

Research Article**Antibacterial activity of dew and Acacia gum on biofilm forming of *Pseudomonas Aeruginosa***Rafat khalaphallah¹ and Hosny·M· Farrag²¹Department of agriculture plant (Microbiology), Faculty of Agriculture, South Valley University, Qena, Egypt²Department of soil and water, Faculty of Agriculture, South Valley University, Qena, Egypt**Abstract**

Antibacterial and antibiofilm activity of natural agents (Nat. ag.) like as dew (D), acacia gum (AG) and mix of dew with acacia gum (MDAG) were investigated on biofilm formnig of *Pseudomonas aeruginosa* (*P.aeruginosa*). *P.aeruginosa* as well as play a role of important opportunistic in water and foodborne pathogen can produce biofilm. Biofilm causes difficulties in various environments, and biofilm causes difficulty in killing and inhibiting bacteria. Novel or natural agents (D,AG and MDAG) are secrete inhibitory substances to prevent infection by pathogenic organisms. The purpose of this study was to evaluate the inhibitory effects natural agent (Nat. ag.) On biofilm formation of *P. aeruginosa*. In this experimental study, the antibacterial effect of (Nat. ag.) was evaluated by agar disc diffusion assay. The minimum inhibitory and minimum bactericidal concentration (MIC),(MBC) were determined by microdilution method. Significant decrease of cell count (50–96%) was observed with increasing time and higher concentration. Percentage biofilm reduction compared to negative control was 96% (dew), 97% (AG), and 99 % (MDAG). For biofilm disruption, biofilms of *P.aeruginosa* were treated with MDAP for 3 hour and attached cells were quantified after staining. Efficacy was experimented by structural quality using Scanning Electron Microscope (SEM). Results showed that (Nat. ag.) has a significant effect on the tested strain and the MIC and MBC values for the strain were the same (256 µl/ml , 312 ul/ml). Furthermore, in case of (Nat. ag.), biofilm production in MIC (88±1.2) and MBC concentration was considerably inhibited. Natural agents are effective in Inhibitory *P. aeruginosa* biofilm. Therefore, this material appears to be a promising agent for prevention of various pseudomonas infections

Keywords: biofilm, dew, acacia gum, *P.aeruginosa*, antibacterial activity

Introduction

Waterborne and foodborne diseases have emerged as the major public health concerns across the globe. Food-borne diseases are major health problems both in developed and developing countries (Abunna et al.2016). Utilization of foods and water contaminated with certain food and water pathogens such as fungi, bacteria, and viruses, is considered as a major source of foodborne illness human. *Salmonella* spp., *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Shigella* spp., and *Listeria monocytogenes*, are the most frequently reported foodborne pathogens from different parts of the world (Hoiby et al.2010 and Hasset et al. 2010). Bacteria are consider one of the causative factors of most food-borne disease though there have been a lot of food-borne diseases (Argaw et al. 2015). One of the primary causes of antibiotic resistance in bacteria is massive misuse of antibiotics, despite warnings regarding overuse the antibiotics are over worldwide (Shields et al. 2014). Exopolysaccharide production and biofilm formation also contribute to antimicrobial resistance among pathogens (Khan et al.2012) and Thus, the development of novel and natural antibacterial agents is needed to combat emergence of resistance among commonly foodborne pathogens. Bacteria biofilms are communities of bacteria living and produced matrix of extracellular polymeric substances which aids the survival of these bacteria (Gupta et al. 2016). Nowadays natural control has created an advanced solution to overcome antimicrobial resistance problems by using novel and natural agents.

