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Composition, Antioxidant, Antibacterial Activities and Mode of Action of Clove (*Syzygium aromaticum L.*) Buds Essential Oil

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Author's contribution

This work was carried out by the main author itself. He designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Subsequently, he managed the analyses of the study in accredited labs, literature searches, read and approved the final manuscript.

Original Research Article

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ABSTRACT

The use of natural antimicrobial compounds, especially extracted from plants, as food preservatives is nowadays widely used, since plant matrices possesses antimicrobial and antioxidant natural products to protect themselves from microbial infection and deterioration as well as for human health benefit properties. The present work was undertaken to examine the antioxidant and antimicrobial activities of clove (*Syzygium aromaticum* L.) buds essential oil. Clove essential oil (CEO) exhibited high amount of total phenolic compounds (TPC) with high radical scavenging activity toward DPPH, ABTS and linoleic acid radicals as well as iron chelating activity. The TPC of CEO was 845 mg GAE g^{-1} demonstrated 509 µmol of TE g^{-1} DPPH radicals scavenging activity and 713 µmol of TE g^{-1} ABTS radicals scavenging activity. The antioxidant capacity of CEO exhibited 94% reduction when evaluated by β-carotene bleaching assay. The Reducing power activity related to iron chelating was 314 µmol of AAE g^{-1}. The composition of CEO exhibit high eugenol content (80.19%) over 16 identified components by GC-MS analysis. Furthermore, CEO exhibited antibacterial activity *in vitro* at low concentrations against tested food borne pathogens applying disc diffusion and microdilutions assays. The minimum inhibitory concentration values (MICs) for tested bacteria which were sensitive to CEO were in the range of 1400–3600µg ml^{-1}. The phenolic components of CEO are

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most active and appear to act principally as membrane permeabilizers which established using staining-DNA fluorescence dye assay. Quantification of membrane permeabilization exudes a positive relationship between the cell membrane damaging and concentrations. Therefore, the CEO can be reliably used in commercial applications as antioxidant, antibacterial and flavoring agent in individual or in combination with common preservatives for controlling the undesirable organoleptic and microbial deteriorations in foodstuffs.

Keywords: Syzygium aromaticum L.; essential oil; total phenolic compounds; antioxidant activity; antibacterial activity; mode of action.

1. INTRODUCTION

Cloves (*Syzygium aromaticum*, syn. *Eugenia aromaticum* or *Eugenia caryophyllata*) are the aromatic dried flower buds of a tree in the family Myrtaceae. Cloves are native to Indonesia and used as a spice in cuisines worldwide [1]. It is well known previously, as an anticarcinogenic [2], as a traditional remedy for asthma [3], disorder of digestive system [4], dental disorders, respiratory disorders, headaches and sore throat in many countries [5]. Besides the reported antimicrobial, antifungal and antiviral properties, the essential oil of *S. aromaticum* shows anti-inflammatory, and anesthetic activities [6].The name of the main constituent of clove essential oil (CEO), is eugenol. Eugenol is a major volatile constituent (45–90%) of clove essential oil obtained through hydro-distillation of mainly (*S. aromaticum*) buds and leaves in addition to acetyleugenol, chavicol, acetyl salicylate and humulenes [1,2]. It is a remarkably versatile molecule incorporated as a functional ingredient in numerous products and has found applications in the pharmaceutical, agricultural, fragrance, flavour, cosmetic and various other industries. Its vast range of activities has been well-researched and includes antimicrobial, anti-inflammatory, antioxidant and anticancer activities. In addition, it is widely used in agricultural applications to protect foods from microorganisms during storage, which might have an effect on human health [7]. Also, eugenol has been classified as 'generally recognized as safe (GRAS)' by the U.S. Food and Drug Administration [8].

Interestingly, natural products have been used to treat microbial growth and numerous essential oils have demonstrated the ability to inhibit the growth of various pathogens. The effect of CEO and eugenol on the growth of Gram-positive (*Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Listeria monocytogenes*) and Gram-negative (*Escherichia coli*; *Salmonella typhi*; *S. choleraesuis*; *Yersinia enterocolitica*, *Pseudomonas aeruginosa*) bacterial strains was investigated [9,10]. At 1000 ppm, eugenol inhibited the growth of the bacteria and complete inhibition was obtained against *P. aeruginosa* at a high concentration of 2000 ppm, which was high in comparison to ampicillin 1mgml^{-1} used as a positive control. The antibacterial activity of eugenol against various pathogens such as *E. coli*, *B. cereus*, *Helicobacter pylori*, *S. aureus*, *S. epidermidis*, *Streptococcus pneumoniae* and *S. pyogenes* was confirmed [11-14]. Moreover, the antimicrobial activity of incorporated eugenol (0.5%) in biofilm with more than 90% against two *P. aeruginosa* pathogens was emphasized. Comparing the antibacterial activity of eugenol to cinnamaldehyde, thymol, carvacrol against *E. coli,* Eugenol possessed the lowest antibacterial activity (MIC value: 1600mgl^{-1}), while their combinations had synergistic interactions resulting in MIC values of 400, 100, 100mgl^{-1} were recorded, respectively [15]. Additionally, the combination of cinnamate and eugenol produced a bactericidal synergistic

