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Antimicrobial and Antioxidant Activities of Clove Essential Oil and Eugenyl Acetate Produced by Enzymatic Esterification

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Abstract This work reports the maximization of eugenyl acetate production by esterification of essential oil of clove in a solvent-free system using Novozym 435 as catalyst. The antimicrobial and antioxidant activities of clove essential oil and eugenyl acetate produced were determined. The conditions that maximized eugenyl acetate production were 60 °C, essential oil of clove to acetic anhydride ratio of 1:5, 150 rpm, and 10 wt% of enzyme, with a conversion of 99.87 %. A kinetic study was performed to assess the influence of substrates' molar ratio, enzyme concentration, and temperature on product yield. Results show that an excess of anhydride, enzyme concentration of 5.5 wt%, 50 °C, and essential oil of clove to acetic anhydride ratio of 1:5 afforded nearly a complete conversion after 2 h of reaction. Comparing the antibacterial activity of the essential oil of clove before and after esterification, we observed a decrease in the antimicrobial activity of eugenyl acetate, particularly with regard to minimum inhibitory concentration (MIC). Both eugenyl acetate and clove essential oil were most effective to the gram-negative than gram-positive bacteria group. The results showed a high antioxidant potential for essential oil before and particularly after the esterification reaction thus becoming an option for the formulation of new antioxidant products.

Keywords Eugenyl acetate · Novozym 435 · Antimicrobial activity · Antioxidant activity · Essential oil of clove · Esterification

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Introduction

During the last years, biocatalytic processes that fall within the concept of "green chemistry" have been gaining greater scope in the flavors and fragrances market that represents a challenging goal for academic and industrial research. Industrial processes based on biocatalytic methods are discussed in terms of its advantages over classical chemical synthesis and extraction from natural sources, as flavorings and fragrances are highly important for food, cosmetics, and chemical and pharmaceutical industries.

Due to the disadvantages of chemical process, research directed its attention to aroma compounds of biological origin, the so-called natural or bioaromas. The terpene alcohols and phenols of natural origin can be widely used in the production of esters in order to obtain aromas with antioxidant properties. Eugenol is a phenol found in sassafras, myrrh, and especially in the essential oil of clove. It has wide use, acting as a stomach and aromatic stimulant and an antiseptic, and is also used as an expectorant in bronchitis and as a versatile condiment in perfumery industries [1–3]. The search for new antimicrobial agents, from natural sources, is intense due to the crescent resistance of pathogenic microorganisms to synthetic products [4, 5].

Enzymatic esterification of the essential oil of clove is a matter of great scientific and technological interest due to the well-known drawbacks of the chemical-catalyzed route as well as the potential use of produced compounds as natural antimicrobials. Although a number of works were presented in the literature about the biological properties of clove oil and eugenol [6–8], a lack was found related to the evaluation of the antimicrobial and antioxidant potential of eugenol esters obtained by enzymatic catalysis for further application in food and cosmetic industries. Several works relate the synthesis of esters from acids and anhydrides by enzymatic catalysis in organic and solvent-free systems; however, just a few use eugenol as substrates [9–13].

In this context, the objective of this work was to maximize the eugenyl acetate production from clove essential oil using Novozym 435 as catalyst in a solvent-free system and also to determine the antimicrobial and antioxidant activities of the essential oil and the eugenyl acetate produced.

Experimental

Substrates and Catalyst

The clove (*Caryophyllus aromaticus*) essential oil (Viafarma, Brazil (85.43 % of eugenol)) and acetic anhydride (Vetec, 97 % purity) were used as substrates for the esterification reactions. The commercial lipase used in this work was *Candida antarctica* (Novozym 435) immobilized on a macroporous anionic resin, purchased from Novozymes Brazil (Araucária, PR, Brazil).

Enzymatic Production of Eugenyl Acetate

The esterification reactions were performed by preparing a reaction mixture of acetic anhydride and eugenol at different molar ratios 1:1 (18 mmol), 3:1 (5.4–18 mmol), and 5:1 (90–18 mmol) in a 50-mL Erlenmeyer flask. After complete dissolution of the substrates, the enzyme was added to the mixture. Experiments were carried out in an orbital shaker at constant agitation of 150 rpm. After the reaction time completion, the biocatalyst was filtered for later measurement of enzyme activity, and samples were kept at 5 °C for further analysis and determination of reaction conversion.

Determination of Reaction Conversion

Quantitative analyses of eugenyl esters produced were carried out in a gas chromatograph (Shimadzu GC-2010) equipped with a data processor, using a capillary column of fused silica INOWAX (30-m length×250-μm i.d.×0.25-μm thickness), and a flame ionization detector, with the following temperature program: 40–180 °C (3 °C/min), 180–230 °C (20 °C/min), 230 °C (20 min), injector temperature 250 °C, detector at 275 °C and with also injection in the mode split, ratio of split 1:100, H_2 (56 kPa) as carrier gas, injected volume of 0.4 μL of sample diluted in *n*-hexane (1:10). Reaction conversion was calculated based on the reduction of area of a limiting reagent on the basis of reaction stoichiometry [10].

