Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2020.26440

207 (2020) 309–320
December

Dual exploitation of clove powder for bioremediation of heavy metals and decontaminating microbes from wastewater

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Received 7 March 2020; Accepted 6 August 2020

A B S T R A C T

Water shortage is an upcoming crisis that most countries will face accordingly. One of the suitable solutions for water management is the treatment and reusing of wastewater. The current proposal is concerning by finding out a potent plant powder that able to remediate heavy metals and represent an antimicrobial effect, successfully. Clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry) fruits, leaves and fruits of *Schinus molle* L. powders were tested for their ability to remediate the heavy metals Co^{2+}, Zn^{2+}, Ni^{2+}, and Pb^{2+} ions. Simultaneously, they were investigated for their antimicrobial activity against *Vibrio cholerae* ATCC700, *Candida albicans* ATCC700, *Pseudomonas aeruginosa* ATCC9027, *Escherichia coli* NCTC10418, *Klebsiella pneumoniae* ATCC13883, *Bacillus cereus* ATCC6633 and *Staphylococcus aureus* ATCC6538. The ion chromatography results showed that clove fruits powder can effectively remove Co^{2+}, Zn^{2+}, and Ni^{2+} ions with removal percentages 39.8%, 37%, and 26.19%, respectively. Whenever, shinus leaves powder showed a 90% percentage of Pb^{2+} ions removal as the highest detected percent compared with shinus fruits powder that was the lowest one. Clove fruits powder was also investigated as the potent antimicrobial agent against all tested microbes compared with shinus leaves and shinus fruits that completely failed to stop the microbial growth of all tested pathogenic microbes. The alginate-immobilized beads of shinus leaves powder showed the highest percentage of removal as 96.67% for shinus leaves against Pb^{2+} ions; while, the free powder of clove fruits was still the best compared with the immobilized one. Gas chromatography–mass spectrometry analysis was investigated to screen the active components of the three powders. Scanning electron microscopy was used to configure the shape of the powders before and after immobilization. *In vivo* testing of the selected plant powders for bioremediation

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of heavy metals and decontamination of microbes in real wastewater samples indicating that clove fruits powder is highly active as an antimicrobial agent; while, shinus leaves' powder is more active regarding heavy metals bioremediation.

Keywords: Heavy metals removal; Antimicrobial activity; Plant powders; GC-MS analysis; Wastewater treatment

1. Introduction

The reusing of treated wastewater for various applications, including irrigation, would be an expected solution for overcoming the prospective water shortage. The untreated wastewater is almost accompanied by pollutants that would harm humans if exposed [1]. The irrigation with untreated wastewater might cause the transfer of heavy metals to the grown vegetables. In addition, the irrigated soil could also be contaminated with large amounts of heavy metals accumulated by irrigation water [2].

Heavy metals are known for their stability, persistence, and un-degradability. They are almost originating from industrial, commercial, or runoff water [3]. They have cytotoxic, mutagenic, and genotoxic effects on humans, animals, and plants [4]. Different chemical and physical methods have been applied to eliminate heavy metals from contaminated water. The most common technologies are adsorption, ion exchange, membrane separation, and precipitation [5–8]. Adsorption is the most preferred method for adsorption of heavy metals from wastewater as it is the most flexible, convenient, and low-cost method [9,10]. In many cases, the adsorbent material can be effectively reused [5].

Pathogenic microbes that pose risk to public health are usually originating from different sources including farms, hospitals, schools, and food processing plants. Frequently, many illnesses are relatively common from wastewater-related sources [3]. Wastewater-related diseases include typhoid, cholera, dysentery, and polio [11].

Vast numbers of methodologies are currently tested for the management of water pollution. Agricultural wastes that contain lignocellulosics have been proved as successful heavy metals adsorbing materials and antimicrobial agents [12]. Herbs are naturally containing active compounds that have antimicrobial activities. Carvacrol, linalool, carnosic acid, and eugenol are examples of these active compounds that able to degrade the microbial cells and disrupt their cytoplasmic membranes [13].

The aim of the current study is to investigate the ability of clove and shinus plant powders to remove heavy metals with simultaneous ceasing the microbial growth in both laboratory and real wastewater samples. The study was extended to investigate the powders' activity in free and immobilized forms and screening their morphological characteristics using scanning electron microscopy (SEM) analysis. The structural components of the plant powders were screened using gas chromatography–mass spectrometry (GC-MS) analysis.

2. Materials and methods

2.1. Sample collection

Clove fruits were purchased from the local market at Abha, Saudi Arabia. Shinus' leaves, and fruits were collected from *Schinus molle* tree that has grown as a weed plant in the Abha area, KSA and shade dried for one month. Each sample was crushed thoroughly using mortar and pestle until a fine powder was developed. It worth mentioning that, unless otherwise stated, all the next experiments were performed in duplicates.

2.2. Sample preparation [14]

Three aqueous extracts of the tested plants were prepared using hot water. Ten grams of each plant powder were weighed and added to 100 mL distilled water. The three suspensions were boiled at 100°C for 5 min followed by cooling at room temperature. The extracts were centrifuged at 6,000 rpm for 10 min to get rid of the plant debris. The obtained supernatants were then kept at 4°C till use.

2.3. Antimicrobial activity

2.3.1. Agar well diffusion method

The antimicrobial activity of clove fruits, shinus leaves, and shinus fruits was tested on Luria-Bertani (LB) agar plates using the agar well diffusion method. Overnight cultures of the pathogenic strains (*Vibrio cholerae* ATCC700, *Candida albicans* ATCC700, *Pseudomonas aeruginosa* ATCC9027, *Escherichia coli* NCTC10418, *Klebsiella pneumoniae* ATCC13883, *Bacillus cereus* ATCC6633, and *Staphylococcus aureus* ATCC6538) were prepared through the inoculation of a pure colony of each strain in 10 mL LB broth followed by incubation at 30°C for 18 h. Each culture was diluted to 0.5 McFarland standard using sterile distilled water. The prepared dilution of each pathogenic culture was used for the inoculation of the prepared LB plates' surfaces using cotton swabs. A central hole was prepared at the center of each plate using a sterile cork porer. A volume of 100 µL of each plant extract was added separately to the prepared holes. On the other hand, three antibiotic discs including ampicillin (10 µg), cefoxitin (30 µg), and cephalothin (30 µg) were used as positive controls, while sterile distilled water was used as a negative control (–Ve). The plates were kept at 4°C for 30 min to allow the diffusion of the tested materials. The plates were then incubated at 30°C for 24 h and the formed clear zones were investigated and recorded in milliliters.

