Journal of Food Measurement and Characterization
https://doi.org/10.1007/s11694-021-01196-y

ORIGINAL PAPER

Evaluation of chemical composition, antioxidant, antibiofilm and antibacterial potency of essential oil extracted from gamma irradiated clove (*Eugenia caryophyllata*) buds

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Received: 6 August 2021 / Accepted: 13 October 2021

Abstract

The efficacy of γ-rays is known as a method for decontamination of food, herbs and spices. In the present study, clove buds (CB) were irradiated at 10 kGy by ^{60}Co irradiator. Essential oils (EOs) recovered from un-irradiated CB and irradiated (ICB) were evaluated for their chemical composition by GC–MS, antioxidant, antibiofilm and antibacterial activity. The minimum inhibitory concentration (MIC) and growth curves against four tested multi-drug resistant (MDR) bacterial pathogens; *S. aureus*, *B. cereus*, *E. coli* and *K. pneumoniae* were also evaluated. The liberation of cellular contents, as well as the alterations in cell membrane permeability, together with ultrastructural changes in the pathogen morphology, have been evaluated utilizing spectrophotometer, scanning electron microscope (SEM) and energy dispersive X-ray fluorescence spectrometry (EDXFS). Results revealed that EOs recovered from ICB leads to a rise in the phenolic and flavonoid contents by 3.82 and 3.44 mg/g of oil, respectively. Antioxidant potency was elevated by 6.63% for ICB recovered EOs compared to EOs from CB. Antibiofilm and antibacterial activities were improved against all tested MDR pathogens accompanied by decline in their cell growth. The MIC of EOs recovered from ICB raised up the release of bacterial intracellular proteins and DNA/RNA contents significantly ($p < 0.05$) in time dependent manner. The leakage of bacterial contents had been supported by the increase in the release of C, O, N, P, Mg with presence of new elements (Ca and S) in *S. aureus* treated with MIC of EOs recovered from ICB. Moreover, cell wall/membrane damages and shrinkage in *S. aureus* cells was clearly observed in SEM images. This study concluded that γ-rays at dose 10 kGy has a significant potential to stimulate antioxidant, antibiofilm and antibacterial potency of EOs recovered from CB due to increase in phenolic and flavonoid contents. This could improve the uses of EOs recovered from ICB will support the efforts to find a natural and more potent antioxidant, antibiofilm, and antibacterial against MDR pathogens, which is useful in food and pharmaceutical industries.

Keywords Multi-drug resistant · Pathogens · Herbal irradiation · Gamma radiation

Introduction

Essential oils (EOs) are volatile substances naturally formed via plants as secondary metabolites [1]. The EOs considered as a fundamental, safe, good for health, cost effective, and powerful source for novel bioactive chemical compounds or mixtures that use in food processes, preservation, therapeutic and medicinal fields. Essential oils extracted from clove (*Eugenia caryophyllata*) buds are well known globally to exhibit pharmacological and biological activities as; antioxidant, anticancer, anti-inflammatory, antibiofilm as well as antimicrobial [2] due to its high content of phenolic and flavonoid derivatives [3, 4]. Clove buds oils show excellent antibacterial, antioxidant along with anti-inflammatory properties that are attributable to the presence of eugenol, which is the major component in

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Published online: 25 October 2021

those plants' essential oils [5]. Generally, these activities need to be improved to face challenges of adverse environmental effects and development of bacterial resistance. Basically, the characteristic pharmacological and biological actions of essential oils might be related to their major and minor constituents and the ratio between them, which play a crucial role in synergistic effect.

Therefore, changing in chemical composition of EOs and the ratio between the active constituents could increase their activities. Irradiation techniques seem to offer this advantage, naturally and safely. Most countries have permitted food irradiation as a method of protecting a variety of foods, particularly since the alternative methods, typically include food fumigation by different chemicals such as, ethylene oxide, ethylene dibromide and methyl bromide [6, 7].

Gamma rays are high energy photons released from nucleus during radioactive delay process and have been used in a variety of situations for many years to inactivate microorganisms e.g. medical products, food products, municipal sewages and sludge sewages [8]. Radiation sometimes has unwanted actions on sensory aspects of a few meals, according to Groth [9], but these effects are usually minor, especially within the approved dose levels. Gamma (γ) rays, is an electromagnetic radiation, arising from the (^{60}Co) with high penetrating power pass through materials without leaving any residues. It has been used globally for more than 40 years as advanced and safe sterilization techniques for medicinal, pharmaceutical, herbs and food products by the International Atomic Energy Agency (IAEA), World Health Organization (WHO) and Food and Agricultural Organization (FAO) [10].

Gaspar et al. [11] discovered a rise in the majority of volatile chemicals as the radiation rate increased, most likely caused by the radiation-induced cell burst. So far, a vast number of studies were investigated the biological behavior of the clove essential oils and others about the potency of gamma radiations on pathogenic bacteria, but studies on chemical composition, antioxidant, antibiofilm and antibacterial efficiency of EOs extracted from irradiated clove buds (CB) are rare. Thus, the focus of the current research was to assess the influence of gamma irradiation on the chemical composition of extracted EOs. Additionally, demonstrate if this dose of gamma radiation (10 kGy), could affect the antioxidant potency of EOs recovered from CB. As well as, their potency as antibiofilm, antibacterial against MDR food borne pathogens (*S. aureus* and *B. cereus*) as Gram positive and *(E. coli* and *K. pneumoniae*) as Gram negative bacteria.

Materials and methods

Clove buds

Clove (*Eugenia caryophyllata*) buds were purchased from traditional and folk medicine plants store in Cairo-Egypt and authenticated in the Department of Botany, Faculty of Agriculture, Cairo-Egypt.

Chemical

Anhydrous sodium sulphate, Folin-Ciocalteu's phenol reagent, gallic acid, aluminum chloride ($AlCl_3$. 6 H_2O), quercetin, 1,1-diphenyl-2-Picrylhydrazyl (DPPH), dimethyl sulfoxide solution (DMSO) and crystal violet dye were purchased from Sigma-Aldrich Co., Milano, Italy, glycerol, glucose, sodium acetate and ethanol were obtained from Roth Company, Overland Park, KS, USA. While, nutrient broth, Mueller–Hinton medium (MHA), tryptic soy broth (TSB), amoxycillin/clavulanic acid (AMC, 30 µg) were purchased from Oxoid, UK.

Gamma irradiation process

Six polypropylene bags, each contained 100 g of mature and intact clove buds (CB), were prepared. Three bags were exposed to 10 kGy of gamma radiation with dose rate 1.1 kGy/h from ^{60}Co source (Gamma cell 220, Canada) in atmospheric air at 25 ± 1 °C. Reference alanine dosimeters traceable to National Physical Laboratory (NPL), Uk was used to measure the dose during irradiation process. The irradiation treatments have been done at the National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Cairo, Egypt. Remaining three bags were used as un-irradiated clove buds (control).

Extraction of essential oil

Both groups; CB and ICB bags, were separately crushed and used for extraction of the essential oils (EOs). Then, separated by hydro-distillation method using a clevenger-type apparatus for 4 h based on the method explained by British Pharmacopoeia [12]. The CB recovered essential oils were dried over anhydrous sodium sulphate. The purified EOs extracted from each group; CB and ICB were stored separately in dark glass vials at 4–8 °C until use in different analysis.

$$\text{Essential oil yield (\%)} = \text{Wt of extracted oil (g)/Wt of dry plant} \times 100 \quad (1)$$

Before each microbiological analysis, the EOs were dissolved in 10% DMSO to reach the required concentration (25 mg/ml).

