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Evaluation of antimicrobial effects of *Rosemary* and *Withania somnifera* methanol extract prepared by ultrasound waveform on *Escherichia coli* biofilm isolated from urinary tract infection

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A B S T R A C T

Generally, bacteria co-exist setting up communities associated to solid superficies, this is to which we refer as biofilms that serve as a survival strategy. This type of formation cause serious sanitary problems for both humans and animals. Nowadays, chemical or natural compounds able to block this formation are looked for. In this project, we have evaluation of antimicrobial effects of *Rosemary* and *Withania somnifera* extract prepared by ultrasound waveform on *Escherichia coli* biofilm isolated from urinary tract infection. *Rosemary* and *Withania somnifera* extracts were obtained by using rotary devices. *E. coli* was collected from the patients being cured in Zabol hospitals. The least hindering and killing concentration of the samples were calculated by lowering their densityin sinks. The growth and biofilm formation of strains were determined by microtiterplate method. Data were analyzed statistically by determination of significant difference using analysis of variance (ANOVA) test. All tests were analyzed at the significance level $P<0.05$. The results revealed that the concentrations of 5 and 10 mg/mL are the most restrain in the biofilm formation of the isolated plates. *Rosemary* and *Withania somnifera* extracts have considerable antimicrobial and ant biofilm effect on the samples of the *E. coli* resistant to antibiotics.

1. Introduction

The use of herbal compounds for the treatment of infections is an old method in many parts of the world, especially developed countries. Paying attention to medicinal herbs with microbial properties can restore the common problems with the use of antibiotics. Rosemary is a plant that is a perennial *lamacae* that forms in the lush greenery of all seasons. The characteristics of this plant are a great smell of leaves, which is known as an aromatic herb. Due to its antimicrobial, anti-mutagenic and chemical precursor properties, it has been recognized as a valuable medicinal plant in the pharmaceutical and medical industries [1, 2].

Essential oils of this plant include boronol, limonene, camphon, camphor and other herbal compounds such as phenolic acids, including daily acids, caffeic acid and chlorogenic acid [3].

Withania somnifera is a plant from the *Solanaceae* family. It grows in Africa, the Mediterranean, and India, and is used as antibacterial, antioxidant, anti-inflammatory and liver diseases [4].

Conventional extraction methods are based on plant placement in a solvent that is used to increase the process speed of mixing or heating. Soxhlet method is a standard method used as the reference for evaluating other methods. This method is general, mainly used to extract low or moderate volatility compounds that are resistant to heat(5). The use of ultrasound in extraction is one of the new methods of extraction that the waves

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penetrating into the material at a frequency of 20 kHz, causing continuous expansion and contractions in the internal molecules resulting in the formation of cavities. These cavities, asymmetrically interconnected, cause the material to be extruded from the cells out of it [5].

Microbial biofilms are communities of bacteria, embedded in a self-producing matrix, forming on living and nonliving solid surfaces([6]. Biofilm-associated cells have the ability to adhere irreversibly on a wide variety of surfaces, including living tissues and indwelling medical devices as catheters, valves, prosthesis, and so forth.

2. Methods

2.1 Extract preparation and investigation of the antimicrobial effects of the plant extract

The wind cheese used in this study was gathered from Sistan and Baluchistan province (fig. 1). They were detected to be wind cheese by a researcher from the University of Zabol. The samples were cut and 10g of the dry plant powder was put into half-liter erlens containing 100ml of methanol. The contents of erlens were shaken 24 hours at room temperature b shaker device with speed of 130 rpm and were kept in ultrasonic carrier for 10 minutes and were filtered with Wattman paper. Separation of solution from extract was done by rotary device and vacuum pump. The obtained extract was weighed and solved in DMSO. The samples were used in fridge at 4 degrees of temperature to be used in antimicrobial tests.

