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Antimicrobial Activity of Some Medicinal Plants Extracts Against Food Industry Isolates

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ABSTRACT

Microbial contamination and biofilm formation in food industries pose a threat to human health worldwide. With the increased use of antibiotics in food industry, the problem of bacterial resistance is emerging, hence leading to serious health issues. Among 24 strains isolated from Shaukat Banaspati Ghee industry (Grw) and Shezan foods (pvt) Ltd, Lahore, Pakistan food industry, 10 highly antibiotic resistant strains were subjected to morphological, physiological and biochemical characterization. 16SrRNA sequencing was performed to identify three highly resistant strains at species level. Antimicrobial activity of aqueous and methanolic plant extracts of *Camellia sinensis* (Green tea), *Syzygium aromaticum* (Clove) and *Mentha piperita* (Peppermint) was evaluated against identified isolates. Agar well diffusion assay was used to monitor the antimicrobial activity of these strains both in mono culture and mixed culture. Streptomycin sulphate (10 μgml^{-1}) and Amphotericin B (5 $mgml^{-1}$) were used as positive controls. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts was also determined. *Syzygium aromaticum* was proven to have excellent antimicrobial activity against all tested microorganisms, while *C. sinensis* and *M. piperita* showed weak antimicrobial activity. Methanolic plant extracts exhibited greater antimicrobial activity than aqueous extracts. Maximum zone of inhibition exhibited by methanolic extracts of *S. aromaticum* was 10 mm and 20 mm in mono and mixed culture bacterial isolates, respectively. The findings from this study warrant further research to help to establish an alternative anti-infective phytotherapeutical approach to control antibiotic-resistant microbial strains in the food industry.

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Authors' Contributions
IL conceived and designed the study. AS performed the experiments. NA, MA, SAM and NMA analyzed the data. IL, NA and MA wrote the article.

Key words
Food industry, Medicinal plants, Antibiotic resistant biofilms, *Mentha piperita, Syzygium aromaticum, Camellia sinensis.*.

INTRODUCTION

The ability of microorganisms to form biofilms is of great importance in nature as well as man-made environments. These microbes may live individually or in colonies performing their functions of life. The formation of a biofilm is determined not only by the nature of the attachment surface, but also by the characteristics of the bacterial cell and by environmental factors (Van Houdt and Michiels, 2010).

Biofilms can be found in almost every environment where bacterial presence occurs (natural, industrial and clinical media). Minimum amount of moisture and nutrients are essential to flourish on a variety of surfaces (Terry *et al*., 2003). The presence of biofilm forming microbial population is very frequent in food industry, on a variety of surfaces including plastic, glass, metal, and wood (Chmielewsky and Frank, 2003). The attachment and biofilm formation on food products or food contact surfaces may result in food poisoning and economical losses to the producer because of food spoilage (Van Houdt and Michiels, 2010).

A major problem in the food industry is the resistance of bacterial population to antibiotics including tetracycline, beta-lactam antibiotics, and sulphamide as well as biocides (*e.g*., disinfectants, food and feed preservatives, or decontaminants) because of poor sanitation of surfaces or materials that come in contact with food directly or indirectly. Dairy animals are continuously exposed to different antibiotics to have good health and yield quality product. While application of disinfection strategies on crops and vegetables pose a serious threat to increasing microbial tolerance to antimicrobial agents (Chorianopoulos *et al*., 2008). Majority of the foodborne biofilm forming bacteria of health and quality concern belong to the genera *Listeria, Staphylococcus, Alcaligenes, Enterobacter, Flavobacterium and Pseudomonas* (Lee *et al*., 2009).

