubilize these extracts, a non-polar solvent ensuring high solubility of the oily extracts (non-polar molecules), or a mixture of polar and non-polar solvents, is required.

Several preliminary tests were carried out to select hexane as the optimal solvent for solubilizing the prepared COE. In addition, the use of a dichloromethane–ethanol mixture (v/v) provided good COE solubility because dichloromethane is a miscible and highly organic solvent, considered effective for solubilizing oils. According to Ketaren,⁷⁴ a high hydrocarbon content, such as pseudocumene, hemimellitene, p-cymene, 3-ethyl-o-xylene, 4-ethyl-o-xylene, 1,2,3,4-tetramethylfulvene, and prehnitene, contained in clove oil, influenced miscibility in 70% ethanol. Higher terpene and oxygenated hydrocarbon contents also influenced miscibility in ethanol. A high terpene content made the clove oil less soluble, whereas a high oxygenated hydrocarbon content made the oil more soluble.

GC-MS Analysis

Quantitative chromatographic analysis by GC-MS allowed the identification and quantification of the main bioactive compounds in the different oily extracts prepared in this study. The results are presented in Table 7 and Figure 1. In addition, chromatographic analysis of the VSO “ELIO” used as the non-polar extraction solvent was performed, and the amounts of saturated and unsaturated fatty acids were quantified (Table 7).

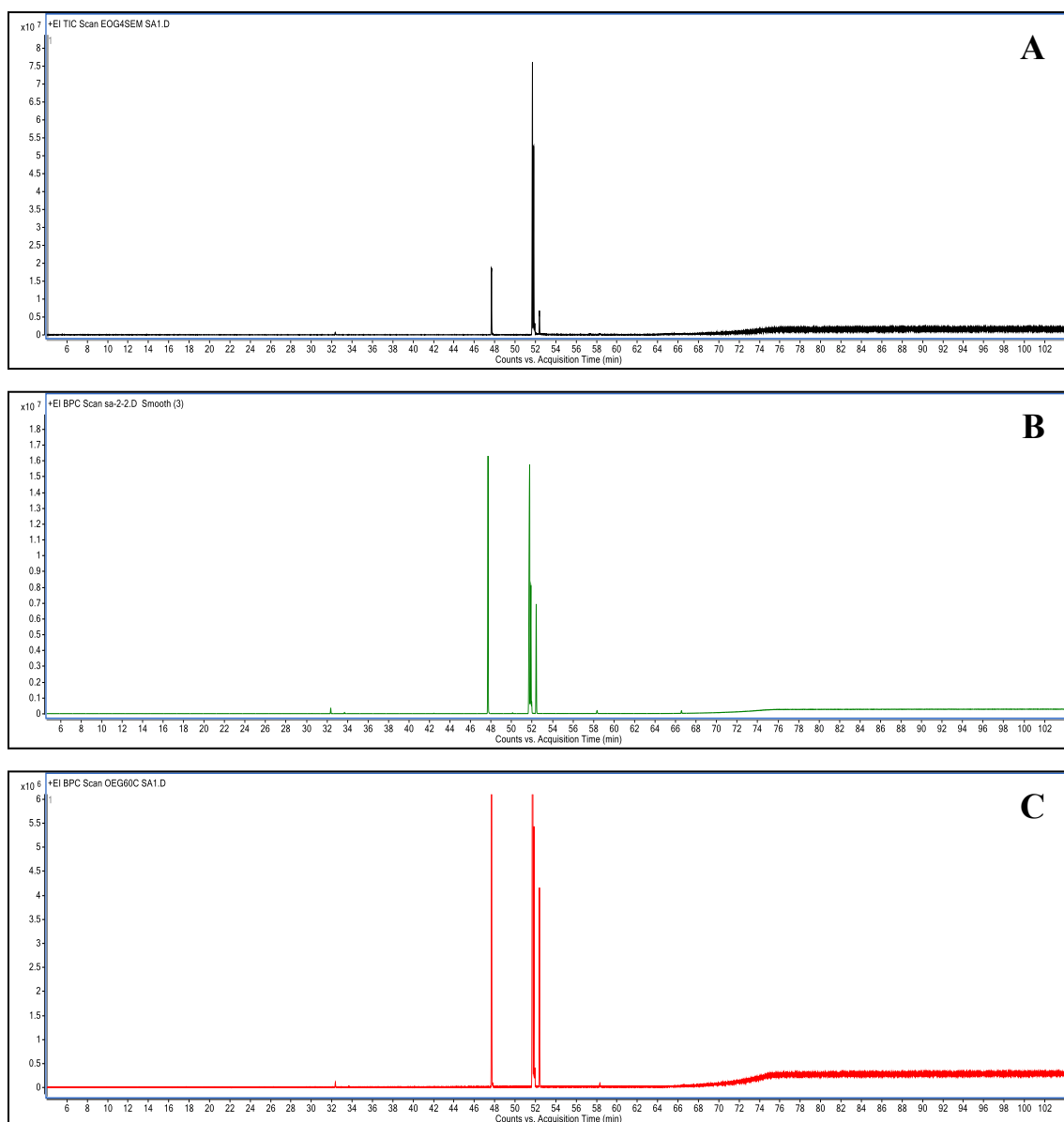
Table 7. Main Bioactive Compounds Identified in the Clove Oily Extracts

Peak	RT (min)	%	Compounds in COE1
1	47.69	10.61	Palmitic acid, methyl ester
2	51.71	50.72	Linoleic acid, methyl ester
3	51.83	33.17	Cis-oleic acid, methyl ester
4	51.93	1.52	Trans-oleic acid, methyl ester
5	52.39	3.99	Stearic acid, methyl ester
Peak	RT	%	Compounds in COE2
1	32.36	0.58	Caryophyllene
2	33.70	0.11	Humulene
3	47.71	27.97	Palmitic acid, methyl ester
4	51.76	42.38	Linoleic acid, methyl ester
5	51.96	0.96	Oleic acid, methyl ester-isomer
6	52.41	11.31	Stearic acid, methyl ester
7	58.34	0.42	Arachidic acid methyl ester
8	66.58	0.35	Docosanoic acid, methyl ester
Peak	RT	%	Compounds in COE3
1	32.36	0.25	Caryophyllene
2	47.70	27.67	Palmitic acid, methyl ester
3	51.74	45.70	Linoleic acid, methyl ester
4	51.86	15.55	Oleic acid, methyl ester
5	51.95	0.87	Oleic acid, methyl ester-isomer
6	52.40	9.95	Stearic acid, methyl ester
Peak	RT	%	Compounds in COE4
1	32.36	0.55	Caryophyllene
2	33.70	0.15	Humulene
3	47.70	27.54	Palmitic acid, methyl ester
4	51.74	44.83	Linoleic acid, methyl ester
5	51.87	15.43	Oleic acid, methyl ester
6	51.95	0.90	Oleic acid, methyl ester-isomer
7	52.40	10.23	Stearic acid, methyl ester
8	58.33	0.25	Arachidic acid methyl ester
9	66.57	0.12	Docosanoic acid, methyl ester
Peak	RT	%	Compounds in VSO
1	47.70	28	Palmitic acid, methyl ester
2	51.71	50.72	Linoleic acid, methyl ester
3	51.83	23	Cis-oleic acid, methyl ester
4	51.92	8	Linolenic acid, methyl ester
5	52.40	12	Stearic acid, methyl ester

Comparative analysis between the VSO and the COE extracts allowed the identification and selection of the bioactive components isolated from clove powder by maceration at different temperatures and extraction periods.

Based on the results presented in Table 7, the clove oily extract prepared by maceration in 100 ml of VSO at room temperature was ineffective for extracting clove bioactive compounds, as its composition was similar to that of VSO. However, quantification of a higher oleic acid content in this extract compared with VSO (23%) indicated that cloves also contain oleic acid (34.69% of cis- and trans-oleic acid) (Table 7). Different amounts of oleic acid, linoleic acid, palmitic acid, and stearic acid were quantified in the various COE extracts, with the highest amount of linoleic acid found in the COE1 extract prepared by maceration at room temperature for 4 weeks (Table 7). Thus, all fatty acids contained in VSO were also identified in the different COE, with the lowest

amounts being stearic acid in COE1 (3.99%) and oleic acid in COE2, COE3, and COE4 (0.96%, 0.87%, and 0.90%, respectively) (Table 7). Two natural terpenes were identified in the extracts labeled COE2, COE3, and COE4: β -caryophyllene (BCP), a highly lipophilic bicyclic sesquiterpene, and humulene, or α -caryophyllene (ACP), both of which contribute to the anti-inflammatory properties of COE (Table 7, Figure 1). β -Caryophyllene (BCP) and humulene, or α -caryophyllene (ACP), have been highlighted in several recent preclinical studies for their ability to enhance chemo-sensitization in chemo-resistant tumors and to efficiently inhibit angiogenesis and the ability of cancer cells to invade and metastasize. Therefore, previous studies have investigated the reasons for the synergistic effect of β -caryophyllene in combination therapy.⁷⁵ β -Caryophyllene has been identified as a promising candidate for cancer treatment due to its antioxidant, immunomodulatory, analgesic, and neuroprotective properties.



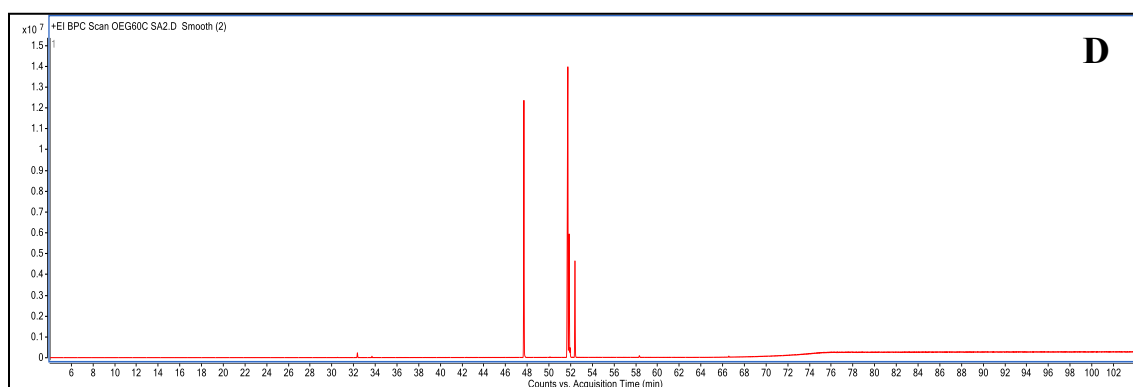


Figure 1. GC-MS Chromatographic Profiles of the Different Clove Oil Extracts: (A) COE1, (B) COE2, (C) COE3, and (D) COE4. The X-axis represents retention/acquisition time (min), and the Y-axis represents signal intensity (counts).

Experimental studies have documented a variety of pharmacological activities associated with BCP, including anti-inflammatory, antioxidant, analgesic, immunomodulatory, cardioprotective, intestinal protective, neuroprotective, and nephroprotective effects.