2.4. GC-MS analysis

The aqueous extract phytochemical investigation was applied on a GC-MS Thermo GC-TRACE ultra ver.: 5.0, Thermo MS DSQ II (Thermo Scientific Co., USA) equipment. GC-MS system experimental conditions were as follows: TR 5-MS capillary dimension: 30 Mts, non-polar column, film thickness: 0.25 µm, ID: 0.25 mm. The mobile phase flow rate (carrier gas: He) had been set at 1.0 mL/min.

In Gas chromatography section, the temperature system (oven temperature), was adjusted to 40°C increased to 250°C at 5°C/min and the injection volume was 1 μL. Chloroform-dissolved samples were entirely tested at a range of 50–650 m/z and the findings were assessed using the library search system Wiley Spectral program [15].

2.5. Heavy metals removal using free plant powders

The ability of the three powders of the tested plants to remove different heavy metals was investigated. Four individual heavy metals of Zn^{2+}, Co^{2+}, Ni^{2+}, and Pb^{2+} ions were prepared in a stock solution of (10,000 mg/L) each and were diluted according to need. A fixed weight of 2% of each plant was tested to remove 50 mg/L of each heavy metal, individually. All the prepared tubes were incubated at 30°C and 150 rpm for 24 h. After incubation, the tubes were centrifuged at 6,000 rpm for 10 min and the supernatants were used for the determination of the residual concentration of each heavy metal using ion chromatography (IC) analysis (Dionex ICS-6000 Ion Chromatography System, Thermo Scientific Fisher (U.S.A.)).

2.6. Immobilization of plant powders

In order to control and easy to collect each plant from the tested heavy metals containing solution, each plant powder was mixed with a sodium alginate solution for subsequent immobilization. The plant-polymer mix was prepared through the amendment of 1% sodium alginate/distilled water solution with 2 g of each plant powder, individually. The mix was stirred for 30 min using a magnetic stirrer till complete homogeneity and fair distribution of the powder inside the polymer. The homogenous mixture was then dropped wisely in $CaCl_2$ solution (3.5%) using a syringe nozzle under stirring conditions. The formed beads were kept stirred for 15 min to allow complete solidification. The beads were filtered using filter paper followed by washing using distilled water to remove excess calcium ions and plant powders.

2.7. Removal of heavy metals using plant-polymer beads

The most promising plant extracts that were able to remove efficiently specific heavy metals were used to test their efficiency to remove the same heavy metals in immobilized forms. The beads of the selected plants were weighed (0.2 g) and added to 10 mL distilled water amended with 50 mg/L of each tested heavy metal, separately. The tubes were incubated at 30°C for 24 h under shaking conditions (150 rpm). After incubation, the beads-free solutions were used for the measurements of the residual heavy metals using IC.

2.8. Ion chromatography analysis

The concentration of the treated and control solutions of tested heavy metals (Co^{2+}, Zn^{2+}, Ni^{2+}, and Pb^{2+}) was detected using IC (Thermo Fisher Scientific Inc., USA). The used eluent was MetPac™ Oxalic Eluent Concentrate, Unites States of America (P/N 046091), while the post-column reagent was PAR reagent (P/N 039672) and MetPac PAR post-column reagent diluent (P/N 046094). The eluent flow rate was 1.2 mL/min and the PCR flow rate was 0.6 mL/min at ambient temperature. The absorbance detection of heavy metals was 520 nm with an injection volume 50 μL of each metal solution.

2.9. Treatment of real wastewater samples

The determination of the ability of the clove fruits and shinus leaves powders to decontaminate real wastewater samples was investigated. Three wastewater samples were used throughout the experiment. The samples were composed of either industrial (one sample named as: S) or industrial mixed with sewage water (two samples named as: Mix1 and Mix2). The antimicrobial activity and heavy metals removal were detected using a specific weight of the selected plant powder. A 2% w/v of each plant powder was prepared and mixed with 10 mL of the contaminated water and followed by incubation at 30°C and 150 rpm for 24 h. The control samples were considered as plant-free water samples. After incubation, the total plate count (TPC), coliform, and *E. coli* count of both control and treated wastewater samples were compared in order to determine the antimicrobial activity of the selected plants in a real wastewater sample. The differentiation and counting of coliform and *E. coli* were achieved through the inoculation of the 1/100 diluted samples into ready to used plates of Compact Dry "Nissui" EC for coliform and *E. coli* (Nissui Pharmaceutical Co., Ltd., USA).

In addition, both the control and treated samples were submitted for IC analysis to screen the type and concentration of existed heavy metals before and after treatment.

2.10. Scanning electron microscopy

The morphology of the most promising plants in free and immobilized forms was characterized by scanning electron microscopy using different magnifications (JEOL, JSM-6360LA, Japan).

3. Results and discussion

3.1. Antimicrobial activity

The antimicrobial activity of the three extracts was investigated against pathogenic bacteria and yeast using the agar well diffusion method. As shown in Table 1 and Fig. 1, only clove fruits extract was able to inhibit the growth of the tested microbes. The potent antimicrobial activity was recorded against *Vibrio cholerae* strain with an 18 mm clear zone. While, moderate antimicrobial activity was recorded against *Candida albicans* strain forming 14 mm clear zone. Furthermore, the growth of the rest of the tested microbes was inhibited by clove fruits extract by 9–10 mm clear zones which could be categorized as lower antimicrobial activity compared with *Vibrio cholerae*. We could summarize the ascending order of the resistivity of the tested pathogens as: *Vibrio cholerae* < *Candida albicans* < (*Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus cereus*) < *Klebsiella pneumoniae*. It worth mentioning that, the clove fruits have a wide spectrum of antimicrobial activity compared with the three tested positive controls of potent antibiotics (ampicillin,

Table 1
Antimicrobial activity of clove fruits, shinus leaves, and shinus fruits aqueous extracts against pathogenic bacteria and yeast

Microbial strains	Clear Zone (mm)						
	AP	FOX	KF	–Ve	Clove fruits	Shinus leaves	Shinus fruits
Vibrio cholerae ATCC700	23	16	0	0	18	0	0
Candida albicans ATCC700	0	0	0	0	14	0	0
Pseudomonas aeruginosa ATCC9027	0	0	8	0	10	0	0
Escherichia coli NCTC10418	0	0	7	0	10	0	0
Klebsiella pneumoniae ATCC13883	0	0	6	0	9	0	0
Bacillus cereus ATCC6633	0	0	0	0	10	0	0
Staphylococcus aureus ATCC6538	0	0	12	0	9	0	0

