Original Article

Biofilm Inhibition and Antibacterial Potential of Different Varieties of Garlic (*Allium sativum*) Against Sinusitis Isolates

Dose-Response:
An International Journal
October-December 2021:1–7

DOI: 10.1177/15593258211050491
journals.sagepub.com/home/dos
SAGE

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Abstract

Sinusitis or rhinosinusitis is inflammation of the paranasal sinuses which can be due to autoimmune, allergy, and infection problems. Current study was aimed to evaluate the antibiofilm and antibacterial potential of different varieties of *A sativum*. Four different varieties (China white, China pink, Desi white, and Desi pink) were used and extracted with methanol and water. Results of antioxidant analysis of *A sativum* extracts showed that all varieties of garlic have considerable quantity of flavonoids with significant DPPH inhibition and reductive potential. Antibacterial activity of *A sativum* extracts was tested against different Gram negative and Gram-positive sinusitis isolates. All the sinusitis isolates were susceptible to both methanolic and aqueous extracts of different varieties of *A sativum* with least MIC values. Antibiofilm potential of extracts against sinusitis isolates was evaluated through crystal violet assay, and all extracts of *A sativum* were significantly effective against destruction of microbial biofilm. In summary, *A sativum* extracts possess effective antibacterial and antibiofilm activity against sinusitis isolates and can be utilized for prevention of drug resistance against sinusitis infections and further evaluation is necessary.

Keywords

Allium sativum, sinusitis, antioxidant, antibacterial, antibiofilm

Introduction

Sinusitis is described as inflammation of one or more of the paranasal sinuses and the condition is considered acute if it lasts less than 4 weeks, subacute if it lasts 4–8 weeks, and chronic if it lasts more than 8 weeks. Three or more episodes of acute sinusitis per year constitute recurrent sinusitis. Viral infection is the main cause of sinusitis, but other microbes (fungi and bacteria) are also present.[1,2] The bacterial invasion of the sinuses by *Moraxella catarrhalis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* is usually preceded by viral infections in the upper respiratory tract. These organisms, as well as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and certain anaerobes, can be identified in chronic sinusitis.[3] The normal flora of the sinusitis includes *Staphylococcus epidermidis*, *S. aureus*, *Propionibacterium acnes*, aerobic diphtheroid, and y-streptococci. Several sinusitis isolates have been reported from healthy nasal cavities including *Streptococcus pyogenes* (0–1%), *H. influenzae* (0–6%), *M. catarrhalis* (0–4%), *S. pneumoniae* (.5–15%) and anaerobic bacteria *Prevotella spp* [6–8%] and *Peptostreptococcus* spp. [7–16%].[4] Fungi are becoming more widely recognized as a cause of

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Received 22 May 2021
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chronic sinusitis, particularly in the southeast and southwest of the United States.[5] Sinusitis infection is increasing dramatically in both developed and developing parts of the world. More than thirty million patients have been reported from the United States alone. Sinusitis may be present in acute to chronic patients and may cause serious adverse reactions.

Medicinal plants make an outstanding contribution to modern therapeutics; around 100 plant-based new drugs were introduced in the United States drug market between 1950 and 1970, including reserpine, deserpidine, vinblastine, and vincristine.[6] *A sativum* (garlic) belongs to the *Alliaceae* family. It contains bioactive compounds that exhibit antimicrobial, anti-inflammatory, anticancer, and antioxidant activities.[7] *A. sativum* is considered one of the most important herbs to promote good health. Allicin (diallyl thiosulfinate) is the most important component of *A. sativum*, and its efficacy against a wide spectrum of Gram-negative and Gram-positive bacteria was studied. In fresh garlic cloves, allicin is not present but released after chopping and crushing of cloves due to the enzyme alliinase activity. Alliums, which are a component of garlic, are mostly cysteine sulfoxides. Cysteine sulfoxides convert alliinase to allicin, resulting in thiosulfates, which are volatile in nature.[8] Allicin, an organosulfur molecule that inhibits lipid biosynthesis, has been shown to reduce synthesis of RNA in bacteria and disrupt the cell wall of fungi. The profile of organosulfur compounds differs in different Allium species. S-amino acids including methionine and cysteine (traces), alk(en)yl cysteine sulfides (ACSOs), and g-glutamyl peptides are also present.[9] The antimicrobial activities of garlic and allicin are explored a large spectrum against *Bacillus, Klebsiella, Cryptocaryon, Clostridium, Helicobacter, Escherichia, Staphylococcus, Salmonella, Proteus, Pseudomonas, Photobacterium,* and *Mycobacterium* spp., but studies on effectiveness of *A. sativum* against sinusitis isolates is very scarce. Therefore, the current study was designed to evaluate the antibiofilm and antibacterial activities of different varieties of *A. sativum* against panel of sinusitis isolates.