BCP has shown significant therapeutic potential in treating diabetes, cardiovascular diseases, inflammatory bowel diseases, ischemia/reperfusion injury, inflammatory disorders, and neurodegenerative diseases.⁷⁶ In addition, Dranski et al.⁷⁷ demonstrated that nanoparticles containing α -humulene improved both behavioral outcomes and memory performance in a neuroinflammatory model, suggesting a potential therapeutic role in addressing deficits associated with β -amyloid-induced neuroinflammation. α -Humulene also exhibits anti-inflammatory, antimicrobial, and anticancer properties and, therefore, therapeutic potential.⁷⁸

Both sesquiterpenes were quantified in the oily extracts prepared by maceration in 50 ml of VSO at room temperature (COE2: 0.58% caryophyllene and 0.11% humulene) and at 60 °C for 3 h (COE4: 0.55% caryophyllene and 0.15% humulene), respectively, which demonstrated the efficacy of both extraction methods in isolating these natural compounds from cloves using small volumes of extraction solvent (Table 7). Furthermore, only β -caryophyllene, without humulene, was quantified in the oily extract prepared by maceration in 100 ml of VSO at 60 °C for 3 h (COE3: 0.25%), whereas both molecules were not detected in the extract labeled COE1, indicating the lower efficacy of maceration at room temperature for 4 weeks using 100 ml of VSO in extracting BCP and ACP (Table 7). The major compounds in all COE were unsaturated fatty acids, especially linoleic acid, the major unsaturated fatty acid present in VSO used as the non-polar extraction solvent, followed by oleic acid in both configurations (cis and trans), palmitic acid, and stearic acid (Figure 1). β -Caryophyllene and humulene were identified as the main minor bioactive components that acted synergistically to contribute to the antioxidant, anti-inflammatory,

and antimicrobial effects of COE (Figure 1). Therefore, the COE extracts were characterized as having a fatty acid chemotype, with the main therapeutic molecules being β -caryophyllene and humulene (Figure 1).

While many studies have focused particularly on clove essential oils, our objective as a research team was to prepare a new topical formulation of clove extracts with therapeutic potential. In a recent study conducted by Tuldjanah et al.,⁷⁹ a eugenol chemotype was determined in clove essential oil, with a percentage of 70%, followed by eugenyl acetate (15%), β -caryophyllene (12%), and other components. Furthermore, Pires Costa et al.⁸⁰ demonstrated that clove essential oil contained a high proportion of eugenol (45–90%). Eugenol is an aromatic natural substance known for its characteristic clove odor. The absence of this compound in the COE extracts prepared in this study could be explained by the inability of VSO to serve as a solvent for its isolation.

Polar Fraction Yields and Phytochemical Screening

The extraction yields of COE polar fractions (COEPF) are shown in Figure 2. The results clearly show that COEPF1 and COEPF3 had high contents of polar compounds such as polyphenols, with a yield of 0.68% from 6 g of COE. In contrast, a very low polar fraction content was recorded for VSO (0.23%), corresponding to the vitamin E (tocopherol) content, the main flavonoid in the VSO composition (Figure 1). This difference reflects the richness of COE in phenolic compounds.

It should be emphasized that the method used (i.e., the choice of solvents) as well as the extraction conditions (hot or cold maceration) both affect the total secondary metabolite content and, consequently, the yield of these compounds and the biological activities they exert.⁸¹ To date, no study on COEPF has been conducted; therefore, no comparison with other works could be made. Phytochemical screening revealed the presence of three classes of secondary metabolites:

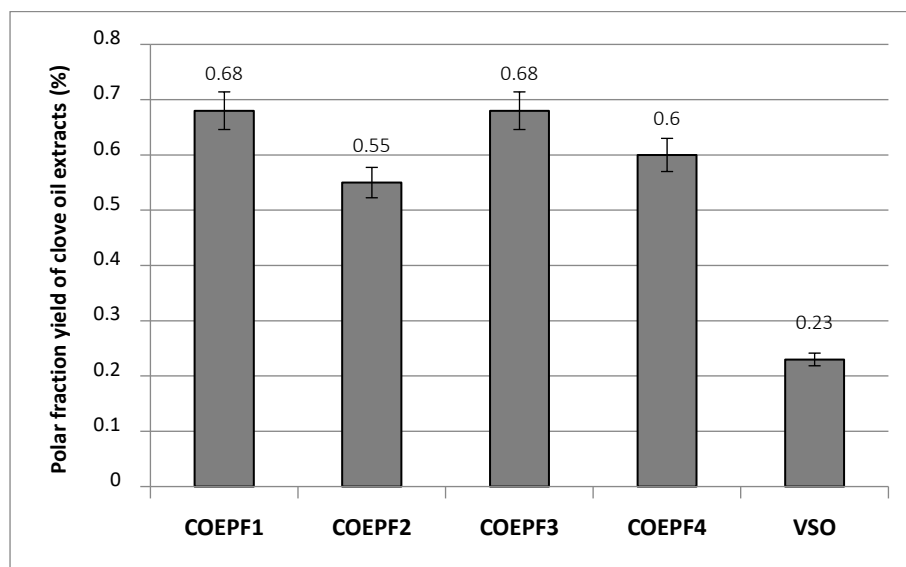


Figure 2. Extraction Yields of Polar Fractions from Clove Oil Extracts (COEPF) and VSO. Data are presented as mean $\pm$ SD ($n = 2$); $p < 0.05$.

polyphenols, flavonoids, and tannins. These bioactive compounds are known to exert diverse biological effects, including antioxidant and anti-inflammatory activities.⁸² The results of the phytochemical screening are summarized in Table 8. The addition of 10% FeCl_3 to the different COEPF samples produced an intense green coloration with a black-green precipitate, indicating the presence of phenols (Table 8).⁸³ For flavonoid detection, the addition of 2% AlCl_3 produced an intense yellowish coloration, reflecting the interaction between aluminum trichloride and flavonoids.⁸⁴ Furthermore, the appearance of a dark green color after the addition of a few drops of FeCl_3 to the different COEPF samples indicated the presence of catechic or condensed tannins (Table 8).⁸⁵

Table 8. Qualitative Phytochemical Screening of Polar Fractions from Clove Oil Extracts (COEPF1–COEPF4) and Vegetable Soybean Oil (VSO). Symbols indicate the intensity of the colorimetric reactions: + weak, ++ moderate, +++ strong, and – absent.

Extraction	Phenols	Flavonoids	Tannins
COEPF1	+++	++	+++
COEPF2	+++	++	+++
COEPF3	+++	++	+++
COEPF4	+++	++	+++
VSO	+	+	-

In comparison with our results, Shehu et al.⁸⁶ demonstrated the presence of phenols and tannins in both aqueous and ethanolic clove extracts, whereas flavonoids were absent only from the aqueous extract.

Polyphenol Quantification in COE Polar Fractions

Phenolic compounds, particularly total polyphenols, flavonoids, and tannins, are considered bioactive secondary metabolites with antiradical capacity. These compounds

have been the subject of numerous recent studies in the field of antioxidant and anti-inflammatory therapeutics.^{87–89} In this context, the COEPF were quantitatively characterized by spectrophotometry to determine total polyphenol, flavonoid, and catechol tannin contents. The results are presented in Figure 3.

According to the results obtained, the extract labeled COEPF4 exhibited the highest TPC (164.61 ± 39.66 mg GAE/g dry extract (DE)), followed by COEPF2, COEPF1, and COEPF3 (108.8 ± 6.89 , 98.06 ± 14.02 , and 39.41 ± 3.20 mg GAE/g DE, respectively), whereas VSO showed the lowest content of these compounds (6.78 ± 1.33 mg GAE/g DE) (Figure 3). Regarding TFC, all polar fractions exhibited higher flavonoid contents, with values exceeding 100 mg QE/g DE, the most representative being COEPF2 (216 ± 37 mg QE/g DE) (Figure 3). The VSO used in this study was rich in tocopherols, the main flavonoid in nutritional oil composition, with a determined value of 104.84 ± 1.85 mg QE/g DE.

Our results further confirm that the extraction solvent volume significantly influenced polyphenol yield, as the highest contents were recorded in the extracts labeled COEPF2 and COEPF4 (Figure 3). In a study conducted by Haouaia and Hamdouche,⁹⁰ aqueous clove extracts obtained by different techniques (maceration, infusion, and decoction) exhibited polyphenol contents of 3.22, 2.84, and 2.94 mg GAE/mg DE, respectively. Similarly, El-Emary et al.⁹¹ reported contents of 83.966 mg/g DE for flavonoids and 222.56 mg/g DE for total phenols in clove methanolic extract, whereas the chloroform extract showed 43.882 mg/g DE for TFC and 126.8 mg/g DE for TPC. Furthermore, our results were superior to those of Adegbola et al.,⁹² who determined a TTC of approximately 1.93 ± 0.03 mg/g DE in clove extracts.

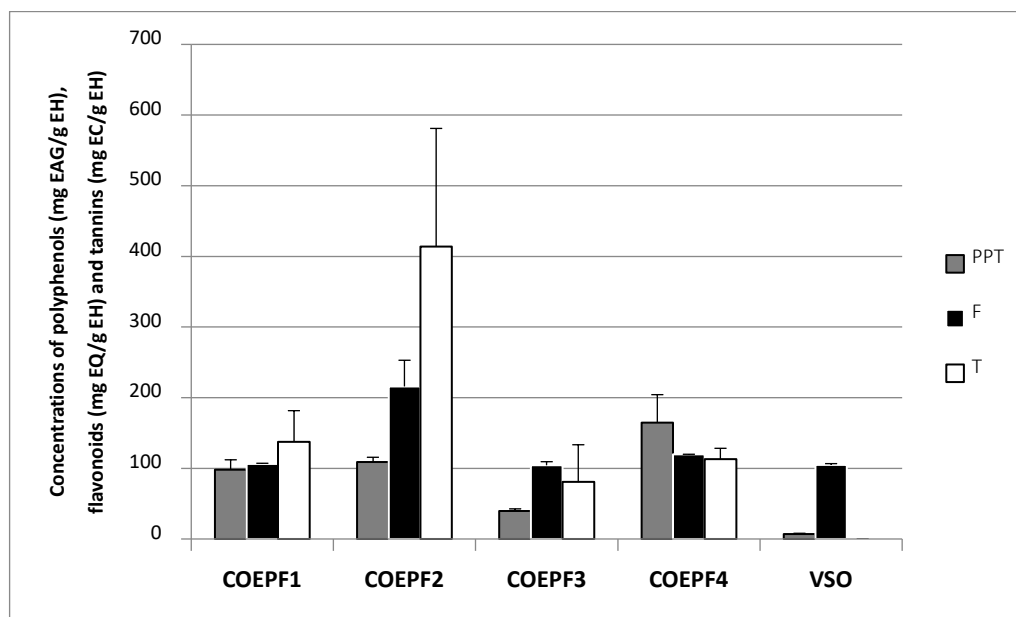


Figure 3. Total Phenolic (TPC), Total Flavonoid (TFC), and Total Tannin (TTC) Contents of Polar Fractions from Clove Oil Extracts (COEPF1–COEPF4) and Vegetable Soybean Oil (VSO). TPC is expressed as mg gallic acid equivalents per gram of dry extract (mg GAE/g DE), TFC as mg quercetin equivalents per gram of dry extract (mg QE/g DE), and TTC as mg catechin equivalents per gram of dry extract (mg CE/g DE). Values are presented as mean $\pm$ SD ($n = 3$); $p < 0.05$.