effect against *E. coli* O157:H7, *S. typhi* and *L. monocytogenes* and an additive effect against *S. aureus* [16]. Fu et al. [17] reported that the CEO showed inhibitory activity against *S. aureus* and *E. coli*. Another study by Betoni et al. [18] showed that the methanol extract of clove possessed antimicrobial activity against of *S. aureus* strains. Furthermore, Yano et al. [19] claimed that the aqueous extract of *S. aromaticum* at a concentration of 0.04 mg ml^{-1} was able to control *Vibrio parahaemolyticus*, a food borne pathogen. The biological activity of essential oils is generally investigated without emphasis on the mechanism of action. In an *in vitro* study, it is revealed that eugenol induced cell lysis through leakage of protein and lipid contents. In addition, both cell wall and membrane of the treated Gram-negative and Gram-positive bacteria were significantly damaged [20]. This synergistic effect could be explained by the fact that eugenol is able to damage the membrane of Gram-positive and Gram-negative bacteria. It was found that a concentration of 1 mM damaged nearly 50% of the bacterial membrane allowing increased penetration antibiotics and therefore a greater antimicrobial effect [21]. Therefore, CEO and eugenol could be widely applied as a novel food antimicrobial agent to inhibit the growth of bacteria and to suppress the production of exotoxins by *S. aureus* [22].

Several antimicrobial and antifungal agents are thus pursued based on chemical, physical, or bio-control properties; however, many of those substances are inefficient except the synthetic form which affect product quality, or cause severe side effects on human health as well as to the environment [23]. It is worth mentioning that until the present; very few studies have been conducted to reveal analyzing the oil composition, the antimicrobial properties as well as the mode of action of CEO to be utilized in food preservation. Therefore, we have come out with an idea of applying natural antimicrobial agents for different food system uses, especially for meat, chicken and fish processed products. Our study was carried out to reveal the potentiality of *S. aromaticum* EO as an antimicrobial and antioxidant agent to be an alternative to commercial food preservatives. To achieve this purpose the composition, antioxidant, antibacterial activities of *S. aromaticum* EO against some food borne pathogens was investigated. Moreover, *In vitro* assay of the potential *S. aromaticum* mode of action was carried out.

2. MATERIALS AND METHODS

2.1 Chemicals

DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2.2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-carboxylic acid; GA, gallic acid; TCA, Trichloroacetic acid; AA, ascorbic acid; DMSO, Dimethylsulfooxids and PLL, Poly-L-Lysine were obtained from Sigma Aldrich, Germany. TSB, Tryptic soy broth; MHA, Müller Hinton Agar; MHB, Müller Hinton Broth; PCA, Plate count Agar were obtained from Biolife, Italy. Antibiotic such as Ampicillin, Kanamycin, Erythromycin, Tetracycline, Neomycin, Vancomycin, Streptomycin, Linkomycen, Gentamycin and Cefotaxime were obtained from Bioanalyse® Ltd. Sti, Turkey and Blank paper disc, 6 mm diameter was obtained from Becton, Dickinson and company sparks, USA. SYTOX-Green (5mM solution in DMSO) was obtained from, Eugene, Oregen, U.S.A.

2.2 Essential Oil

The pure essential oil of clove *S. aromaticum* L. (CEO) was obtained from the Fragrance and Extraction Factory, Sugar Industrial Integrated Company (SIIC), Cairo, Egypt. According

the company procedure, CEO was extracted from the matured buds after harvesting using the hydro-distillation system. The clove buds was imported from India as a yield of session 2011.

2.3 Bacterial Strains and Propagation

Gram-negative bacterial strains such as (*E. coli, E. coli* O157:H7, *P. aeruginosa, P. fluorescens, S. typhimurium* and *S. typhi*) and gram-positive bacterial strains (*B. cereus, B. megaterium, B. subtilis, Clostridium botulinum, C. perfringens, L. monocytogenes, S. aureus, S. warneri and E. faecalis*) were obtained from the Institute for Fermentation (Institut für Gärungsgewerbe, Berlin, Germany). The propagation of each strain immediately before inoculation using TSB medium at 37°C for 24 h twice under aerobic and anaerobic condition (CO_2 GasPak System) depending on the strain was carried out.

2.4 Determination of Total Phenolic Content (TPC)

The total phenolic content of CEO was analyzed using the Folin–Ciocalteu reagent according to the method of [24] and modified by [25]. Briefly, 0.1ml of appropriate CEO dilution was mixed with 2.5ml of 10-fold diluted Folin-Ciocalteu's phenol reagent and allowed to react for 5 min. Then 2ml of 7.5% Na_2CO_3 solution was added and the final volume was made up to 10ml with distilled H_2O. After 1h of reaction at room temperature, the absorbance at 760nm was measured. The measurements were compared to a prepared standard curve of gallic acid (GA) solution, and the total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of CEO (mg of GAE g^{-1}).