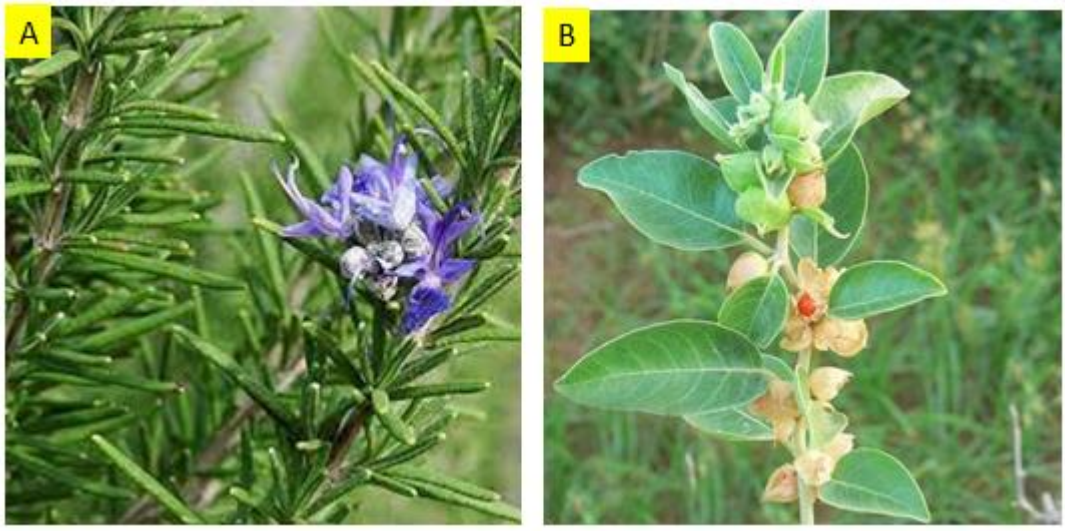

Fig. 1. Characteristic of *Rosemary* (A) and *Withania somnifera* (B)

2.2 The antimicrobial effect of the extract on E. coli

Sensitivity of the bacteria samples with multiple resistances to the wind cheese extract against *E. coli* was analyzed by dilution method in broths. To this end, seven broths of microtitre plates were injected 100 ml of MHB. The first broth was added 100ml of the diluted extract. Then, 100ml of the first broth was transferred to the second one and the same was done to the last broth. 100ml of the last broth was removed and 100ml of the microbial suspension with 107 units per ml was added to all broths. The mixture was kept 24 hours at temperature of 37 degrees. The first broth inhibiting the growth of bacteria after being positioned in the incubator was considered as MIC and for more precision; 10 ml of the light broths was transferred to Moller environment. After 24 hours, the first concentration removing 99.9% of the bacteria was regarded as MBC.

2.3 Biofilm formation assay in presence of the biocides:

After performing the procedure described above, the microplate was covered and

incubated aerobically for 24 h at suitable temperature. At first, the OD (Optical Density) was measured (600 nm) by using an automated ELISA counter, then, the content of each well of the microplate was aspirated and each well was washed three times with 250 µL of sterile physiological saline. The remaining attached bacteria were fixed with 200 µL of 99% methanol per well and after 15 min all of the wells were emptied and left to dry. Then, each well was stained for 5 minute with 0.2 mL of 2% crystal violet. Excess stain was rinsed off by washing the plate slowly with distilled water. After the plate was air dried, the dye bound to the adherent cells was re-solubilized with 160 mL of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 492 nm by using an automated ELISA counter.

2.4 Antibiotic activity

Four pure strains of Escherichia coli were determined by Kirby-Bauer antibiogram and their susceptibility to antibiotics was evaluated. Antibiotics used in this study included gentamicin, amoxiclav, azithromycin, and amikacin (manufactured by Antibody Medicine). After 24 hours of incubation at 37 ° C, the diameter of the inhibitory halos was measured and the sensitivity and resistance of the strains were determined and the results were compared with the NCCLS standard table[7].

2.4 Statistical analyses:

The growth was compared at each experiment using analysis of variance (ANOVA) repeated measures (GraphPad 8). The level of statistical significance was set at $p< 0.01$.

3. Results

The results of this study showed that the highest resistance to ceftazidime, gentamicin, amoxiclav and azithromycin were 75%, 50%, 25% and 25%, respectively. While the highest sensitivity to antibiotics azithromycin, gentamicin and amoxiclav was equal to 50% (Table 1).