Ampicillin (AP); cefoxitin (FOX); cephalothin (KF) were used as positive controls, while sterile distilled water was used as a negative control (–Ve)

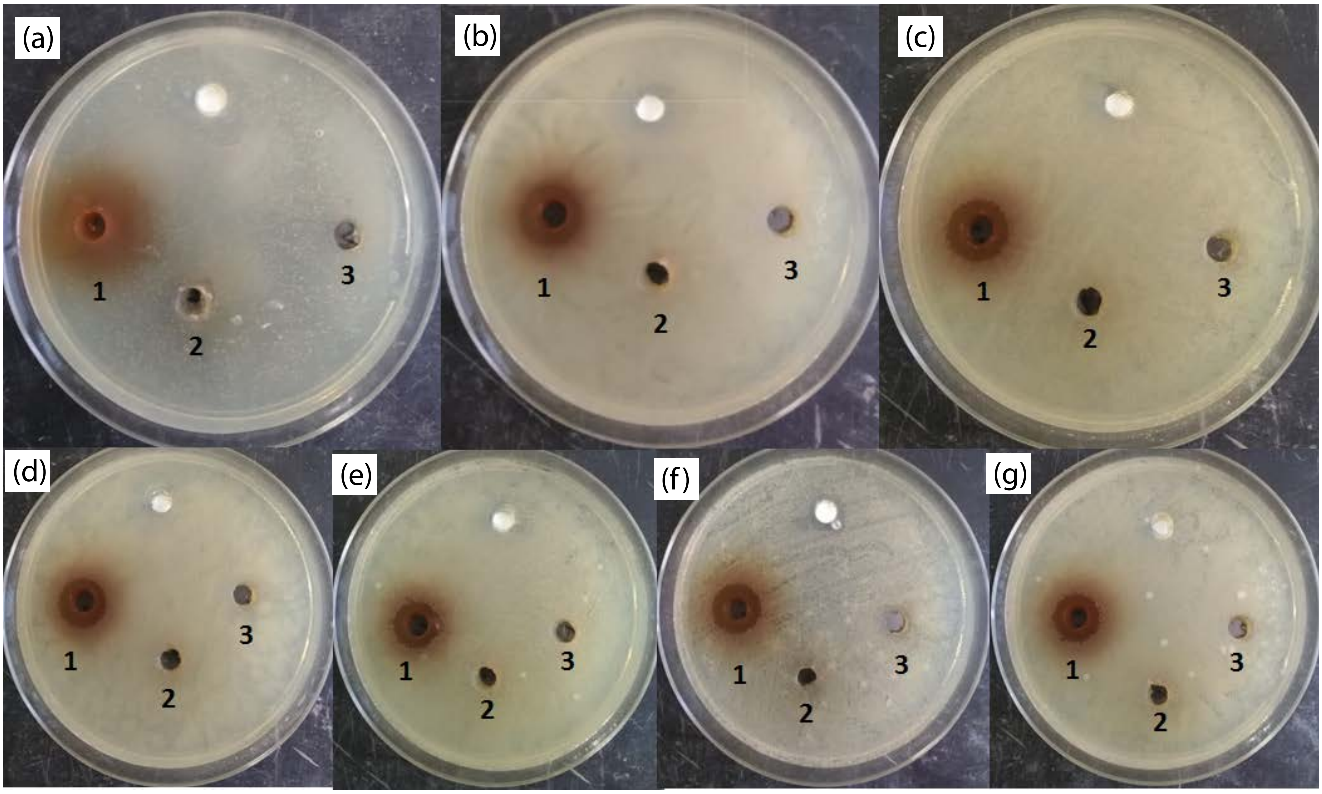

Fig. 1. The detected clear zones of clove fruits, shinus leaves, and shinus fruits extracts against pathogenic microbes. (a) *Vibrio cholerae*, (b) *Candida albicans*, (c) *Pseudomonas aeruginosa*, (d) *Escherichia coli*, (e) *Klebsiella pneumoniae*, (f) *Bacillus cereus*, and (g) *Staphylococcus aureus*; while (1) clove fruits extract, (2) shinus leaves extract, and (3) shinus fruits extract.

cefoxitin, and cephalothin). The clove fruits extract showed antimicrobial activity against all the tested pathogenic microbes, while the tested positive controls showed a narrow spectrum and partially affect the tested pathogens (Table 1).

On the other hand, shinus leaves and shinus fruits extracts failed to cease the growth of all tested pathogenic microbes as observed for negative control (Table 1 and Fig. 1). The potency of the three tested plant extracts as antimicrobial agents could be summarized as: clove fruits > (shinus leaves and shinus fruits). Various studies showed that clove has a strong antimicrobial activity [16–18]. Moreover, it has been used as anticarcinogenic, antiallergic, antioxidant, insecticidal, and antifungal agents [19–22].

3.2. GC-MS analysis

Characterization of the active chemicals found in the aqueous extracts of dry fruits of *Schinus molle* and *Syzygium aromaticum* and leaves of *Schinus molle* is presented in (Fig. 2 and Table 2). GC-MS chromatogram analysis showed five peaks (fruits of *Syzygium aromaticum*), nine peaks (leaves