A number of the antimicrobial agents such as chlorine dioxide, sodium nitrate and ionizing radiations have been used to eliminate the bacteria in nature, household, and for bacterial infection treatment over the past five decades (Chorianopoulos *et al*., 2008). Due to the continued

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application of antimicrobial agents, microbial resistance to conventional antibiotics also increases (Capita and Alonso-Calleja, 2013). It is one of the top priority matters of food industry to have well cleaned and proper sanitizing/ disinfection method to avoid biofilm contamination. However, used methods of disinfectants are not so ideal due to poor penetration ability in biofilm matrix. Search for new substances as disinfectants is the target area in food industry. The negative approach of consumers to the artificial synthetic chemicals as disinfectants have shifted our focus more towards "naturals". It has been documented previously that essential oils and extracts of edible and medicinal plants, herbs and spices act as very potent natural antibacterial agents (Chorianopoulos *et al*., 2008). Advancements in sciences and technology enable the innovative development of new pharmaceuticals with better remedial activity and less side-effects by the use of natural plants. Substances and compounds extracted from plant parts (leaves, roots, stems and seeds) are now the basis of innovative research for potential applications of antimicrobial activity (Shokeen *et al*., 2009).

Plant products, mostly spices and extracts of a range of plant parts have been used comprehensively as natural antimicrobials and antioxidants (Oskay *et al*., 2009). *S. aromaticum* (Clove) is an evergreen aromatic plant belonging to the Myrtaceae family (Chaieb *et al*., 2007) that was found to have antibacterial and anti-inflammatory activities against food borne pathogens like *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli*. It is reported that eugenol is the major antimicrobial component of *S. aromaticum* which make its way to check the antimicrobial activity to food borne harmful bacteria (Naveed *et al*., 2013).

Camellia sinensis (Green tea) possesses antibacterial, antioxidant, anti-inflammatory and anticarcinogenic activity against various pathogens such as *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, *Enterococcus* spp.), some fungi (*e.g*., *Candida albicans*), and a variety of viruses (*e.g*., HIV, herpes simplex, influenza) (Wanda, 2014). *C. sinensis* is reported to contain 4000 bioactive compounds, out of which one third is attributed to catechin (polyphenols) (Tariq *et al*., 2010). Methanolic extracts of *C. sinensis* have already been reported to limit the growth of food borne pathogens (Kim *et al*., 2001).

Mentha piperita (piper mint) is a medicinally significant herbaceous plant belonging to family Labiate. *M. piperita* is very important as its leaves and extracts are used in foods, cosmetics and medicines (Scavroni *et al*., 2005). *M. piperita* extracts are able to cease the growth of various pathogens as well as fungi extending the shelf life of food products (Tiwari *et al*., 2009).

This study was aimed to assess the resistance of food borne bacterial isolates against commercial antibiotics and their biofilm forming ability. Additionally, antibacterial and antibiofilm potential of aqueous and methanolic extracts from medicinal herbs and spices (*S. aromaticum*, *C. sinensis*, *M. piperita*) was also evaluated against food industry isolates.

MATERIALS AND METHODS

Sample collection

Eighteen samples were collected from Shaukat Banaspati Ghee industry (Grw) and Shezan foods (pvt) Ltd, Lahore, Pakistan. Sampling was done using sterilized cotton swabs on different food contact surfaces like floor, drains, and stainless steel surfaces of food industries where the food was processed. Samples were taken to the Immunology Lab, Zoology Department GCU, Lahore under sterile conditions and processed within two hours.

Isolation and characterization of purified strains

Bacterial strains were isolated and purified by various rounds of streaking and restreaking on nutrient agar plates. Purified microbes were characterized morphologically, physiologically and biochemically following Gerhardt *et al*. (1994). Gram's staining, acid fast staining and motility assays were performed. Biochemical test included catalase test, citrate utilization, H_2S production, methyl red, voges proskauer, indole, denitrification, urease, TDA test, and carbohydrate fermentation. Growth curves were generated in nutrient broth for 15 h to determine the generation time and specific growth rate of the strains. Impact of pH, temperature and antibiotics was monitored in nutrient agar. Five different disks with different concentrations including Ampicillin (Am-10µml-1), Oxacillin (Ox-1µml-1), Carbenicillin (Py-100µml-1), Tetracycline (Te-30µml-1), and Amoxicillin (Ax-25µml-1) were used to check the antibiotic resistance profile on Mueller Hinton agar using dis diffusion agar method (Bauer *et al*., 1966).