Acacia gum is widely used in the food industry as an emulsifier and listed as a food additive (Mortensen et al. 2017). In general, AG has been shown to establish prebiotic efficacy (Calame et al.2008). AG has been used as a traditional herbal medicine for hundreds of years. In this context, the use of AG in traditional medicine has been reported to treat inflammation of the intestinal mucosa, and to cover inflamed surfaces (Gamal et al. 2003). In the Middle East, AG is used as a sanitary substance that has antibacterial effects against most of pathogens (Clark et al. 1993). Altogether, AG has been shown that influences the outcome of many different metabolic diseases or other non-communicable diseases (Patel et al.2015 and Ali et al.2009). Based on

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the emergence of antibiotic-resistant bacteria which have a biofilm, deeper insight into alternative antibacterial and immunomodulatory strategies is needed. These studies highlight the value of combining natural agent technologies to advance our understanding of the action mechanisms of novel antibacterial and anti-biofilm therapies.

Materials and Methods

Dew and acacia gum preparation

Dew drops is water in the form of water droplets that appears on thin, Dew drops are collected in the early morning from the alfalfa plant, collected by a sterile towel and squeezed into a sterilized glass bottle. Some elements, salinity, alkalinity and acidity are estimated. Glassware is kept in the refrigerator at a temperature until use in the experiments. Mixing for (Nat.ag.) is calculated amount of acacia gum with a size of dew drops to form an anti-bacterial paste. Concentration of dew preparation was used to 32 to 624µml/ml., also, for acacia gum was cleaned from impurities and then washed with sterile distilled water, and concentration of AG preparation was used from 32 to 624µg/ml. this solution was termed as natural agents of AG and incubated at 5 °C for 24h, The solution was centrifuged at 2500 x g for 20min at 5 °C.

Bacteria and Culture Conditions

Waterborne and foodborne bacterial isolate *P. aeruginosa* was collected from the wastewater treatment station, Egypt. *P. aeruginosa* is gram negative bacteria (G-) and aerobic (and at times facultative anaerobic), rod-shaped bacterium with unipolar motility. It has been identified as an opportunistic pathogen of both humans and plants. *P. aeruginosa* isolate was cultured in TTC tergitol 7 agar (solid media) for 24 hours at 37 °C and cultured in nutrient broth (liquid media) with continuous agitation at 100 rpm. *P. aeruginosa* grown at 37°C for 24 hours in nutrient broth (NB) with shake-flasks at 60 rpm and TTC7with tregetol agar for solid media. 1ml of bacterial broth was removed, and bacteria were washed twice at 6000rpm for 3 minutes in deionized water to remove any growth media. 20 µl of the bacterial suspension was added to 1 ml of the relevant solution in the presence or absence of natural agent (0.5–10%). Samples were then incubated for 20 min before being subjected to further washing and centrifugation at 6000 x g for 6 min to remove excess natural agents for sizing, and morphology analyses.

Minimum inhibition and bactericidal concentration on *P. aeruginosa*

For the MIC and MBC assayed, MDAG was serially diluted with *P. aeruginosa* cell suspension (107 CFU/ml) and was incubated at 37 °C overnight. 2 fold serial dilutions prepare of natural agents up to 7 row and last row negative control (NC) without bacteria in microdilution plate. Put the inoculum a few colonies from an agar plate *P. aeruginosa*, preparing a Mac Farland standard, and then diluting this standard into media. Distribute *P. aeruginosa* into the microdilution plate with serial dilution test of dew and acacia and incubate the microdilution plate. Plate a portion of each well on an agar media, incubate the agar and check for colonies to determine MBC and read the microdilution plate to determine MIC value.

Evaluation of Antibacterial Activity of natural agents

Antibacterial activity of dew, Acacia gum and mixed dew with acacia gum (MDAG) against pathogenic bacteria was determined using the High Throughput Spot Culture Growth Inhibition Assay (HT-SPOTi) as described by (Danquah et al. 2016). Briefly, a double serial dilution of a stock solution of the test extracts and fractions (1000 µg/mL in 2% dimethyl sulphoxide, DMSO) was done in a PCR half-twisted plate to give a concentration range of 7.8–500 µg/mL. Each dilution aliquots (100µL) were dispensed into corresponding wells of a 96-well plate and mixed with 200µL of molten agar. The plates were stirred and spotted with 2 µl of a standard microbial suspension of *P. aeruginosa*. The plates were stand and closed for 20 min, and incubated at 37 °C for 24 h. The presence or absence of growth was determined by visual inspection compared to control wells.