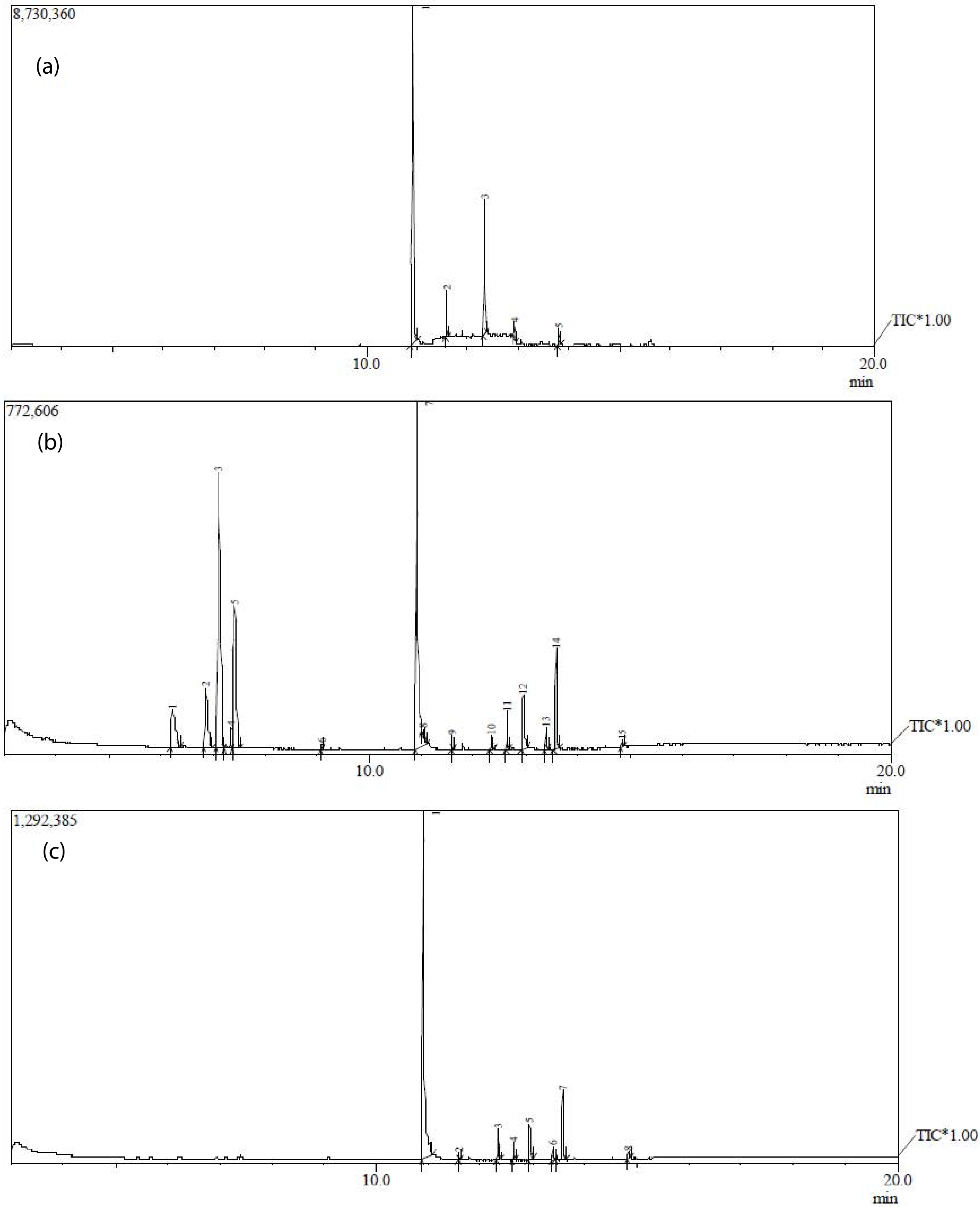

Fig. 2. GC-MS chromatograms illustrating the peaks of the three tested plant powders. (a) Clove fruits, (b) shinus leaves, and (c) shinus fruits.

Table 2
GC-MS analysis of the three tested plant powders (clove fruits, shinus leaves, shinus fruits)

Plant powder	Peak number	*R*. time	Area %	Compound name
Clove fruits	1	10.924	40.21926	3-allyl-6-methoxyphenol
	2	10.924	40.21926	Eugenol
	3	11.592	1.068959	Caryophyllene
	4	12.337	14.14907	Phenol, 2-methoxy-4-(2-propenyl)-, acetate
	5	12.934	2.654533	Diethyl phthalate
Shinus leaves	1	10.904	5.8427672	Eugenol
	2	10.904	58.428235	3-allyl-6-methoxyphenol
	3	11.593	0.4449219	1,3,6,10-dodecatetraene,3,7,11-trimethyl-, (Z,E)-
	4	12.339	7.4275271	Phenol, 2-methoxy-4-(2-propenyl)-, acetate
	5	12.644	0.9300456	Cyclohexanemethanol, 4-ethenyl-α,α,4-trimethyl-3-(1-methylethenyl)-
	6	13.388	17.059394	Diethyl phthalate
	7	13.584	1.3215904	Guaiol
	8	10.904	7.5231329	2-naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methylene-
	9	10.904	1.0223857	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19
Shinus fruits	1	10.907	17.20825525	Eugenol
	2	6.224	6.276082068	α-pinene
	3	6.865	5.82714565	β-myrcene
	4	7.104	33.97701934	α-phellandrene
	5	0	13.10379809	Benzene, 1-methyl-2-(1-methylethyl)-
	6	7.425	0.136669125	Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)-
	7	9.091	17.20825525	(S)-3,4-dimethylpentanol
	8	10.907	0.250742705	Phenol, 2-methoxy-3-(2-propenyl)-
	9	11.034	0.227677507	Isooctanol
	10	11.592	1.232735673	1,3,6,10-dodecatetraene,3,7,11-trimethyl-, (Z,E)-
	11	12.343	0.663724549	Acetyl eugenol
	12	12.646	0.498563122	Cyclohexanemethanol, 4-ethenyl-α,α,4-trimethyl-3-(1-methylethenyl)-, [1R-(13 12.946 149.00 163228 51124 diethyl phthalate
	13	13.389	3.107539658	5-Azulenemethanol, 1,2,3,4,5,6,7,8-octahydro-α,α,3,8-tetramethyl-
	14	13.586	0.281635458	2-naphthalenemethanol, decahydro-α,α,4a-trimethyl