16S ribosomal RNA gene sequencing

16S RNA gene sequencing was performed to identify the taxonomic position of the isolates. The forward primer (-3′) and reverse primer (16S-27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-1522R (5′- AAGGAGGTGATCCAGCCGCA-3′) (Penicon) were used. Amplification reactions were performed under standard conditions (Wojtyczka *et al*., 2014). The PCR products were gel purified using the QIAquick Gel purification kit (QIAgen, Germany) and subjected to cycle sequencing. Obtained data were analysed with the Chromas Lite (Technelysium Pty Ltd., Tewantin, QLD, Australia) software and compared with the sequences deposited in the GenBank database. A phylogenetic tree

was constructed using the ClustalW software.

Quantification of biofilm formation

Biofilm formation of the identified bacterial isolates was monitored by using the congo red assay (Mathur *et al*., 2006), test tube method (Liaqat *et al*., 2009), and air-liquid interface coverslip assay (Mathur *et al*., 2006). Experiments were carried out in triplicates.

Preparation of aqueous and methanolic plant extracts

Syzygium aromaticum (clove), *M. piperita* (peppermint) and *C. sinensis* (green tea) were used for the preparation of plant extracts. The leaves of *M. piperita* were washed with distilled water and sun dried before grinding. Aqueous and methanolic extracts of plants were prepared and stored at 4°C (Somchit *et al*., 2003). For *S. aromaticum* aqueous extract, cloves were dried for 3-4 days in an incubator at 37°C and ground into fine powder using pestle and mortar. 40 g of clove powder was dissolved in 200 ml of autoclaved distilled water, boiled, centrifuged and stored at 4°C until use (Badhe *et al*., 2013). Methanolic extracts of plants were prepared by dissolving 60 g of plant powder in 360 ml methanol then placed in a shaker at 37°C for 24 h. The extracts were filtered, concentrated on a rotary vacuum evaporator (Agrawal, 2011) and stored at 4°C. For *S. aromaticum* methanolic extract, 40 g of *S. aromaticum* powder was mixed in 300 ml of 80 % methanol. The mixture was then filtered with Whatman filter paper no.1 and placed in an incubator at 37°C until methanol evaporated completely. The concentrated clove extract was then dissolved in 2X tris HCl (pH 8.0) and stored at 4°C until use (Pandey and Singh, 2011).

Minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) determination

MIC of the extracts was determined by broth dilution method (Pirbalouti *et al*., 2010). The aqueous and methanolic extracts of *S. aromaticum*, *M. piperita* and *C. sinensis* were diluted to concentrations ranging from 5 to 45 mg ml^{-1} in 3 ml nutrient broth. 150 µl of overnight bacterial suspension with a turbidity of 0.5 MacFarland standard (1.5 x 10^8 CFU/ml) was inoculated in the test tubes. Streptomycin sulphate (10 µgml^{-1}) and Amphotericin B (5 mgml^{-1}) while extract free media were used as positive and negative controls respectively were run in parallel to each concentration. After incubation for 48 hrs OD was determined at 523 nm. MIC was recorded as the lowest concentration which showed no visible growth. For MBC determination, 10 µl from the tubes with MIC and higher concentrations of extract were spread on nutrient agar plates, incubated plates at 37°C for 24 h. The concentration at which the 99% of the growth was inhibited was recorded as MBC (Okoli *et al*., 2002; Hassan *et al*., 2016).

Antibacterial activity

Plant extracts are popular for their antimicrobial activity. Antibacterial screening of plant extracts was carried out by measuring zone of inhibition by agar well diffusion method (Rios *et al*., 1998). Four dilutions (100%, 75%, 50% and 25%) of both aqueous and methanolic extracts were prepared. 100 µl of fresh bacterial suspension [with OD approx. 1.5 x 10^8 CFU/ml by comparing turbidity to 0.5 MacFarland standard (Koneman *et al*., 1997)] was spread on labeled nutrient agar plates with a sterilized glass spreader. Wells were made on each agar plate, labeled and filled with 200 µl plant extracts. Experiments were run in triplicates. Antibacterial activity of plant extracts was also tested against mixed cultures. Mixed cultures were prepared by adding equal volume of *E. cloacae* + *E. coli, E. cloacae* + *E. ludwigii, E. coli* + *E. ludwigii* and *E. cloacae* + *E. coli* + *E. ludwigii*.