The results of this study showed that the lowest inhibitory concentration of cheese extract was 2.5 mg/ml, while the highest inhibitory concentration was 5 mg/ml, the highest lethal concentration was 10 mg. mg/ml, in which two strains were completely eliminated at this concentration, and the lowest lethal concentration was equal to 2.5 mg/ml, in which one strain was eliminated in this concentration.

The lowest and highest inhibitory concentrations of rosemary extract were 5 and 10 mg/ml, in which 3 and 1 strains were inhibited, while the highest lethal concentration was 20 mg/ml, which was one. The strain is eliminated at this concentration (Table 2).

The results of this study showed that by increasing the concentration of the extract, the amount of biofilm formation decreases. As the concentration of the *Withania somnifera* extract increased, biofilm formation decreased more than rosemary. The results revealed that the concentrations of 5 and 10 mg/mL are the most restrain in the biofilm formation of the isolated plates (fig. 3 and 4).

Table 1. Antibiotic pattern of Escherichia coli strains (%)

Resistance level	Ceftazidime	Amoxiclav	Amikacin	Gentamicin	Azithromycin
S	0	50	25	50	50
I	25	25	75	0	25
R	75	25	0	50	25

S= sensitive; I= Intermediate; R= resistant

Table 2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) (mg/ml)

Bacterial Strains	*Rosemary*		*Withania coagulans*	
	MBC	MIC	MBC	MIC
1	10	5	10	5
2	10	5	5	5

3	5	5	2.5	2.5
4	20	10	10	5

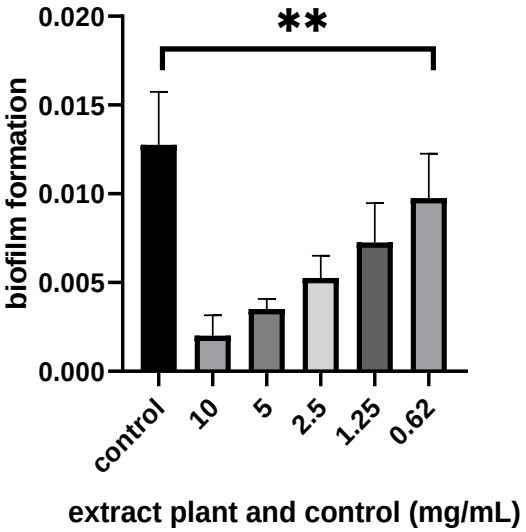

Fig. 2. The effects of different concentrations of extract plant of *Withania somnifera* on the biofilm formation of bacterial and control

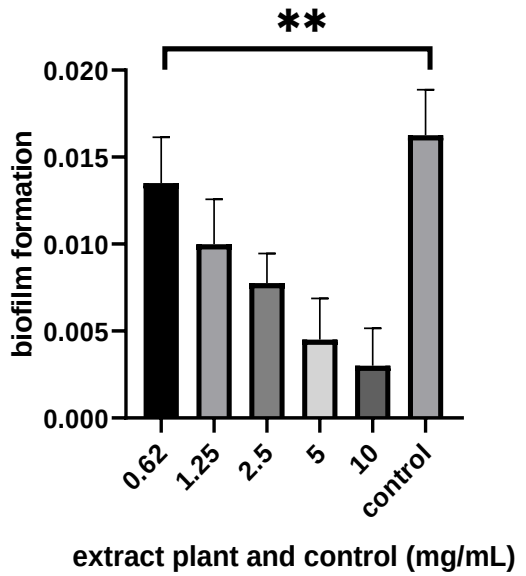

Fig. 2. The effects of different concentrations of extract plant of *Rosemary* on the biofilm formation of bacterial and control

4. Discussion

Natural products derived from medicinal plants are abundant source of biologically active compounds. Many plant compounds have been used for development of new antimicrobial agents [8, 9]**.** However, few plant extract have been investigated for their antibiofilm activity.