of *Schinus molle*), and fourteen peaks (fruits of *Schinus molle*) which indicates the presence of various phytochemicals compounds in each plant. 3-Allyl-6-methoxyphenol and eugenol were quantitatively the most dominant compound found equally (40.22%) in fruits of *Syzygium aromaticum* followed by phenol, 2-methoxy-4-(2-propenyl)-, acetate (14.15%). Diethyl phthalate and caryophyllene were found in a little amount (2.65%) and (1.07%), respectively. The various components present in the entire herb shinus leaves that were detected by the GC-MS are 3-allyl-6-methoxyphenol (58.43%) represented that major compounds followed by diethyl phthalate (17.60%), phenol, 2-methoxy-4-(2-propenyl)-, acetate (7.43%), 2-naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methylene- (7.52%) and eugenol (5.84%) as an intermediate compound. Minor compounds are guaiol (1.32%), oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19 (1.02%), cyclohexanemethanol, 4-ethenyl-α,α,4-trimethyl-3-(1-methylethenyl)- (0.93%) and 1,3,6,10-dodecatetraene,3,7,11-trimethyl-, (Z,E)- (0.44%). Shinus fruits had α-phellandrene (33.98%) as major chemicals followed by eugenol, (S)-3,4-dimethylpentanol (17.21%) and benzene, 1-methyl-2-(1-methylethyl)- (13.10%). α-pinene, β-myrcene, cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)-, phenol, 2-methoxy-3-(2-propenyl)-, isooctanol, 1,3,6,10-dodecatetraene,3,7,11-trimethyl-, (Z,E)-, acetyl eugenol, cyclohexanemethanol, 4-ethenyl-α,α,4-trimethyl-3-(1-methylethenyl)-, [1R-(13 12.946 149.00 163228 51124 diethyl phthalate, 5-Azulenemethanol, 1,2,3,4,5,6,7,8-octahydro-α,α,3,8-tetramethyl- and 2-naphthalenemethanol, decahydro-α,α,4a-trimethyl found in a little compound in the range 6.28% and 0.14%.

The existence of antimicrobial agents among the detected phytochemical compounds of a plant extract means the possibility of such a plant to be a potent antimicrobial material. However, the concentration of this agent is an important factor in expressing its antimicrobial activity. Eugenol as an antimicrobial agent has been detected in the three tested clove and shinus extracts; however, its concentration was varied. As shown in Table 2, eugenol was detected at 40.2% for clove fruits, 5.8% for shinus leaves, and 17.2% for shinus fruits. These results indicate that the concentration of eugenol is much higher in clove fruits than shinus fruits and leaves, indicating that clove fruits can represent antimicrobial activity better than shinus fruits and leaves, which is perfectly matched with the obtained antimicrobial activity data.

3.3. Removal of heavy metals

3.3.1. Using free plant powders

The free plant powders were tested for their ability to remove four different heavy metals at concentration 50 mg/L. The bioremediation activity was varied according to the type of used powder. As shown in Fig. 3, clove fruits were the most promising plant powder that able to achieve the highest successful removal of 75% of the tested metal ions. It was able to remove Co^{2+}, Zn^{2+}, and Ni^{2+} ions with percentages of removal 39.8%, 37%, and 26.19%, respectively. However, shinus leaves and shinus fruits recorded lower percentages of removal than clove fruits. Between them, shinus leaves were the best that showed percentages of removal 37.16%, 30.3%, and 23.69% for Co^{2+}, Zn^{2+}, and Ni^{2+} ions, respectively. The lowest percentage of removal was recorded as 12.6%, 22.6%, and 0.17% for the ions of Co^{2+}, Zn^{2+}, and Ni^{2+}, respectively, that have been treated by shinus fruits powder.

On the other hand, the Pb^{2+} ions were effectively remediated by shinus leaves powder with a percentage of removal 90% compared with 67.5% and 72.77% for clove fruits and shinus fruits powders, respectively. We could represent the potency of the three tested powders to remediate heavy metals as: clove fruits > shinus leaves > shinus fruits. Recent researches proved the successful exploitation of plant powders for the adsorption of heavy metals from aqueous solutions. Water hyacinth (*Eichhornia crassipes*) has effectively been used to remove lead, copper, cadmium and chrome from aqueous solutions via biosorption [23]. Biosorption is much preferred than other used methods such as ion exchange, chemical precipitation, and reverse osmosis. These methods are more expensive and can generate undesired secondary components, in addition to their inability to remove heavy metals with concentrations lower than 100 mg/L [24]. However, biosorption is much safer and can be used for the remediation of other pollutants such as dyes and organic compounds [25].

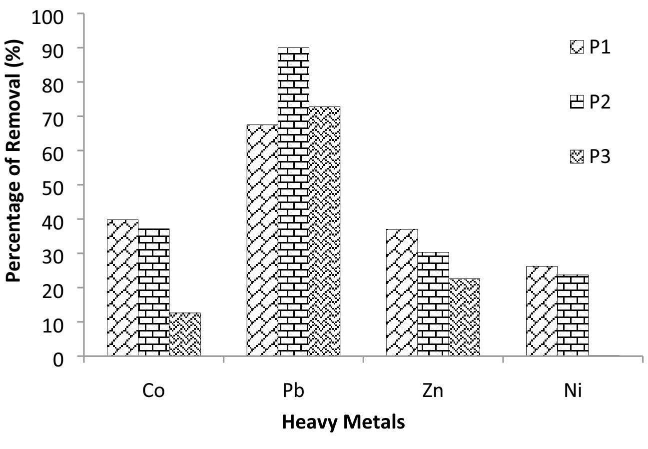

Fig. 3. Comparison among the three tested plant powders (clove fruits, shinus leaves, and shinus fruits) for their ability to remove heavy metals (Co^{2+}, Pb^{2+}, Zn^{2+}, and Ni^{2+}).

3.3.2. Using plant-immobilized beads

The selected plant powders that showed a promising remediation activity against the tested heavy metal ions were submitted for beads formation through the immobilization into alginate polymer. The immobilized powder of clove fruits was tested for the removal of 50 mg/L of Co^{2+}, Zn^{2+}, and Ni^{2+} ions; while, the immobilized powder of shinus leaves was tested for the removal of the same concentration of Pb^{2+} ions. As shown in Fig. 3, the immobilized clove fruits powder failed to remediate the three tested metal ions (Co^{2+}, Zn^{2+}, and Ni^{2+}) successfully compared with its free powder. Whenever the immobilized shinus leaves powder was able to remediate Pb^{2+} ions effectively than its free powder. The free alginate beads were slightly able to remediate some of the tested metal ions better or with the same activity as clove fruits immobilized powders (Fig. 4). In the case of Co^{2+} ions, clove fruits-immobilized beads were able to remediate 11% compared with 7.45% for free alginate beads, indicating the remediation activity of the powder alone was 3.55%. While the manipulation of Zn^{2+} ions slightly differed. The clove fruits-immobilized beads showed remediation activity with a percentage of removal 8.5% compared with 4.07% for alginate free beads, which indicates the ability of clove fruits alone to remove 4.43% of Zn^{2+} ions. The remediation of Ni^{2+} ions was much better than the former two metal ions. The free alginate beads remediate 0% of the tested Ni^{2+} ions, indicating that the recorded percentage of removal (25.7%) is totally accounted for the clove fruits powders.