Materials and Method

Collection and Maintenance of Sinusitis Isolates

Four microbial cultures of sinusitis isolates (*Klebsiella pneumonia*, *Proteus mirabilis*, *S. aureus* and *Enterococcus faecalis*) were collected from Medicinal Biochemistry Lab, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. For purification of bacterial culture, sterilized petri plates with nutrient agar medium were developed and plates were streaked with respective sinusitis isolate under sterilized conditions. Plates were incubated 37°C for 24 h.

Collection of Plant Material and Extract Preparation

Four different varieties of *A. sativum* (China white, China pink, Desi white, and Desi pink) were collected from the local market of Faisalabad, Pakistan, and identified by Department of Botany, University of Agriculture, Faisalabad, Pakistan. The plant materials were dried and blended into powder form. The dried powder of *A. sativum* was extracted with methanol and water as extraction solvents. For extraction, the 50 g of dried plant powder was mixed with 500 mL of solvents (methanol and water separately) and kept on orbital shaker at room temperature for 76 h. Then, extracts were filtered using Whatman filter paper 1 and solvent was reduced on rotary evaporator.[10]

Total Flavonoid Contents

The total flavonoid contents of *A. sativum* extracts were determined using the method used by Pranuthi et al.[11] The reaction mixture was prepared by adding .15 mL of $NaNO_2$ (5%) to 2 mL of distilled water and 1 mL of *A. sativum* extracts and incubated at room temperature for 6 min. After incubation, .15 mL of $AlCl_3$ (10% solution) and 0.3 mL of NaOH (6% solution) were added to the reaction mixture. Methanol was added to make the total volume of reaction mixture up to 5 mL. Reaction mixture was incubated for 10 min, and optical density (OD) was measured at 510 nm. TFC in extracts were calculated from calibration curve of catechin.

Antioxidant Potential of Extracts

DPPH Inhibition Assay. Antioxidant activity of different varieties of *A. sativum* extracts was determined by measuring their ability to scavenge 1,1-diphenyl-2 picryl hydrazyl stable free radicals (DPPH). The DPPH test was performed as described by Noor et al.[12] with slight modification. A 10 µL of *A. sativum* extract was added to 1 mL of methanolic solution of DPPH (.0004%) (.1 mM). After 30 min of incubation in darkness at room temperature, the absorbance was recorded at 517 nm. The BHT was used as a standard. The inhibition of DPPH radical by *A. sativum* extracts was calculated as follows.

$$\text{DPPH Inhibition (\%)} = 100 \times (\text{A Blank} - \text{A Sample/A Blank})$$

Reducing Potential of Extracts. To measure the reductive potential, reaction mixture was prepared by adding 2.5 mL of each potassium ferricyanide (1%) and phosphate buffer (pH = 6.6) and 1 mL plant extract. The blank contained 2.5 mL phosphate buffer (pH = 6.6) and 2.5 mL of 1% potassium ferricyanide solution. After this, the reaction mixture was incubated for 20 min at 50°C. Then, 2.5 mL of 1% trichloroacetic acid solution was added into reaction mixture and centrifuged for 10 min at 3000 rpm. After centrifugation, the upper layer was separated and mixed into .2 mL of .1% ferric chloride and 2.5 mL distilled water. The optical density was measured at 700 nm.[13]