Effect of Process Variables on the Enzymatic Production of Eugenyl Acetate

A 2^3 full experimental design with triplicate runs at the central point was employed to assess the experimental conditions that maximize the reaction conversion. Reaction time was fixed at 6 h, and experiments were carried out as described before. The variables studied were temperature (40 to 60 °C), acetic anhydride to eugenol molar ratio (1:1 to 5:1), and enzyme concentration (1 to 10 wt%—based on the substrate amount).

The software Statistica® 6.0 (StatSoft Inc.) was used to assist the design and the statistical analysis of experimental information, adopting, in all cases studied, a confidence level of 95 % ($p<0.05$).

Kinetics of Enzymatic Production of Eugenyl Acetate

After maximizing the experimental conditions, kinetic experiments were performed with substrate molar ratios of 1:1, 3:1, and 5:1; enzyme concentrations of 1, 5.5, and 10 wt% (by weight of substrates); and temperature ranging from 40 to 60 °C. It may be important to emphasize that in all cases, destructive experiments, without sampling, were carried out at times of 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h.

Determination of Antimicrobial Activity

The sensitivity degree or resistance of ten microorganisms against clove essential oil (*C. aromaticus*) and eugenyl acetate was tested by measuring the size of the zones of antimicrobial effect (size of halo formed). For this purpose, the whole reaction system was submitted to vacuum microdistillation to remove any residue of eugenol and acetic anhydride at the end of the reaction.

The following microorganisms were tested: *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Enterococcus faecalis* as gram positives and *Citrobacter freundii*, *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* as gram negatives. The microorganisms were previously grown in medium Luria Bertani broth (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) during 24 h at 36±1 °C.

For this purpose, Whatman number 3 paper disks with 7-mm diameter were placed over Mueller–Hinton agar (Merck) culture medium. Five, 5, 10, and 20 μL of eugenyl acetate and oil of clove were used in each disk.

Results are expressed as millimeter and were calculated as the arithmetic mean of total halo diameter obtained after triplicate for each microorganism and concentration. Means were compared by Tukey's test at 5 %, using the software Statistica 8.0.

Antioxidant Activity

The method is based on measuring the extinction of absorption of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) at 515 nm. Determination of antioxidant activity was performed in triplicate by a spectrophotometric method. The technique consisted of incubation for 10 min in 500 μL of an ethanol solution of DPPH 0.1 mmol/L with 500 μL of solutions containing increasing concentrations of clove essential oil (150, 250, 500, 1,000, 2,500, 5,000, 7,500, and 10,000 mg/mL) in ethanol. We proceeded similarly to the preparation of the solution called "control" but substituting 500 μL of sample to 500-μL ethanol solvent. To a solution called "white", ethanol was used and the oil in the same concentration range studied. The percent uptake of the DPPH radical was calculated in terms of percentage of antioxidant activity (AA%), according to the following equation:

$$AA\% = 100 - \left\{ \left[\left(Abs_{sample} - Abs_{blank} \right) \times 100 \right] \quad Abs_{control} \right\} \tag{1}$$

The determination was made in a UV-Visible spectrophotometer (515 nm) (Agilent Technologies brand, model 8453E). After evaluating the optimal concentration range, we calculated the concentration of essential oils needed to capture 50 % of the free radical DPPH (IC_{50}) by linear regression.

Results and Discussion

Maximization of Eugenyl Acetate Production

To evaluate the effects of reaction variables on eugenyl acetate production, a 2^3 factorial design was conceived taking into account the reaction temperature (T), enzyme concentration [E] (by the weight of substrates, wt%), and acetic anhydride to eugenol molar ratio (RM). Table 1 presents the coded and real independent variable values of the experimental matrix generated as well as the response, expressed here as eugenyl acetate yield. It can be noticed from this

Table 1 Matrix of the experimental design (coded and real values) with responses in terms of eugenyl acetate conversion

Run	Temperature (°C)	Acetic anhydride to clove oil molar ratio	Enzyme concentration (wt%)	Conversion (%)
1	−1 (40)	−1 (1:1)	−1 (1 %)	59.16
2	1 (60)	−1 (1:1)	−1 (1 %)	86.81
3	−1 (40)	1 (5:1)	−1 (1 %)	89.99
4	1 (60)	1 (5:1)	−1 (1 %)	93.87
5	−1 (40)	−1 (1:1)	1 (10 %)	44.73
6	1 (60)	−1 (1:1)	1 (10 %)	82.30
7	−1 (40)	1 (5:1)	1 (10 %)	90.47
8	1 (60)	1 (5:1)	1 (10 %)	99.87
9	0 (50)	0 (3:1)	0 (5.5 %)	96.98
10	0 (50)	0 (3:1)	0 (5.5 %)	96.96
11	0 (50)	0 (3:1)	0 (5.5 %)	97.41

At 150 rpm and 6 h of reaction

table that high reaction conversions were reached for all experimental levels investigated, at different conditions of temperature, enzyme content, and substrate molar ratio.