Chemical composition of clove essential oil

GC–MS analysis

Gas chromatography/mass spectrometry (GC–MS) analysis of EOs extracted from CB and ICB was achieved via an Agilent 7000 Series Triple, Quad gas chromatograph interfaced to a mass spectrometer (GC/MS) outfitted with an Elite-5MS (5 percent diphenyl/95 percent dimethyl poly siloxane) fused a capillary column (30 × 0.25 µm ID × 0.25 µm DF). For GC–MS recognition an electron ionisation system with ionising energy of 70 eV was used. Helium gas (99.999 percent) was utilized as carrier gas at a constant flow rate was 10.0 ml/min and injection volume of 2.0 µl was employed (split ratio of 10:1); the injector temperature 250 °C; ion-source temperature 200 °C. The oven temperature was programmed from 110 °C (iso thermal for 2 min) with a rise of 10 °C/minutes to 200 °C, then 5 °C/minutes to 280 °C, ending with a 9 min iso thermal at 280 °C, mass spectra were acquired at 70 eV: a scan interval of 0.5 s and fragments from 45 to 450 Da. The relative % quantity of each constituent was considered by multiplying it by the total areas. Turbomass software was supported for handling mass spectra and chromatograms. Clarification of the components were done by comparing their recorded mass spectra to standard mass spectra from the National Institute of Standards and Technology (NIST) libraries, The name, structure and molecular weight of the components of the test materials were determined [13]. Whereas, the spectrum of the unknown components were kept in the NIST library.

Total phenolic content (TPC)

Total phenolic contents in EOs recovered from CB and ICB were spectrophotometrically assessed with Folin Ciocalteu reagent method using gallic acid standard curve according to Singleton and Rossi [14]. The absorbance of TPC values were measured by spectrophotometer (Jasco V530, Japan) at 725 nm. The results for TPC were expressed as mg of gallic acid equivalent per gram of extracted oils (mg GAE/ g oil).

Total flavonoid content (TFC)

Aluminium chloride ($AlCl_3$. 6 H_2O) method with quercetin standard curve according to Marinova et al. [15] was utilized. The absorbance values were determined at 510 nm by spectrophotometer (Jasco V530, Japan). Standard curve of quercetin was utilized and the results of TFC were expressed as mg of quercetin per gram of extracted oils (mg QE/ g oil).

Antioxidant activity using DPPH radical as scavenging activity

Antioxidant activity of both extracts; CB–EOs and ICB–EOs was resulted in terms of hydrogen donating or radical-scavenging capability using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay and the samples calculated using the following formula of Salmanian [16]. The reaction mixtures were read via UV–visible spectrophotometer (Jasco V530, Japan) at 517 nm. Radical scavenging activity was recorded as percent of inhibition and was calculated by means of the following formula.

$$\text{Antioxidant activity (\%)} = \text{A(blank)} - \text{A (sample)/A(blank)} \times 100 \quad (2)$$

where A: is the light absorbance, blank: use DPPH reagent only and sample: use DPPH reagent in addition to the EOs.

Bacterial strains and culture preparation

The antibacterial and antibiofilm efficiencies of EOs from CB and ICB were evaluated against four MDR pathogens; two Gram positive; *Staphylococcus aureus* (*S. aureus*) and *Bacillus cereus* (*B. cereus*), besides two Gram negative; *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*). These strains were obtained from drug radiation research department at NCRRT, Cairo, Egypt. They were pre-identified by MALDI-TOF mass spectrometry at Children's Cancer Hospital 57357, Giza, Egypt. All strains were stored as 15% glycerol stocks at − 20 °C until use. To achieve optimal growth, 100 µl of the thawed microbial stock suspension into 5 ml of nutrient broth, followed by overnight incubation at 35 ± 2 °C for 18 h. For the experiments, 100 µl of overnight culture added to 10 ml of TSB and left at the same previous conditions for 2–3 h. Then, adjusted to 0.5 McFarland standard (1–2 × 10^7 cfu/ml). This prepared strain culture became ready to use in the following assays.

Antibacterial activity assays

Disc diffusion assay and minimum inhibitory concentration (MIC) determination

The antibacterial activity of essential oils extracted from CB and ICB against *S. aureus, B. cereus, E. coli* and *K. pneumoniae*, was evaluated by the disk diffusion method described by CLSI [17]. Briefly, Whatman No.1 sterile filter paper discs, 6 mm in diameter were placed on the surface of freshly inoculated MHA plates with 100 µl of pre-adjusted tested pathogen. Then, each disc loaded with 20 µl of EOs from CB or ICB (25 mg/ml). Moreover, DMSO and

antibiotic disc (AMC, 30 µg) were utilized as controls. All plates were incubated at 35 ± 2 °C for 18 h. The degree of antibacterial activity of both EOs was evaluated by measuring the diameter of inhibition zone (DIZ) around each disc in mm scale. On the other hand, the minimum inhibitory concentration (MIC) of both oils, CB–EOs and ICB–EOs against the four tested MDR pathogens was determined [18]. Different concentrations (25, 12.5, 6.25, 3.125, 1.5, 0.78, 0.39, 0.19 µl/ml) of CB–EOs or ICB–EOs were inoculated into MHA plates. One hundred µl of pre-adjusted suspension of the tested pathogen was spread over the surface of the plate and incubated at 35 ± 2 °C for 18 h. The MIC is described as the lowest concentration of the tested EOs which has no visible microbial growth after the incubation period. The tests were performed three times and the average of three values was calculated.

Growth curve assay

The growth curve experiment at different time intervals was performed to examine the bactericidal effect of MIC and double MIC (2 × MIC) of CB–EOs and ICB–EOs, on the bacterial growth at different time (h). Each of the four pathogen suspensions, prepared as mentioned before, was divided into five flasks treated with MIC and 2 × MIC of EOs from CB beside, MIC and 2 × MIC of EOs from ICB, plus the control, treated with 10% DMSO. All were incubated at 35 ± 2 °C for 18 h with shaking 200 (rpm). At selected time intervals; 0, 2, 4, 6, 8 and 24 h, 1.0 ml of each flask was inoculated to test tube contain 9.0 ml of sterile PBS and serial dilution was done (10^{-1}–10^{-12}). After that, 100 µl from each dilution was inoculated on separate MHA plate and incubate at 35 ± 2 °C for 18 h for manual colony counting. The obtained colony number from each plate was expressed as colony forming unit (cfu) per ml. The average of three counts was used to calculate $\log_{10}$ cfu/ml. The $\log_{10}$ cfu/ml was plotted against treatments in different time intervals to check the antibacterial activity rate and potency of various EOs concentrations.

Antibiofilm activity

The antibiofilm activity of selected concentration of EOs from CB or ICB was evaluated through testing its ability to prevent the pathogen adherence using spectrophotometric method, as illustrated previously with Barapatre et al. [19]. Each well in microtiter plate was filled by 180 µl of TSB supplemented with 0.1% glucose (v/v), and inoculated with 10 µl of pre-adjusted pathogenic culture. Then, 10 µl of selected concentration of EOs from CB or ICB was added. After incubation for 48 h at 35 ± 2 °C, content of every well was rinsed 3 times by PBS, pH 7.2 to eliminate free-floating 'planktonic' bacteria. After that, the biofilm formed by adhering bacteria was fixed with sodium acetate (2% w/v) and stained for 20 min with crystal violet dye (0.1% w/v). The overload stain was washed away with distilled H_2O. After drying, 200 µl of 95% ethanol was added to the wells to dissolve the remaining dye that had enclosed within the biofilm. Finally, an ELISA reader (Huma Reader HS, Human, Wiesbaden, Germany) was used to determine the absorbance at 570 nm. The achieved absorbance readings were considered as an indicator of an index of bacterial adhering to the surface of the well walls, which represent intensity of formed biofilms. The percentage (%) of antibiofilm potency was calculated using the Eq. (3):

$$\text{Antibiofilm potency } \% = \text{A(control)} - \text{A (sample)}/\text{A(control)} \times 100 \quad (3)$$

where A is the absorbance reading, control is TSB inoculated with tested pathogen alone, and sample is TSB with tested pathogen and selected EOs concentration.