Antioxidant Activities of Clove Oil Extracts

In this study, four methods were used to evaluate the *in vitro* antioxidant activity: the DPPH free radical scavenging assay, total antioxidant capacity (TAC), ferric reducing antioxidant power (FRAP), and the β -carotene bleaching test coupled with linoleic acid autoxidation.

DPPH Free Radical Scavenging Assay

The results of DPPH free radical inhibition percentages

(antiradical activity) induced by the different COE samples, as well as the IC_{100} values (mg/ml), are summarized in Tables 9 and 10. Lower IC_{100} values indicated more effective antioxidant activity of the COE samples. In this study, the concentrations achieving 100% DPPH free radical inhibition were determined, demonstrating the potent antioxidant power of the COE samples even at low doses, with free radical inhibition percentages exceeding 50% (Table 9).

Table 9. Antiradical Activity of Clove Oil Extracts (COE1–COE4), Vegetable Soybean Oil (VSO), and Ascorbic Acid, Expressed as DPPH Free Radical Inhibition Percentages (%) at Different Concentrations and IC_{100} Values (mg/ml). Values are presented as mean $\pm$ SD ($n = 3$); $p < 0.05$

Extract samples	Concentrations (mg/ml)					
	0.25	0.5	1	2	4	IC_{100}
COE1	18.89 $\pm$ 0.08	65.65 $\pm$ 0.246	65.84 $\pm$ 0.112	62.70 $\pm$ 0.497	82.30 $\pm$ 0.214	6.31
COE2	27.83 $\pm$ 0.587	51.14 $\pm$ 0.1	74.64 $\pm$ 0.455	75.02 $\pm$ 0.017	78.64 $\pm$ 0.452	6.07
COE3	40.63 $\pm$ 0.263	67.60 $\pm$ 0.281	79.69 $\pm$ 0.222	83.16 $\pm$ 0.112	85.01 $\pm$ 0.01	5.76
COE4	76.07 $\pm$ 0.05	76.40 $\pm$ 0.285	84.68 $\pm$ 0.225	87.30 $\pm$ 0.211	97.67 $\pm$ 0.234	5.88
VSO	23.83 $\pm$ 0.117	36.06 $\pm$ 0.043	35.39 $\pm$ 0.279	35.16 $\pm$ 0.111	35.49 $\pm$ 0.346	7.25
Ascorbic acid	86.68 $\pm$ 0.227	88.77 $\pm$ 0.161	90.2 $\pm$ 0.141	91.48 $\pm$ 0.342	96.29 $\pm$ 0.205	10.51

For VSO samples, DPPH free radical inhibition did not exceed 35.49%, even at the highest dose, reaching 35.49 $\pm$ 0.346% at 4 mg/ml; therefore, its efficacy was lower than that of the different COE samples (Table 9). The COE samples exhibited notable antiradical activity, with inhibition percentages reaching 82.30 $\pm$ 0.214% for COE1, 78.64 $\pm$ 0.452% for COE2, 85.01 $\pm$ 0.01% for COE3, and 97.67 $\pm$ 0.234% for COE4, compared with 96.29 $\pm$ 0.205% for ascorbic acid at 4 mg/ml (Table 9). VSO samples showed the lowest antioxidant activity against DPPH free radicals. Among the COE samples, COE4 exerted a notable

effect on DPPH free radicals at low concentration, reaching 76.07 $\pm$ 0.05% at 0.25 mg/ml (Table 9).

Thus, the COE prepared by maceration at 60 °C for 3 h exhibited the highest antioxidant activity, with potent antiradical activity and the lowest IC_{100} values compared with the standard antioxidant used (COE3: 5.761 mg/ml; COE4: 5.880 mg/ml; ascorbic acid: 7.250 mg/ml) (Table 9). According to these results, all COE extracts were considered effective antioxidants, exerting more pronounced antiradical activity against DPPH free radicals than the standard used (ascorbic acid), for which the IC_{100} value was higher than

those of the COE samples ($IC_{100} = 7.250$ mg/ml) (Table 9).

Many recent studies have determined the effectiveness of clove extracts as antioxidants.^{93,94} Hung⁹⁵ reported that eugenol, the major compound in clove extracts and oils, had antioxidant capacity and DPPH free radical scavenging activity. In contrast, in this study, we identified two compounds known for their antioxidant and anti-inflammatory effects, caryophyllene and humulene. The antioxidant effect was therefore attributed to the presence of these bioactive substances in COE, which act through synergistic effects with the other identified components.

In their studies, Santos et al.⁹⁶ demonstrated that clove extracts obtained by supercritical fluid and pressurized liquid extraction exerted notable antioxidant activity with high contents of phenolic compounds. Our results were superior to those of Al-Zereini et al.,⁹⁷ who determined an antioxidant effect of clove essential oil with an IC_{50} value of 1.6 ± 0.1 μ g/ml. Subsequently, Kiki⁹⁸ determined that the antioxidant potential of clove essential oil was maximal, with a DPPH free radical inhibition percentage of 98.6% at a concentration of 800 μ g/ml. Thus, based on the results of our study, we confirmed that the COE obtained by maceration at room temperature and at 60 °C were more effective and qualified as extracts with good antioxidant effects expressed at low doses, given their lower IC_{100} values.

Baghshahi et al.⁹⁹ demonstrated that clove ethanolic extract at concentrations of 0.1, 0.5, 1, 5, and 12.5 μ g/ml exerted DPPH free radical inhibition with percentages of 5.9%, 13.1%, 25.2%, 72.5%, and 97.9%, respectively. Furthermore, they demonstrated that the antioxidant effect was greater than that induced by vitamin E. Our results

were superior and noteworthy because we recorded higher DPPH free radical inhibition percentages at very low COE doses, which demonstrated the richness of the extracts in antioxidant molecules compared with other clove extracts or oils obtained by other extraction techniques.

Total Antioxidant Capacity Assay

The total antioxidant capacity (TAC) was evaluated using the ascorbic acid method, with ascorbic acid employed as the standard at concentrations ranging from 0.25 to 4 mg/ml. The results are presented in Figure 4. The results indicated that each COE exhibited antioxidant capacity, qualifying it as a potent antioxidant. The extraction technique and solvent volume influenced this antioxidant effect. The extract labeled COE1 exhibited the highest antioxidant effect, with a total antioxidant compound concentration of approximately 109.05 ± 0.035 mg AAE/g COE (Figure 4). The total antioxidant capacities of the other COE extracts were approximately 95.02 ± 0.016 mg AAE/g COE for COE2, followed by COE3 (92.61 ± 0.276 mg AAE/g COE), VSO (76.62 ± 0.268 mg AAE/g), and COE4 (63.21 ± 0.14 mg AAE/g COE) (Figure 4). Ivanović et al.¹⁰⁰ demonstrated that clove extracts obtained by supercritical carbon dioxide extraction exhibited antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl free radicals and peroxide formation. In addition, Valarezo et al.¹⁰¹ demonstrated the effectiveness of clove essential oil as an antioxidant and highlighted its potential as a natural alternative for preserving meat products, thereby responding to consumer demand for safer foods free of synthetic preservatives.

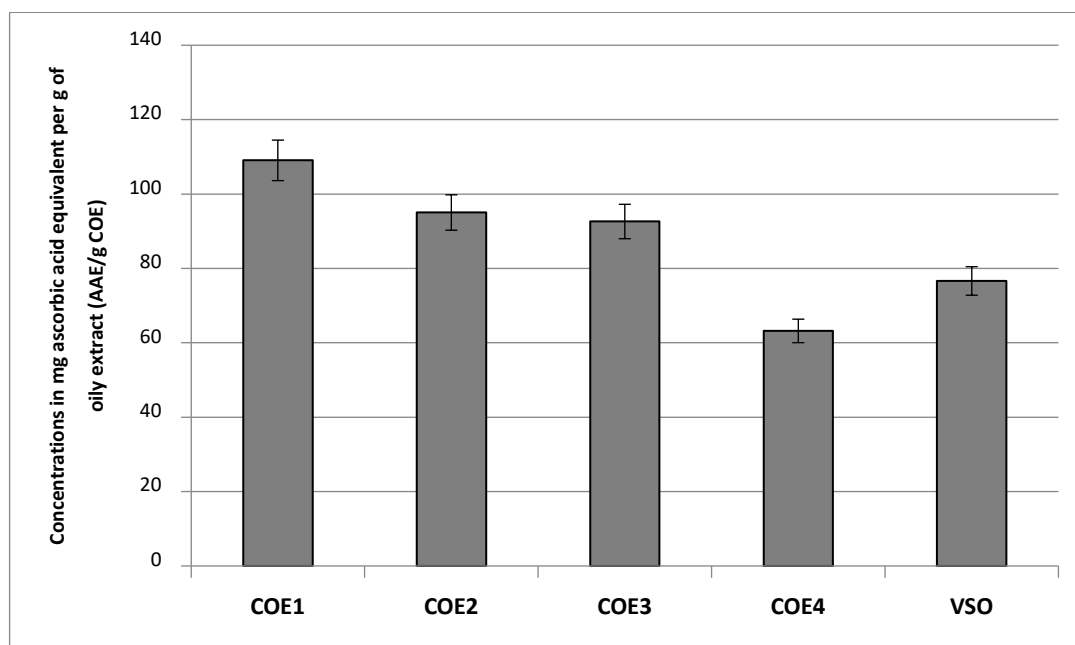


Figure 4. Total Antioxidant Capacity (TAC) of Clove Oil Extracts (COE1–COE4) and Vegetable Soybean Oil (VSO), Expressed as mg Ascorbic Acid Equivalent per gram of Oily Extract (mg AAE/g extract). Values are presented as mean $\pm$ SD ($n = 3$); $p < 0.05$.

Ferric Reducing Antioxidant Power Assay

The antioxidant activity of COE was also evaluated using the FRAP method. The results are presented in Figure 5. Higher optical density values indicated a more pronounced ferric reducing antioxidant power exerted by the COE. The results showed that all COE samples exhibited lower ferric reducing power than the standard used, ascorbic acid (Figure 5). The reducing power of the extract labeled COE2 was the highest among the extracts (0.88 ± 0.06), and FRAP was proportional to the increase in extract concentration (Figure 5). All COE exhibited greater ferric reducing power at a concentration of 4 mg/ml than VSO used as the extraction solvent. The ferric reducing antioxidant power of VSO, used as the non-polar extraction solvent, was much weaker than that of the COE. This iron-reducing power induced by VSO was due to the vitamin E contained in the oil ingredients, which acts as a preservative agent

preventing VSO oxidation. Thus, after maceration of clove powder in VSO, the latter becomes enriched with clove active ingredients, which enhances its antioxidant capacity, as evidenced by the FRAP assay results. The optical density values obtained were 0.17 ± 0.05 at the highest VSO concentration (4 mg/ml), followed by 0.653 ± 0.037 , 0.709 ± 0.006 , 0.784 ± 0.059 , and 0.885 ± 0.06 for COE3, COE1, COE4, and COE2, respectively (Figure 5).