Inspection of the results at the central point of the design (assays 9, 10, and 11) demonstrates the good reliability and reproducibility of the experiments. Higher conversion (~100 %) was obtained at the substrate molar ratio of 5:1, higher enzyme concentration (10 wt%) and temperature (60 °C).

Results were statistically analyzed, and the effects are presented as a Pareto chart, shown in Fig. 1, where it can be observed that after 6-h reaction, the substrate molar ratio and temperature presented a significant positive effect on reaction yield, while enzyme concentration affected negatively eugenyl acetate production ($p<0.05$). Such results indicate that the reaction may be conducted using lower enzyme concentration, which means cost-effectiveness regarding the catalyst.

Kinetics of Eugenyl Acetate Production

Reaction kinetics toward eugenyl acetate production was carried out with destructive experiments varying substrate molar ratio, temperature, and enzyme concentration. Data scattering observed for the kinetic results presented in this work may be explained in terms of experimental errors associated and the fact that destructive experiments were carried out without sampling, which may be viewed as an important internal consistency test of the results.

Effect of Acetic Anhydride to Eugenol Molar Ratio

In this case, the experiments were performed at a fixed temperature of 50 °C and 5.5 % enzyme concentration. Figure 2 presents the results obtained for acetic anhydride: eugenol ratios of 1:1, 3:1, and 5:1. From this figure, one can note that the highest conversions (~95 %) were reached after 2 h and a substrate molar ratio of 5:1. The maximum yield of eugenyl acetate (~100 %) was reached after 5-h reaction at the molar ratios of 3:1 and 5:1.

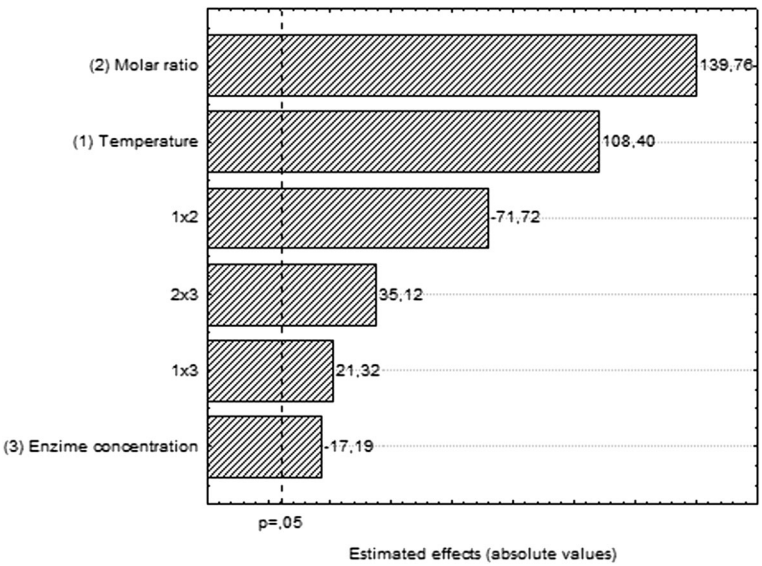

Fig. 1 Pareto chart of eugenyl acetate production (6 h of reaction)

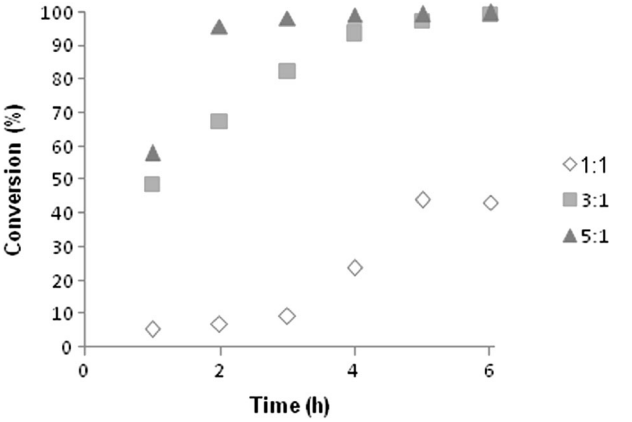

Fig. 2 Effect of substrate acetic anhydride to eugenol molar ratio on the solvent-free production of eugenyl acetate at 50 °C, enzyme concentration of 5.5 wt%, and 150 rpm

Effect of the Enzyme Concentration

The effect of enzyme concentration on reaction yield was evaluated keeping temperature at 50 °C and the acetic anhydride to eugenol molar ratio 3:1 at 150 rpm, varying enzyme content at 1, 5.5, and 10 % (by the weight of substrates, wt%). As can be clearly seen from Fig. 3, enzyme concentration did not present a relevant effect on eugenyl acetate production. After 4 h of reaction, the enzyme concentration does not exhibit any effect for practical purposes, with a maximum conversion reached after 6 h (>90 %).