2.5 Antioxidant Activity

2.5.1 DPPH radical scavenging assay

Radical scavenging activity of CEO was determined by a spectrophotometric method based on the reduction of a methanol solution of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) using the method of [27] modified by [25]. Aliquot of 0.1 ml from each CEO dilution was added to 2.9 ml of $6x10^{-5}$ mol methanolic solution of DPPH. The absorbance at 517 nm was measured after the solution had been allowed to stand in the dark for 60 min. The Trolox calibration curve was plotted as a function of the percentage of DPPH radical scavenging activity. The final results were expressed as micromoles of Trolox equivalents (TE) per gram of CEO (µmol TEg^{-1}).

2.5.2 ABTS radical cation scavenging activity

The radical scavenging activity of CEO against ABTS radical cation was measured using the method of [27] which was modified by [25]. ABTS was dissolved in water to a 7mmol l^{-1} concentration. ABTS radical cation was produced by reacting ABTS stock solution with 2.45mmol l^{-1} potassium per-sulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16h before use. The ABTS radical cation solution was diluted with ethanol to an absorbance of 0.70±0.02 at 734nm and equilibrated at 30°C. Aliquot of proper CEO dilution (0.1ml) was mixed with 2.9ml of diluted ABTS radical cation solution. After reaction at 30°C for 20 min, the absorbance at 734nm was measured. The Trolox calibration curve was plotted as a function of the percentage of ABTS radical cation

scavenging activity. The final results were expressed as micromoles of Trolox equivalents (TE) per gram of CEO (μmol of TEg^{-1}).

2.5.3 β-carotene–linoleic acid bleaching assay

The antioxidant activity of CEO was evaluated by the spectrophotometric β-carotene bleaching test [28] with minor modification. A stock solution of β-carotene–linoleic acid mixture was prepared as follows: 0.5mg β-carotene was dissolved in 1ml of chloroform (HPLC grade); 25μl linoleic acid and 200mg Tween 40 were added. Chloroform was completely evaporated by using a vacuum evaporator. Then, 100ml of distilled water was added with oxygen (30 min at a flow rate of 100ml min^{-1}) vigorous shaking. Aliquots (2.5ml) of this reaction mixture were dispensed to test tubes and 0.5 ml of CEO dilution prepared at 4g l^{-1} concentration was added and the emulsion system was incubated for up to 48h at room temperature. The same procedure was repeated with the synthetic antioxidant, BHT as positive control, and a blank containing only 0.5ml of ethanol. After this incubation period, the absorbance of the mixtures was measured at 490nm. The antioxidative capacity of CEO was compared with BHT and blank, the results were expressed as BHA-related percentage.

2.5.4 Reducing power assay

The determination of reducing power was carried out as described by [29]. Briefly, 1 ml of methanolic diluted CEO was mixed with 2.5ml phosphate buffer (0.2 mol, pH 6.6) and 2.5ml ($K_3Fe(CN)_6$,1%). The mixture was incubated in screwed cap tubes at 50°C for 20 min. A portion (2.5ml) of TCA (10%) was added to the mixture, which was then centrifuged at 10.000xg for 10 min The upper layer of the solution (2.5ml) was mixed with deionized water (2.5ml) and 0.5ml ($FeCl_3$,0.1%) then the absorbance was measured at 700nm. The measurements were compared to a standard curve of prepared ascorbic acid (AA) solution, and the final results were expressed as micromoles of ascorbic acid equivalents (AAE) per gram of CEO (μmol of $AAEg^{-1}$).

2.6 Gas Chromatography Mass Spectrometry (GC–MS)

The chemical composition of the essential oil was analyzed using GC–MS technique according to [30] with minor modification. The chromatographic procedure was carried out using a Shimadzu QP2010-GC-MS with autosampler. The sample was diluted 25-30 times with acetone, with 1μL injected into the column. A fused silica capillary column HP5-MS (30mx0.32 mm, film thickness 0.25μm) was used. Helium was used as the carrier gas 1.25 ml min^{-1}, and a split ratio of 1/100 was applied. The oven temperature used was maintained at 60°C for 8 min. The temperature was then gradually raised at a rate of 3°C per min to 180°C per min and maintained at 180°C for 5 min. The temperature at the injection port was 250°C. The components of the test solution were identified by comparing the spectra with those of known compounds stored in the internal library.