The test was performed in triplicates, then the data was averaged, and the standard error was calculated.

Antibacterial and antibiofilm mechanism of EOs extracted from CB and ICB

Determination of cell content leakage

Quantification of proteins, DNA/RNA and elements leakage from the bacterial cells was applied as a marker for bacterial cell membrane breakdown. The release of cell contents from the selected MDR pathogens into the extracellular media was monitored by different methods. The first step in all methods was carried out as follows: after adjusting the log phase of the tested pathogen growth to 0.5 McFarland standard, each pathogen culture (250 ml) were divided into 5 portions. Each portion was treated with one of following; MIC or 2 × MIC of EOs from CB or MIC or 2 × MIC of EOs recovered from ICB, and the control (without treatment). Then, all flasks were incubated at 35 ± 2 °C for 0, 2, 4 and 8 h. From each portion at a selected time, about 10 ml of the suspension were immediately centrifuged at 10,000×*g* for 10 min at 4 °C. Then, the supernatant was separated into three tubes. First one was used to calculate the contents of proteins released from the bacterial cytoplasm spectrophotometrically by measuring the absorbance at 595 nm, as described by Bradford [20]. While, the second tube was filtered through membrane filter with 0.2 µm pore size (Gelman Sciences, Ann Arbor, Mich.) to determine the amounts of the DNA and RNA released from the bacterial cytoplasm at optical density 260 nm [21, 22]. Additionally, the third tube of selected pathogen only was used to determine the change in the cell morphology using scanning electron

microscope (SEM) and to detect the released elements from pathogen cell using energy dispersive X-ray fluorescence spectrometry (EDXFS).

Scanning electron microscope (SEM) and energy dispersive X-ray fluorescence spectrometry (EDXFS)

Scanning electron microscope (SEM) (Philips XL 30 ESEM, Jeol, Japan) was used to examine the morphological alterations of the highest sensitive bacteria before and after treated with MIC of EOs extracted from CB and ICB as the method already described by Bajpai et al. [23].

Energy dispersive X-ray fluorescence spectroscopy (EDXFS) is applied to examine the elements qualitatively and quantitatively by measuring the re-emitted characteristic X-rays from the elements. Energy dispersive XRF spectrometers are useful for non-destructive purposes, since they can measure a broad variety of elements. The mapping of elements intensity was plotted as a count per second (cps) on Y-axis, while energy of electron volt (eV) was plotted on X-axis. Where, cps stands for intensity of elements and eV stands for position on X-axis when intensityof the element was taken.

Statistical analysis

All tests were conducted in a completely randomized design in independent triplicates to confirm the reproducibility of the results. The report of the data was given as mean ± SE. Duncan's multiple range test determined significant differences, at the 95% confidential level ($p < 0.05$) using statistical SPSS software version 25 (Chicago, SPSS Inc., USA) [24, 25]

Results and discussion

Essential oil yield and chemical composition of CB–EOs and ICB–EOs

As shown in Table 1, exposing clove buds (CB) to 10 kGy of gamma irradiation emitted from ^{60}Co has a noticeable change in the oil yield, concentration of phenols and flavonoids as well as antioxidant activity.

The percentage yield of volatile oil extracted from CB and ICB were 10.68 and 12.74%, respectively. Also, a noticeable increase in the TPC for oils recovered from ICB equal to 13.30 mg GAE/g oil relevant to oils recovered from the control sample (CB) 9.30 mg GAE/g oil. Furthermore, the TFC increased by 3.44 mg QE/g oil compared to CB recovered oil. In addition, the antioxidant potency increased by 6.63%.

Table 1 Impact of gamma irradiation on the volatile oil yield %, phenolic content, flavonoids content and antioxidant activity % by (DPPH%) of clove buds (CB) as a control and after exposed to 10 kGy (ICB) irradiated clove buds (ICB)

Oil characterization	(CEO) Control	(ICEO) 10 kGy
Oil yield %	10.68 ± 0.558	12.74 ± 0.611
Phenolic content (mg GAE/g oil)	9.32 ± 0.350	13.14 ± 0.095
Flavonoids content (mg QE/g oil)	6.51 ± 0.316	9.95 ± 0.523
DPPH %	87.30 ± 0.490	93.93 ± 0.600

mg GAE/g oil: mg of gallic acid equivalent per gram of extracted oil, mg QE/g oil: mg of quercetin per gram of extracted oil. Values are mean ± SE (n = 3)

The dose of gamma radiation (10 kGy) is recommended by many global organizations as Codex Alimentarius Commission has certified the utilizing of gamma radiation as a mainly secure approach for food microbiological sanitization, counting herbs and spices. Also, the World Health Organization and scientists concurred that γ-rays rate ≤ 10 kGy not create any toxicological alterations or enhancement in irradiated food [26, 27]. The higher oil yield, TPC and TFC in EOs recovered from ICB compared to that recovered from CB may be related to radiation-induced breakdown of cell wall structure, as reported by other authors before [28, 29]. In the same concern, El-beltagi et al. [30] found that, the highest yield of essential oils extracted from celery seeds was registered at dose level of 10 kGy of gamma irradiation. While, the noticeable elevation in TPC and TFC of ICB–EOs could be due to gamma irradiation's breakdown of bigger phenolic substances into minor ones, as suggested by Suhaj and Horváthová [31]. These findings are consistent with those of Aly et al. [28], who found that γ-rays up to 10 kGy enhanced phenolic and flavonoid contents in *Moringa oleifera* oil. Also, they assumed that, the increase in total phenolic contents may be attributed to activity of phenylalanine ammonia-lyase, the key enzyme for the metabolism of phenolic compounds. Likewise, increasing the flavonoid content may be due to the damages induced by irradiation [32]. Consequently, increasing the phenolic and flavonoid of EOs content, causing increase in the antioxidant potency. These results are in accordance with Daneshzadeh et al. [33] who revealed that when testing the ethanolic extract from aerial parts of *Eryngium billardieri* the TPC and TFC as well as antioxidant activity were increased by increasing in extraction concentration. Furthermore, Daneshzadeh et al. [33] reported that according to statistical analysis there was a significant ($P < 0.01$) linear correlation between DPPH free radicals scavenging activity and TPC (with r value 93%) and TFC (with r value 93%). Same observation about increasing in antioxidant activity is

derived from the phenolic compounds and there is a direct correlation between the antioxidant activity and the amount of phenolic compounds as well as between the antioxidant activity and the amount of TFC [34–37].

The higher DPPH scavenging activity of EOs recovered from ICB compared with EOs recovered from CB that observed in the present study may be elucidated by a synergistic action between phenolic compounds, after they had increased in EOs recovered from ICB. However, phenolic compounds act as free radical terminators throughout scavenging or chelating procedure [22]. The current results are in accordance with Fatemi et al. [38] who reported an increase in antioxidant activity of EOs extracted from irradiated *Rosmarinus officinalis* at dose level 10 kGy measured by DPPH. Gülçin et al. [39] one of several authors who reported that clove essential oil has high percentage of antioxidant compounds as food preservative. But in difference with other authors as Suhaj and Horváthová [31], who reported no changes in the TPC of irradiated clove EOs at doses 5, 10, 20 kGy compared to un-irradiated one. These differences may be attributed to the chemical composition of clove and the portion that exposed to radiation.