Similar results were obtained by Chiaradia et al. [6]. The authors demonstrated that after the first 30 min of reaction, the highest conversions were obtained for the enzyme concentrations of 1, 5.5, and 10 %, respectively, and 20, 46, and 51 %. After 1 h of reaction, the enzyme concentration did not exhibit any significant effect.

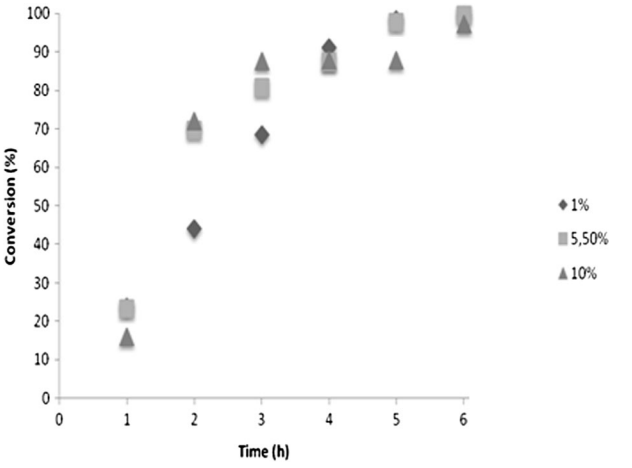

Fig. 3 Effect of enzyme concentration on the solvent-free production of eugenyl acetate at 50 °C, acetic anhydride to eugenol molar ratio of 3:1, and 150 rpm

A possible explanation might be related to the fact that an excess of enzyme in the reaction medium could not contribute to the conversion enhancement, since high enzyme concentration may lead to the formation of aggregates, thus not making the enzyme active site available to the substrates [10, 14–16]. The enzyme molecules on the external surface of such particles are exposed to high substrate concentrations, but the mass transport could drastically limit the substrate concentration inside the particles. Lower activities of the biocatalyst reduce the efficiency of the enzyme, not enhancing the reaction conversion [17].

Effect of Temperature

Toward assessing the effect of temperature on reaction conversion, experiments were performed at a fixed acetic anhydride to eugenol molar ratio of 3:1, enzyme concentration of 5.5 %, and varying system temperature at 40, 50, and 60 °C. It can be noticed from Fig. 4 that higher conversions were obtained in the first 2-h reaction for the temperatures of 50 and 60 °C, 72 and 83 %, respectively. After such time, reaction conversion increases over 95 % at 50 and 60 °C after 6 h of reaction.

In an attempt to compare the results obtained here in terms of eugenyl acetate production by enzymatic esterification of eugenol and acetic anhydride in a solvent-free system, one can observe that no work could be found in the literature, evidencing the real contribution of the present study. Some recent works related to this subject were found and were the basis for our discussion. Horchani et al. [18] studied the synthesis of eugenyl benzoate by enzymatic esterification using a non-commercial immobilized *S. aureus* lipase as biocatalyst. The maximum conversion (75 %) was obtained using 240 IU of immobilized catalyst and a benzoic acid/eugenol molar ratio of 1:22 dissolved in 4.6 mL of chloroform as solvent at 41 °C. Lower conversions were obtained compared to our work, and it is also important to observe that the authors used chloroform as organic solvent, what can enhance the cost of the process, due to need of solvent separation after the reaction completion. A recent work [19] relates the optimization of enzymatic synthesis of eugenol ester using statistical approaches. Eugenol and caprylic acid were used as substrates and Lipozyme TL IM as catalyst. The maximum conversion yield (72.2 %) was obtained at the optimal conditions of 65 °C, 250 rpm, 259 min, 100-mg enzyme, and 2:1 molar ratio of eugenol/caprylic acid in a solvent-free system. Again, the higher conversion obtained by these authors is considerably

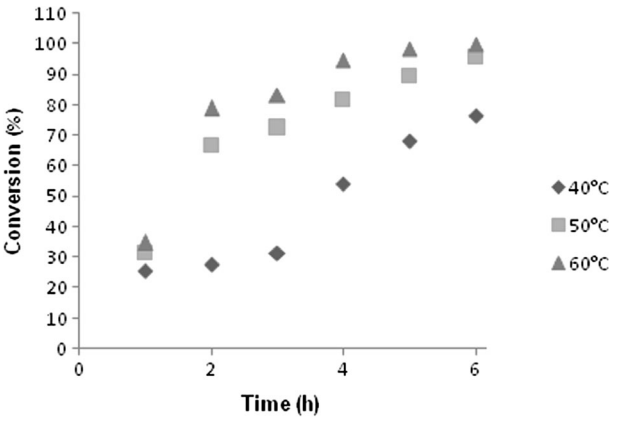

Fig. 4 Effect of temperature on the solvent-free production of eugenyl acetate at acetic anhydride to eugenol molar ratio of 3:1, enzyme concentration of 5.5 wt%, and 150 rpm

lower compared to the results presented here. Another work was found in this subject, but a chemical catalyst was used instead of a biocatalyst [20]. The authors evaluated the eugenol benzoate production by esterification of eugenol with benzoic acid using several solid acids as catalysts, among which super acid UDCaT-5, a zirconia-based catalyst, was found to be the best, giving a conversion of about 90 % under a temperature of 110 °C. The authors also verified that the catalyst was highly active and reusable. Note that high temperatures were required to achieve considerable conversion.