2.7 Antibacterial Activity

2.7.1 Inhibitory effect by agar disc diffusion method

The determination of the inhibitory effect of CEO on test bacterial strains was carried out by the agar disc diffusion method [31]. Each strain was freshly propagated for 20-24hr then the culture suspensions were adjusted by comparing against 4 McFarland. Petri dishes with

15ml of Mueller-Hinton Agar (MHA) were prepared, previously inoculated with the culture suspension to produce 10^5Cfuml^{-1}. After the solidification of the media (4°C for 2h), Sterilized filter paper discs (6.0mm) were impregnated with 10µl of diluted CEO with 10% dimethylsulfoxide (DMSO). While, incorporated paper disc with DMSO was used as a control. The inoculated plates were incubated at 37°C for 48h under aerobic conditions except the clostridium dishes were incubated under anaerobic conditions (CO_2 Gas Pak System). At the end of the incubation period, the diameters (mm) of the inhibition zones were measured with caliper. The measurements were done basically for the inhibition zone diameter.

2.7.2 Determination of minimum inhibitory concentration (MIC)

The microdilution broth susceptibility assay was used according to [32] with modification. A stock solution of CEO was prepared in 10% DMSO and then serial dilutions of CEO were made in a concentration range from 0.5 to 20mg ml^{-l}. The 96-well plates were prepared by dispensing into each well, 95µl of 2-fold Mueller-Hinton Broth (MHB), 100µl of CEO from each CEO dilution and 5µl of the inoculants. The inoculums of microorganisms were prepared using 24h cultures and suspensions were adjusted to 4 McFarland standard turbidity. The final volume in each well was 200µl. A positive control (containing inoculum but no CEO) and negative control (containing CEO but no inoculums) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at 37°C for 48 h under aerobic conditions except the clostridium microplate were incubated under anaerobic conditions (CO_2 GasPak System). Additionally, the inhibitory effect of 10 antibiotics was tested against the tested bacterial strains under same condition as positive control. Three replicates of each microassay were carried out and the experiment was carried out in triplicate. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms as mentioned by [33].

2.8 Determination the Mode of Action

The SYTOX-Green uptake assay was performed according to the method described by [34,35] with minor modification. One thousand cells of the each test strains were cultivated in 96-well microtiter plate containing 200µl MHB. After 20h of incubation at 37°C, CEO and SYTOX-Green were added to final concentration of 10mgml^{-1} and 0.2µM, respectively. After additional incubation period for 1h, the cells were washed with Tris buffer (pH, 6.0) and fixed with Poly-L-Lysine (PLL) on glass slid then covered with slid cover. The results were recorded by Microscope (ZEISS, Axioskop 50) equipped with an Olympus digital camera C-4000. Fluorescence color images were captured using Argus X1 software under fluorescence light in absence and presence of CEO. Furthermore, in order to determine the time of CEO induce membrane per meabilization as well as to quantify the performed florescence dye the same cultivation procedure with black 96-well microtiter plate was done. After 20h, CEO at concentrations of 10, 20 and 40mg ml^{-l} and SYTOX-Green at 0.2µmml^{-1} were added. Fluorescence was quantified 10min immediately after application. Measurements were carried out for 300min each 30min interval using a SpectraMaxM2 fluorescence measurement system at an excitation wavelength of 488 nm and an emission wavelength of 540nm. Fluorescence values were corrected by subtracting the fluorescence value of incubated samples in the absence of CEO. Measurements were carried out in triplicate.

2.9 Statistical Analysis

The statistical analysis was carried out using SPSS program with multi-function utility regarding to the experimental design under significance level of 0.05 for the results according to [36].

3. RESULTS AND DISCUSSION

3.1 Total Phenolic Content and Antioxidant Activity of CEO

The amounts of total phenolic content TPC in the CEO was determined spectrometrically and calculated as milligrams of Gallic acid equivalents (GAE) per gram as well as the antioxidant activities of CEO by the DPPH radical scavenging, ABTS, the β-carotene–linoleic acid bleaching and the reducing power were tested. As seen in Table 1, the amount of TPC founded in the CEO was very high reached to 845.04±1.15mg $GAEg^{-1}$ of CEO. Whereas, obtained results exhibited that the DPPH radical cation scavenging activity (RSA) of CEO was 509.45±4.82µmol of TE g^{-1}. Moreover ABTS-RSA was used to determine the evolution of antioxidant activity of CEO, and results are presented in Table 1. Compared with the DPPH radical scavenging activity, the ABTS-RSA of CEO samples was affected similarly to present 713.12± 5.64µmol of TE g^{-1}. Furthermore, the relative antioxidative activity (RAAs) of CEO is given in Table 1. The inhibition values of linoleic acid radicals were estimated as 94.19±1.05% when compared to BHA. A relationship between the DPPH scavenging ability, ABTS and β-carotene bleaching extent was found. Data in Table 1 illustrated the evolution of reducing power of CEO which was 314.24±7.41µmol of $AAEg^{-1}$. It is worth mentioning that, according to these results, there is a relationship between total phenol contents and antioxidant activities. Phenolic compounds, biologically active components, are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups [37]. The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability or radical scavenging activity. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form diphenypicrylhydrazine (non radical) with the loss of this violet color [38]. However, high correlation had been found between TPC, DPPH and ABTS as shown in Table 1 and recorded by [39].