The ascorbic acid as standard was the most potent antioxidant compound, as indicated by the highest optical density values obtained, and exhibited the highest ferric reducing antioxidant power (Figure 5).

Our results are in agreement with those of Gülçin et al.,¹⁰² who reported that clove oil possessed more potent antioxidant activity in DPPH free radical scavenging, hydrogen peroxide scavenging, and chelation of ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron ions.

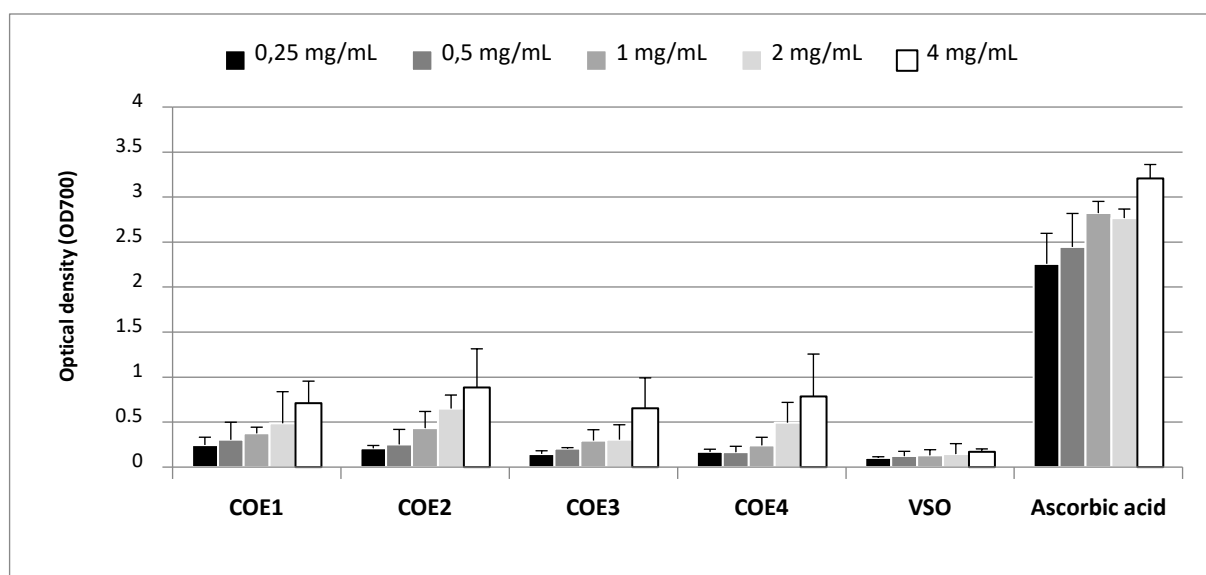


Figure 5. Ferric Reducing Antioxidant power (FRAP) of Clove Oil Extracts (COE1–COE4), Vegetable Soybean Oil (VSO), and Ascorbic Acid at Different Concentrations (0.25–4 mg/ml), Expressed as Optical Density (OD) Values at 700 nm. Data are presented as mean $\pm$ SD ($n = 3$); $p < 0.05$.

β -Carotene Bleaching Test Coupled with Linoleic Acid Autoxidation

The antioxidant activity of COE extracts was also evaluated using the β -carotene bleaching test coupled with linoleic acid autoxidation. Linoleic acid autoxidation releases peroxide radicals that attack the β -carotene structure by binding to its double bonds, resulting in the disappearance of the initial color of the β -carotene solution. The results of the β -carotene bleaching inhibition percentages are presented in Figure 6, and the IC_{50} and IC_{100} values of the different COE, expressed as peroxide radical reduction, are also reported. The β -carotene bleaching inhibition percentages were proportional to COE concentrations. All COE extracts inhibited β -carotene bleaching, with inhibition percentages exceeding 100% (Figure 6). At a concentration of 4 mg/ml,

all COE extracts exerted a potent antioxidant effect by inhibiting the radicals released during linoleic acid autoxidation, thereby limiting β -carotene bleaching, with inhibition percentages of approximately $136.255 \pm 0.18\%$, $134.305 \pm 0.22\%$, $123.325 \pm 0.23\%$, and $120.66 \pm 0.47\%$ for the extracts labeled COE3, COE1, COE2, and COE4, respectively (Figure 6). At the same concentration, ascorbic acid showed an inhibition percentage not exceeding 80% ($72.475 \pm 0.34\%$), indicating that the antioxidant potential of COE in this assay was more effective than that of ascorbic acid (Figure 6).

The β -carotene bleaching inhibition percentages were higher at the lowest dose (0.25 mg/ml), reaching $130.515 \pm 0.36\%$ for COE3, $109.025 \pm 0.018\%$ for COE2, and $100.11 \pm 0.078\%$ and $95.565 \pm 0.4\%$ for COE4 and COE1, respectively,

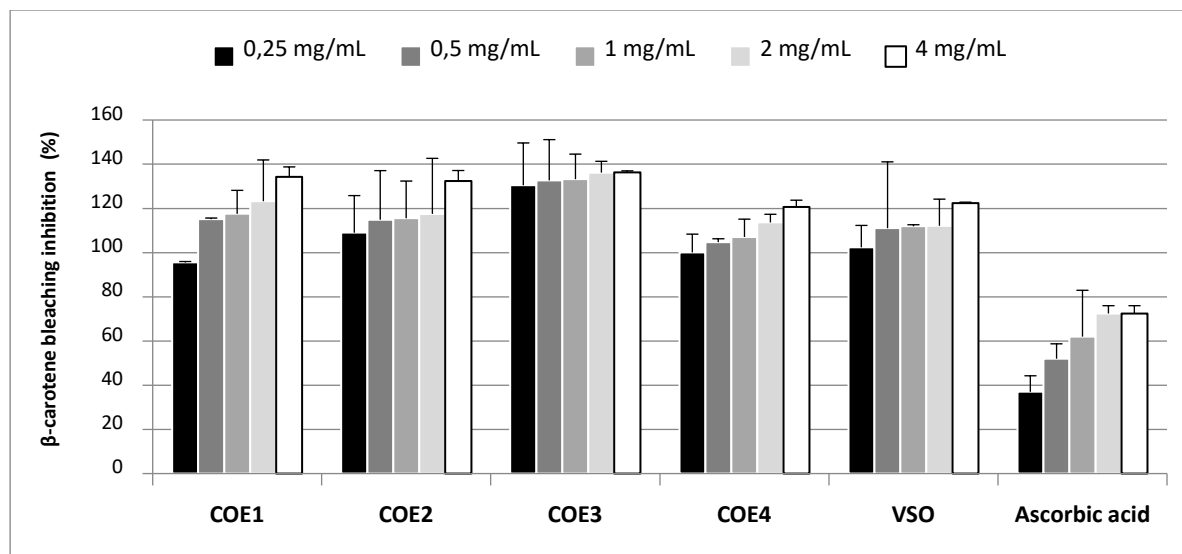


Figure 6. β -Carotene Bleaching Inhibition (%) of Clove Oil Extracts (COE1–COE4), Vegetable Soybean Oil (VSO), and Ascorbic Acid at Different Concentrations (0.25–4 mg/ml), Evaluated by the β -carotene Bleaching Test Coupled with Linoleic Acid Autoxidation. Values are presented as mean $\pm$ SD ($n = 3$); $p < 0.05$.

whereas ascorbic acid gave an inhibition percentage of approximately 37% (Figure 6). VSO, used as the non-polar extraction solvent, also exhibited ferric reducing power, attributed to the oil's richness in vitamin E. All COE extracts were characterized as effective antioxidants, exerting a good inhibitory effect on peroxide radicals, with IC_{100} values lower than that of the standard, reaching 0.995 mg/ml and 1.151 mg/ml for COE1 and COE4, respectively. An IC_{50} value of 2 mg/ml was determined for ascorbic acid. Our results are consistent with those of Abo El-Maati et al.,¹⁰³ who reported that clove phenolic extracts exerted a notable effect on β -carotene bleaching inhibition. In a study, Lee and Shibamoto¹⁰⁴ identified the major components in clove extracts by chromatographic analysis (GC-MS), namely eugenol (24.3 mg/g) and eugenyl acetate (2.35 mg/g), and demonstrated that the antioxidant potential of clove extracts and their major compounds (eugenol and eugenyl acetate) was comparable to that of α -tocopherol.

Antimicrobial Activity of Clove Oil Extracts

Agar Disc Diffusion Method

The results, presented in the figures below, are expressed as the diameter of the microbial growth inhibition zone in millimeters against Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*), Gram-positive bacteria (*Streptococcus* sp., *S. aureus*), and a fungus (*C. albicans*). The presence of these inhibition zones indicates the presence of antimicrobial substances within the extracts. Microbial sensitivity was classified based on the inhibition zone diameter: resistant (< 8 mm), moderately sensitive (8–14 mm), sensitive (14–20 mm), and extremely sensitive (> 20 mm). Based on the results obtained, the COE1 extract exhibited low antimicrobial activity. The pure, undiluted

COE1 extract produced inhibition zones with diameters ranging from 8 to 10 mm against most strains, with the notable exception of *S. aureus* 1, which showed no sensitivity and was therefore considered highly resistant. At a concentration of 1000 mg/ml, COE1 affected only *E. coli*, with an inhibition diameter of 7 mm, *S. aureus* 3, with an inhibition diameter of 8 mm, and *C. albicans*, with an inhibition diameter of 8 mm (Figure 7).

In contrast, the COE2 extract demonstrated a broader antimicrobial effect, inhibiting the growth of all tested strains except *S. aureus* 3. Specifically, the pure, undiluted COE2 extract induced inhibition zones of 8 mm against *K. pneumoniae*, 8 mm against *P. aeruginosa*, 11 mm against *P. mirabilis*, and 7 mm against *S. aureus* 2, classifying these strains as moderately sensitive to the extract (Figure 8).

The undiluted COE3 extract showed moderate antimicrobial activity, producing inhibition zones of 7 mm against *E. coli*, 8 mm against *K. pneumoniae*, 10 mm against *P. mirabilis*, 7 mm against *S. aureus* 1, and 8 mm against *S. aureus* 3 and *C. albicans*. However, it had no effect on *P. aeruginosa*, *Streptococcus* sp., or *S. aureus* 2 (Figure 9). In contrast, the pure, undiluted COE4 extract demonstrated a broader but weak effect against all tested strains. It produced small inhibition zones of 7 to 8 mm for most strains, with a slightly larger zone of 11 mm observed for *P. mirabilis* (Figure 10). At a concentration of 1000 mg/ml, COE4 exhibited low activity against *E. coli*, *P. aeruginosa*, *P. mirabilis*, *Streptococcus* sp., and *S. aureus* 3, with inhibition zones of 7 to 9 mm. A concentration of 500 mg/ml also showed a minimal effect on *E. coli* and *P. aeruginosa*, with an inhibition zone of approximately 8 mm. Notably, *S. aureus* and *C. albicans* were found to be resistant to COE4 (Figure 10).