Biofilm Inhibition Assay

The effect of natural agents separate and MDAG on biofilm formation by *P. aeruginosa* was investigated using the microplate crystal violet stain retention method (Abidi et al. 2014]. *P. aeruginosa* cultures prepared in nutrient broth (NB) were used for the assay. The test samples were solubilized in DMSO (2%) and reconstituted in NB to achieve a concentration range of 32–624 µg/mL. Aliquots of the microbial culture (180 µL) and the test samples (20 µL) were pipetted into a flat bottom 96-well microtiter plate and incubated at 37°C for 24 hours. Negative controls were included in the plate; the experiment was performed in triplicate. After microtiter plate incubation, the supernatant was captured and each well was washed with phosphate buffer saline (pH 7.2) to remove capture cells. The biofilms were fixed by drying at 50°C for 30 minutes and stained with 0.1% crystal violet aqueous solution for 10 minutes at 25° C. The wells were carefully washed with sterile water and the stain bound to cells solubilized with 95% ethanol. Absorbance was measured at 600 nm using an automated microplate reader. The mean absorbance was determined, and the percentage inhibition of biofilm was calculated as:

$$\text{Biofilm inhibition\%} = \frac{\text{ABS (control)} - \text{ABS(test)}}{\text{ABS (control)}} * 100 \quad (\text{Eq1})$$

For the biofilm formation quantification of viable cells assayed in plastic micro plates, this method was investigated by (Stepanovic et al. 2004). A 20 µl portion of broth culture of *P. aeruginosa* was added to each well of a 24-well plate which was incubated aerobically, with mild agitation at 70 rpm, for 72 h at 37 °C. Every 12 h, the bacterial cell replaced by fresh media nutrient broth supplemented by 2% sucrose. Negative controls were obtained by incubating the micro plates with media without inoculum. After supernatant media removed, the biofilm was treated with AG and MDAG 64, 128, 256, 312 and 624 µg/ml. but, in case of treated with dew to bacterial biofilm concentration was 64, 128, 256, 312 and 624 µl/ml, at room temperature and without agitation. Same concentrations of natural agents were used as positive control. Following treatment, the extract was removed and the wells were gently washed twice with sterilized distilled water.

Statistical Analysis

The statistical analysis was presented using a completely randomized design and the general linear models procedure of SAS of experiments.

Statistical significance was compared for each treated group with the control and determined by Duncan multiple range tests were used to compare means. Histograms were created in Microsoft Excel 2010.

Results

The dew and Acacia powder chemical composition

The chemical composition of dew revealed the abundance of major cations Na⁺ and Mg²⁺, Ca²⁺. The acidity from dissolved CO₂, SO_x, and NO_x is mostly neutralized by Ca²⁺, thus giving an alkaline character to the dew. The mean dew electrical conductivity (EC) was in the order to 730µs/cm, the dew pH was between to 6.7-7.9, this consider is acidic for exposed to air and adsorption of gaseous CO₂, SO_x, and NO_x. Acacia consists principally of gum, which is a complex mixture of calcium, magnesium and potassium salts of acid.