The study of W,AL Shahwany, The result showed that the isolates behaved differently in their sensitivity to the different extracts added to their growth medium. Among the different plant phenolic extracts tested, *Z. officinale* showed antibiofilm efficacy against *k. pneumoniae* and *S. aureus* followed by T. vulgaris and *C. zeylanicum* extracts exhibited [10].

The study of Nikolic, The antibacterial and anti-biofilm activity of ethanolic extract from the rhizome of *Zingiber officinale* were evaluated. The values were in the range from 0.0024 to > 20 mg/ml. The most sensitive bacteria were Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923. Anti-biofilm activity was tested by crystal violet assay. *Pseudomonas aeruginosa* ATCC 27853, Proteus mirabilis and *E. coli* ATCC 25922 were used as the test organisms. Ethanolic extract showed the best result on Proteus mirabilis biofilm where biofilm inhibitory concentration (BIC50) was 19 mg/ml [11].

Yahya et al. (2013) found out that the ethanolic extract of *Z. officinale* inhibited *P. aeruginosa* biofilm formation under both aerobic and anaerobic environments [12].

Lycium chilense and *Schinus fasciculatus* were the most effective antimicrobial plant extracts (15.62µg/ml and 62.50µg/ml for *Staphylococcus* sp. Mcr1 and *Bacillus* sp. Mcn4, respectively). The highest (66%) anti-biofilm activity against *Bacillus* sp. Mcn4 was observed with *T. absinthioides* and *L. divaricate* extracts. The highest (68%) anti-biofilm activity against *Staphylococcus* sp. Mcr1 was observed with *L. chilense* extract. *T. minuta, T. absinthioides,* and *L. divaricata* showed percentages of anti-biofilm activity of between 55% and 62%. The anti-adherence effects of *T. minuta* and *L. chilense* observed in *Bacillus* sp. Mcn4 reflected a difference of only 22% and 10%, respectively, between anti-adherence and biofilm inhibition. Thus, the inhibition of biofilm could be related to cell adherence. In *Staphylococcus* sp. Mcr1, all

plant extracts produced low anti-adherence percentages [13].

The study of Geethashri, demonstrate the efficacy of aqueous extract of *Azadirachta indica*, *Mangifera indica*, *Piper betel* and *Pepper nigrum* for antibiofilm activity against *E. faecalis* and *S. aureus.* The aqueous extracts were obtained by cold percolation method. The antibiofilm activity of plants extract was evaluated at 30, 15 and 7.5 mg/ml concentration. The percentage yield of extract was maximum in *P. nigrum*. The aqueous extract of *A. indica* significantly suppressed *E. faecalis* and *S. aureus* biofilm at 7.5 mg/ml at $p<0.01$ and $p<0.001$ significance level. *P. betel* significantly ($p<0.001$) disintegrated the *E. faecalis* biofilm at 30 mg/ml and *S. aureus* at 15 mg/ml ($p<0.01$). *P. nigrum* disintegrated *E. faecalis* and *S. aureus* biofilm significantly ($p<0.05$ and $p<0.001$) at 30 and 15 mg/ml respectively. *M*. *indica* significantly ($p<0.05$) suppressed *S. aureus* biofilm at 30 mg/ml [14].

The study of Varposhti, biofilm formation of *P. aeruginosa* strain 214 was determined in presence of three plant extracts, *Cyclamen coum*, *Dianthus orieltalis* and *Origanum majorana*, and *Zataria multiflora* Bio essential oil. The *C. coam* extract and *Z. multiflora* Bio essential oil inhibited biofilm formation completely at concentrations<0.062 mg/ml and 4 7l/ml, respectively. The *D. orientalis* and *O. majorana* extracts did not inhibit biofilm formation at the used concentrations (0.003 – 8 mg/ml) [6].

The study of Gaetti-Jardim, antimicrobial activity of plants extracts on microbial biofilms was determined in microplates. *Psidium cattleianum* and *Myracrodruon urundeuva* extracts demonstrated significant inhibitory activity on all bacterial strains tested; alcoholic and aqueous extracts showed similar results [15].