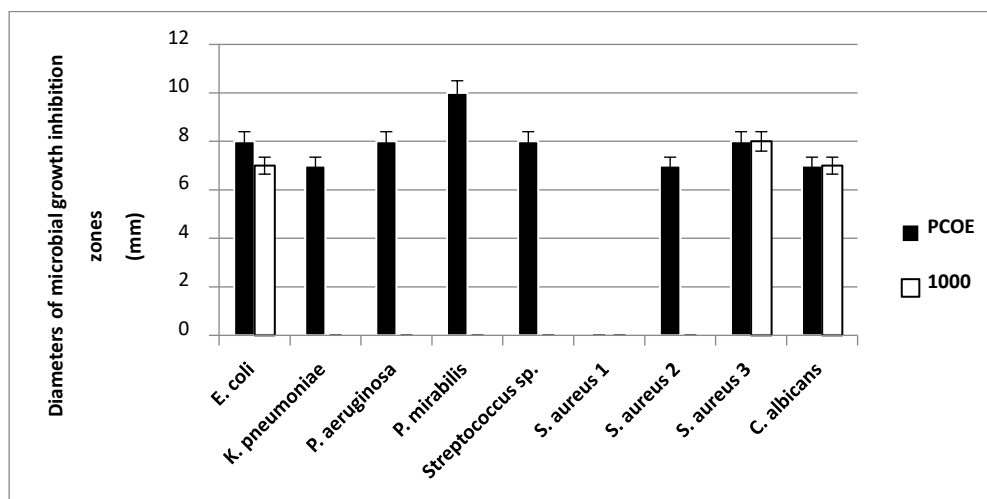


Figure 7. Antimicrobial Activity of COE1 Against Selected Microbial Strains, Evaluated by the Agar Disc Diffusion Method. Inhibition zone diameters (mm) are shown for the pure extract (POCE) and at 1000 mg/ml. Values are mean $\pm$ SD ($n = 3$). Microbial sensitivity was classified as resistant (< 8 mm), moderately sensitive (8–14 mm), sensitive (14–20 mm), and extremely sensitive (> 20 mm).

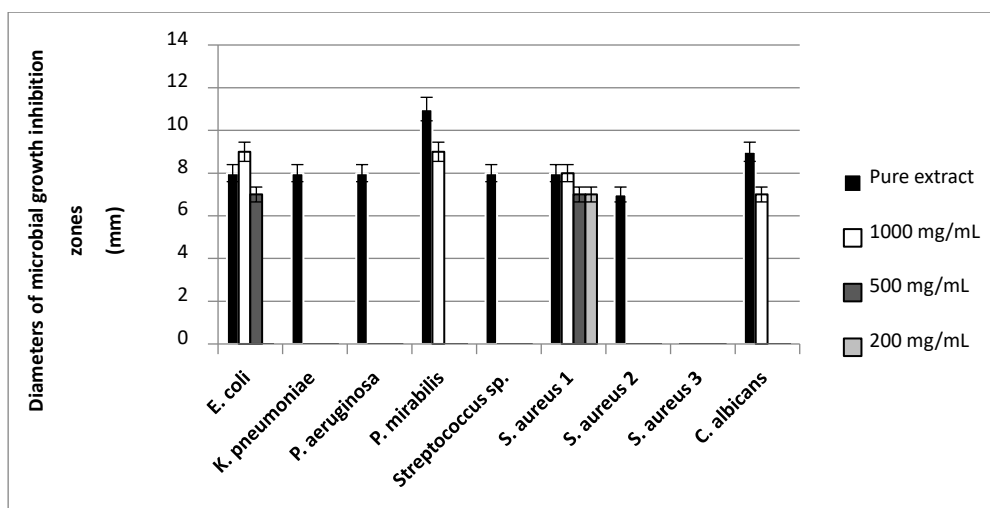


Figure 8. Antimicrobial Activity of COE2 Against Selected Microbial Strains, Evaluated by the Agar Disc Diffusion Method. Inhibition zone diameters (mm) are shown for the pure extract and at 1000, 500, and 200 mg/ml. Values are mean $\pm$ SD ($n = 3$).

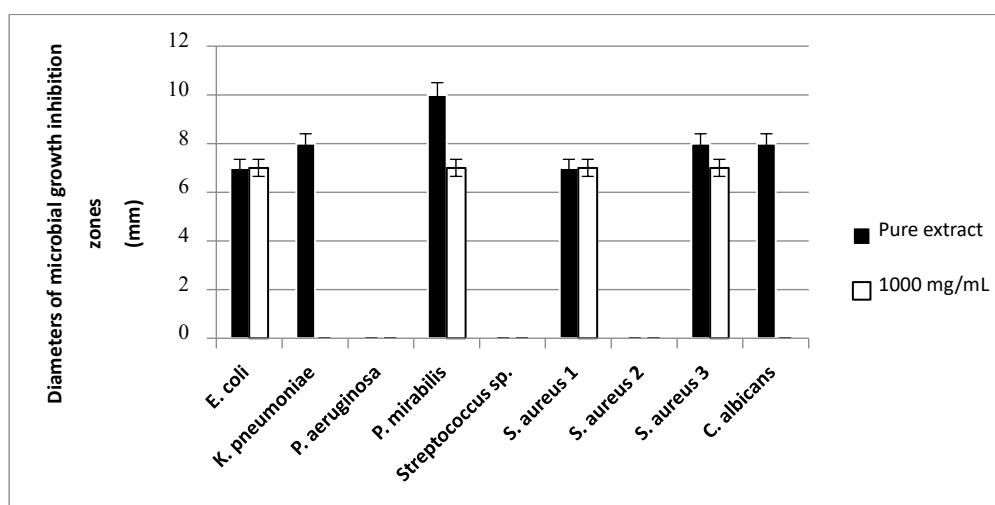


Figure 9. Antimicrobial Activity of COE3 Against Selected Microbial Strains, Evaluated by the Agar Disc Diffusion Method. Inhibition zone diameters (mm) are shown for the pure extract and at 1000 mg/ml. Values are mean $\pm$ SD ($n = 3$).

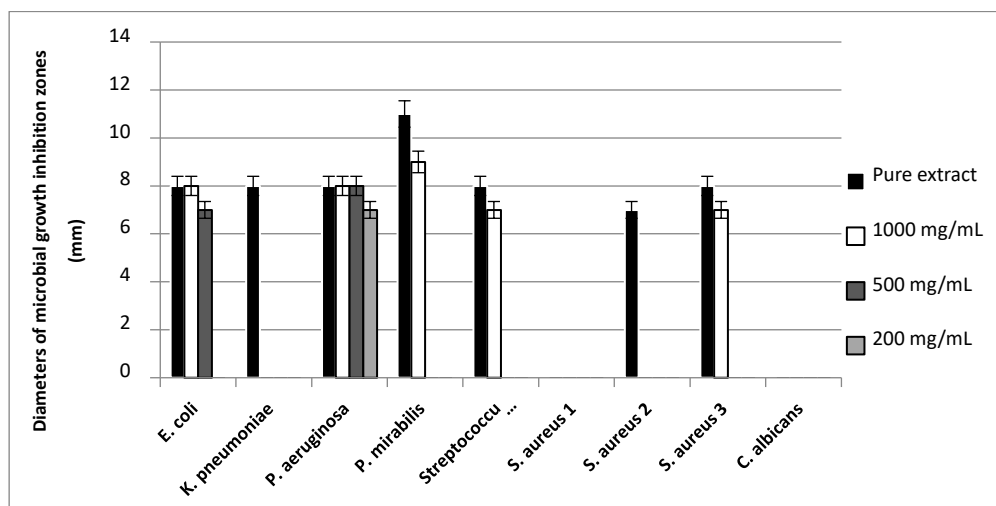


Figure 10. Antimicrobial Activity of COE4 Against Selected Microbial Strains, Evaluated by the Agar Disc Diffusion Method. Inhibition zone diameters (mm) are shown for the pure extract and at 1000, 500, and 200 mg/mL. Values are mean $\pm$ SD (n = 3).

Overall, the observed inhibition zones, ranging from 8 to 11 mm, indicate that the oily extracts of *S. aromaticum* exhibited modest antibacterial and antifungal activity, with the tested microorganisms showing only low sensitivity.

The variations in inhibition zone diameters observed in our study were consistent with the findings of Assam et al.¹⁰⁵ and Oshomoh et al.,¹⁰⁶ who suggested that differences in bioactive molecule composition or their mechanism of action are likely causes. The antimicrobial activity of the clove oil extracts may be attributed to polyphenolic compounds, as noted by Funatogawa et al.¹⁰⁷ and Buzzini et al.¹⁰⁸

However, our results showed a significantly weaker effect compared with previous research. For example, a study by Boukhatem¹⁰⁹ demonstrated that clove essential oil produced inhibition zones of 16 mm and 14 mm against *E. coli* and *P. aeruginosa*, respectively, which was a much stronger effect than that observed in our study. Similarly, Yassin et al.¹¹⁰ found that clove essential oil had a powerful antifungal effect on *C. albicans*, with inhibition zones ranging from 22 to 40 mm. In contrast, our extracts produced only a maximum inhibition zone of 9 mm against this same fungal strain, indicating a very weak effect.

In a recent study conducted by Wadi¹¹¹ on the antibacterial activity of clove aqueous extract, it was found that the extract exhibited a broad-spectrum antimicrobial effect against ten common bacteria, including both Gram-

positive and Gram-negative types. Specifically, *E. coli* (33 ± 1 mm) and *K. pneumoniae* (32 ± 1 mm) were the most sensitive to the extract. The study also highlighted that the extract's antibacterial properties were largely due to the presence of eugenol, which was found to be the most abundant compound. Other studies have also shown that while aqueous extracts of clove are effective, they may be less potent compared with other types of extracts, such as those prepared with oil.¹¹¹

Determination of MIC Values

Based on the microbial growth curves and the corresponding minimum inhibitory concentration (MIC) values listed in Table 10, the lowest concentration of clove oil extracts required to inhibit the growth of each specific microorganism was determined. The results showed that each microbial strain had a unique MIC value, indicating that their sensitivity to the clove oil extracts varied. Several microbial strains exhibited very low MIC values when treated with the clove oil extracts, including *E. coli* (0.488 mg/ml), *K. pneumoniae* (0.488 mg/ml), *P. aeruginosa* (0.488 mg/ml), *P. mirabilis* (0.488 mg/ml), *S. aureus* 1 (1.953 mg/ml), *S. aureus* 2 (1.953 mg/ml), and *C. albicans* (0.488 mg/ml) (Table 10). These low MIC values are a strong indicator of the potent antimicrobial activity of the extracts, meaning that only a small amount is required to inhibit the growth of these microorganisms.

Table 10. Minimum Inhibitory Concentrations in mg/ml of Oil Extracts of Cloves for the Different Microbial Strains Tested

		MIC values (mg/ml)							
		S1	S2	S3	S4	S5	S6	S7	S8
COE	COE2	0.488	0.488	0.488	0.488	31.25	1.953	1.953	0.488
	COE4	1000	62.5	31.25	1000	500	1000	1000	1000

S1: *E. coli*, S2: *K. pneumoniae*, S3: *P. aeruginosa*, S4: *P. mirabilis*, S5: *Streptococcus* sp, S6: *S. aureus* 1, S7: *S. aureus* 2, S8: *C. albicans*.