Table 1: Physical and chemical composition of dew and acacia gum

Dew Parameters		Acacia gum Parameters	
EC	530µs/cm ^a	EC	61µs/cm
pH	6.6-7.9	PH	4.9-5.5
TDS	154 mg/ml ^b	Ash	2.6-4%
Cations		Cation	
Ca ⁺	85 µeq/L ^a	Ca ⁺	58ppm ^a
Mg ⁺	970 µeq/L ^a	Mg ⁺	2700ppm ^a
Na ⁺	155 µeq/L ^e	Na ⁺	8ppmb ^e
K ⁺	79 µeq/L ^d	K ⁺	7500ppm ^b
Anions		Component	
SO ₄	1689 µeq/L ^a	Nitrogen%	0.34%
NO ₂	200 µeq/L ^e	Uronic acid	14.5%
Cl ⁻	125 µeq/L ^d	Moisture%	10%
F ⁻	110 µeq/L ^d	Volatile matter	62%

Acacia gum is consist of polysaccharide that yields L-arabinose, D-glucuronic acid, D-galactose and L-rhamnose on hydrolysis. 1, 3-Linked D-galactopyranose units form the backbone chain of the molecule and the terminal residues of the 1, 6-linked side chains are uronic acids 14.5%. Acacia contains 12–15% of water and several occluded enzymes such as oxidases, peroxidases and pectinases, the total ash content in the range of 2.6–4.0% (Musa et al. 2019).

Effect of Natural agents on bacterial cell morphology

The direct surface interaction of Natural agents separate (D and AG) and MDAG with P.aeruginosa was studied using intensity percentage to visualize the effect of MDAG on the morphology of the P.aeruginosa cells. Figures 1 clearly demonstrated the binding of MDAG to the bacterial cell size 1000nm at intensity is 30%, while the bacterial cell size with AG 900 nm at 20% intensity. Intensity and cell size 28 % and 1100, respectively. In comparison to negative and positive control shown in Figure 6 a positive control is 15 % and 1200 nm. So, the cell size in positive control bigger than cell size with negative control.

Antibacterial Activity of dew, Acacia gum and mix formula

Antibacterial activity for dew or acacia gum separate or together on P.aeruginosa (fig. 2) showed that when the antibacterial activity of MDAG against p.aeruginosa was quantitatively assessed by inhibition zone, MDAG was effective with the inhibition zones ranging from 40 to 70 mm, The highest inhibition zone was recorded at concentra-

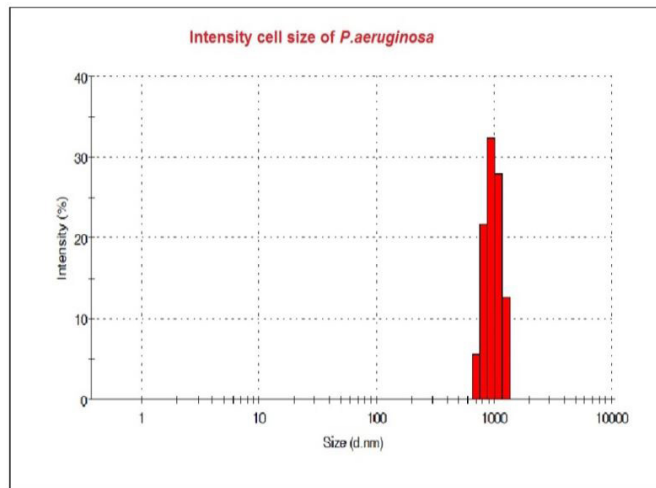


Figure 1: The direct surface interaction of Natural agents on Intensity cell size of P. aeruginosa