Antimicrobial Activity of A. sativum Against Sinusitis Isolates

Agar Well Diffusion Assay. For determination of antibacterial activity of *A. sativum* extracts, agar well diffusion assay was

used. Nutrient agar plates were developed by pipetting fresh bacterial culture of sinusitis isolate into sterilized agar medium. After solidification, wells of 6 mm diameter were made and different extracts of *A. sativum* (100 µL) were added into the wells. Ampicillin was used as standard. The antimicrobial activity was calculated in terms of zone of inhibition in millimeter (mm).[14]

Minimum Inhibitory Concentration. For the determination of MIC of *A. sativum* extracts, resazurin-based microdilution assay was performed. Briefly, 100 µL of *A. sativum* extracts (50 µg/mL) was added into first well of each row while nutrient broth (50 µL) was added into the 2–10 wells of each rows. Last two wells in each row contained growth control (diluted standardized inoculum) and nutrient broth only (for broth sterility control). To transfer *A. sativum* extracts into 2–10 wells of each row, a multichannel micropipette was used in such a way that each well has twice the concentration of final test. Bacterial suspension (50 µL) was then added into each well. Following 24 h incubation, resazurin solution (30 µL) was added into each well and followed by 2–4 h incubation. MIC was the lowest concentration before color change while column with blue color was scored as above the MIC value. Broth microdilutions were achieved precisely according to the protocol of Clinical and Laboratory Standards Institute.[15,16]

Microbial Biofilm Inhibition

Biofilm inhibition of *A. sativum* extracts was carried out using method of Nadaf et al.[17] The well of the sterile 96 well flat-bottomed plastic tissue culture plate was filled with 100 µL of sterilized nutrient broth, *A. sativum* extract (100 µL) and bacterial culture (20 µL). Negative control well contains only nutrient broth and bacteria. Ampicillin was used as standard. The plates were covered and incubated at 37°C for 24 h. After 24 h, content of each well was discarded, and plates were washed thrice with sterile phosphate buffer saline solution (pH, 7.4) to remove all the planktonic microbes. After washing, 200 µL of ethanol (99.9%) was added into each well and plates were incubated for 15 min. After incubation, 200 µL crystal violet stain was added to each well and plates were again incubated for 10 min. After incubation, the excess stain was discarded and plates were washed again with distilled water (200 µL) and 33% of glacial acetic acid (200 µL) was added. Absorbance of plate was measured at 600 nm. For microscopic analysis of biofilm inhibition, microscopic slides were prepared by following the same method.

Statistical Analysis

Obtained data were analyzed through statistical software SPSS, version 20. Results are expressed as Mean ± SD.

Results and Discussion

In Vitro *Antioxidant Activities of Extracts*

The results of *in vitro* antioxidant activities of different varieties of *A. sativum* are presented in Table 1. Results indicated that all varieties of *A. sativum* had significant antioxidant potential, but methanolic extracts of *A. sativum* showed the best antioxidant activities. Among different varieties of *A. sativum*, the methanolic extract of China pink (CP) showed the highest amount of total flavonoid contents (71.90 ± .51) followed by methanolic extract of Desi white (DW) (69.80 ± .24). From results, it was found that methanol is good solvent for the extraction of antioxidant compounds from *A. sativum* as compared to water. Similarly, the methanolic extract of China Pink (CP) showed maximum inhibition (77.79 ± .51) of DPPH radical and the results were comparable with standard (79.89 ± .06). Similarly, the methanolic extract of Desi white (DW) has greater reductive potential (2.94 ± .56) as compared to all other varieties of *A. sativum*. Chung[8] reported the antioxidant activity of garlic extract and found that garlic extract to give protection to the body against free radical damage. Four main compounds from garlic, that is, allyl disulphide, allyl cysteine, alliin, and allicin were reported for antioxidant activity by lipid peroxidation and hydroxyl scavenging activity, and it was concluded that all the four compounds from garlic are active against free radicals.