Chiaradia et al. [6] maximized the eugenyl acetate production by esterification of eugenol and acetic anhydride in a solvent-free system using Novozym 435 as catalyst. The operating conditions that maximized eugenyl acetate production were 50 °C, eugenol to acetic anhydride ratio of 1:3, 150 rpm, and 5.5 wt% of enzyme, with a conversion of 99 %. A kinetic study was performed to assess the influence of substrate molar ratio, enzyme concentration, and temperature on eugenyl acetate yield. Results show that an excess of anhydride, low enzyme concentration (1 wt%), and 60 °C afforded a nearly complete conversion after 6 h of reaction.

Synthesis of geranyl and citronellyl esters from citronella essential oil with a commercial lipase from *C. antarctica* was investigated by Paroul et al. [21]. The optimized experimental conditions were 60 °C, 6 h of reaction time, molar ratio of essential oil/acid of 1:1, and concentration of enzyme of 10 wt%. Yields higher than 90 % were achieved for both tested acids.

Evaluation of Antimicrobial Activity

According to Katzung [22], the disk diffusion is a satisfactory methodology to determine the sensibility of several microorganisms against many pharmaceutical substances and is adequate when the resistance mechanism is due to the degradation of the antimicrobial agent by the microorganism.

Tests employing the diffusion disk technique were carried out aiming at providing a comparison in terms of antimicrobial activity between pure oil (before esterification reaction) and eugenyl acetate (which was previously distilled).

In the antimicrobial experiments, the concentrations of eugenyl acetate and eugenol tested were 5, 10, and 20 μL, as shown in Table 2. It can be noted from this table that the highest and similar antimicrobial activities using clove essential oil and eugenyl acetate were observed at higher concentrations. However, at lower doses, eugenyl acetate shows lower activity compared to clove oil.

The hydrophobicity of phenol limits the value of agar disc/diffusion tests for estimating antimicrobial potency accurately [23]. According to Klancnik et al. [24], the methodology of the disks has the greatest importance to provide initial data of the antimicrobial action of natural products, the ease and speed of execution. Thus, Table 3 presents the results of minimum inhibitory concentration (MIC) for the essential oil of clove and eugenyl acetate ester.

In assessing the results of MIC (Table 3), it was observed that the essential oil of clove showed a better performance than the acetate ester eugenyl for both gram-positive and -negative bacteria. After esterification, we observed a decrease of the effective antimicrobial activity of approximately sevenfold. Differently from our study, Dorman and Deans [25] noted that the addition of an acetate moiety to the molecule appeared to increase the antibacterial activity; geranyl acetate was more active against a range of gram-positive and -negative species than geraniol.

Lachowicz et al. [26] found crude essential oil of basil more effective than components linalool and methyl chavicol either separately or together. Pei et al. [27] studied the

Table 2 Antimicrobial activity of eugenyl acetate and oil of clove against gram-positive and gram-negative bacteria using 5, 10, and 20 μL of sample per disk