The chemical profile of EOs recovered from CB and ICB were characterized by GC–MS analysis. The irradiation dose level, 10 kGy affects the oil constituent ratios as declared in Table 2. A total of 11 compounds were recognized in CB representing 98.92% of total percentage. While, in EOs recovered from ICB the total number of compounds was decreased to 6 representing 98.23% of total percentage. The major compounds in both EOs were euginol 72.27% and 63.5%, respectively. Followed by caryophyllene (11.04%) and phenol, 2-methoxy-4-(2-propenyl)-, acetate (6.31%) in CB. On the other hand, in EOs recovered from ICB the euginol followed by phenol, 2-methoxy-4-(2-propenyl)-, acetate (26.32%), then caryophyllene (4.27%). In addition, five minor compounds in EOs recovered from CB; anethole, germacrene-D, isoeugenol, delta-cadinene and methyl salicylate were not detected in ICB–EOs. This in consent with Gaspar et al. [11] findings, who reported that γ-rays up to 30 kGy doesn't modify the qualitative content of clove aroma or flavour, as well as no new compounds are generated in the EOs recovered from irradiated clove. Clove essential oil's have a high biological activity due to having several bioactive compounds [40]. Several investigations have found that one of the major components in EOs extracted from clove is eugenol (4-allyl-2-methoxyphenol), which has antifungal action. According to the United States Food and Drug Administration (FDA), eugenol is used as a food additive and is a safe compound [11, 41]. The bioactive substances found in essential oils of clove buds given their relevance as food preservation materials for the food manufacturing [42]. Furthermore, caryophyllene oxide was detected in the both of EOs recovered from un-irradiated and irradiated samples, but it was highest in the EOs recovered from ICB. The oxidation of caryophyllene is due to its oxide as it is well documented and explains the result mentioned earlier [43, 44]. While, the difference between the present findings and other findings in the amount of oil yield or in the amount or values of major and minor contents could be due to the source or the portion or the basic weight of the clove buds used to extract the EOs.

Table 2 Chemical composition of essential oils (EOs) recovered from un-irradiated clove buds (CB) and irradiated clove buds (ICB) at dose level 10.0 kGy of gamma radiation

Name of oil constituent	Chemical formula	(CB) Control	(ICB) 10 kGy	Structure	References
Anethole less lipophilic properties	$C_{10}H_{12}O$	3.38	ND	Monoterpenes	Bhavaniramya et al. [45]
Germacrene-D less lipophilic properties	$C_{15}H_{24}$	0.18	ND	Sesquiterpenes	Bhavaniramya et al. [45]
Eugenol high lipophilic properties	$C_{10}H_{12}O_2$	72.27	63.50	Monoterpenes Phenylpropene compound	Bhavaniramya et al.[45] and Latifah-Munirah et al. [46]
Caryophyllene lipophilic volatile	$C_{15}H_{24}$	11.04	4.27	Sesquiterpenes	Bhavaniramya et al. [45]
Humulene less lipophilic properties	$C_{15}H_{24}$	1.52	0.56	Sesquiterpenes	Bhavaniramya et al. [45]
Isoeugenol lipophilic properties	$C_{10}H_{12}O_2$	2.43	ND	Monoterpenes	Bhavaniramya et al. [45]
Delta-Cadinene	$C_{15}H_{24}$	0.23	ND	Sesquiterpenes	Bhavaniramya et al. [45]
Phenol, 2-methoxy-4-(2-propenyl)-, acetate high lipophilic properties	$C_{12}H_{14}O_3$	6.31	26.32	Carboxylic Acids	Bhavaniramya et al. [45]
Caryophyllene oxide lipophilic volatile	$C_{15}H_{24}O$	0.46	2.35	Sesquiterpenes	Bhavaniramya et al. [45]
α-Copaene lipophilic properties	$C_{15}H_{24}$	0.40	1.23	Sesquiterpenes	Bhavaniramya et al. [45]
Methyl salicylate soluble in organic solvents	$C_8H_8O_3$	0.70	ND		
Total		98.92	98.23		

ND not detected

Antibacterial Activity

Diameter of inhibition zone (DIZ) and minimum inhibitory concentration (MIC)

Both EOs recovered from CB and ICB were tested for their putative antibacterial activity against four MDR pathogens. The existence and diameter of the inhibitory zone revealed that the EOs recovered from ICB was more efficient than both, the EOs recovered from CB and the used antibiotic (AMC) as shown in Table 3. In addition, the same pathogens were more sensitive to the MICs of EOs recovered from ICB (ranged between 0.39 and 1.5 µl/ml) than the MICs of EOs extracted from CB (ranged between 1.5 and 6.25 µl/ml). The higher antibacterial potency of EOs recovered from ICB than from CB in present study may be attributed to the slight difference between their chemical compositions and the ratio between the major and minor components of the EOs. Daneshzadeh et al. [33] reported that the antimicrobial efficacy of the essential oils is originated from the interaction between the major and the minor components, as well as the activity of the EOs constituents.

The largest difference in DIZ between EOs recovered from CB and ICB was observed for *S. aureus* (4 mm) and the lowest was for *E. coli* and *K. pneumoniae* (2 mm). Also, the EOs recovered from ICB was more effective against *S. aureus*. Moreover, Table 3 shows that the MIC of EOs recovered from ICB were two fold lower than MIC of EOs recovered from CB with Gram positive bacteria and lower by one fold with Gram negative. The DIZ and MIC values reported in present study are in accordance with Silvestri et al. [47] who reported a differences in clove essential oil MICs' values between Gram positive and Gram negative bacteria (0.2–0.6 mg/ml). While, in contrast with Radünz et al. [48] who demonstrated that the clove essential oil had independent effect on the the microorganism membranes, where it showed similar antibacterial action up to the 0.304 mg/ ml against *S. aureus, E. coli*, *L. monocytogenes* and *S. Typhimurium*. The alterations in MICs values between the present results and other researcher results might be accredited to the type of then used bacteria, differences in EO extraction methods, and differences in concentration of oil constituents' ratio. In the present study, *E. coli* and *K. pneumoniae* (as Gram negative bacteria) were more resistance to both EOs than *S. aureus* and *B. ce*reus (as Gram positive bacteria) which in consistent with the study done by Araby et al. [10]. This could explain by the presence of external membrane encircling the cytoplasm (cell membrane) in Gram negative bacteria, which functions as a barrier, preventing some materials/compounds from passing through the bacterial cell. Additionally, the existence of efflux pump porins, also acts as an imperative function in intrinsic resistant in Gram negative bacteria [10, 48]. In current study, the antibacterial effects of EOs recovered from CBs and ICB have been evaluated and also established their mechanism of action by using different state of art assays including growth curve assay and leakage of protein and nucleic acids assays for the four MDR pathogens. Alongside with the SEM and EDXFS images for *S. aureus*, the highly susceptible tested pathogen for both EOs recovered from CB and ICB. Herein, the growth curves for the tested MDR pathogens revealed that as compared with the same pathogens treated with MIC of EOs recovered from CB or ICB, the control showed fast elevation in bacterial ($\log_{10}$ cfu/ml). Moreover, MIC of both EOs had nearly the same inhibitory effect on tested MDR pathogens as 2 × MIC. This in contrast with Fu et al. [49] who reported that MIC and 2 × MIC of clove essential oils had different effects; antibacterial and bactericidal, respectively on *S. epidermidis*, *E. coli* and *Candida albicans*. The obtained results could be contributed to that both concentrations; MIC and 2 × MIC of the same extracted oil which had the same chemical constituents but with different concentrations due to the increase in its volume. This means that antibacterial potency of the clove essential oils may be depending on its constituents not on its volume. Based on growth pattern (Fig. 1), all the treated pathogens showed drop in their colony count ($\log_{10}$cfu/ml) after 2 h compared to initial count, then their $\log_{10}$ cfu/ml were slightly raised up after 4–8 h, this may be due to the repairing mechanisms done by all the microorganisms to