Susceptibility of biofilms against plant extracts

Plant extracts were supplemented to the 3ml nutrient broth with concentration according to their MIC. 30 µl of standard bacterial inoculum was added to the test tubes. Controls inoculated only with bacterial suspension were run in parallel. After incubation, culture was discarded and tubes were dried at 37ºC for 10 min. 5 ml of 0.1% crystal violet was added and then tubes were washed with 0.85% saline solution. O.D was measured at 523 nm. Experiments were run in triplicates.

Statistical analysis

Data was analyzed in SPSS (Version 13.0) using one way ANOVA followed by post hoc Turkey test. Bars having no common superscript are significantly different at $p<0.05$.

RESULTS AND DISCUSSION

Morphological and biochemical characteristics of isolated strains

Twenty four morphologically distinct strains were isolated from 18 samples collected from food industries. These isolates were characterized on the basis of color, texture, margin and elevation. Majority of the strain (79%) Gram-negative while 21% were Gram-positive. All strains showed negative result for acid fast staining. Whereas 70% of the strains showed good motility. Ten strains showing resistance against ampicillin, carbenicillin, and amoxicillin were subjected to the biochemical characterization, identified strains were found to belong to genera; *Enterobacter, Shigella, Pseudomonas, Escherichia* and *Klebsiella* (Data not shown).

16S rRNA gene sequencing and physiological characterization

Molecular characterization was done by ribotyping of

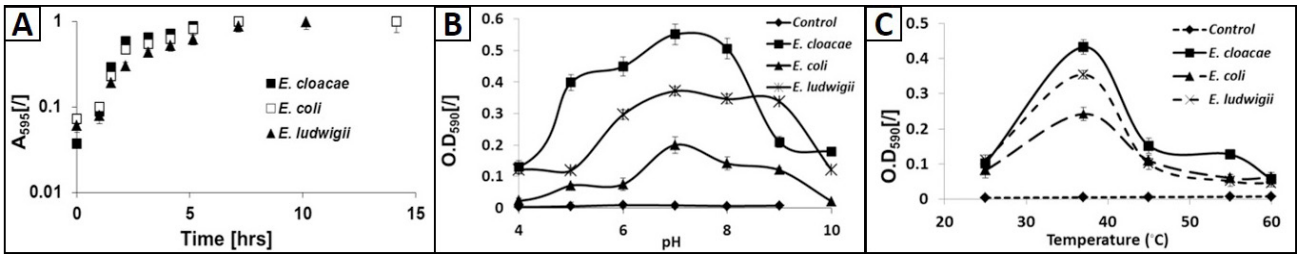

Fig. 1. **A,** Growth curves of antibiotics resistant isolates; **B,** Growth of antibiotics resistant isolates at different pHs (4,5,6,7,8,9,10); **C,** Growth curves of antibiotics resistant isolates at different temperatures (25°C, 37°C, 45°C, 55°C and 60°C) for 24 h.

16S rRNA gene of three highly resistant strains. Theses strains were resistant against all the five antibiotics studied here (Data not shown) and were identified as *E. coli* (AC: KM658273), *E. cloacae* (AC: KM658274), and *E. ludwigii* (AC: KM658275). This is in agreement with Van Houdt and Michiels (2010), who isolated bacteria from a variety of industries including the food industry and reported their ability to form biofilms on a variety of biotic and abiotic surfaces. Physiological characterization of the three strains indicated that all strains have a log phase of 1.5 up to 5.15 h. Afterwards, stationary phase was observed (Fig. 1A). pH preference remains same in all three strains growing best at 7. All strains showed optimum growth at 37°C temperature with a specific growth rate ranging from 0.59±0.02 to 0.42±0.05 h^{-1}. Growth ceased at 45-60°C (Fig. 1B, C). It has been reported previously that when the temperature falls beyond the optimum, the enzymatic activity would be inhibited due to destabilization of three-dimensional structure of enzymes, causing denaturation. This process decreases the reaction velocity (Bisswanger, 2014).