Table 1. Total phenolic content and potential antioxidant activities of Clove EO (mean±SE)

Item	Clove EO
TPC (mg GAE g^{-1})	845.04±1.15
DPPH (µmol of TE g^{-1})	509.45±4.82
ABTS (µmol of TE g^{-1})	713.12± 5.64
B-carotene bleaching[a] (RAA) %	94.19±2.05
Reducing power (µmol of AAE g^{-1})	314.24±7.41

[a]: calculated depending on BHA activity as100%

The β-Carotene bleaching method is based on the loss of the yellow color of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants [40]. On the other hand, Eminagaoglu et al. [41] reported that β-carotene bleaching of the EO was higher than

that of the methanolic extract from *S. cuneifolia*. While, Oke et al. [42] found that the antioxidant activity of methanolic extract was higher than the EO of *S. cuneifolia*. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelating potential [43]. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen peroxide and lipid peroxides to reactive free radicals. Fe^{3+} ion also produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+} ion [44]. The production of these radicals can lead to lipid peroxidation, protein modification and DNA damage. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes [45]. Chelating activity of the extract was determined by the reducing power assay. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of the rate of color reduction therefore allows estimation of the chelating activity of the coexisting chelators [46].

3.2 Chemical Composition of *S. aromaticum* EO

Sixteen signal peaks related to separated components were obtained by GC-MS in *S. aromaticum* essential oil, where only compounds more than 1% were descendingly shown in Table 2. Eugenol (80.19%), eugenyl acetate (7.91%), caryophyllene (3.79%), furan, tetrahydro-3-methyl (2.26%) and 2-propanone, methylhydrazone (1.54%) were observed in content higher than 1% and considered to be 95.69% of CEO composition. Eugenol, eugenyl acetate and caryophyllene were estimated as major components of *S. aromaticum* essential oil. However, our study supports the view that eugenol is the major component for the *S. aromaticum* EO. Whereas, the differences in chemical composition of essential oils depend on climatic, seasonal, and geographic conditions were mentioned before [47]. Essential oils are rich in phenolic compounds such as Eugenol, carvacrol and thymol are widely reported to possess high levels of antioxidant and antimicrobial activity [48,49]. In present studies, eugenol is a major volatile constituent (45-90%) of CEO which is a phenolic component was found as the major compound therefore the EO of *S. aromaticum* showed high antioxidant [2] and antimicrobial activities [50,30].

Table 2. Major components of *S. aromaticum* essential oil determined by GC-MS

Compounds	%
Eugenol	80.19
Eugenyl acetate	7.91
Caryophyllene	3.79
Furan, tetrahydro-3-methyl	2.26
2-propanone, methylhydrazone	1.54

3.3 Inhibitory Effect of CEO by Agar Disc Diffusion Method

Recently, there has been considerable interest in essential oils with antimicrobial activities for controlling pathogens and/or toxin producing microorganisms in foods [51,52]. A few preservatives containing essential oils are already commercially available such as 'DMC Base Natural', 'Protecta One' and 'Protecta Two' which are classified as generally recognized as safe (GRAS) food additives [42,53]. To study the antimicrobial susceptibility,

the agar disc diffusion method using CEO impregnated paper discs against some food borne pathogens was investigated, Table 3. The inhibition zone values of discs for bacterial strains which were sensitive to CEO were in the range of 13.00–25.33mm, Table 3. The highest inhibitory activity was against *L. monocytogenes* and *S. aureus* which showed the lowest MIC (1400 and 1500µg ml^{-1}) and largest growth inhibition zones for agar disc diffusion assay (25.33 and 22.67mm), respectively. While, the lowest inhibitory activity was against *S. typhimurium* which showed the highest MIC (3600µgml^{-1}) and lowest growth inhibition zones for agar disc diffusion assay (13.00mm), Table 3. The inhibition zones of the essential oil for each assay on test bacteria showed a significant correlation with MIC values, Table 4. Control treatment (DMSO) did not show an inhibitory effect on any of the tested bacteria. This active essential oil for all tested bacteria will be helpful devising antimicrobial formulations with which to protect foods against infection by multiple pathogens [42].

Table 3. Inhibitory effect of CEO by agar disc diffusion assay against some food borne pathogenic bacterial strains *In vitro*

Organism	Inhibition zone diameter (mm)	
	Gram	CEO
B. cereus.	+	16.67±0.19
B. megaterium	+	19.33±0.38
B. subtilis	+	15.00±0.33
CL. botulinum	+	18.33±0.19
CL. perfringens	+	19.33±0.19
E. coli	−	21.67±0.19
E. coli O157:H7	−	21.00±0.33
L. monocytogenes	+	25.33±0.51
E. faecalis	+	21.33±0.38
P. aeruginosa	−	19.00±0.33
P. flurocence	−	19.67±0.19
S. typhimurium	−	13.00±0.33
S. typhi	−	15.67±0.38
S. aureus	+	22.67±0.38
S. warneri	+	19.00±0.33