		Inhibition zone (mm)					
		Essential oil of clove			Eugenyl acetate		
	ATCC	5 μL	10 μL	20 μL	5 μL	10 μL	20 μL
Gram-positive							
Listeria monocytogenes	7644	$13.00^{a}\pm1.00$	$14.00^{a}\pm1.32$	$14.50^{a}\pm1.00$	$10.53^{b}\pm0.15$	$12.10^{ab}\pm0.26$	$12.13^{ab}\pm0.41$
Staphylococcus aureus	25923	$12.33^{b}\pm0.28$	$13.33^{a}\pm0.28$	$14.16^{a}\pm1.60$	$10.46^{c}\pm0.30$	$11.06^{c}\pm0.35$	$12.60^{ab}\pm0.30$
Bacillus subtilis	6633	$11.50^{a}\pm0.86$	$12.00^{a}\pm0.72$	$12.08^{a}\pm1.32$	$11.96^{a}\pm0.35$	$12.46^{a}\pm0.35$	$12.03^{a}\pm0.45$
Enterococcus faecalis	29212	$13.16^{b}\pm0.76$	$17.16^{a}\pm0.28$	$17.66^{a}\pm0.76$	$10.96^{c}\pm0.35$	$12.00^{bc}\pm0.50$	$13.33^{b}\pm0.30$
Bacillus cereus	11778	$10.66^{b}\pm1.15$	$10.67^{b}\pm1.12$	$10.68^{b}\pm1.16$	$10.56^{b}\pm0.30$	$11.03^{b}\pm0.35$	$12.03^{a}\pm0.45$
Mean		12.13	13.43	13.81	10.89	11.73	12.42
Gram-negative							
Pseudomonas aeruginosa	27853	$12.16^{c}\pm0.28$	$13.50^{b}\pm0.50$	$15.00^{a}\pm0.08$	$11.05^{d}\pm0.30$	$12.10^{c}\pm0.23$	$12.21^{c}\pm0.10$
Serratia marcescens	8100	$12.33^{b}\pm0.44$	$13.83^{a}\pm0.76$	$14.33^{a}\pm0.28$	$12.00^{b}\pm0.20$	$12.30^{b}\pm0.43$	$13.16^{a}\pm0.15$
Citrobacter freundii	8090	$12.00^{b}\pm0.28$	$13.08^{ab}\pm0.76$	$14.00^{a}\pm0.29$	$11.02^{b}\pm0.80$	$11.41^{b}\pm0.63$	$13.02^{ab}\pm0.80$
Escherichia coli	25922	$15.16^{b}\pm0.57$	$20.66^{a}\pm1.75$	$21.16^{a}\pm1.46$	$12.13^{c}\pm0.23$	$13.06^{c}\pm0.40$	$14.26^{b}\pm0.40$
Klebsiella pneumoniae	10031	$13.66^{a}\pm0.57$	$13.83^{a}\pm0.76$	$14.10^{a}\pm1.32$	$10.53^{b}\pm0.35$	$11.03^{b}\pm0.35$	$12.90^{a}\pm0.20$
Mean		13.06	14.98	15.72	11.35	11.98	13.11

Means followed by the same letter in rows do not differ among them, by the Tukey's test, at 95 % confidence level

ATCC American Type Culture Collection, USA

Table 3 Minimum inhibitory concentration (MIC) of the oil of clove and eugenyl acetate on gram-positive and gram-negative bacteria

	ATCC	MIC (μL/mL)	
		Clove oil	Eugenyl acetate
Gram-positive bacteria			
Listeria monocytogenes	7644	0.3	2.5
Staphylococcus aureus	25923	0.7	4.8
Bacillus subtilis	6633	0.5	4.2
Enterococcus faecalis	29212	0.5	4.3
Bacillus cereus	11778	1.2	5.4
Mean		0.64	4.2
Gram-negative bacteria			
Pseudomonas aeruginosa	27853	0.7	4.7
Serratia marcescens	8100	0.5	4.0
Citrobacter freundii	8090	0.2	2.1
Escherichia coli	25922	0.8	4.9
Klebsiella pneumoniae	10031	0.2	2.0
Mean		0.48	3.5

antibacterial effects in isolated compounds and combinations of eugenol, cinnamaldehyde, carvacrol, and thymol against *E. coli* and report the existence of synergism in all combinations tested. Thus, the synergism may be the cause of the higher activity of the clove oil in relation to eugenyl acetate.

On the other hand, Ultee et al. [28] concluded that to be effective against vegetative cells of *B. cereus* antimicrobials, the compound should have both a hydroxyl group on the phenolic ring as well as a system of delocalized electrons (i.e., the presence of α–β double bonds) to elicit strong antimicrobial activity. Thus, the change in the structure of eugenol by esterification may partially explain the reduction in antimicrobial activity observed for eugenyl acetate.

From the average presented (Tables 2 and 3), the results showed that the clove oil has a higher inhibitory activity against gram-negative bacteria. Although there are exceptions in the literature, in general, essential oils are more effective against gram-positive than gram-negative bacteria, which can be attributed, in part, to the greater complexity of the double-membrane-containing cell envelope of these organisms in contrast with the single-membrane glycoprotein/teichoic acid of gram-positive bacteria [29].

The higher activity of clove oil to gram-negative bacteria suggests that it is more likely that the effects of differences in hydrophobicity between these two bacterial groups, with gram-negative cells having more hydrophobic surfaces, can be offset by the presence of porin proteins in the outer membrane of gram-negative cells. These can create channels large enough to allow restricted passage of small-molecular-mass compounds, like the phenolics present in essential oils, allowing their access to the periplasmic space, the glycoprotein layer, and the cytoplasmic membrane [23].

The increase in the dose of the essential oil and eugenyl acetate caused a significant increase of effect for various bacteria. In others studies, the essential oil of clove showed strong antimicrobial activity when tested for *S. aureus*, *L. monocytogenes*, *Staphylococcus epidermidis*, *E. coli*, *Campylobacter jejuni*, and *Salmonella enteritidis* [8] and *B. subtilis*, *P. aeruginosa*, *E. faecalis*, *C. freundii* [7], showing to be effective against both groups [30].