However, the COE4 extract required much higher concentrations, ranging from 500 to 1000 mg/ml, to inhibit

the growth of several strains. These high MIC values suggested that COE4 had a much weaker antimicrobial

effect, and in some cases, the high values indicated that the microorganisms were resistant to it. Therefore, it can be concluded that the COE2 extract possessed more powerful antimicrobial activity than COE4 (Table 10).

Based on our results and comparison with the control (microbial strains without oil extract), it can be concluded that COE exerted a microbiostatic effect on the different microbial strains tested (bacteria and the fungal strain *C. albicans*), causing a slowdown in microbial growth. The exception was the oily extract labeled COE2, for which a bactericidal effect was recorded, with a significant decrease in the bacterial cell concentration of *K. pneumoniae* induced by bacterial cell destruction (Table 10).

Mostafa et al.¹¹² demonstrated that clove ethanolic extract exerted inhibitory effects against several bacteria, with a MIC value of 10 mg/ml. They also determined that the extract's effectiveness against *B. cereus*, *S. aureus*, and *E. coli* was greater than that exerted by gentamicin. Notably, they found that the clove extract's inhibition zone against *P. aeruginosa* (13.4 ± 0.11 mm) was very similar to that of gentamicin (13.1 ± 0.35 mm).

Moreover, Al-Tawalbeh et al.¹¹³ found that clove extract showed a strong antimicrobial effect against *P. aeruginosa* comparable to the antibiotic imipenem, and when combined with antibiotics, a significant reduction in their respective MICs was observed. The effectiveness of the clove extract is believed to result from a synergistic effect among its various bioactive compounds.

A recent study showed that clove extract has antifungal properties against *Candida* species, specifically *C. albicans*, *C. glabrata*, and *C. tropicalis*. The researchers tested four different solvents, methanol, ethyl acetate, n-hexane, and diethyl ether, to extract the active compounds from cloves. All four extracts demonstrated antifungal activity; however, the ethyl acetate extract was the most effective. The primary antifungal agents found to be active against *C. albicans* were eugenol and eugenol acetate.¹¹⁴

Eugenol demonstrated potent activity against *C. albicans* keratitis, an inflammation of the cornea, in a rabbit model. The MIC of eugenol against the fungus was determined to be 2 mg/ml, which was less potent than fluconazole, which had an MIC greater than 0.4 mg/ml. Despite this, a significant 75% of the rabbits treated with eugenol successfully recovered from the infection, highlighting its therapeutic potential. The study used a maximum non-toxic dose of 4 mg/ml for treatment.¹¹⁵

The antimicrobial effect of the COE was notably weaker than that reported for clove essential oils (CEO) (e.g., *C. albicans* inhibition zones of only 9 mm compared with 22–40 mm in the literature). This discrepancy can be critically analyzed through two lenses. First, regarding eugenol concentration: while clove buds typically contain up to 85–90% eugenol in their EO, the non-polar maceration in

vegetable oil used in this study may not have efficiently captured this major antimicrobial component. GC-MS analysis of the COE primarily identified caryophyllene and humulene, whereas eugenol is often the primary driver of the high antimicrobial activity (e.g., inhibition zones of 28 mm) reported in other studies. Second, regarding extraction solvent polarity: the use of soybean oil as a non-polar solvent may have excluded polar antimicrobial compounds, such as certain phenolic acids, that are more effectively extracted with ethanol or hydrodistillation. Thus, regarding the microbiostatic nature, the study found that the oily extracts primarily exerted bacteriostatic and fungistatic effects rather than bactericidal ones (with the exception of *K. pneumoniae*). This suggests that the current COE formulation serves better as a preventive or supportive agent rather than a primary antimicrobial treatment.

In Vitro Anti-inflammatory Activity Assay

The *in vitro* anti-inflammatory activity of COE was evaluated using two different methods: inhibition of bovine albumin denaturation and antihemolytic activity. The results are presented in the Figures 11 and 12. All COE exhibited notable anti-inflammatory potency in inhibiting bovine albumin denaturation, with inhibition percentages exceeding 80% at high doses. The highest inhibition percentages were $87.87 \pm 0.62\%$, $86.75 \pm 0.54\%$, $82.46 \pm 0.33\%$, and $78.06 \pm 0.044\%$ for COE2, COE1, COE3, and COE4, respectively, at a concentration of 4 mg/ml (Figure 11). For the standard (1% sodium diclofenac), the denaturation inhibition percentages were approximately $31 \pm 0.67\%$ at 0.25 mg/ml, $62 \pm 0.46\%$ at 0.5 mg/ml, $72 \pm 0.19\%$ at 1 mg/ml, and $82 \pm 0.69\%$ at 2 mg/ml, reaching a maximum of $85.37 \pm 0.26\%$ at 4 mg/ml (Figure 11). Thus, COE1 ($87 \pm 0.54\%$) and COE2 ($88 \pm 0.62\%$) exhibited higher inhibition percentages of bovine albumin denaturation than diclofenac ($85 \pm 0.26\%$) at the same concentration (4 mg/ml) (Figure 11). These results indicated that these extracts were more effective as anti-inflammatory agents at 4 mg/ml than the standard product used. Ozuah¹¹⁶ demonstrated the anti-inflammatory effect of clove phenolic extracts, and Bennabi et al.,¹¹⁷ in their study, proved the effectiveness of clove oil in inhibiting BSA protein denaturation and highlighted the use of a clove oil topical formulation as a novel and effective alternative to conventional treatments. The results of the antihemolytic activity and erythrocyte membrane stabilization indicating the hemolysis percentages (%) and the red blood cells (RBC) protection percentages are mentioned in Figures 12 and 13. The results showed that all tested concentrations of the different COE extracts exerted antihemolytic activity, with protection against red blood cell hemolysis exceeding 60%. Diclofenac sodium (1%) exhibited the strongest antihemolytic effect, which increased with dose, reaching $84.46 \pm 0.38\%$ at 4 mg/ml

(Figure 12). In contrast, the COE samples exerted a protective effect against red blood cell hemolysis, with hemolysis inhibition percentages of $51.36 \pm 0.25\%$, $50.15 \pm 0.1\%$, $48.8 \pm 0.56\%$, and $48.76 \pm 0.54\%$ for COE1, COE3, COE4, and COE2, respectively (Figure 13). COE1, COE3,

and COE4 exerted an antihemolytic effect superior to that of diclofenac ($40.78 \pm 0.16\%$), with hemolysis inhibition percentages reaching $45.17 \pm 0.12\%$, $47.47 \pm 0.33\%$, and $43.66 \pm 0.46\%$, respectively, at a dose of 0.25 mg/ml (Figure 13).

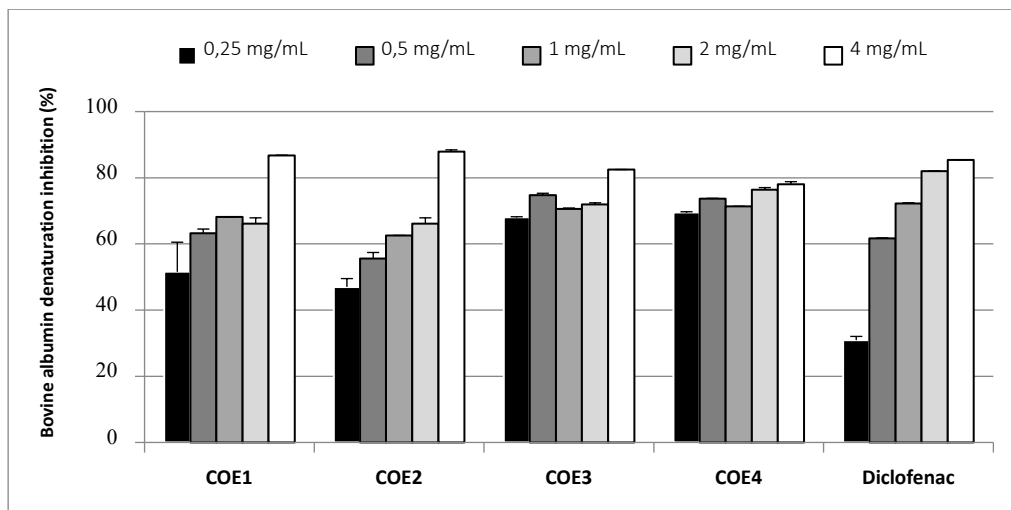


Figure 11. Inhibition of Bovine Serum Albumin (BSA) Thermal Denaturation by Clove Oil Extracts (COE1–COE4) and Diclofenac Sodium (standard) at Different Concentrations (0.25–4 mg/ml). Data are expressed as mean $\pm$ SD ($n = 3$); $p < 0.05$.

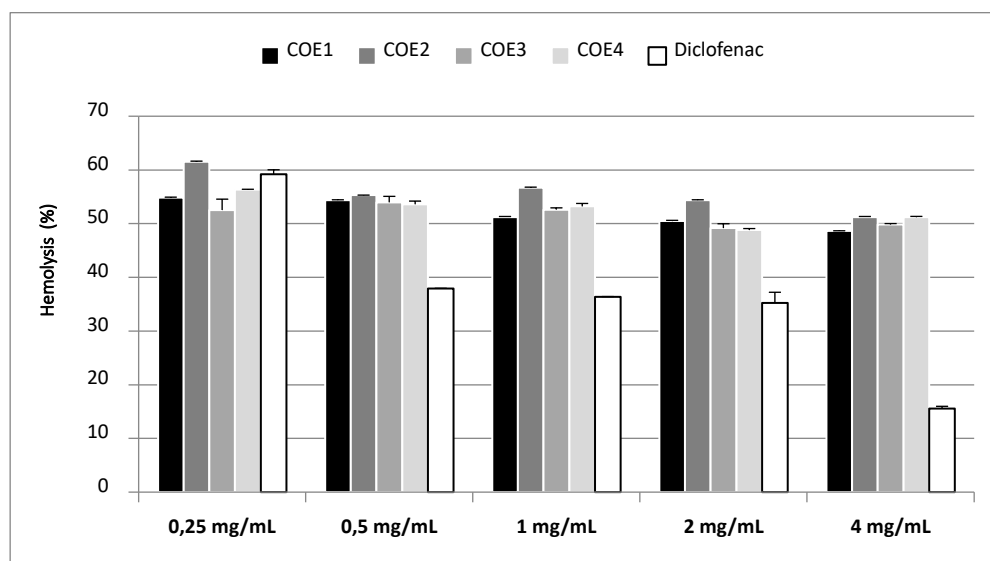


Figure 12. Hemolysis Percentages (%) of Red Blood Cells Induced by Hypotonic Solution in the Presence of Clove Oil Extracts (COE1–COE4) and Diclofenac Sodium (1%) at Different Concentrations (0.25–4 mg/ml). Data are expressed as mean $\pm$ SD ($n = 3$); $p < 0.05$.

Several recent studies have demonstrated the anti-inflammatory effect of *S. aromaticum*.^{118,119} Han and Parker¹²⁰ demonstrated that *S. aromaticum* essential oil had antioxidant and anti-inflammatory effects attributed mainly to its major compound, eugenol. Elbestawy et al.¹²¹ proved that clove essential oil of the eugenol chemotype possessed more potent anti-inflammatory activity, with human erythrocyte hemolysis inhibition percentages of approximately 53.04%, 58.74%, 61.07%, and 63.64% at concentrations of 4, 8, 16,

and 32 $\mu\text{g/ml}$, respectively. In addition, Al-Ameedi et al.¹²² demonstrated that clove alcoholic extract had an anti-inflammatory effect in a mouse inflammation model, and Koh et al.¹²³ determined that clove eugenol exerted notable anti-inflammatory activity.