The study of Shafiei, The PEM compared with its respective constituent plants showed the lowest MIC towards *S. sanguinis* (3.81 mg/ml) and *S. mutans* (1.91 mg/ml) and exhibited a synergistic effect. The *Psidium* sp. (15.24 mg/ml) and, PEM and *Psidium* sp. (30.48mg/ml) showed the lowest MBC towards *S. sanguinis* and *S. mutans* respectively. The anti-adherence effect of the PEM and its respective constituent plants (except *Psidium* sp.) was different for the two bacteria in the single-species biofilm [16].

The study of Mohsenipour and Hassanshahian, was aimed to examine the effect of *Thymus vulgaris* (*T. vulgaris*) extracts on the planktonic form and biofilm structures of six pathogenic bacteria. According to disc diffusion test (MIC and MBC), the ability of *Thymus vulgaris* (*T. vulgaris*) extracts for inhibition of bacteria in planktonic form was confirmed. In dealing with biofilm structures, the inhibitory effect of the extracts was directly correlated to their concentration. Except for the inhibition of biofilm formation, efficacy of each extract was independent from type of solvent [17].

The study of Chevalier, Saponin concentrations reached 0.7 and 0.95 mg ml”1 in S. virgaurea subsp. virgaurea and S. virgaurea subsp. Alpestris extracts, respectively. C. albicans was grown in liquid medium and cells were counted by microscopic examination after 0, 4 and 24 h of incubation. Solidago extracts did not inhibit the growth of *C. albicans* (four strains), *E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, *S. mutans*, *Streptococcus salivarius* or *Enterococcus faecalis*. When inocula were incubated with Solidago extract for 4 and 24 h, we observed a decrease in Candida yeast–hyphal transition. Candida biofilms were then prepared in microtitre plates and treated with plant extracts at 0 h, to estimate biofilm formation, or at 18 h to estimate the effect of the saponin on pre-formed biofilms [18].

The study of Bhunu, the effect of the leaf extracts of P. curatellifolia on *M. smegmatis* growth and biofilm formation was investigated in order to determine the basis of its use in traditional medicinal use. The minimum inhibitory concentrations (MIC) of the extracts were found to be 6.2 µg/ml for the acetone extract, 12.5 µg/ml for both the ethanol and the total extract and 50 µg/ml for both the methanol and ethyl acetate extracts. The ethanol extract, dichloromethane extract and water extract were the only extracts that effectively inhibited biofilm formation in *M.*

smegmatis. Combining the ethanol extract with kanamycin enhanced the effect of the ethanol extract in terms of inhibition of biofilm formation [19].

The study of Sasirekha, an attempt has been made to screen different plant extracts against microbial biofilms. Out of 33 isolates BF5 was found to be a high biofilm producer by crystal violet assay with high hydrophobicity. On studying the factors influencing biofilm production increase in glucose concentration, acidic and alkaline pH, high osmolarity and low iron concentration promoted BF 5 biofilm formation. Among the different plant extracts tested, turmeric showed 100% efficacy against BF5 followed by cloves (97%), Indian borage (91%) and *Aloe vera* (84%)[20].

The study of Teanpaisan, evaluate the antimicrobial and antibiofilm activity of A. lakoocha extract against oral pathogens by an in vitro method. All tested strains were susceptible to A. lakoocha extract with variable degrees of antimicrobialminhibition. The extract was effective against both Gram-negative bacteria (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*) and Gram-positive bacteria (*Streptococcus mutans*, Streptococcus sobrinus), with MIC ranging from 0.10 – 0.39 mg/ml and MBC from 0.10 – 3.12 mg/ml. Killing activity depended on time and concentrations of the extract. The extract acted as a potent antibiofilm agent with dual actions, preventing biofilm formation and also eradicating the existing biofilm [21].