Table 3 Diameter of inhibition zones and minimum inhibitory concentrations of essential oils recovered from un-irradiated clove buds (CB) and irradiated clove buds (ICB) against chosen Gram positive and Gram negative multi-drug resistant (MDR) pathogens

Group of pathogens	Pathogen strain	Diameter of inhibition zone (DIZ) (mm)			Minimum inhibitory concentration (MIC) (µl/ml)	
		CB–EOs	ICB–EOs	AMC	CB–EOs	ICB–EOs
Gram positive	*S. aureus*	8.0	12	0.0	1.5	0.39
	B. cereus	7.0	10.0	0.0	6.25	1.5
Gram negative	*E. coli*	8.0	10.0	0.0	1.5	0.78
	K. pneumoniae	7.0	9.0	0.0	3.12	1.5

CB–EOs: essential oils recovered from un-irradiated clove buds (control), ICB–EOs: essential oils recovered from irradiated clove buds at dose 10 kGy of gamma radiation, AMC = amoxicillin/clavulanic acid disc (30 µg)

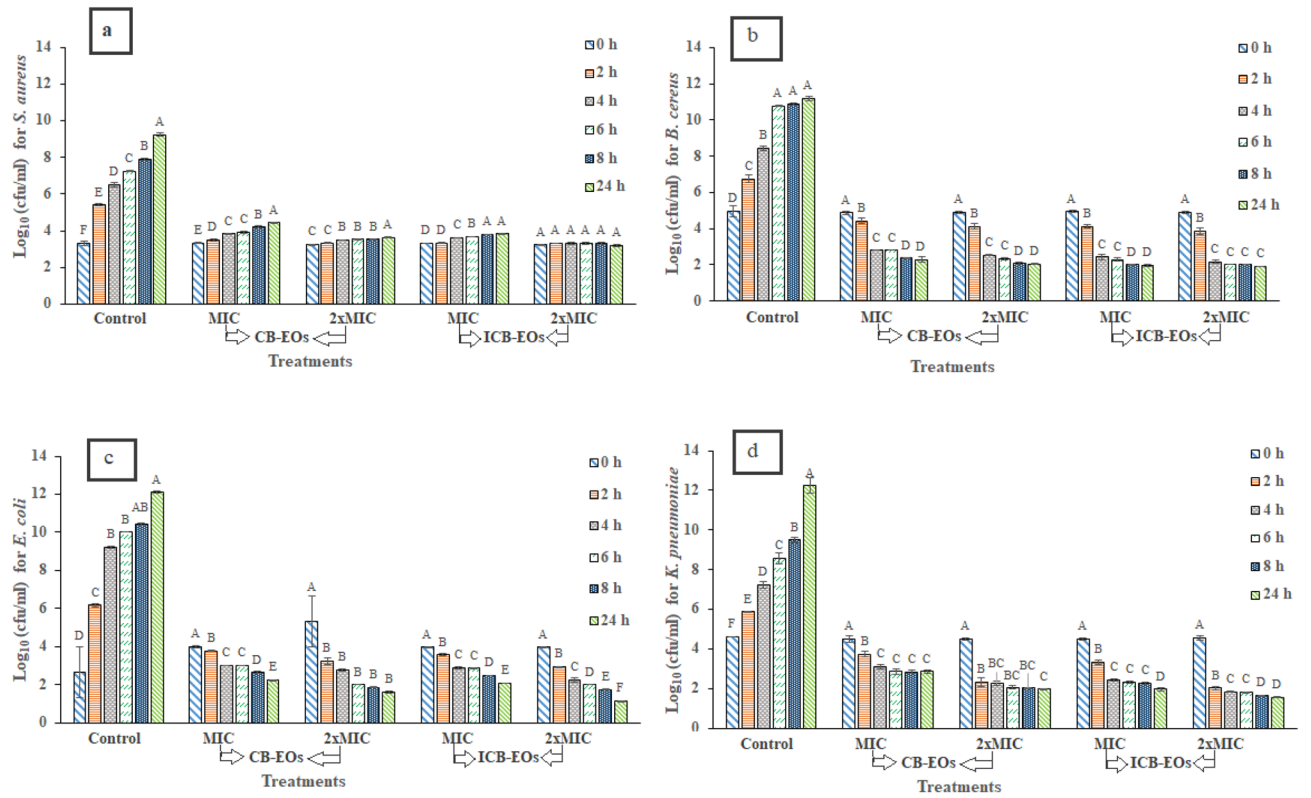

Fig. 1 Growth curves represented by the log number of colony forming unite per ml (cfu/ml) of *S. aureus* (**a**), *B. cereus* (**b**), *E.coli* (**c**) and *K. pneumonia* (**d**) as a control and after treated with concentrations (MIC and 2 × MIC) of clove essential oil recovered from un-irradiated clove buds (CB) and irradiated clove buds (ICB) at different incubation times by hours (h). Vertical bars ± SE (n = 3) and various letters on the bars in each treatment indicate significantly differences at ($p \leq 0.05$)

overcome their environmental stress as reported by Nada et al. [43]. Overall, the growth of the tested pathogens was suppressed significantly by both EOs. But, EOs recovered from ICB had greater antibacterial activity than EOs recovered from CB. Phothisuwan et al. [44] reported that the shelf life of eggs was extended by approximately five fold when their paper trays were exposed to UV-C radiation after sprayed by clove EOs containing (eugenol/eugenyl acetate at 7:1). This means that antimicrobial activity of clove EOs spray was enhanced by UV-C radiation. Therefore, the antibacterial mechanism beside the action of MIC (CB–EOs) and MIC (ICB–EOs) against the four tested MDR pathogens were further investigated in the current study and *S. aureus*, as the more sensitive tested pathogen, was selected for SEM and EDXFS images.

As the biofilms are challenging to be eradicated by usual antibacterial treatments due to excessive antibiotic resistance occurs in pathogens. The results in this study showed highly significant effect ($P \leq 0.05$) of both EOs recovered from CB and ICB on biofilm inhibition of tested pathogens with higher influence of MIC (ICB–EOs) than MIC (CB–EOs) as shown in (Fig. 2). Which may increase the importance and usage of ICB–EOs as antibacterial and antibiofilm in clinical, pharmacy, cosmetics and food industries. Kim et al. [50] reported, the first step of bacterial infection engages bacterial cells adhering to host cells and the production of biofilms which can be found on/in mammalian cells, plants, stainless steel, glass, and polymers. These biofilms are resistant to conventional antimicrobial agents, host defenses, and external stresses. As a result, managing of biofilms are urgently needed in clinical and food-processing contexts.

Determination of cell content leakage

Mechanisms of EOs recovered from CB and ICB as antibacterial were determined by measuring the cell content's release, as protein and DNA/RNA of the tested MDR pathogen supernatant. The obtained results (Figs. 3 and 4) revealed that later the addition of the appropriate EOs MIC to pathogen, the release of cell components raised significantly ($P \leq 0.05$) by incubation time. Both Gram positive and Gram negative pathogens had a similar pattern. However, the protein concentrations increased in time depending manner as shown in (Fig. 3). Compared to control at 8 h, the MIC of EOs recovered from ICB showed higher release of bacterial proteins ranged between 5.9% in *B. cereus* and ~ 32% in