Table 1. Antioxidant Potentials of Bioactive Compounds Extracted From Different Varieties of *Allium sativum*.

Sample	Extract	TFC (µg/mL)	DPPH Inhibition (%)	Reducing Potential (%)
CW	Methanol	45.17 ± .17	64.26 ± .34	1.19 ± .11
	Water	35.19 ± .23	21.54 ± .28	1.06 ± .25
CP	Methanol	71.90 ± .51	77.79 ± .51	2.59 ± .67
	Water	29.57 ± .32	22.02 ± .25	.94 ± .82
DW	Methanol	69.80 ± .24	54.18 ± .19	2.94 ± .56
	Water	33.51 ± .34	38.62 ± .82	.71 ± .17
DP	Methanol	50.85 ± .45	45.45 ± .39	2.15 ± .89
	Water	40.84 ± .76	64.24 ± .83	.59 ± .11
Ascorbic acid	—	—	79.89 ± .06	2.99 ± .65

CW; China white, CP; China pink, DW; Desi white, and DP; Desi pink.

Antibacterial Potential of Extracts

Determination of the antibacterial activity of medicinal plants as strong agents is critical in both animal and human health due to increased resistance of micro-organisms against antibiotics. In this study, different varieties of *A. sativum* extracts were tested for antibacterial activity against sinusitis isolates. The agar well diffusion assay is a qualitative method that is mostly employed for the screening of plant extracts for their antimicrobial activity, especially when the diameter zones of inhibition are less than 10 mm. The zone of inhibition of different extracts can vary depending on the polarity of the compounds used, as a more diffusible but less active extract can have a larger diameter of inhibition than a non-diffusible but more active extract. The results of antibacterial activity of different varieties of *A. sativum* are presented in Table 2 and Figure 1 while the results of minimum inhibitory concentration (MIC) are given in Table 3. All extracts showed significant antibacterial activities against sinusitis isolates. Methanolic extracts of all verities were active against all the bacteria, while aqueous extracts showed mild to moderate antibacterial activity against tested microorganism. The highest diameter of inhibition was observed with methanolic extract of Desi pink (DP) followed by methanolic extract of China pink (CP) against *E faecalis*. Meanwhile, aqueous extracts of all varieties of *A. sativum* were less effective against sinusitis isolates as compared to methanolic extracts. Both Desi white (DW) and Desi pink (DP) are more effective against sinusitis isolates as compared to China white and China pink varieties. Against *K. pneumonia*, the highest antibacterial activity was shown by methanolic extract of DP, while the least activity was shown by water extract of China pink (CP). Similarly, against *P. mirabilis* and *S. aureus* the highest antibacterial activity was shown by methanolic extract of China pink (CP) and Desi pink (DP), while the least activity was observed in case of water extract of China pink (CP). Likewise, against *E faecalis* methanolic extract of Desi pink (DP) and water extract of China pink (CP) were most and least

Table 2. Antibacterial Activities of Different Varieties of *Allium sativum* Against Sinusitis Isolates.

		Zone of inhibition (mm)			
Sample	Extract	*Klebsiella pneumonia*	*Proteus mirabilis*	*Staphylococcus aureus*	*Enterococcus faecalis*
CW	Methanol	20 ± .37	21 ± .52	17 ± .94	25 ± .18
	Water	13 ± .58	16 ± .27	15 ± .38	13 ± .26
CP	Methanol	21 ± .57	20 ± .49	28 ± .29	27 ± .37
	Water	8 ± .55	7 ± .62	13 ± .44	11 ± .53
DW	Methanol	27 ± .86	25 ± .31	25 ± .31	26 ± .28
	Water	17 ± .43	18 ± .98	15 ± .35	14 ± .11
DP	Methanol	26 ± .47	24 ± .66	27 ± .19	28 ± .76
	Water	19 ± .38	8 ± .87	16 ± .72	12 ± .39
Ampicillin	—	30.20 ± .94	29.38 ± .28	27.34 ± .58	31.53 ± .31

CW; China white, CP; China pink, DW; Desi white, and DP; Desi pink.