In the study of Fu et al., Which investigated the antimicrobial effects of Rosemary essential , it has been determined that the amount of non-growth of this essential oil on *S. aureus* is 18 mm and MIC is 0.125 [22]. Tsai et al. In a study on Streptococcus subbrinus, the effect of rosemary ethanoic extract was found to be 1.42 mg / ml [23]. In the study of Soltan Dallal et al., The antimicrobial effects of rosemary essential oil were evaluated by disk diffusion method and dilution in tubes on methicillin-resistant *S. aureus*. The diameter of the pathogen's inhibition zone was about 20 mm. The minimum inhibitory concentration was 1.4 mg / ml and the lowest lethal concentration of growth was 2.8 [24].

The study of Moreno, The antibacterial efficacy of phytochemicals as 1,8-cineole, monoterpene constituent of *Rosmarinus officinalis* (rosemary) essential oils, and carnosic acid, main diterpene of rosemary leaves, was evaluated against nosocomial strains of MDR-*Klebsiella pneumoniae* and methicillin-resistant *S. aureus* (MRSA) grown in planktonic culture and biofilm. The fluorescent SYTOX green dye was used to assess the integrity of the bacterial plasma membranes. Results showed that 1,8-cineole decreased planktonic growth of MDR-*K. pneumoniae* cells, exhibited a clear permeabilizing effect on the bacterial plasma membrane, and disrupted biofilms developed by all MDR-*K. pneumoniae* strains tested. Carnosic acid inhibited MRSA strains, having up 8 antibiotic resistance, growing in a planktonic state [25].

The study of Elhariry, determine the bioactive compounds in two rosemary water extracts (RWE1 and RWE2) and to assess their antimicrobial, anti-adhesive and antibiofilm potentials against the food-related *Bacillus* and *Pseudomonas* species at concentrations; 4, 8, 12, 16 and 20 mg mL^{-1}. The anti-adhesive and antibiofilm doses were higher than MIC_{90}. RWE1 and RWE2 showed potential reduction in the bacterial cell adhesion to HEp-2 cells – 17.5–64.7 and 12.2–52.9%, respectively[26].

The Sandasi, The antibiofilm activity of extracts obtained from selected herbs, spices, beverages and commercially important medicinal plants was investigated on Listeria monocytogenes. The respiratory activity was assessed using the 2, 3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) reduction assay. The majority of extracts tested prevented cell adhesion to the polyvinyl chloride (PVC) surface. Seven of the 15 extracts reduced biofilm adhesion of both the clinical and the type strains by at least 50%. In contrast, inhibition of a preformed biofilm was more difficult to achieve, with only three extracts (*Rosmarinus officinalis*, *Mentha piperita* and

Melaleuca alternifolia) inhibiting the growth of both strains by at least 50% [27].

The study of A. Abdulhasan, The ability to form biofilm formation was performed and antimicrobial activity for antibiotics and rosemary EO were investigated by minimal inhibitory concentration (MIC) in broth microdilution method. The results showed that all isolates had the ability to produce biofilm (100%). The MIC value of gentamicin, trimethoprim/sulfamethoxazole, ciprofloxacin and Rosemary EO against *E. coli* were 256, 2000, 500 and 104 µg/ml, respectively for strong biofilm producer isolates while the MIC values were 512, 2000, 1000 and 104 µg/ml, respectively for moderate biofilm producer isolates. Combination of gentamicin and ciprofloxacin with EO were reduced the MIC of antibiotics when used alone in both strong and moderate biofilm producer isolates [28].

5. Conclusion

This is the first report on evaluation of plants extracts for their anti-biofilm activity against oral pathogens. Our study results highlight the scientific evidence for the use of plants in oral care and treat the emergence of multidrug resistant microorganisms and potential side effects of allopathic health care products. Further this study result requires support by the evaluation of antimicrobial activity against drug resistant clinical isolates and cytotoxicity on human gingival fibroblast cells.