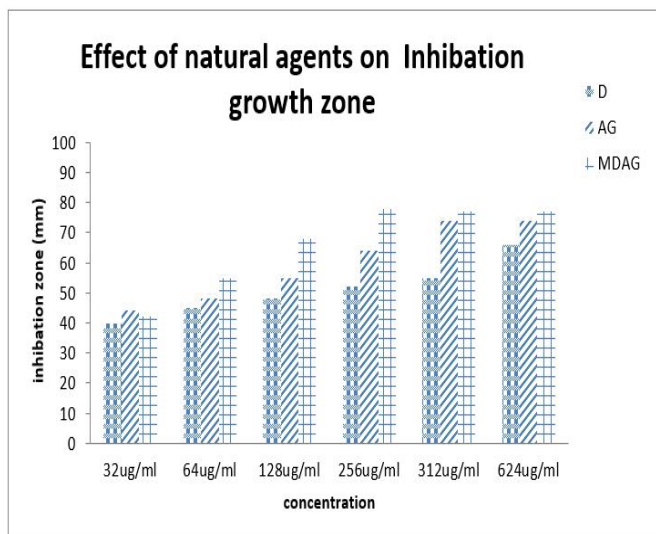


Figure 2: Effect of natural agents on P. aeruginosa inhibition growth zone

Table 2: Minimum inhibitory and bactericidal concentration of dew and acacia gum

		128 ug, uml/ml	256 ug, uml/ml	312 ug, uml/ml	624 ug, uml/ml
MIC	D	48	52	55	66
	AG	55	64	74	74
	MDAG	68	78	77	77
MBC	D	48	58	60	60
	AG	65	75	75	75
	MDAG	54	75	76	75

tion of 75 µg/ml.

MIC for P.aeruginosa was between 256 and 312 µg/ml, while the MBC values ranged from 128 to 256 µg/ml and Inhibitory concentration of MDAG was three fold higher than D or AG.

The Natural agent separate and natural agent together showed varying degrees of antibacterial activity against Gram-negative bacteria in the HT-SPOTi test. The MICs for susceptible bacteria ranged from 256 to ≥624 µg/mL (Table 2). The Natural agent separate showed the highest inhibition towards the test organisms with P.aeruginosa being most susceptible at MIC 624 µg/mL. The test samples inhibited the growth of P.aeruginosa showed varying inhibitory activities on the microorganisms with the highest effect against P.aeruginosa (MIC- 312 µg/

mL).

Biofilm Formation Inhibitory and disruption by natural agents

The Natural agent separate (D and AG) and natural agent together (MDAG) were tested for biofilm formation inhibitory effect against *P. aeruginosa* at a concentration range of 32 –624µg/mL. The percentage biofilm inhibition for all samples is demonstrated in Figures 1 and 2. For all test, the antibiofilm effect was not concentration-dependent the ethyl acetate fraction (AEF) demonstrated the highest activity with 59–69% inhibition of *P. aeruginosa* biofilm formation (Figure 2(a)). Much lower inhibition of biofilm formation was expressed against *p. aeruginosa* with the highest effect given by the pet-ether fraction, (45–67%) (Figure 2(b)).

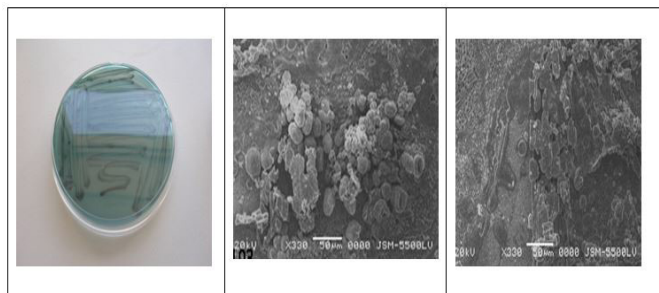


Figure 3: Scanning electron micrographs of control (no treatment) and MDAG-treated *P. aeruginosa* biofilm.

Effect of mix formula on the biofilm disruption and removal, MDAG exhibited highest activity against *P. aeruginosa* biofilm compared to that of the other dew and acacia gum separate (Fig. 4). Percentage biofilm reductions by MDAG when compared with control (without treatment) were 76%, 71% (dew drops), and 70% (acacia gum). In assessing the ability of MDAG to remove *Paeruginosa* biofilm, 24 hour exposure gave maximum biofilm reduction. Biofilm removal increased significantly ($P < 0.05$) with increasing MDAG concentration with highest activity observed at the highest concentration tested (156 µg/ml).