Activity Capture of Free Radicals of DPPH Test

The results obtained after the determination of antioxidant activity (DPPH test) of essential oil before and after esterification at different concentrations are shown in Table 4. The results demonstrate that the antioxidant concentration increases proportionally to the percentage of oil or eugenyl acetate added, reaching the maximum value of 61.12 and 81.59 %, respectively, for the antioxidant activity concentration of 500 μg/mL.

After identifying the concentration range with a linear increase on antioxidant activity, the equation of the line and the determined IC_{50} of the essential oil of clove and eugenyl acetate were traced. The correlation between antioxidant activity (%) and the concentration of oil used (Y=0.133x+1.113) with R^2=0.919 gave an IC_{50} of 367.5 μg/mL. The correlation between antioxidant activity (%) and the eugenyl acetate concentration used that is eugenyl (Y= 0.167x+2.581) with R^2=0.970 gave an IC_{50} of 283.9 μg/mL. The percentage of antioxidant activity and IC_{50} obtained indicate a high antioxidant activity of the essential oil of clove and especially the eugenyl acetate produced. According to Mensor et al. [31], the plant *Ginkgo biloba* is considered a plant with high antioxidant activity, as it has an IC_{50} of 38.91 μg/mL.

The phenolic antioxidants act as radical scavengers and, sometimes, as chelators of metals, acting as an initiator and propagator on the oxidative process [32, 33]. The phenolic compounds have a potent antioxidant action, in spite of not having a conclusive study about their mechanism in vivo. Eugenol, the major compound of clove essential oil, can be classified as a primary antioxidant. It has an inhibitory capacity of lipid peroxidation on initial and propagation phases by the interference on chain reactions, sequestering the active O_2, and, when metabolized to a dimer (dieugenol), it inhibits the peroxidation at levels of propagation of a chain reaction of free radicals with α-tocopherol [4], demonstrating a potential antioxidant action and strategic application in the industry. By the substitution of a hydroxyl group from eugenol by acetate, the clove essential oil presented a higher antioxidant activity, which can be explained by the inductive effect exerted by the group under the aromatic ring, which could facilitate the liberation of hydrogen atoms from the ring. However, more studies aiming at elucidating the antioxidant action mechanism of esters are necessary to corroborate this hypothesis.

This demonstrates that the essential oil *C. aromaticus* as well as the oil enriched with eugenyl acetate have a high antioxidant activity with promising potential for application in fine chemical, cosmetic, and food industries.

Table 4 Percentage of DPPH neutralization of the essential oil of clove and eugenyl acetate

Concentrations (μg/mL)	(AA%)	
	Oil of clove	Eugenyl acetate
10	0.56	1.12
25	2.81	4.26
50	6.76	9.21
75	8.73	15.64
100	12.95	21.78
250	49.55	53.99
500	61.12	81.59

Conclusions

The technical viability of eugenyl acetate enzymatic synthesis from crude clove essential oil was evaluated in this work as well as the antimicrobial and antioxidant activities of the oil and the ester. High esterification yields were verified with the use of Novozym 435 as catalyst. It was experimentally observed that the best reaction yield, 99 % for 6-h reaction, was reached at 60 °C, 150 rpm, acetic anhydride/eugenol molar ratio of 5:1, and enzyme concentration of 10 wt%. From the kinetic study, one could verify that high yields were obtained after 2 h of reaction, reducing the enzyme concentration, at higher temperatures and substrate molar ratio of 5:1.

The crude clove essential oil and the eugenyl acetate enzymatically produced presented higher antimicrobial activities against gram-negative bacteria. The esterified oil showed a lower antimicrobial activity compared to the crude clove essential oil. The antioxidant activity showed a linear correlation with the concentration of essential oil. The results showed a high antioxidant potential for essential oil before (IC_{50} of 367.5 μg/mL) and particularly after the esterification reaction (IC_{50} of 283.9 μg/mL) thus becoming an option for the formulation of new antioxidant products.