3.4 Minimum Inhibitory Concentrations (MIC) of CEO against Some Food Borne Pathogens

The MICs of CEO and some common antibiotics (ampicillin, erythromycin, linkomycen, cefotaxime, vancomycin, kanamycin, neomycin, tetracycline, streptomycin and Gentamycin) against some food borne pathogens were shown in Table 4. All the tested bacteria seem to be sensitive to CEO. However, *L. monocytogenes* was more sensitive to the essential oil than the other tested bacteria. The highest inhibitory activity was against *L. monocytogenes* followed by *S. aureus* which showed the lowest MIC 1400 and 1500µgml^{-1}, respectively. While the highest resistance bacteria to CEO was *S. typhimurium* and *S. typhi* where 3600 and 3200µg ml^{-1} were recorded, respectively. The sensitivities of studied bacteria against ampicillin, erythromycin, linkomycen, cefotaxime, vancomycin, kanamycin, neomycin, tetracycline, streptomycin and gentamycin are given in Table 4. The MIC values of tested antibiotics were in the range of 09 – 40µg ml^{-1}, Table 4. Comparing the effect of CEO with tested antibiotics against the tested bacterial strains, the activity of the antibiotics is around 100-fold of the CEO.

Table 4. Minimal inhibitory concentrations of CEO and common antibiotics against some pathogenic bacterial strains *In vitro*

Organism	MIC[a] µg ml^{-1}											
	Gram	CEO	Ampicillin	Erythromycin	Linkomycen	Cefotaxime	Vancomycin	Kanamycin	Neomycin	Tetracycline	Streptomycin	Gentamycin
B. cereus.	+	2500	--	32	20	22	40	30	26	38	28	24
B. megaterium	+	2200	--	26	24	28	38	32	26	34	26	22
B. subtilis	+	2800	--	28	20	24	22	23	24	26	24	28
CL. botulinum	+	2600	22	23	24	25	25	29	19	21	13	17
CL. perfringens	+	2400	24	24	23	23	22	26	23	18	12	24
E. coli	−	2000	--	20	20	--	25	25	21	25	21	15
E. coli O157:H7	−	2300	19	--	--	--	26	19	18	15	18	22
L. monocytogenes	+	1400	21	27	22	29	29	20	24	26	28	17
E. faecalis.	+	2400	21	19	19	15	29	18	14	16	18	11
P. aeruginosa	−	2200	2	22	21	19	25	26	14	22	18	16
P. flurocence	−	2500	21	22	10	28	28	12	23	21	14	19
S. typhimurium	−	3600	19	16	--	--	25	17	15	09	18	16
S. typhi	−	3200	21	23	19	26	27	27	23	23	13	24
S. aureus.	+	1500	22	25	23	23	22	26	22	26	15	27
S. warneri.	+	2700	21	22	28	21	29	26	22	26	25	17

[a]: the MIC was calculated as µg ml^{-1} medium--: not detected

On the other hand, the CEO shown to be active against all tested strains. In contrast, some of those strains were not susceptible to some antibiotics even used in very high concentrations. This active CEO for all test bacteria will be helpful devising antimicrobial formulations with which to protect foods against infection by multiple pathogens as well as make the food flavor much better. Some of tested strains such as *E. coli* O157:H7 is a concern to public health on a global scale and is found in a wide variety of foodstuffs including meat and meat products, milk, yogurt, water, salad vegetables, fruits, fruit juices and cider. Pasteurization and cooking are adequate methods of ensuring that viable cells are eliminated, but heat treatment is not desirable for all foods and cross-contamination cannot always be prevented [54]. Controlling the numbers and growth of those pathogens, therefore remains an important objective for sectors of the food production industry.

3.5 Potential Mode of Action of *S. aromaticum* EO

The CEO mode of action related to membrane permeabilization by the SYTOX-Green uptake to check the activity of CEO against foodborne pathogens was detected. In a qualitative approach, an assay based on the uptake of the fluorogenic SYTOX-Green dye to check the binding of CEO to the cell wall and plasma membranes of sensitive foodborne pathogens was followed by permeabilization of the membrane was used. The bacterial strains *B. cereus, B. megaterium, B. subtili, Cl. botulinum, Cl. perfringens, E. coli, E. coli* O157:H7, *L. monocytogenes, P. aeruginosa, P. flurocence, S. typhimurium, S. typhi, S. aureus, S. warneri* and *E. faecalis* were tested for CEO induce membrane per meabilization. Therein, one example of effect of CEO on *E. coli* O157:H7 was shown as result of this investigation in Fig. 1. A strong SYTOX-Green fluorescent related to uptake of the SYTOX-Green fluorogenic dye and staining of the nucleic acid was observed when all strains were incubated with SYTOX-Green and 10 mg CEO ml^{-1} Fig. 1-B. In contrast, no DNA-SYTOX-Green fluorescence was detected when *E. coli* O157:H7 was incubated with SYTOX-Green in the absence of CEO Fig. 1-D. the SYTOX Green dye had no effect on bacterial growth and only displays a very faint fluorescence when not bound to DNA as shown Fig. 1-D. This observation confirmed that CEO cause intercellular uptake of the dye under these conditions. In order to test the CEO mode of action against some food borne pathogens the influx of fluorogenic SYTOX-Green dye through the fungal cell membrane was established by [34]. Likewise, inducing membrane per meabilization was shown by the application of this technique [35]. Hence, only at concentrations those are several ten to hundredfold higher than the minimal inhibitory concentration, the antimicrobial agents can be detected inter cellulary [55,56]. It could qualitatively be explained that CEO readily per meabilizes the membrane of all tested strains at $10mgml^{-1}$ Fig. 1. Therefore, antimicrobial activity of CEO might be due to pores formation through the cell wall or penetrate the cell membranes by aiding of specific CEO receptors.