Quantification of biofilm formation

Biofilm formation was determined qualitatively by congo red assay in which all the strains showed black crystalline colonies indicating positive biofilm forming potential. Quantitatively, the test tube method proved to be the most reliable method (Liaqat *et al*., 2009) among all methods of biofilm formation. Experiments were conducted for 3, 5 and 7 days. Highest biofilm formation was achieved at 72 h of incubation. *E. coli* exhibited highest biofilm formation by tube method compared to *E. cloacae* and *E. ludwigii* (Fig. 2A). Air-liquid interface coverslip assay revealed again *E. coli* as strongest biofilm former after 72 h (Fig. 2B). Also the increased biofilm formation exhibited by different strains may be related to different properties of the attachment surface such as surface roughness, disinfectability, hydrophobicity and vulnerability to wear (Van Houdt and Michiels, 2010).

MIC and MBC determination

MIC and MBC of aqueous and methanolic plant extracts against bacterial strains isolated from food industry was determined. Results showed that MIC of aqueous plant extracts was in the range of 5-40 mg ml^{-1} against *E. cloacae, E. coli,* and *E. ludwigii* and of methanolic extracts was in the range of 15-45 mg ml^{-1}. Usually MIC value was in the range below MBC. Our study revealed that in *E. cloacae, S. aromaticum* methanolic extract showed MIC value

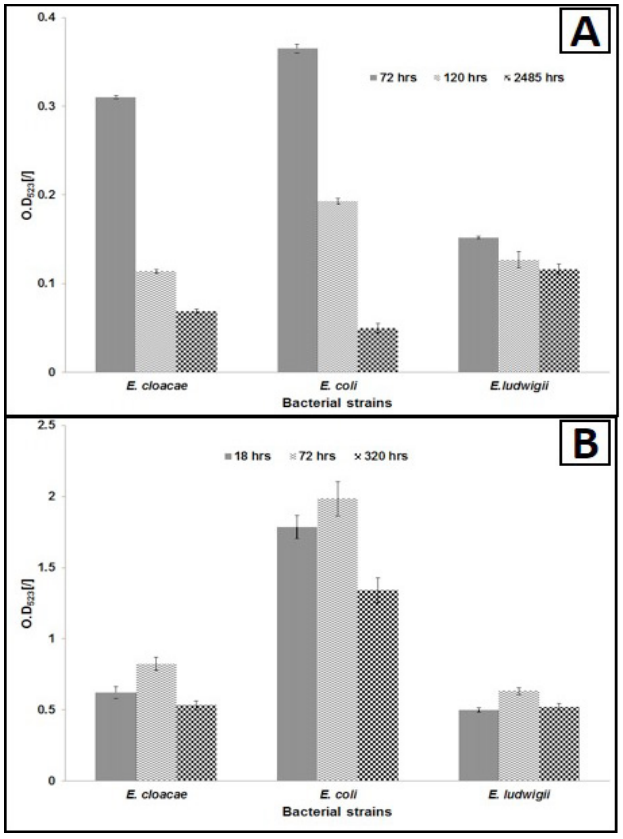

Fig. 2. **A,** Time course of biofilm formation by the three food industry isolates *E. Cloacae* sp. (GenBank accession no. KM658273), *E. coli* (KM658274) and *E. ludwigii* (KM658275) by test tube method; **B,** By cover slip assay. Data were obtained from the average of three independent experiments.

of 5.0 mg ml^{-1} (Fig. 3A) which is in accordance with the MIC values of ethanolic and aqueous extract of *S. aromaticum* ranging from 0.5-5.5 mg ml^{-1}, 0.8-5.5 mg ml^{-1}, respectively (Hoque *et al*., 2008). In contrast, aqueous extract of *S. aromaticum* proved to be more effective showing significantly low MIC (5, 15 mg ml^{-1}) and MBC values (20, 25 mg ml^{-1}) for *E. coli* and *E. ludwigii,* respectively (Fig. 3B, C). Plant extracts have the ability to inhibit bacterial growth due to the presence of some biologically active compounds like phenolics, alkaloids and terpenoids that interfere with the metabolic machinery of the bacterial cell (Dybey and Bhadauria, 2009).