Galenic Formulation Based on Clove Oil Extracts

To ensure safety, the final cream formulation was subjected to a full set of analytical controls, including physicochemical

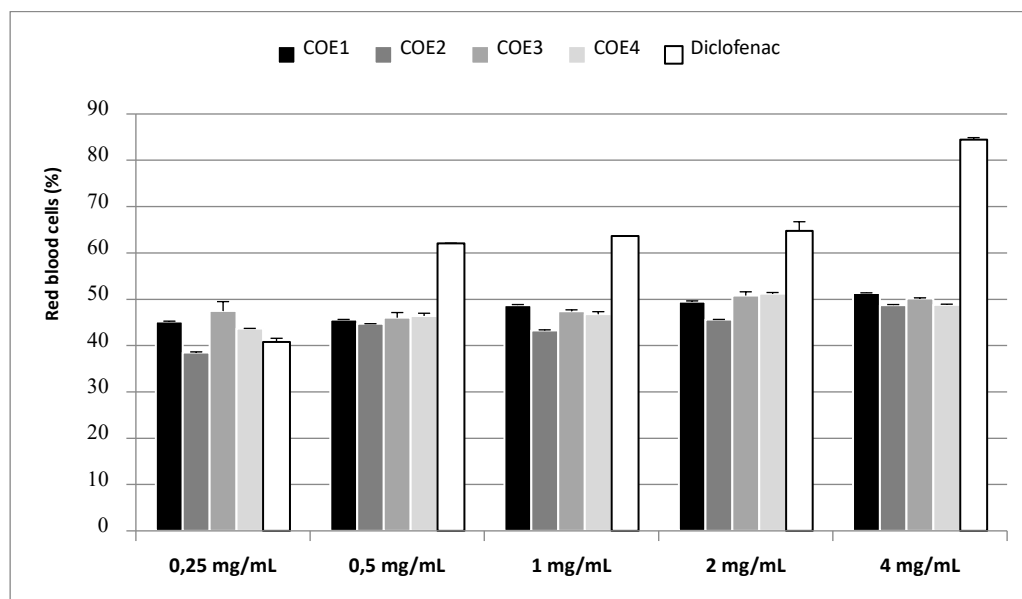


Figure 13. Inhibition of Hypotonic Solution-Induced Red Blood Cell Hemolysis (protection percentages, %) by Clove Oil Extracts (COE1–COE4) and Diclofenac Sodium (1%) at Different Concentrations (0.25–4 mg/mL). Data are expressed as mean $\pm$ SD (n = 3); $p < 0.05$.

and microbiological quality control, as well as an assessment of its cutaneous toxicity.

Quality Control of the Cream

Organoleptic and Physicochemical Characteristics

Analysis of the cream's organoleptic properties revealed variations linked to the extraction method (Table 11). The cream formulated with clove oil extract prepared by maceration at room temperature as the active ingredient had an ecru color, indicating successful preservation of polyphenols and natural pigments during the extended four-week extraction period. Conversely, the hot extraction

method (COE2) yielded a COEC with a white hue, likely attributable to thermal degradation of the chromophoric compounds. Both COEC formulations demonstrated a smooth, lump-free, and homogeneous texture, confirming the emulsification quality and the stability of the extracts in both final products (Table 11). Both clove creams were characterized by a pleasant odor reminiscent of cloves and their bioactive volatile compounds (Table 11). In addition, both products penetrated the skin easily after cutaneous application, indicating a crucial property for a topical anti-inflammatory product and suggesting excellent compatibility with the skin's hydrolipidic film (Table 11).

Table 11. Organoleptic Characteristics and pH of COEC

Parameters	COEC4	COEC1
Colour	Ecru	White
Texture	Smooth	Smooth
Smell	Pleasant smell referring to cloves smell	Pleasant smell referring to cloves smell
Homogeneity	Uniform, Absence of lumps	Uniform, Absence of lumps
Penetration	Easily penetration	Easily penetration
pH	5	5

COEC1: Cream prepared using COE obtained by maceration at room temperature; COEC4: Cream prepared using COE obtained by maceration at 60 °C.

These findings are consistent with those of Dorman and Deans,¹²⁴ who reported the stability of aromatic compounds in topical formulations. The pH analysis of both COECs revealed an optimal value (pH 5), perfectly suited for dermatological applications, ensuring excellent local tolerance while preserving the skin's barrier function.¹²⁵

The acidic pH of both COECs was mainly attributed to the stability of their bioactive compounds, especially fatty acids, caryophyllene, and humulene, the major compounds in cloves, thereby maintaining the anti-inflammatory efficacy of both creams. Additionally, this pH value could

act as a potent preservative in both products, ensuring optimal protection against microbial contamination, which is in agreement with European Pharmacopoeia standards.

Microbiological Control

Microbiological analysis confirmed the excellent quality of the COECs and their compliance with current standards by demonstrating a complete absence of contamination (Table 12). No growth of viable aerobic microorganisms was detected on Plate Count Agar (PCA), and no yeast or mold colonies were detected on Sabouraud agar. Furthermore, no colonies

of *S. aureus* or *P. aeruginosa* were detected on Chapman agar and Cetrimide agar, respectively, indicating the high

microbiological quality of both prepared products in accordance with European Pharmacopoeia standards (Table 12).

Table 12. Microbiological Analysis of the Anti-Inflammatory COECs Formulated with Clove Oil Extracts (COE1 and COE4), Including Total Aerobic Germ Count (TAGC), Total Yeast and Mold Count (TYMC), and Detection of *S. aureus* and *P. aeruginosa*

	TAGE (CFU/g)	TYME (CFU/g)	<i>S. aureus</i>	<i>P. aeruginosa</i>
COEC1	0	0	Absence	Absence
COEC4	0	0	Absence	Absence

In Vitro Anti-inflammatory Effect of the Clove Cream

To demonstrate the anti-inflammatory effect of the topical formulation prepared in this study, several tests were performed by applying the formulation to a bovine serum albumin (BSA) solution to assess its effectiveness in inhibiting the thermal denaturation of this protein. The results are presented in Figure 14.

The results showed that higher COEC1 concentrations resulted in greater inhibition of bovine serum albumin thermal denaturation (Figure 14). At a concentration of 0.25

mg/ml, the albumin denaturation inhibition was $78.34 \pm 0.016\%$, and it increased to $79.37 \pm 0.099\%$ at 0.5 mg/ml, reaching $86.03 \pm 0.065\%$ at 1 mg/ml (Figure 14). At higher COEC1 concentrations (2 and 4 mg/ml), the inhibition percentages were more pronounced, reaching $88.33 \pm 0\%$ and $90.94 \pm 0\%$, respectively (Figure 14). The results obtained with the COEC1 were higher and demonstrated greater inhibition of albumin denaturation than those obtained with COE alone, indicating that the use of COE in topical formulations enhances its therapeutic effects (Figure 14).

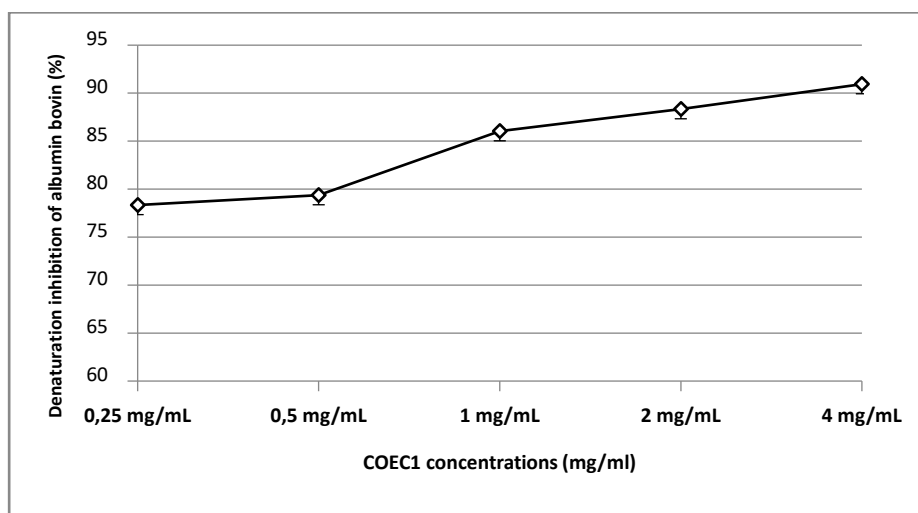


Figure 14. *In vitro* Inhibition of Bovine Serum Albumin (BSA) Thermal Denaturation (%) by the clove Oil Extract Cream (COEC1) at Different Concentrations (0.25–4 mg/ml). Data are presented as mean $\pm$ SD (n = 3); $p < 0.05$.

Primary Skin Irritation Test: Cutaneous Toxicity

Before the therapeutic application of pharmaceutical products, their toxicity must be assessed to ensure *in vivo* safety. In this study, a clove-based cream was developed, and a cutaneous toxicity evaluation was conducted to confirm that the product is safe for topical application without causing adverse effects in the host organism. The results are presented in Table 13.

The results of the primary skin irritation test demonstrated excellent local tolerance, as a complete absence of erythema (redness) and edema (swelling) was observed in mice treated with the COEC1, COEC4 and in the control group throughout the incubation period (Table 13). These findings confirmed the safety profile of the final product for cutaneous application in therapeutic treatments.

The excellent skin tolerance to COEC was due to

several factors, including the use of an optimized concentration of the active ingredients, namely the COE at optimal concentrations that did not cause skin toxicity, particularly erythema and edema, thereby ensuring that its effects were well controlled.

Our results are consistent with those of Batiha et al.,¹²⁶ who reported that clove essential oil was safe for cutaneous application due to its composition of natural anti-inflammatory compounds.

While these preclinical results are promising, additional studies are needed to confirm safety in humans. Patch tests on human volunteers, as recommended by Frosch and John,¹²⁷ would be a crucial next step. These tests could also include objective measures of skin barrier function, such as transepidermal water loss, to provide a more detailed assessment.