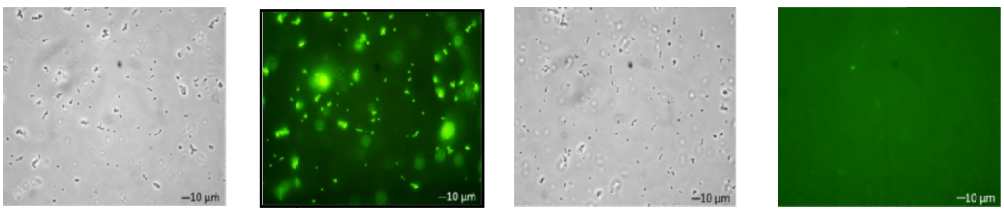

Fig. 1. *E. coli* O157:H7 SYTOX-Green dye uptake in absences or presence of CEO as exemplary shown *In vitro*. (A)-Light photo and (B)-Florescence photo for treated *E. coli* O157:H7 strain with 10mg ml^{-1} CEO and 0.2µM SYTOX-Green. While, (C)-Light photo and (D)-Florescence photo for untreated *E. coli* O157:H7 strain. The same finding was shown for all tested bacterial strains

The SYTOX-Green uptake assay was also used to analyze the kinetics of AFP induced membrane permeabilization in order to quantify the membrane per meabilizing potential of CEO against all tested strains. For all test strains Fig. 2 illustrated the obtained results from *E. coli* O157:H7, *E. faecalis, L. monocytogenes, B. cereus, S. aureus, Cl. botulinum, P. flurocence* and *S. typhi* selected from tested strains. Therein, obtained data suggested that the degree of CEO activity can be directly correlated with increasing of measured fluorescence values. The results indicated that measuring of fluorescence at certain points in time revealed that the plasma membrane of all foodborne pathogens which were partly per meabilized even after 10min. During subsequence incubation time, the fluorescence values were changed depends on the strain and the concentration exude a positive relation between the fluorescence values with increasing the time was found. Generally, CEO at 10 and 20mg ml^{-1} exhibit remarkably effect on the cell wall of all tested strains as well as at 40 mg ml^{-1} Fig. 2. Significant differences (P<0.05) have been found between 20 and 40 mg ml^{-1} applied concentration after 0.5 and 2 hours even till the end of incubation period mean that strains membranes were per meabilized. The increasing value is not the same because this dependent on each strain membrane but the trend of the per meabilization was similarly shown. The most dramatically effect with the same trend was shown after 5 hr. Our results were associated together indicated that CEO has highly antibacterial activity against tested foodborne pathogens. Although, the antimicrobial properties of essential oils and their components have been previously reviewed. The mechanism of action of some other EO has not been studied in great detail [57]. Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell [58]. An important characteristic of EOs and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria disturbing the structures and rendering them more permeable [59]. Leakage of ions and other cell contents can then occur [60]. Generally, the EOs possessing the strongest antibacterial properties against foodborne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol [57]. It seems reasonable that their mechanism of action would therefore be similar to other phenolics; this is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force (PMF) electron flow, active transport and coagulation of cell contents [61]. Locations and mechanisms in the bacterial cell thought to be sites of action for EO components: degradation of the cell wall [62], damage to cytoplasmic membrane [60], damage to membrane proteins [63], leakage of cell contents [57], coagulation of cytoplasm [64] and depletion of the proton motive force [65], hydroxyl group in phenolic compounds such as carvacrol and thymol has been confirmed [60].

Components of EO also appear to act on cell proteins embedded in the cytoplasmic membrane. Enzymes such as ATP ases are known to be located in the cytoplasmic membrane and to be bordered by lipid molecules. Two possible mechanisms have been suggested whereby cyclic hydrocarbons could act on these. Lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and distort the lipid–protein interaction; alternatively. Direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible [61,66].