Conflict Of Interest

All authors disclose any financial and personal relationships with other people or organizations and the authors declare that there are not any potential conflicts of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article

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References

1. Tavassoli S, Mousavi S, Emam-Djomeh Z, Razavi S. (2011). Comparative study of the antimicrobial activity of *Rosmarinus officinalis* L. essential oil and methanolic extract. Middle-East Journal of Scientific Research, 9(4): 467-471.
2. Fazeli-nasab B, Fooladvand Z. (2016). A Review on Iranian *Carum copticum* (L.): Composition and Biological Activities. European Journal of Medicinal Plants, 12(1): 1-8. https://doi.org/10.9734/EJMP/2016/17584
3. Tiwari R, Bharti S, Kaur H, Dikshit R, Hoondal G. (2005). Synergistic antimicrobial activity of tea & antibiotics. Indian Journal of Medical Research, 122(1): 80.
4. Mehmood F, Shahzadi I, Ahmed I, Waheed M T, Mirza B. (2020). Characterization of *Withania somnifera* chloroplast genome and its comparison with other selected species of Solanaceae. Genomics, 112(2): 1522-1530. https://doi.org/10.1016/j.ygeno.2019.08.024
5. Luque-Garcıa J, De Castro M L. (2003). Ultrasound: a powerful tool for leaching. TrAC Trends in Analytical Chemistry, 22(1): 41-47. https://doi.org/10.1016/S0165-9936(03)00102-X
6. Varposhti M, Abdl A, Mohammadi P, Saboora A. (2013). Effects of extracts and

an essential oil from some medicinal plants against biofilm formation of *Pseudomonas aeruginosa*. Journal of Medical Microbiology and Infectious Diseases, 1(1): 36-40.

7. Wikler M A. (2006). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. CLSI (NCCLS), 26: M7-A7. NII Article ID (NAID): 20001404762
8. Sobhanizadeh A, Solouki M, Fazeli-Nasab B. (2017). Optimization of callus induction and effects of biological and non-biological elicitors on content of phenol/flavonoid compounds in *Nigella sativa* under in-vitro conditions. Journal of Cell & Tissue, 8(2): 165-184. https://doi.org/10.29252/JCT.8.2.165
9. Valizadeh M, Beigomi M, Fazeli-Nasab B. (2020). Antibacterial and Anti biofilm effects of ethanol and aceton leaf extract of *Momordica charantia* and *Tecomella undulata* against *Acinetobacter baumannii*. Int. J. Adv. Biol. Biomed. Res., 8(4): 403-418. https://doi.org/10.33945/sami/ijabbr.2020.4.6
10. Shahwany A, Tawfeeq H K, Hamed S E. (2016). Antibacterial and anti-biofilm activity of three phenolic plant extracts and silver nanoparticles on *Staphylococcus aureus* and *Klebsiella pneumoniae*. J. Biomed. Biotechnol, 4(1): 12-18. https://doi.org/10.12691/bb-4-1-3
11. Nikolić M, Vasić S, Đurđević J, Stefanović O, Čomić L. (2014). Antibacterial and anti-biofilm activity of ginger (*Zingiber officinale* (Roscoe)) ethanolic extract. Kragujevac Journal of Science, 2014(36): 129-136. https://doi.org/10.5937/KgJSci1436129N
12. Yahya M, Saifuddin N, Hamid U. (2013). *Zingiber officinale* ethanolic extract inhibits formation of *Pseudomonas aeruginosa* biofilm. Int. J. Pharm. Bio. Sci, 3(46): e54.
13. Romero C M, Vivacqua C G, Abdulhamid M B, Baigori M D, Slanis A C, Allori M C G d, Tereschuk M L. (2016). Biofilm inhibition activity of traditional medicinal plants from Northwestern Argentina against native pathogen and environmental microorganisms. Revista da Sociedade Brasileira de Medicina Tropical, 49: 703-712. https://doi.org/10.1590/0037-8682-0452-2016
14. Geethashri A, Manikandan R, Ravishankar B, Shetty A V. (2014). Comparative evaluation of biofilm suppression by plant extracts on oral pathogenic bacteria. Journal of Applied Pharmaceutical Science, 4(3): 20. https://doi.org/10.7324/JAPS.2014.40305
15. Gaetti-Jardim Júnior E, Landucci L F, Arafat O K K, Ranieri R V, Ramos M M B, Ciesielski F I N, Schweitzer C M, Okamoto A C. (2011). Antimicrobial activity of six plant extracts from the Brazilian savanna on periodontal pathogens. International Journal of Odontostomatology, 5(3): 249-256. http://dx.doi.org/10.4067/S0718-381X2011000300008
16. Shafiei Z, Rahim Z H A, Philip K, Thurairajah N. (2016). Antibacterial and anti-adherence effects of a plant extract mixture (PEM) and its individual constituent extracts (*Psidium* sp., *Mangifera* sp., and *Mentha* sp.) on single- and dual-species biofilms. PeerJ, 4: e2519. PubMed: 27761322
17. Mohsenipour Z, Hassanshahian M. (2015). The inhibitory effect of *Thymus vulgaris* extracts on the planktonic form and biofilm structures of six human pathogenic bacteria. Avicenna journal of phytomedicine, 5(4): 309. PMCID: PMC4587606; PMID: 26442753
18. Chevalier M, Medioni E, Preˆcheur I. (2012). Inhibition of *Candida albicans* yeast–hyphal transition and biofilm formation by *Solidago virgaurea* water extracts. Journal of medical microbiology, 61(7): 1016-1022. https://doi.org/10.1099/jmm.0.041699-0
19. Bhunu B, Mautsa R, Mukanganyama S. (2017). Inhibition of biofilm formation in *Mycobacterium smegmatis* by *Parinari curatellifolia* leaf extracts. BMC Complementary and Alternative Medicine, 17(1): 1-10. https://doi.org/10.1186/s12906-017-1801-5
20. Sasirekha B, Megha D, Chandra M, Soujanya R. (2015). Study on effect of different plant extracts on microbial biofilms. Asian Journal of Biotechnology, 7(1): 1-12.