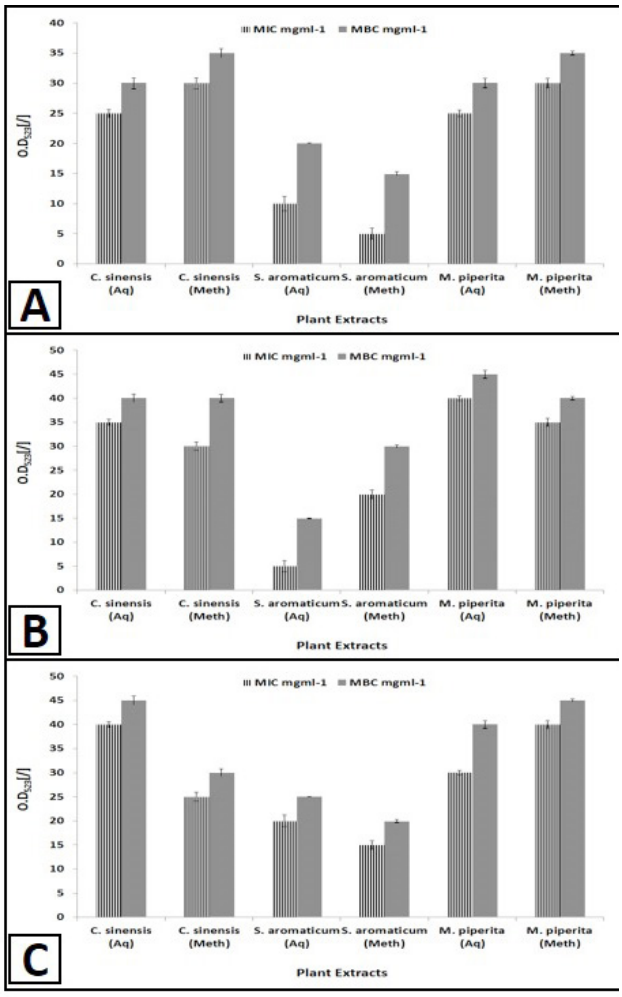

Fig. 3. MIC and MBC determination of aqueous and methanolic plant extracts against food industry isolates. **A,** *E. Cloacae* sp. (GenBank accession no. KM658273); **B,** *E. coli* (KM658274) and C, *E. ludwigii* (KM658275).

Antibacterial activity of plant extracts in planktonic and biofilm mode

Aqueous and methanolic extracts of *C. sinensis, S. aromaticum* and *M. piperita* were tested for their antibacterial activity against three highly resistant and good biofilm forming bacteria both in planktonic and biofilm mode. Methanolic extracts were found to have more potent antimicrobial activity than aqueous extracts against all tested strains. Extracts of *C. sinensis* and *M. piperita* showed weak antibacterial activity against tested mono culture bacterial strains as compared to *S. aromaticum* extract (Figs. 4, 5). Methanolic extract of *C. sinensis* showed highest antibacterial activity against *E. coli* in planktonic monoculture compared to mixed culture where aqueous extract was most effective. In biofilm mode, *C. sinensis* methanolic extract was found to have highest antibacterial activity both in mono and mixed culture food industry isolates (Fig. 5A, B). It has been experimentally shown that extracts and essential oil from peals of citrus fruits