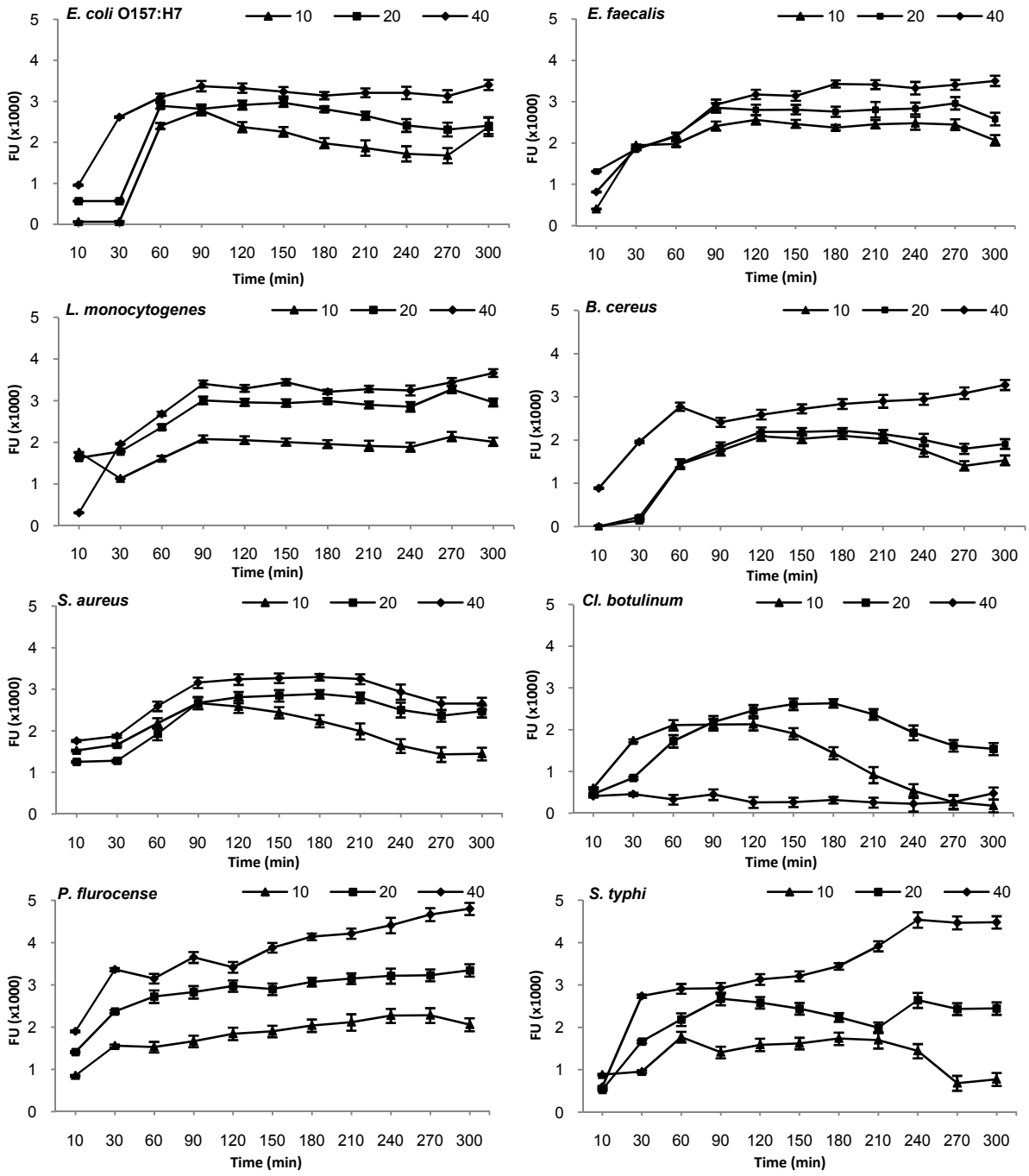

Fig. 2. SYTOX-Green uptake by different pathogenic bacterial strains conducted to plasma membrane permeabilization. Strains were incubated in the presence of 0.2µM SYTOX-Green and 10, 20 and 40mg ml^{-1} CEO. Fluorescence values were corrected with the baseline fluorescence which was determined by incubating the strains in the absence of CEO. Measurements were carried out in triplicate and results expressed as (mean±SE)

4. CONCLUSION

Cloves (*S. aromaticum*) buds essential oil (CEO) exhibited high amount of TPC with high radical scavenging activity toward DPPH, ABTS and linoleic acid radicals as well as chelating activity toward iron element. The composition of CEO exhibit a high eugenol content over 16 identified components by GC-MS analysis. The CEO exhibited antibacterial activity at low concentrations against tested food borne pathogens *In vitro*. The phenolic components are most active and appear to act principally as membrane per meabilizers which established using staining-DNA fluorescence dye. The CEO can be reliably used in commercial applications as antibacterial and flavoring agent in individual or in combination with common preservatives for controlling the undesirable organoleptic and microbial deterioration in some food modules such as meat, chicken and fish products.

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COMPETING INTERESTS

Author has declared that there are no competing interests.

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