On the other hand, the manipulation of Pb^{2+} ions was completely different. As shown in Fig. 4, the percentage of removal of Pb^{2+} ions was 96.67% for shinus leaves-immobilized beads compared with 65.84% percentage of removal of free alginate beads. These percentages of removal indicate that the shinus leaves powder was able to remediate 30.83% of the tested Pb^{2+} ions concentrations. It worth mentioning that, these results prove that alginate polymer has higher selective remediation activity against Pb^{2+} ions, and there is a synergistic effect of remediation when both alginate polymer and shinus leaves powder combined together in the form of beads.

Immobilization technique has been used intensively for enzymes [26,27], microbes [28–30], and nanoparticles [31,32]. However, to our knowledge, this is the first time to use immobilization technology for the removal of heavy metals using plant powders.

3.4. Testing of plant powders for wastewater samples treatment

3.4.1. Antimicrobial activity

The proved *in vitro* antibacterial activity of clove fruits powder against pathogenic microbes was also tested *in vivo* through the addition of clove fruits powder to real wastewater samples. Fig. 5 illustrates the ready to used plates of Compact Dry "Nissui" EC for counting coliform and *E. coli*, in two of the tested wastewater samples. Both

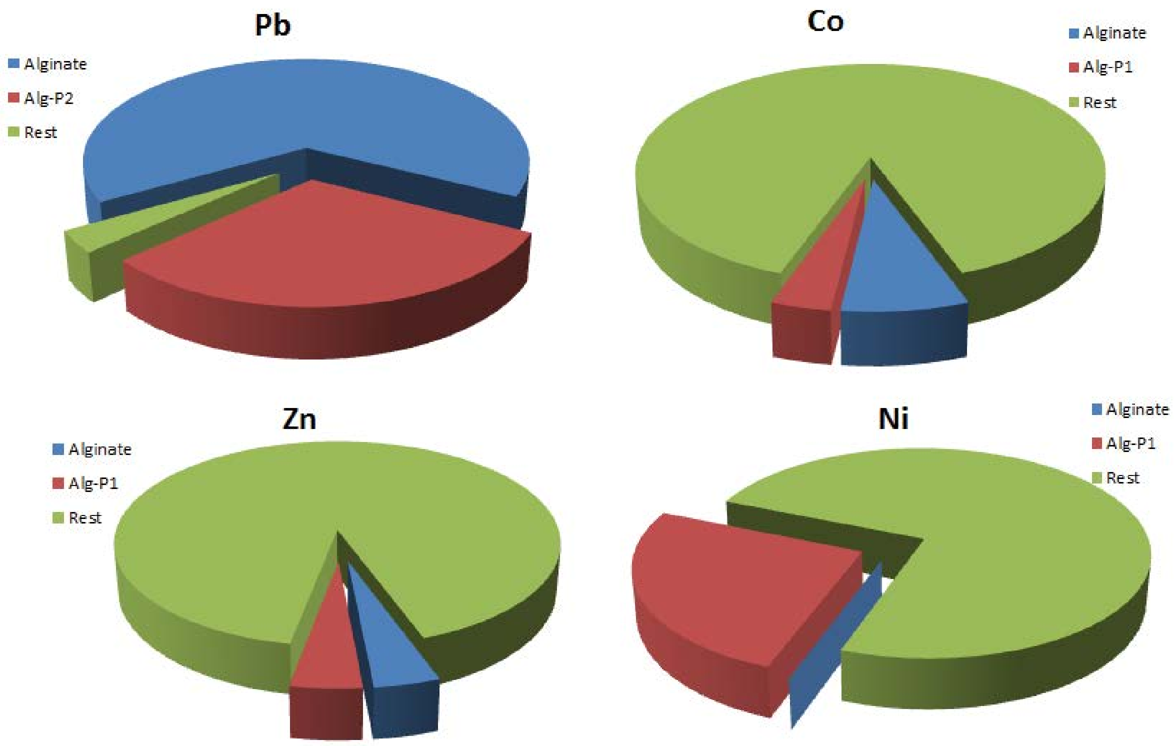

Fig. 4. Percentage of heavy metal removal using free and plant-immobilized alginate beads.

Fig. 5. Representative photo of ready to used plates of Compact Dry "Nissui" EC for counting of coliform and *E. coli*.

of clove fruits and shinus leaves were tested as positive and negative antimicrobial agents, respectively. As shown in Table 3, *E. coli* was absent from industrial wastewater sample (S), but was inhabiting the mixed industrial and sewage samples (Mix1 and Mix2) with different concentrations of 1 × 10^5 and 172 × 10^5 cfu/mL, respectively. Clove fruits powder was able to completely inhibit the growth of *E. coli* in sample Mix1 or reduce its concentration to reach 212 × 10^2 cfu/mL as in sample Mix2. However, the *E. coli* count of Mix1 and Mix2 samples was increased after the treatment with shinus leaves powder, indicating that this powder might have nutrient materials that support bacterial growth. In the same context, clove fruits powder succeeded to completely inhibit the growth of coliforms in samples S and Mix1, but reduced their count from 44 × 10^5 to 56 × 10^2 cfu/mL in sample Mix2.

On the other hand, the TPC which reflects the overall microbial count in samples reflected the potential antimicrobial activity of clove fruits that minimized the bacterial count from 831 × 10^3, 512 × 10^5, and 480 × 10^5 to 210, 600, and 176 × 10^3 cfu/mL in samples S, Mix1, and Mix2, respectively. These data are strongly matched with the data obtained by the in vitro antimicrobial activity of clove fruits powder. It worth mentioning that, clove fruits powder can be effectively used as an antimicrobial agent for the treatment of microbes inhabiting real water samples. It has been used for a long time as an antimicrobial agent against pathogenic microbes [33,34].