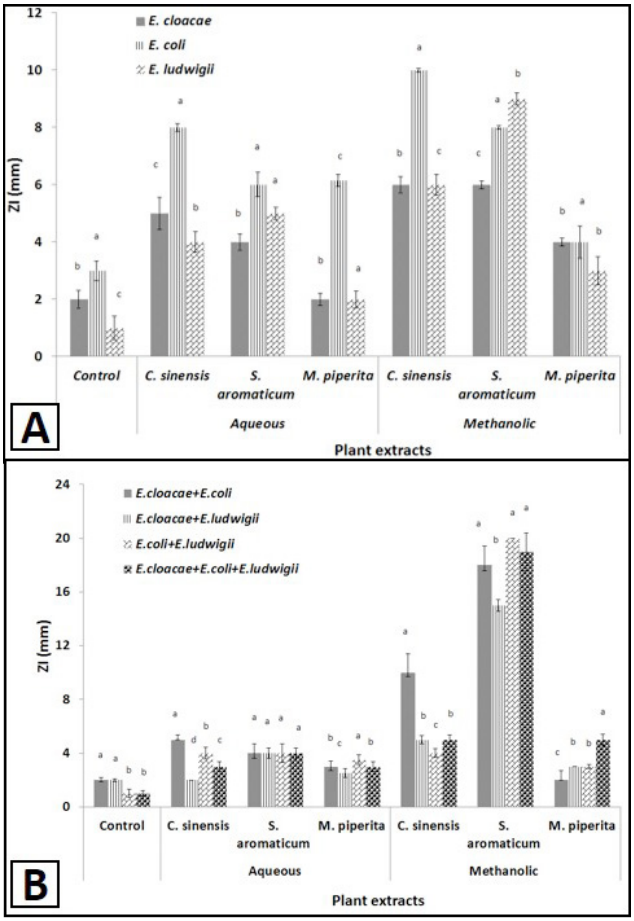

Fig. 4. Comparison of antibacterial activity of plant extracts on monoculture (**A**) and mixed culture (**B**) food industry isolates in planktonic mode. Data was analyzed in SPSS (Version 13.0) using one way ANOVA followed by post hoc Turkey test. Bars having no common superscript are significantly different at $p<0.05$.

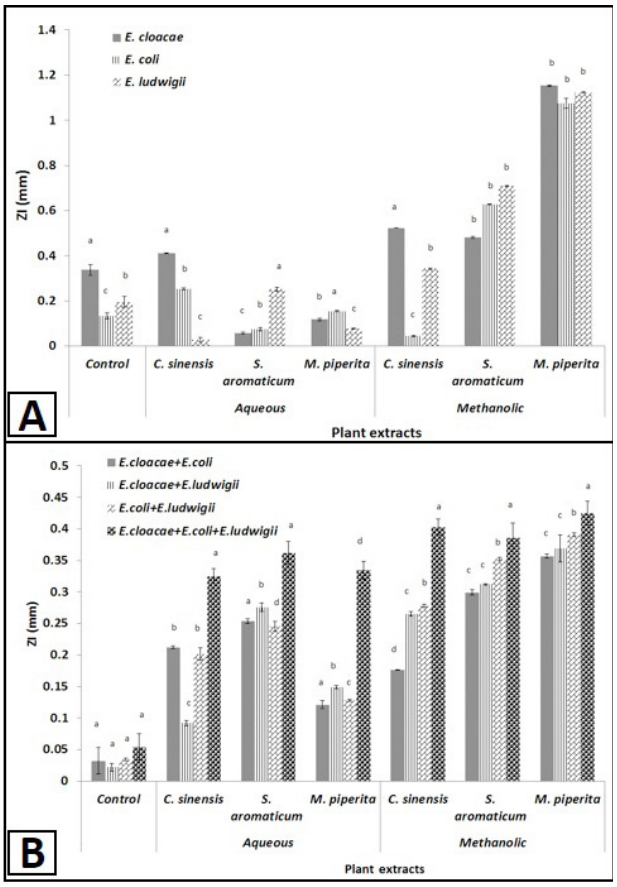

Fig. 5. Comparison of antibacterial activity of plant extracts on monoculture (**A)** and mixed culture (**B**) food industry isolates in biofilm mode. Data was analyzed in SPSS (Version 13.0) using one way ANOVA followed by post hoc Turkey test. Bars having no common superscript are significantly different at $p<0.05$.

exhibit inhibitory activity against microorganisms. Mandal *et al*. (2011) assessed the *in vitro* antibacterial activity of *C. sinensis* ethanolic extract and found it useful in combating emerging drug-resistance among enteropathogens including *S. typhi* and *V. cholerae* Ogawa. In another study, Maduhri *et al*. (2014) evaluated the antibacterial activity of peel extracts from *C. sinensis* and *C. aurantium* and observed that *C. sinensis* had a greater antibacterial activity than *C. aurantium*.