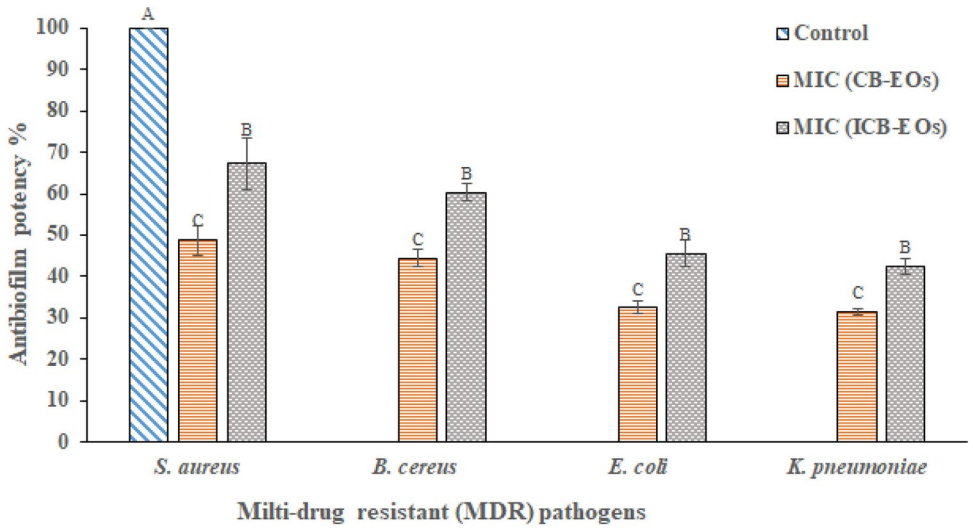

Fig. 2 Antibiofilm potency percentage of minimum inhibitory concentration (MIC) of essential oils recovered from un-irradiated clove buds (CB) and irradiated clove buds (ICB) on *S. aureus, B. cereus*, *E. coli* and *K. pneumoniae*. Control, any of the four tested pathogen alone without treatment = 100% biofilm formation (used to compare the antibiofilm activity of the treatments). Vertical bars ± SE (n = 3) and various letters on the bars in each treatment indicate significantly differences at ($p \leq 0.05$)

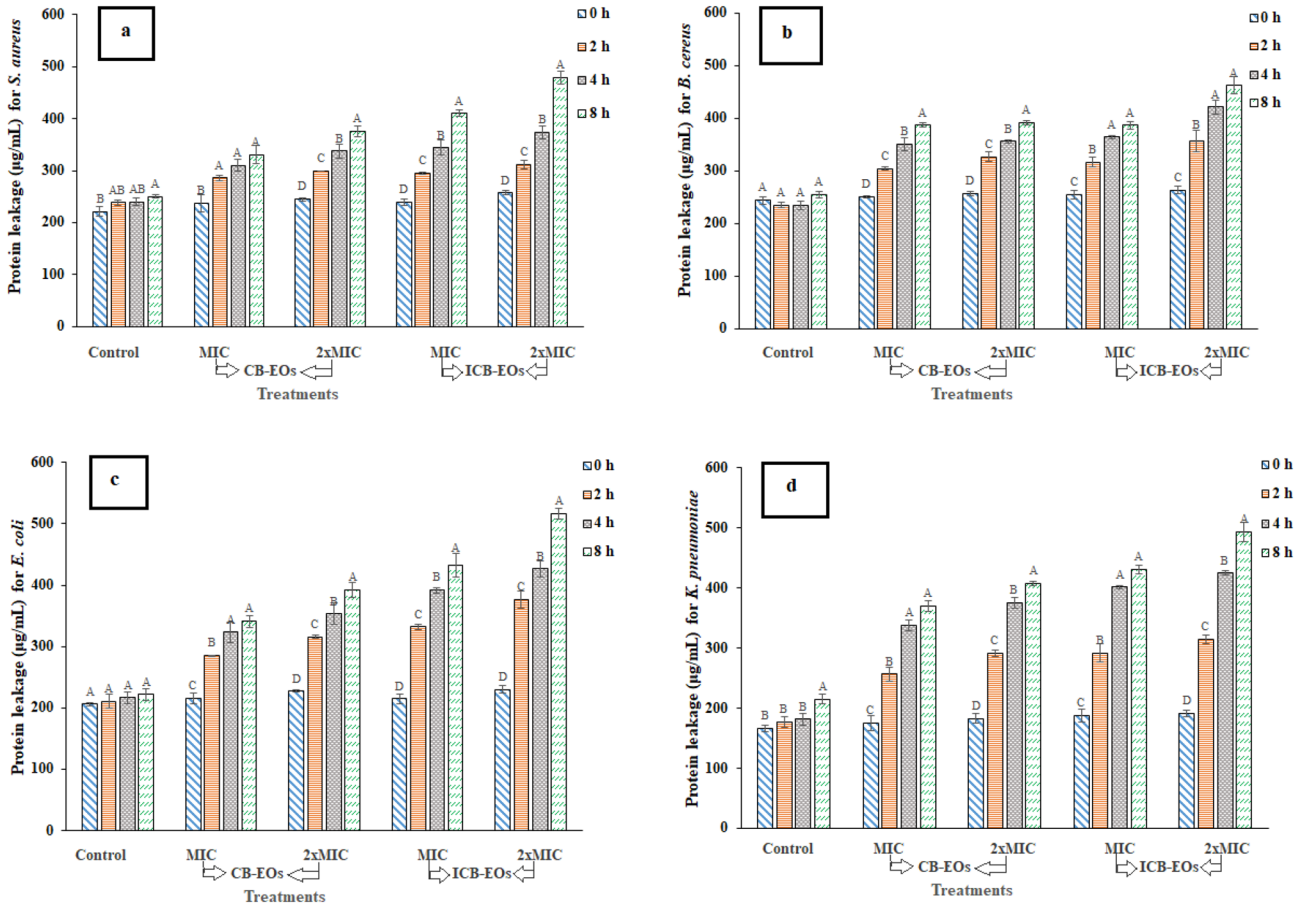

Fig. 3 Protein leakage in µg/ml from *S. aureus* (**a**), *B. cereus* (**b**), *E.coli* (**c**) and *K. pneumonia* (**d**) as a control and after treated with MIC of essential oil recovered from un-irradiated clove buds (CB) and irradiated clove buds (ICB) at different incubation times (0, 2, 4 and 8 h). Vertical bars ± SE (n = 3) and various letters on the bars in each treatment indicate significantly differences at ($p \leq 0.05$)