3.4.2. Heavy metals removal

The activity of clove fruits and shinus leaves samples to bio-remediate heavy metals from real wastewater samples (S, Mix1, and Mix2) was investigated. Unexpectedly, the bioremediation activity of shinus leaves powder was higher than clove fruits through the three tested wastewater samples and the four investigated heavy metals. As shown in Fig. 6, clove fruits powder was inactive to remove Pb^{2+} ions from sample S; while shinus leaves powder was able

Table 3
Antimicrobial activity of clove fruits and shinus leaves powders against real wastewater samples

Sample description	Sample code	Total plate count (TPC) (CFU/mL)	Coliform (CFU/mL)	*E. coli* (CFU/mL)
Industrial wastewater (S)	S (control)	831×10^3	18×10^2	Absent
	S-clove fruits	210	Absent	Absent
	S-shinus leaves	140×10^6	800×10^5	Absent
Industrial and sewage wastewater (Mix1)	Mix1 (control)	512×10^5	2×10^5	1×10^5
	Mix1-clove fruits	600	Absent	Absent
	Mix1-shinus leaves	176×10^5	320×10^5	$1,040 \times 10^5$
Industrial and sewage wastewater (Mix2)	Mix2 (control)	480×10^5	44×10^5	172×10^5
	Mix2-clove fruits	176×10^3	56×10^2	212×10^2
	Mix2-shinus leaves	149×10^5	240×10^5	$1,600 \times 10^5$

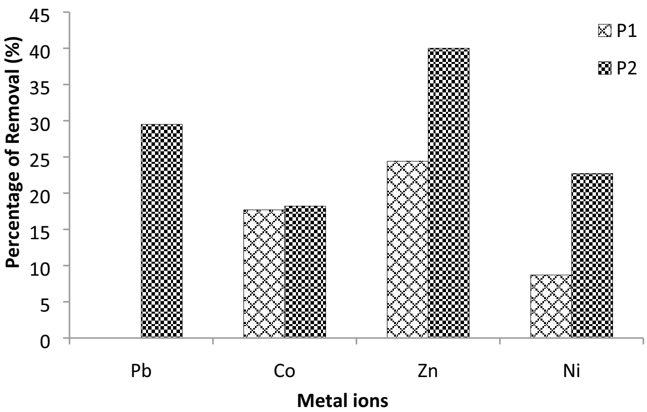

Fig. 6. Bioremediation activity of clove fruits and shinus leaves against heavy metals existed in wastewater sample S.

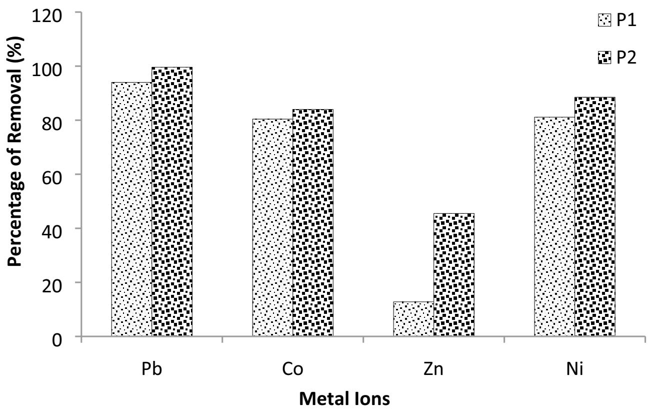

Fig. 7. Bioremediation activity of clove fruits and shinus leaves against heavy metals existed in wastewater sample Mix1.

to remove 29.5% of the same metal ions, which is greatly matched with the data reported for lab experiments. Furthermore, the bioremediation activity of shinus leaves powder was significantly higher than clove fruits powder for the removal of Zn^{2+} and Ni^{2+} ions with percentage removal of 40% and 22.7%, respectively. The percentage of removal of Co^{2+} ions was almost close for both clove fruits and shinus leaves powders and recorded as 17.7% and 18.2%, respectively.

In addition, the activity of shinus leaves powder to remediate Pb^{2+}, Co^{2+}, and Ni^{2+} ions from Mix1 sample was a little bit superior to clove fruits activity (Fig. 7). For shinus leaves powder, the percentage of removal was 99.6%, 84%, and 88.5% compared with 94%, 80.4%, and 81.12% for clove fruits powder when removing the mixture of Pb^{2+}, Co^{2+}, and Ni^{2+} ions, respectively. However, it was significantly able to remove Zn^{2+} ions with a percentage of 45.5% compared with 12.8% for clove fruits powder. Moreover, the lowest bioremediation activity of shinus leaves and clove fruits powder was recorded through the removal of heavy metals from sample Mix2. As shown in Fig. 8, both powders failed to remove Zn^{2+} and Co^{2+} ions from the tested wastewater sample, in addition to failing of clove fruits powder to remediate Pb^{2+} ions from the same sample. On the other hand, shinus leaves powder succeeded to remediate Pb^{2+} and Ni^{2+} ions with activity 28.8 and 42.4%, respectively. In this sample (Mix2), clove fruits powder succeeded only to remediate Ni^{2+} ions with the percentage of removal 32.2%, which is significantly lower than the activity percentage of shinus leaves powder regarding the same metal.

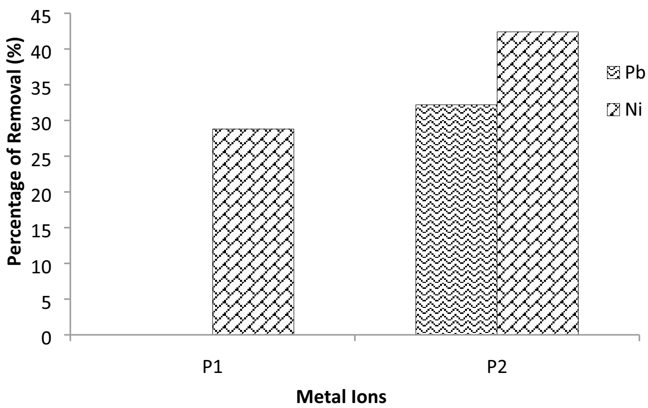

Fig. 8. Bioremediation activity of clove fruits and shinus leaves against heavy metals existed in wastewater sample Mix2.

As mentioned earlier, these data (*in vivo* data) are contrary to the data obtained *in vitro* as represented in Fig. 2.