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References

1. Paoli, S., Giani, T. S., Presta, G. A., Pereira, M. P., Fonseca, A. S., Brandão Neto, J., et al. (2007). *Brazilian Archives of Biology and Technology, 50*, 175–182.
2. Guenette, S. A., Rodd, A., Marier, J. F., Beaudry, F., & Vachon, P. (2007). *European Journal of Pharmacology, 562*, 60–67.
3. Guenette, S. A., Helie, P., Beaudry, F., & Vachon, P. (2007). *Veterinary Anaesthesia and Analgesia, 34*, 164–170.
4. Affonso, R. S., Rennó, M. N., Slana, G. B. C. A., & França, T. C. C. (2012). *Revista Virtual de Química, 4*, 146–161.
5. Chaieb, K., Hajlaoui, H., Zmantar, T., Kahla-Nabki, A. B., Rouabhia, M., Mahdouani, K., et al. (2007). *Phytotherapy Research, 21*, 501–506.
6. Chiaradia, V., Paroul, N., Cansian, R. L., Júnior, C. V., Detofol, M. R., Lerin, L. A., et al. (2012). *Applied Biochemistry and Biotechnology, 168*, 742–751.
7. Silvestri, J. D. F., Paroul, N., Czyewski, E., Lerin, L., Rotava, I., Cansian, R. L., et al. (2010). *Revista Ceres, 57*, 589–594.
8. Scherer, R., Wagner, R., Duarte, M. C. T., & Godoy, H. T. (2009). *Brazilian Journal of Medicinal Plants, 11*, 442–449.
9. Paroul, N., Grzegozeski, L. P., Chiaradia, V., Treichel, H., Cansian, R. L., Oliveira, J. V., et al. (2011). *Bioprocess and Biosystems Engineering, 34*, 331–337.
10. Paroul, N., Grzegozeski, L. P., Chiaradia, V., Treichel, H., Cansian, R. L., Oliveira, J. V., et al. (2010). *Journal of Chemical Technology and Biotechnology, 85*, 1636–1641.
11. Liaw, E. T., & Liu, K. J. (2010). *Bioresource Technology, 101*, 3320–3324.
12. Aguedo, M., Belo, I., Ly, M. H., Teixeira, J. A., Belin, J. M., & Waché, Y. (2004). *Food Technology and Biotechnology, 42*, 327–336.
13. Bartling, K., Thompson, J. U. S., Pfromm, P. H., Czermak, P., & Rezac, M. E. (2001). *Biotechnology and Bioengineering, 75*, 676–681.
14. Valério, A., Fiametti, K. G., Rovani, S., Franceschi, E., Corazza, M. L., Treichel, H., et al. (2009). *Journal of Supercritical Fluids, 49*, 216–220.
15. Yang, T., Rebsdorf, M., Engelrud, U., & Xu, X. (2005). *Journal of Agricultural and Food Chemistry, 53*, 1475–1481.

16. Watanabe, T., Shimizu, M., Sugiura, M., Sato, M., Kohori, J., Yamada, N., et al. (2003). *Journal of the American Oil Chemists Society, 80*, 1201–1207.
17. Karra-Châabouni, M., Ghamghi, H., Bezzine, S., Rekik, A., & Gargouri, Y. (2006). *Process Biochemistry, 41*, 1692–1698.
18. Horchani, H., Salem, N. B., Zarai, Z., Sayari, A., Gargouri, Y., & Chaâbouni, M. (2010). *Bioresource Technology, 101*, 2809–2817.
19. Chaibakhsh, N., Basri, M., Anuar, S. H. M., Rahman, M. B. A., & Rezayee, M. (2012). *Biocatalysis and Agricultural Biotechnology, 1*, 226–231.
20. Yadav, G. D., & Yadav, A. R. (2012). *Chemical Engineering Journal, 192*, 146–155.
21. Paroul, N., Grzegozeski, L. P., Chiaradia, V., Treichel, H., Cansian, R. L., Oliveira, J. V., et al. (2011). *Applied Biochemistry and Biotechnology, 166*, 13–21.
22. Katzung, B. G. (2003). *Farmacologia Básica e Clínica* (8th ed.). Koogan: Rio de Janeiro – Guanabara.
23. Holley, R. A., & Patel, D. (2005). *Food Microbiology, 22*, 273–292.
24. Klancnik, A., Piskernik, S., Jersek, B., & Mozina, S. S. (2010). *Journal of Microbiological Methods, 81*, 121–126.
25. Dorman, H. J. D., & Deans, S. G. (2000). *Journal of Applied Microbiology, 88*, 308–316.
26. Lachowicz, K. J., Jones, G. P., Briggs, D. R., Bienvenu, F. E., Wan, J., Wilcock, A., et al. (1998). *Letters in Applied Microbiology, 26*, 209–214.
27. Pei, R. S., Zhou, F., Ji, B. P., & Xu, J. (2009). *Journal of Food Science, 74*, 379–383.
28. Ultee, A., Bennik, M. H. J., & Moezelaar, R. (2002). *Applied and Environmental Microbiology, 68*, 1561–1568.
29. Burt, S. A. (2004). *International Journal of Food Microbiology, 94*, 223–253.
30. Skandamis, P., Tsigarida, E., & Nychas, G. J. E. (2002). *Food Microbiology, 19*, 97–103.
31. Mensor, L. L., Menezes, F. S., Leitão, G. G., Reis, A. S., Santos, T. C., Coube, C. S., et al. (2001). *Phytotherapy Research, 15*, 127–130.
32. Ramalho, V. C., & Jorge, N. (2006). *Quimica Nova, 29*, 755–760.
33. Balasundram, N., Sundram, K., & Samman, S. (2006). *Food Chemistry, 99*, 191–203.