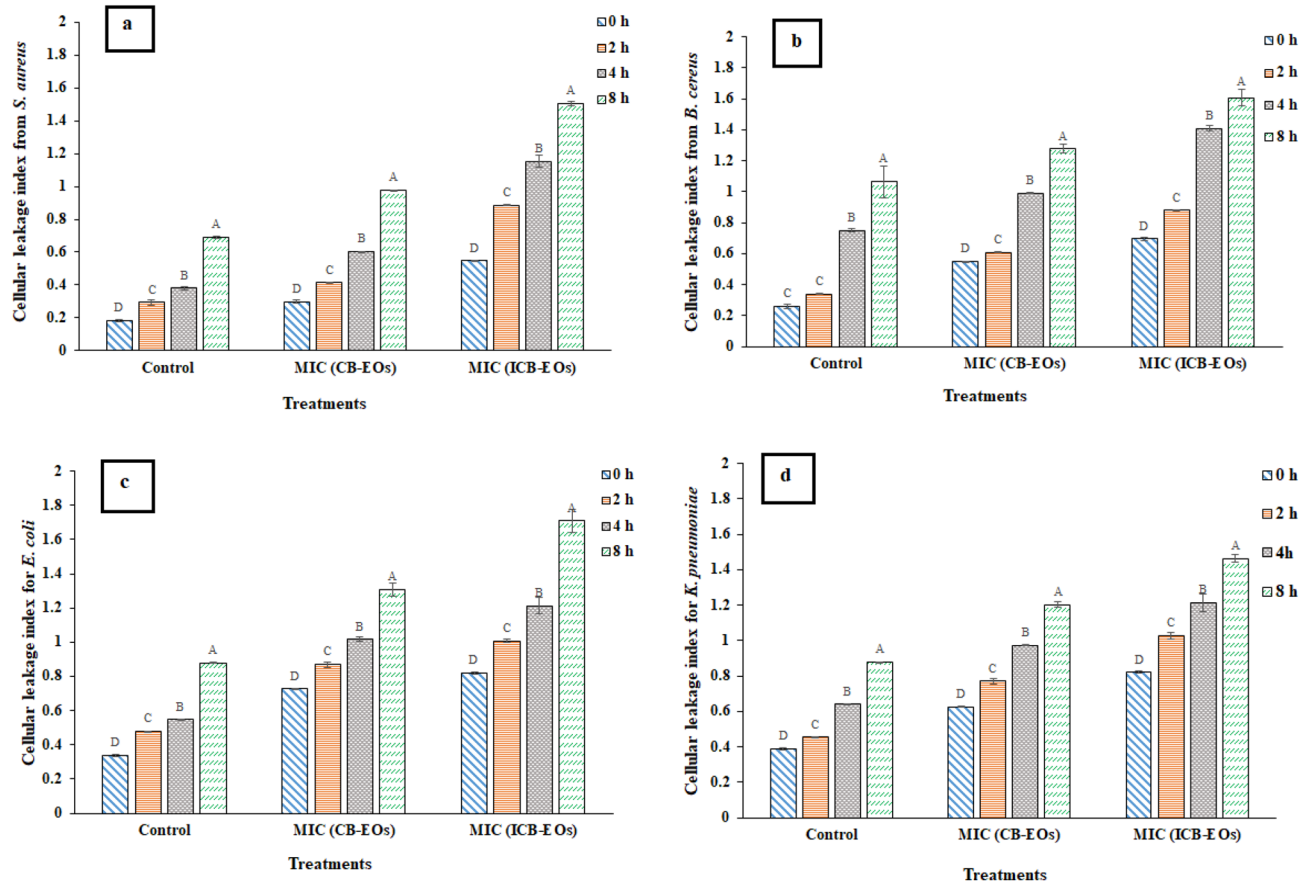

Fig. 4 Leakage of DNA and RNA from *S. aureus* (**a**), *B. cereus* (**b**), *E.coli* (**c**) and *K. pneumonia* (**d**) as a control and after treatment with MIC of essential oil recovered from un-irradiated clove buds (CB) and irradiated clove buds (ICB) at different incubation times (0, 2, 4 and 8 h). Vertical bars ± SE (n = 3) and various letters on the bars in each treatment indicate significantly differences at ($p \leq 0.05$)

S. aureus and *E. coli* than the MIC of CB EOs did. Similar trend by both Gram positive and Gram negative strains were seen in DNA/RNA leakage, as shown in (Fig. 4). The present results indicate the presence of damage in the bacterial cell wall/membrane causing the release of its content into the medium. It's well known that, the bacterial proteins and genetic materials (DNA and RNA) are the most important crucial targets to inhibit bacterial growth. Since these cell constituents (proteins, DNA and RNA) are the initiator, originator and architects for many cellular functions [51]. Addtionally, Wu et al. [52] reported that membrane integrity is a vital factor to maintain and regulate the cell morphology, functions, metabolism and energy transduction. Consequently, even slightly damage or rupture in this structure could harmfully affect different crucial cell systems, resulting in inhibition of cell growth followed by death. Therefore, in present study, mechanisms of EOs recovered from CB and ICB as antibacterial were confirmed by determining the integrity of *S. aureus* membranes by SEM images and EDXFS analysis.

Scanning electron microscope (SEM) and energy dispersive X-ray fluorescence spectrometry (EDXFS)

Scanning electron microscope was used to observe, evaluate and confirm the ultrastructural features, morphological changes and damages in *S. aureus* cell wall, the most sensitive strain among our chosen MDR pathogens. The results of this assay are shown in Fig. 5. The SEM images support the results of the growth curves and the leakage of protein, DNA/RNA from the studies pathogens after treatments. *S. aureus* (Fig. 5A) showed spherical, regular and intact morphology with uniform shape and size. The SEM images showed irreversible damage in *S. aureus* cell wall when treated with both EOs recovered from CB and ICB. But, treatment with ICB–EOs (Fig. 5C) led to more deformed cell morphology, shrunken in cells compared to the *S. aureus* treated with MIC of EOs recovered from CB (Fig. 5B). Wongsawan et al. [53] reported that eugenol is

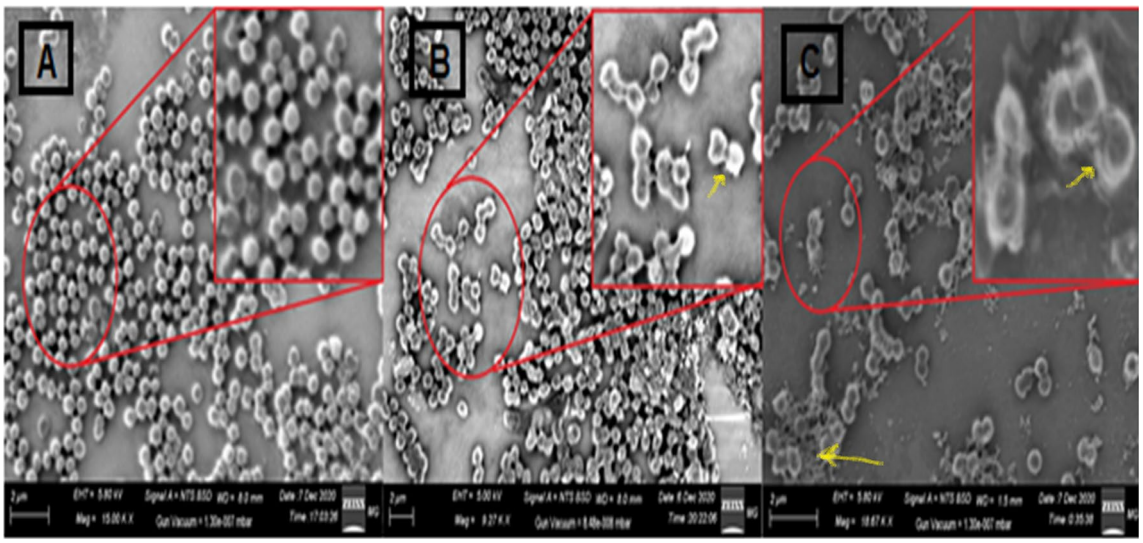

Fig. 5 The SEM images of *S.aureus* before (**A**) and after treated with MIC of EOs recovered from CB (**B**) and MIC of EOs recovered from ICB. The arrows indicated some deformed morphology and rupture in *S. aureus* cell wall leading to leakage of proteins, DNA/ RNA and other cellular content as elements through the membrane

a phenolic compound with antimicrobial properties. It can easily pass through Gram positive bacteria cell walls, causing cell wall disintegration, and subsequently damaging the cytoplasmic membrane, protein destruction, bacterial enzyme system impairment, increased permeability, and eventually cell lysis.