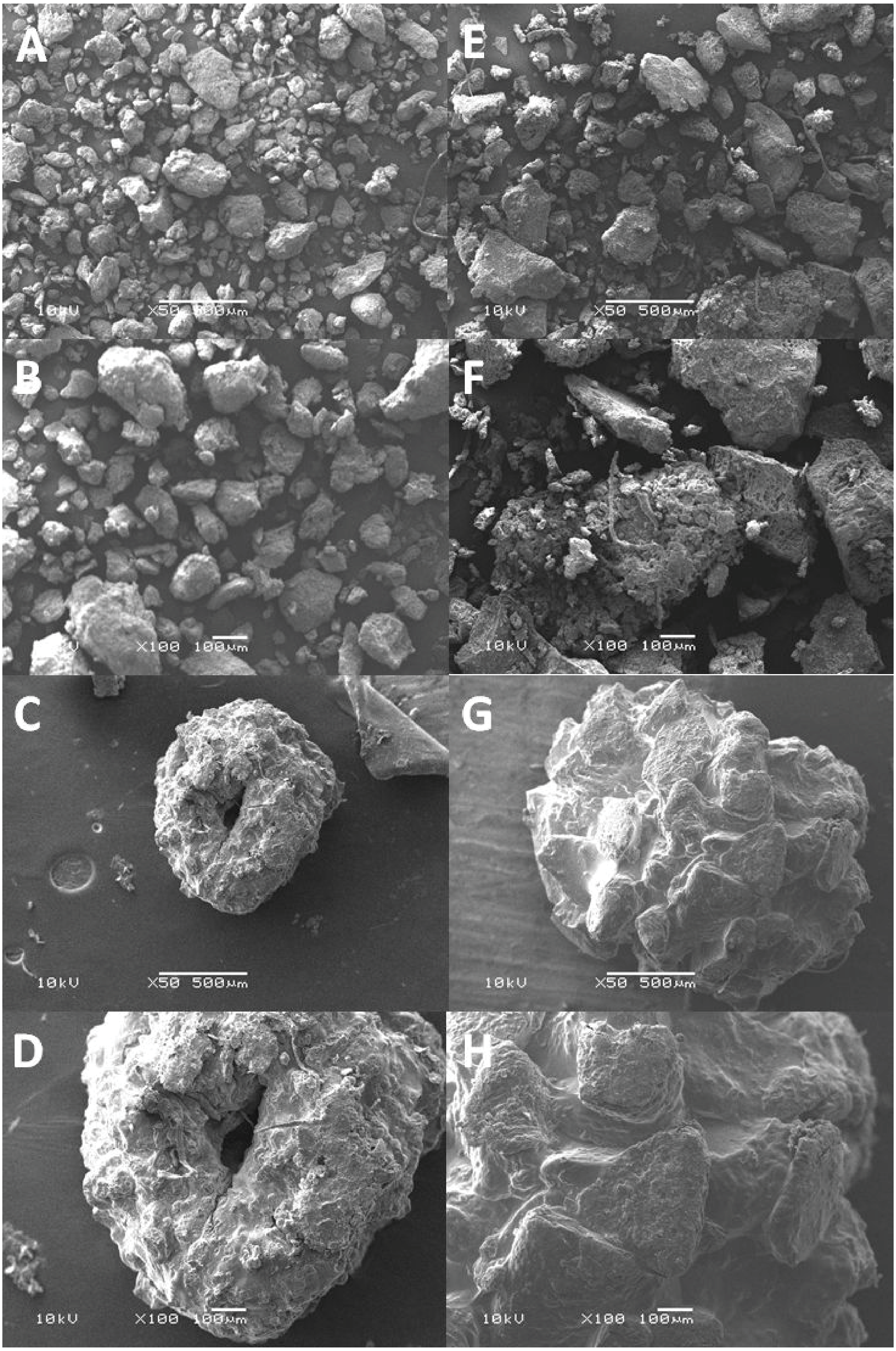

Fig. 9. SEM for the free and alginate-immobilized powders of clove fruits and shinus leaves. (A and B) Free powders of clove fruits, (C and D) alginate-immobolized powders of clove fruits, (E and F) free powders of shinus leaves, and (G and H) alginate-immobolized powders of shinus leaves.

However, the metal ions *in vitro* were examined individually; while, the metal ions tested *in vivo* were examined together. The test *in vivo* water samples are either industrial or industrial/sewage wastewater, which are highly contaminated with the organic load. Some of these organic molecules might have metals chelating activity. In such a case, the large organic molecule/metal ions structure might need large substrates to carry or to be easy to adhere to. We could attribute the ability of shinus leaves powder to remediate metal ions better than clove fruits powder to its large pieces as will be shown by SEM micrographs.

3.4.3. Scanning electron microscopy

The micrographs of clove fruits and shinus leaves in the form of free and immobilized forms were investigated using SEM. Different magnifications of both forms were obtained to detect the actual shape and size of the plant powders before and after immobilization. As shown in FFigs. 9A and B, the particle size of clove fruits powder were significantly smaller than the particle size of shinus leaves powder (Figs. 9E and F). This observation indicates that the surface area of clove fruits powder is larger than shinus leaves, which would enhance its catalytic activity, especially for small entities. This is perfectly matched with the data obtained in Fig. 3, which showed higher activity for the heavy metals removal of Co^{2+}, Zn^{2+}, and Ni^{2+} ions by clove fruits powder than shinus leaves powder. However, large pieces of shinus leaves powder can be more active for adhesion of large organic molecule/metal ions structures and enhance its remediation activity, which is matched with data obtained in Figs. 6–8.

On the other hand, Figs. 9C and D show the surface shape of an alginate bead that entraps the clove fruits powder. As observed, the photographed bead is approximately impermeable and lacks the distinct appearance of pores. This indicates that the inner part of the bead is not available for interaction with the materials that existed outside. This observation is perfectly matched with data of Fig. 4 which shows the lower activity of clove fruits-immobilized beads against the three tested metal ions (Co^{2+}, Zn^{2+} and Ni^{2+}). This also indicates that the outer plant powders are the only ones that have the chance to perfectly interact with the surrounding metal ions. In the same context, the beads that entrap shinus leaves powder have the same observation of being impermeable (Figs. 9G and H). On contrary to the lower ability of clove fruits-immobilized beads to remediate heavy metals, shinus leaves-immobilized beads showed the higher ability for the remediation of Pb^{2+} ions than free powder. This is might be explained that, even if the beads are not impermeable, but the alginate polymer has a selective ability to remediate the Pb^{2+} ions, and thus it does not matter the whole shinus leaves power is available for the metal ions or not.

4. Conclusion

It would be concluded that clove fruits powder is the most preferred one among the other two shinus powders to absorb a large number of tested heavy metals and to inhibit the pathogenic microbes in wastewater samples. The free powder was much potent than the immobilized powders for the removal of Zn^{2+}, Co^{2+}, and Ni^{2+}, while, the adsorption of Pb^{2+} ions was much higher in the case of shinus-immobilized powder. The overall results summarize the successful usage of clove fruits powder for the bioremediation of heavy metals and pathogenic microbes from wastewater, in order to be used for multiple purposes including irrigation.

Acknowledgment

We would like to thank King Khalid University for funding under the Research Group Number (R.G.P.1/134/40).

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