In planktonic mode, *S. aromaticum* methanolic extract showed maximum zone of inhibition 8.0 ± 0.0 (ZI±S.D) against monoculture of *E. coli*, and this result is in accordance with a study that showed *E. coli* exhibited inhibition zone of 8±1 when exposed to clove extract (Saeed and Tariq, 2008). Sethi *et al*. (2013) reported that *S. aromaticum* extract was proven to be an active inhibitor of food borne bacteria. This is also what we observed in our study where *S. aromaticum* methanolic extracts resulted in highest zone of inhibition 20.00 ± 0.02 against mixed culture of *E. coli* and *E. ludwigii* (Fig. 4A, B). Likewise, methanolic extract of *S. aromaticum* exhibited significantly higher anticaterial activity both in mono and mixed culture biofilm (Fig. 5A, B). Lopez *et al*. (2005) also proved that *S. aromaticum* oil is equally effective against food borne Gram positive and Gram negative bacteria.

M. piperita showed antimicrobial activity in the form of aqueous and methanolic extract in both mono and mixed culture isolates. In comparison with methanolic extract of *M. piperita*, aqueous extract against *E. coli* showed a greater zone of inhibition (6.2±0.2). While in combination of *E. cloacae, E. coli,* and *E. ludwigii,* zone of inhibition was 4.9±0.4 (Fig. 4A, B). Antibacterial activity of *M. piperita* is in correlation with the study that its oil has strong antibacterial and antioxidant activities against both Gram negative and Gram positive bacterial strains (Lopez *et al*., 2005). Methanolic extracts of *M. piperita* were found to be more effective in monoculture while no significant difference was observed in antibacterial activity of aqueous or methanolic extracts in planktonic mode (Fig. 4A, B). Sandasi *et al*. (2011) also reported that *M. piperita* showed highest antimicrobial activity against *P. aeruginosa* and *Candida albicans* both in planktonic and biofilm mode. A similar pattern was observed in biofilm mode in our study where methanolic extracts were observed to have more potent antibacterial activity in mono or mixed culture in biofim mode than aqueous extract (Fig. 5A, B). Saharkhiz *et al*. (2012) examined antifungal activity of essential oil of *M. piperita* and observed that it inhibited the biofilm formation of *C. albicans* and *C. dubliniensis*. Considering the wide range of the antifungal activities, he concluded that it might be used as a potential antifungal agent in food industry.

CONCLUSIONS

Food hygiene is the first and foremost concern for both developing and developed countries. The presence of diseases causing biofilm forming microbes in food contact surfaces offers alarming situation for food hygiene. Bacterial population develop resistance more and more and ultimately having genetic variability due to the excessive use of commercial antibiotics for the treatment of diseases. Due to this increased resistance microbial biofilms are difficult to eradicate. There is an urgent need to identify effective alternatives to overcome this problem. Results reported in our study contributed to the knowledge of antibacterial and therapeutic properties of Pakistani medicinal plants. Both aqueous and methanolic plant extracts of *C. sinensis, S. aromaticum,* and *M.*

piperita proved to have considerable antibiofilm activity and thus used as a substitute way for treatment of food-borne diseases. The results of present investigation clearly indicate that the antibacterial and antifungal activity vary with the species of the plants, plant material, and type of extract used. Hence, the present study ascertains the value of plant extract before applying in food industry, which could be of considerable interest to the development of new drugs. On the other hand more research is needed on medicinal benefits and possible harmful effects of herbal plants to confirm the safety of their effective concentrations for human consumption.

Statement of conflict of interest

Authors have declared no conflict of interest.

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