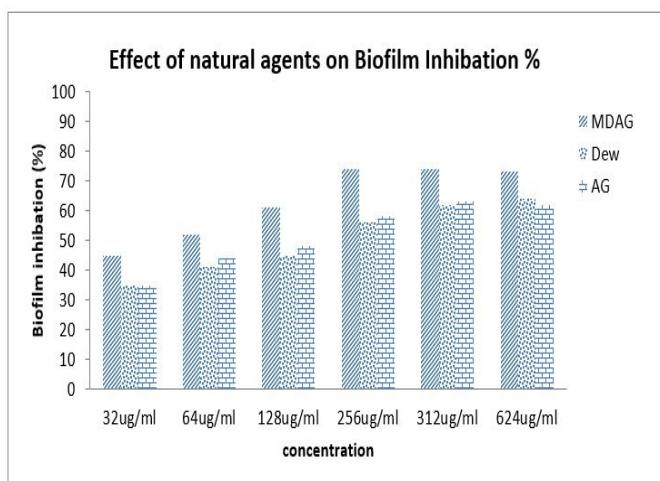


Figure 4: Effect of natural agents on *P. aeruginosa* percentage of biofilm inhibition

Discussion

The inhibition of biofilm formation observed for mix MDAG formula could be attributed to possible quenching of quorum sensing mechanisms in the bacteria. Paste from acacia medicinal plants have been shown to interrupt bacteria biofilm formation through mechanisms such as damaging microbial membrane structures, inhibiting pepti-

doglycan synthesis (Gross et al.2001 and Smith et al. 2005). Acacia species including *A. nilotica* have demonstrated remarkable antibiofilm-forming effects against *P. aeruginosa* (Elamary et al. 2020 and Ouedraogo et al. 2019).

Biofilm growth or interrupt is investigated by a number of physical, chemical, and biological processes. The initial stages of bacterial adhesion involve the transport of cells to a surface. Initial adhesion is governed by “long-range forces,” including double-layer electrostatic interaction, steric interactions, van der Waals forces (attractive), and hydrophobic/hydrophilic interactions (Garrett et al. 2008 and Jenkins et al. 2005).

Acacia plant contains a peroxidase enzyme, which is typically destroyed by brief exposure to heat. The enzyme forms colored complexes with certain amines and phenols and promotes the destruction of many products including alkaloids and easily oxidizable compounds, like as some vitamins. Antibacterial activity of methyl-p-hydroxybenzoate against *Pseudomonas aeruginosa* reduces by Acacia gum likely protecting to the microbial cells from the effect of the preservative (Banin et al.2006).

Acacia gum may affect the phagocytosis of normally killed intracellular bacteria. *P. aeruginosa* infection can lead to many diseases, such as infections of burn injuries and the outer ear, also pathogen for animals and plant. *Pseudomonas* can be spread by equipment that is contaminated and not cleaned properly. *Pseudomonas* can, in rare circumstances, it causes pneumonia (Fine et al. 1996) in good association with the AG-mediated increase in ROS production, AG treatment of human whole blood enhanced uptake and killing of pathogenic strain cells, *Paeruginosa* meningitis Better partially intracellular killing of the laboratory strain.

The dew is exhibit near neutral pH close to 7. The dew chemical composition revealed the abundance of major cations Na^+ and Mg^{2+} , Ca^{2+} . The acidity from dissolved CO_2 , SO_4 , and NO_x is mostly neutralized by Ca^{2+} , thus giving an alkaline character to dew water. These alkaline characters are lead to remove *P.aeruginosa* biofilm. According to Borges et al.(2012), Kaur et al. (2009), and Yang et al.(2012) found that group B *Streptococcus* produced a greater amount of biofilm at pH 6.5 than at pH 4.2.

Conclusion

This study has provided the first evidence of the anti-biofilm formation inhibitory potentials of the dew and Acacia gum. This gives scientific credence to the traditional uses of the plant for managing infections and highlights the dew and acacia gum as a potential source of bioactive compounds to both anti-biofilm and inhibitory activity.

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