21. Teanpaisan R, Senapong S, Puripattanavong J. (2014). In vitro antimicrobial and antibiofilm activity of *Artocarpus lakoocha* (Moraceae) extract against some oral pathogens. Tropical Journal of Pharmaceutical Research, 13(7): 1149-1155. https://doi.org/10.4314/tjpr.v13i7.20
22. Fu Y, Zu Y, Chen L, Shi X, Wang Z, Sun S, Efferth T. (2007). Antimicrobial activity of clove and rosemary essential oils alone and in combination. Phytotherapy research, 21(10): 989-994. https://doi.org/10.1002/ptr.2179
23. Tsai P-J, Tsai T-H, Ho S-C. (2007). *In vitro* inhibitory effects of rosemary extracts on growth and glucosyltransferase activity of *Streptococcus sobrinus*. Food chemistry, 105(1): 311-316. https://doi.org/10.1016/j.foodchem.2006.11.051
24. Dallal M S, Mashkani M G, Yazdi M H, Amiri S A, Mobasseri G, Mohtasab T A, Harati F A. (2011). Antibacterial effects of *Rosmarinus officinalis* on Methicillin-resistant *Staphylococcus aureus* isolated from patients and foods. Scientific Journal of Kurdistan University of Medical Sciences, 16(1): 73-80.
25. Moreno S, Galván E, Vázquez N, Fiorilli G, Guido P. (2015). Antibacterial efficacy of *Rosmarinus officinalis* phytochemicals against nosocomial multidrug-resistant bacteria grown in planktonic culture and biofilm. The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs. 1st edition. Badajoz: Formatex: 3-8.
26. Elhariry H, Abuzaid A A, Khiralla G M, Gherbawy Y. (2014). Antibiofilm formation and anti-adhesive (to HE p-2 cells) effects of rosemary water extract against some food-related pathogens. International Journal of Food Science & Technology, 49(4): 1132-1141. https://doi.org/10.1111/ijfs.12409
27. Sandasi M, Leonard C, Viljoen A. (2010). The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. Letters in applied microbiology, 50(1): 30-35. https://doi.org/10.1111/j.1472-765X.2009.02747.x
28. Abdulhasan G. (2017). Synergism effect of rosemary essential oil and some antibiotic against *Escherichia coli* isolated from clinical samples. IOSR Journal of Pharmacy and Biological Sciences, 12(2): 39-42. https://doi.org/10.9790/3008-1202033942