Moreover, EDXFS was used also to confirm the leakage of the cell content into the external medium by characterizing the released atomic elements in the sample. Figure 6 (A1, B1 and C1) shows the availability of the automatically detected elements; C, O, N, Na, Si, Mg, P, Ca and S outside the *S. aureus* cells. The mapping of these elements in scanned area resulted in the elemental spectra shown in Fig. 6 (A2, B2, C2) and in Table 4. Clearly, the intensity of all the detected elements (C, O, N, Na, Si, Mg, P, Ca and S) outside the *S. aureus* cell were increased gradually from *S. aureus* before treatment < after treated with MIC of EOs recovered from (CB) < after treated with MIC of EOs recovered from (ICB). Where, there was duplication in the elements recorded by counts per second per electron volt (cps/eV) in *S. aureus* after treated with MIC of EOs recovered from (ICB). Moreover, an interesting finding in this study was illustrated by EDXFS images and analysis, the release of Ca and S ions outside the *S. aureus* cells after treated with EOs recovered from ICB, while complete absence of both ions when the same MDR pathogen was treated with MIC of EOs recovered from CB. This finding supports our results, the EOs recovered from ICB have more antibacterial potency against the MDR pathogen compared to a counterparts treated with MIC (CB). These findings may be explaining the mechanism of clove bud essential oils action as antibacterial. Where, The EOs recovered from ICB causing complete rupture in the bacterial cell membrane leading to leakage of a lot of its crucial elements as calcium, which is responsible for maintaining the integrity and structure of bacterial cell wall as mentioned by Gupta et al. [54]. Calcium ions are incorporated in numerous mechanisms in prokaryotes as, maintaining the cell structure, sporulation, fruiting body formation, motility and transportation. In addition, there are many suggestions that a number of calcium-binding proteins CaBPs are acting as signaling transduction in bacteria [55]. On the other hand, sulfur (S) is an integral of numerous crucial biomolecules, as cysteine, methionine, thiamine, biotin, and coenzyme A [56]. The current study demonstrated that the release of S may be due to destruction in iron-sulfur clusters, which mainly associate to proteins and involved in numerous vital bacterial physiological processes, as energy metabolism, repairing, and DNA replication [57]. Herein, this study declared the causing of impairment of numerous pathways, replication and repairing of bacterial cells due to the release of essential elements especially calcium and sulfur, which consequently leading to cell lysis.

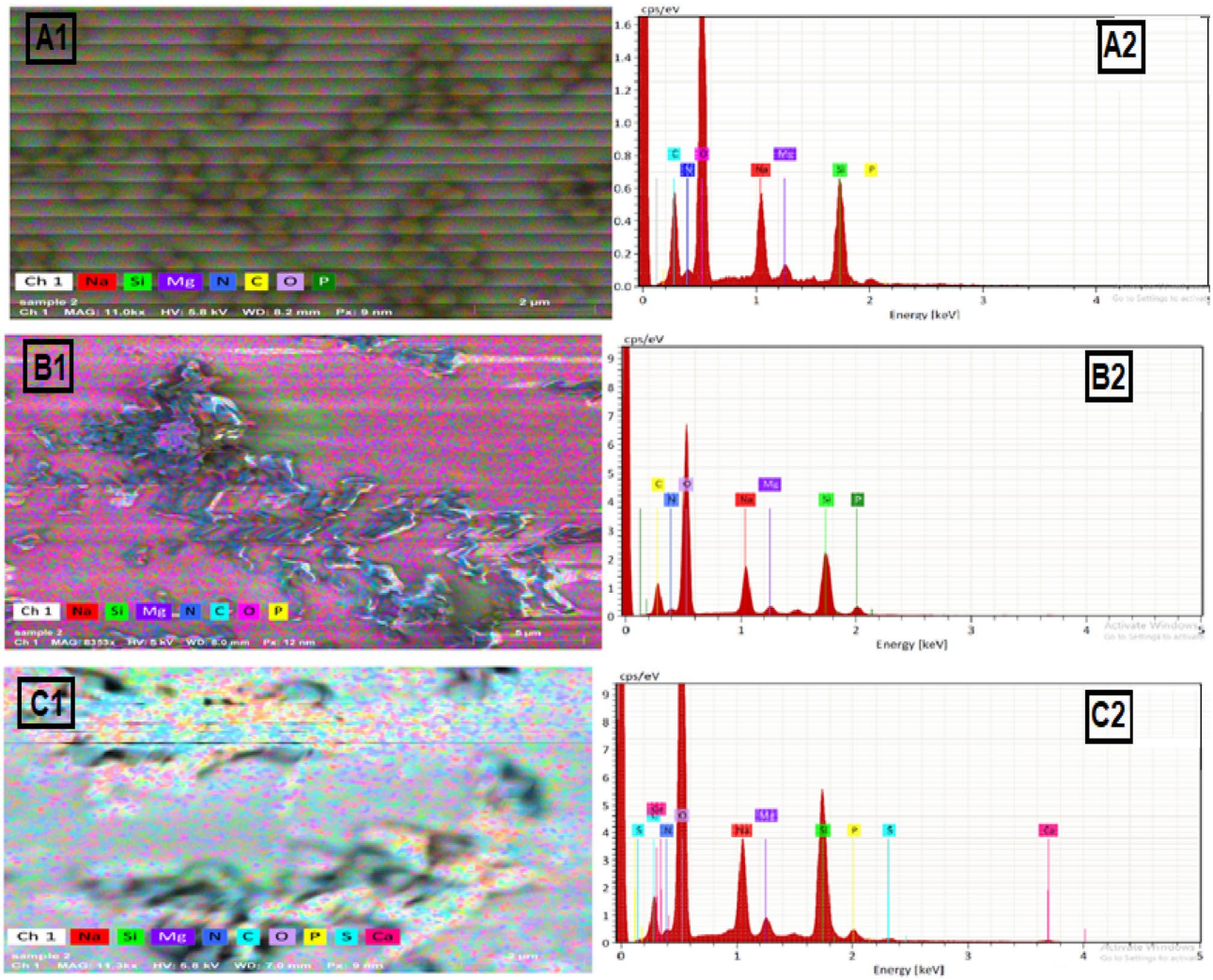

Fig. 6 Energy dispersive X-ray fluorescence spectrometry (EDXFS) analysis. Automatically detected elements; C, O, N, Na, Si, Mg, P, Ca as well as S and cellular components outside the scanned *S. aureus* cells (control) (**A1**), *S.aureus* treated with MIC of EOs recovered from CB (**B1**), and *S. aureus* treated with MIC of EOs recovered from ICB (**C1**). **A2**, **B2** and **C2**; elemental mapping spectra of detected elements in the same scanned area

Conclusion

In brief, the current study reports the chemical composition of the essential oils extracted from un-irradiated and irradiated (at dose 10 kGy) clove buds along with their antioxidant, antibiofilm and antibacterial activity against four tested multi-drug resistant pathogens. The EOs recovered from ICB exhibited slight change in the ratio between the compounds compared to EOs recovered from un-irradiated CB, which may be related for adverse effects on cell wall/membrane permeability, leakage of pathogen cell content and leaded to a detectable increase in EOs recovered from ICB for antioxidant, antibiofilm and antibacterial potency compared to EOs recovered from CB. For that reason, extensive researches on the biological activities of each fraction alone and in combination with other constituents of EOs recovered from ICB in vivo and in vitro are needed. This will support the efforts to find a natural and more potent antioxidant, antibiofilm, and antibacterial against MDR pathogens, which is useful in food and pharmaceutical industries.

Table 4 Intensity by counts per second per electron volt (cps/eV) of elements detected by energy dispersive X-ray fluorescence spectrometry (EDXFS) in nontreated sample of *S. aureus* (control), *S. aureus* treated with MIC of recovered oil from un-irradiated clove buds (CB) and *S. aureus* treated with MIC of recovered oil from irradiated clove buds (ICB)

Element	*S. aureus* (control)	*S. aureus* + MIC (CEO)	*S. aureus* + MIC (I-CEO)
C	0.50	1.20	1.90
O	2.00	6.80	12.00
N	0.10	0.50	0.70
Na	0.60	2.00	4.20
Si	0.80	2.50	5.50
Mg	0.10	0.50	1.00
P	0.03	0.50	0.80
Ca	ND	ND	1.80 & 0.10
S	ND	ND	0.20 & 0.10

ND not detected

Acknowledgements The authors would like to thank the Egyptian Atomic Energy Authority and October University for Modern Science and Art (MSA), Faculty of Biotechnology, Egypt for supporting and funding this study.

Declarations

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

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