Table 13. Erythema and Edema Scores in the Control and COEC-Treated Groups (COEC1 and COEC4) at 24, 48, and 72 Hours After Cutaneous Application (primary skin irritation test)

	Erythema			Edema		
	24h	48h	72h	24h	48h	72h
Control group	0	0	0	0	0	0
Treated groups (COEC1 & COEC4)	0	0	0	0	0	0

Table 14. Inflammation Inhibition Percentages (%) Over Time (30–360 min) in Formalin-Induced Inflammation in Mice. Data are presented as mean $\pm$ SD (n = 5); $p < 0.05$

Time (min)	30	60	90	120	180	210	240	270	300	330	360
Batch 3	4 $\pm$ 0	5.55 $\pm$ 0.03	7.69 $\pm$ 0.006	9.31 $\pm$ 0.03	11.11 $\pm$ 0.05	12.60 $\pm$ 0.03	14.06 $\pm$ 0.05	15.49 $\pm$ 0.05	17.10 $\pm$ 0.06	17.88 $\pm$ 0.05	19.04 $\pm$ 0.05
Batch 4	14.28 $\pm$ 0.01	14.62 $\pm$ 0.02	15.38 $\pm$ 0.02	15.86 $\pm$ 0.04	16.33 $\pm$ 0.01	16.97 $\pm$ 0.03	17.42 $\pm$ 0.05	17.87 $\pm$ 0.05	18.19 $\pm$ 0.04	18.36 $\pm$ 0.04	18.64 $\pm$ 0.04
Batch 5	38.09 $\pm$ 0.02	38.88 $\pm$ 0.02	39.44 $\pm$ 0	40.12 $\pm$ 0.006	40.89 $\pm$ 0.01	41.48 $\pm$ 0.06	42.09 $\pm$ 0.06	43.26 $\pm$ 0.06	44.01 $\pm$ 0.006	44.82 $\pm$ 0.01	45.59 $\pm$ 0.03
Batch 6	29.76 $\pm$ 0.03	30.55 $\pm$ 0.03	30.95 $\pm$ 0	31.21 $\pm$ 0.03	31.99 $\pm$ 0.06	32.340.06	33.03 $\pm$ 0.06	33.79 $\pm$ 0.06	34.11 $\pm$ 0.06	34.38 $\pm$ 0.06	34.87 $\pm$ 0.07
Batch 7	23.8 $\pm$ 0.02	25.22 $\pm$ 0.05	27.33 $\pm$ 0.03	28.91 $\pm$ 0.04	29.42 $\pm$ 0.05	30.01 $\pm$ 0.05	30.69 $\pm$ 0.01	31.28 $\pm$ 0.01	31.86 $\pm$ 0.05	32.36 $\pm$ 0.08	33.04 $\pm$ 0.1

Batch 3: COE1; Batch 4: COE4; Batch 5: COEC1; Batch 6: COEC4; Batch 7: FLUCIDAL® 3%

Despite the need for further human trials, the complete lack of an irritant reaction in our animal model supports the safety profile of the cream. This paves the way for its potential development in dermatological applications, particularly for sensitive or inflamed skin.

In Vivo Anti-inflammatory Properties of the COEC

The table below summarizes the results of edema diameter measurements obtained during the evaluation of the anti-inflammatory effect of COE, both COEC1 and COEC4, and the anti-inflammatory ointment used as the standard (niflumic acid, FLUCIDAL® 3%).

Since all COE and the COEC formulations were considered safe for cutaneous application, as indicated by a primary irritation index of zero, the selection of the COE concentration used for cream preparation (10%) was based on efficacy studies. A 10% COE concentration in the COEC was found to be effective in treating inflammation and induced significant edema inhibition in mice.

Analysis of the results demonstrated significant anti-inflammatory activity of the COE and COEC formulations, consistent with recently elucidated biological mechanisms.

Compared with the positive control (Batch 1), the oily macerate obtained at room temperature (COE1) used to treat 1% formalin-induced inflammation (Batch 3) showed increasing inhibition, reaching 19.04% at $t = 360$ min (Table 14). The hot macerate (Batch 4), in contrast, exhibited a faster but less sustained effect, peaking at 18.64%.

Notably, the COEC formulations proved to be more effective than the extracts alone. The COEC1 was particularly effective, achieving 45.59% inflammation inhibition in Batch 5. This result significantly surpassed that of the reference treatment, FLUCIDAL® ointment, which achieved only 33.04% inhibition (Table 14).

The exceptional anti-inflammatory performance of the COEC was therefore attributed to two key mechanisms acting together. First, caryophyllene and humulene, the primary active compounds known as potent anti-inflammatory

agents, act on TRPA1 receptors to reduce the release of pro-inflammatory mediators such as TNF- α and IL-6, which are triggered by formalin. Second, the cream excipients improve the cutaneous bioavailability of these bioactive compounds, allowing more effective absorption through the skin. This enhanced delivery mechanism mirrors findings from other studies, such as Salem et al.,¹²⁸ which highlighted the benefits of using nano-lipid systems to increase the therapeutic effects of clove bioactive compounds.

More specifically, the significant *in vivo* anti-inflammatory activity of the COEC, which surpassed that of the reference drug FLUCIDAL® (niflumic acid) (45.59% vs. 33.04% inhibition), is largely driven by the major sesquiterpenes identified: β -caryophyllene and α -humulene. These compounds likely exert a synergistic effect by targeting multiple points in the inflammatory cascade triggered by formalin. Regarding TRPA1 and nociception, caryophyllene and humulene are known to interact with transient receptor potential (TRP) channels, specifically TRPA1. Formalin induces pain and edema through a biphasic response in which the late phase is characterized by the release of inflammatory mediators; these sesquiterpenes may desensitize TRPA1 channels, thereby reducing the neurogenic and inflammatory signals associated with formalin injection. Furthermore, as noted in the introduction, clove bioactive components suppress cyclooxygenase-2 (COX-2) and inflammatory cytokines such as TNF- α and IL-6. Caryophyllene, a selective CB2 receptor agonist, and humulene likely work in tandem to downregulate these pathways, inhibiting prostaglandin production and preventing the recruitment of inflammatory cells to the site of injury.

Membrane stabilization: The observed erythrocyte membrane stabilization (antihemolytic activity) further suggests that these compounds protect lysosomal membranes. *In vivo*, this stabilization prevents the release of hydrolytic enzymes and inflammatory mediators from neutrophils, which are primary drivers of formalin-induced edema.

Synergistic inhibition: While BCP targets CB2 receptors

to suppress inflammatory signaling, α -humulene provides complementary action by inhibiting COX-2 expression and reducing prostaglandin production. This “dual-track” inhibition, addressing both cytokine release and enzymatic pathways, explains why the COEC outperformed the standard niflumic acid (45.59% vs. 33.04% inhibition).

The COEC1 used in Batch 5 demonstrated a slightly superior anti-inflammatory effect compared with the COEC4 in Batch 6. This difference suggests that room-temperature extraction may better preserve synergistic terpenes, a finding consistent with the conclusions of Ahmadiani et al.¹²⁹ Collectively, these results indicate that clove oil extract-based creams, which combine an effective delivery system with multi-targeted active ingredients, offer a promising alternative to conventional non-steroidal anti-inflammatory drugs (NSAIDs) for treating skin inflammation caused by chemical agents such as formalin.

A key finding of this study was that the clove cream exhibited superior efficacy compared with the raw oily extracts. This improvement is rooted in pharmacotechnical principles. Enhanced cutaneous penetration: the use of Tween 80 and stearic acid as emulsifiers facilitates the formation of a stable O/W (oil-in-water) emulsion. These surfactants reduce interfacial tension, allowing the bioactive sesquiterpenes to bypass the skin's lipophilic barrier more effectively. Tween 80, which can temporarily disrupt the lipid bilayer of the stratum corneum, acts as a penetration enhancer. This increases the cutaneous bioavailability of the sesquiterpenes, allowing them to reach deeper dermal layers where the inflammatory cascade occurs.

Bioavailability: the smooth, homogeneous texture of the formulation ensures a consistent distribution of the active COE on the skin surface. Agents such as paraffin oil and beeswax act as occlusives, preventing transepidermal water loss and increasing the contact time of BCP and humulene with the dermis, thereby maximizing their cutaneous bioavailability. Compatibility: the measured pH of the cream (aligned with natural skin pH) ensures that the skin barrier remains intact during treatment, preventing irritation (skin irritation index = 0) while promoting absorption of the active ingredients.

Conclusion

In a context where the search for new natural resources with therapeutic properties is gaining increasing importance, this research focused on cloves (*Syzygium aromaticum*). The primary objective was to explore the potential of clove oil extracts (COE), obtained via maceration in a non-polar vegetable oil solvent (“Elio”), as a source of bioactive compounds. These compounds were specifically investigated for their antioxidant, antimicrobial, antihemolytic, and anti-inflammatory properties, with the ultimate goal of their application in new therapeutic formulations.

The antioxidant activity of COE was evaluated using four complementary methods. The Total Antioxidant Capacity (TAC) assay assessed their overall power by measuring the neutralization of free radicals and oxidizing species. In parallel, the DPPH test was used to specifically quantify their ability to scavenge the DPPH radical, along with the ferric reducing antioxidant power (FRAP) assay and the β -carotene bleaching test coupled with linoleic acid autoxidation. The combined results from these analyses clearly demonstrate the strong antioxidant properties of the extracts.

The evaluation of the antimicrobial activity of clove oil extracts revealed low efficacy against the majority of microbial strains tested. Using a microdilution technique, it was determined that the extracts primarily exerted a bacteriostatic effect on most Gram-positive and Gram-negative bacterial strains and a fungistatic effect on *C. albicans*. A notable exception was *K. pneumoniae*, on which a bactericidal effect was observed, with a MIC of 0.488 mg/ml and a MBC of 1000 mg/ml. These results suggest that despite the well-documented antimicrobial properties of cloves, the effectiveness of their oily extracts can vary significantly depending on the extraction conditions, concentrations, and specific microbial strains investigated.

In light of our preliminary findings, it is clear that additional research is necessary to fully understand the antimicrobial potential of *S. aromaticum* oily extracts. To strengthen our results, future studies should investigate the specific mechanisms of action of these extracts on micro-organisms and explore the effects of different concentrations and combinations with other antimicrobial agents. This approach would help improve their effectiveness and fully explore their therapeutic potential.

A topical formulation (cream) based on clove oil extracts was developed to demonstrate its *in vivo* anti-inflammatory efficacy using a Swiss mouse model. The cream prepared with extracts from both extraction methods exhibited an anti-inflammatory effect superior to that of the COE alone. Notably, the COEC formulated with the COE1 demonstrated a more potent effect than the cream containing the COE4 extract. The efficacy study was coupled with an evaluation of skin toxicity, which determined the Primary Irritation Index (PII) to be zero. This result confirms that the cream is non-irritating upon cutaneous application and can therefore be safely used for the treatment of inflammatory pathologies without causing undesirable side effects.

In conclusion, the successful development of novel clove oil extracts via vegetable oil maceration was demonstrated. These extracts were considered potent antioxidant and anti-inflammatory products linked to β -caryophyllene and humulene, the major compounds in the COE. We validated the superior *in vivo* efficacy and safety of the COEC formulation after therapeutic application for treating

inflammation induced by formalin in a mouse model. Furthermore, additional studies should be conducted on the antimicrobial effect of the COE using the same extraction design as described in the present study against other microbial strains with different antibiotic resistance profiles.

Authors' Contributions

Conceptualization, NEHB and FB; methodology, NEHB, BM, FB, HY, HZ, LM, MOA, RBRN, BH, IY, and RE; software, NEHB and FB; validation, NEHB; writing—original draft preparation, NEHB, FB, and BM; writing—review and editing, NEHB and FB; visualization, NEHB and FB; supervision, NEHB; project administration, NEHB and BM; funding acquisition, BM. All authors have read and agreed to the published version of the manuscript.

Ethical Approval

For animal experimentations, adequate measures were taken to minimize pain and discomfort of the animals, and all experimental procedures were in accordance with the ethical guidelines of the organization for economic cooperation and development (OECD).

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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