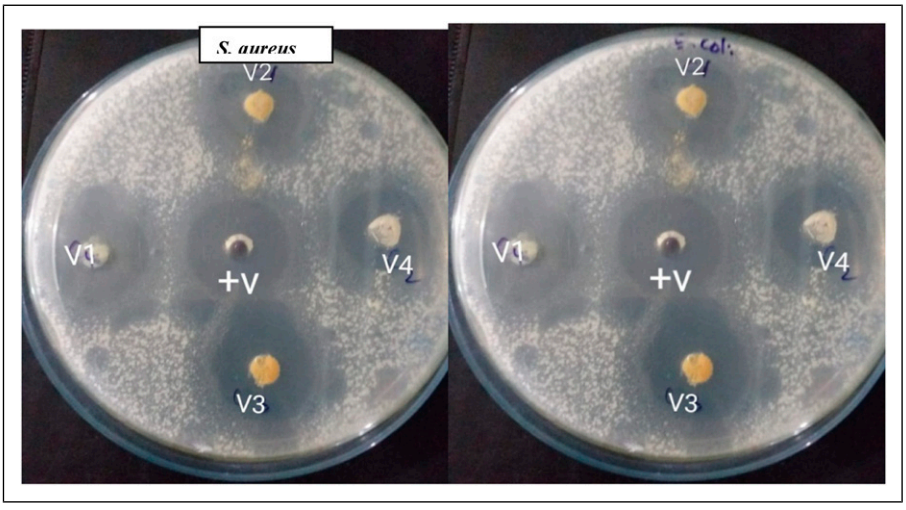

Figure 1. Antibacterial activity of methanolic extracts of different varieties of *Allium sativum* against *Staphylococcus aureus* and *Enterococcus faecalis*. Note: V1–V4 indicate varieties of garlic as China white (V1), China pink (V2), Desi white (V3), and Desi pink (V4).

Table 3. Minimum Inhibitory Concentrations of Different Varieties of *Allium sativum* Against Sinusitis Isolates.

		Minimum inhibitory concentration (μg/mL)			
Sample	Extract	*Klebsiella pneumonia*	*Proteus mirabilis*	*Staphylococcus aureus*	*Enterococcus faecalis*
CW	Methanol	6.31 ± .68	6.72 ± .66	4.66 ± .71	5.16 ± .27
	Water	7.67 ± .23	9.18 ± .30	3.88 ± .45	3.27 ± .90
CP	Methanol	8.17 ± .30	6.79 ± .30	5.85 ± .41	7.16 ± .50
	Water	9.20 ± .31	8.17 ± .54	9.85 ± .27	9.52 ± .28
DW	Methanol	7.81 ± .04	9.76 ± .23	8.61 ± .08	3.58 ± .18
	Water	9.76 ± .23	8.61 ± .08	3.58 ± .18	4.07 ± .30
DP	Methanol	5.71 ± .27	2.10 ± .68	3.79 ± .71	3.13 ± .22
	Water	8.52 ± .30	9.6 ± .23	8.21± .45	4.44 ± .88
Ampicillin	—	4.11 ± .50	3.91 ± .41	4.07 ± .30	8.61 ± .08

CW; China white, CP; China pink, DW; Desi white, and DP; Desi pink.

Table 4. Antibiofilm Potential of Aqueous and Methanol Extracts of *Allium Sativum* Against Sinusitis Infection.

		Microbial biofilm inhibition (%)			
Sample	Extract	*Klebsiella pneumonia*	*Proteus mirabilis*	*Staphylococcus aureus*	*Enterococcus faecalis*
CW	Methanol	22.08 ± .10	64.57 ± .37	62.38 ± .45	43.67 ± .23
	Water	19.08 ± .30	37.37 ± .90	34.98 ± .95	38.67 ± .53
CP	Methanol	61.82 ± .66	55.76 ± .06	49.76 ± .71	61.91 ± .68
	Water	56.82 ± .16	51.76 ± .27	45.76 ± .11	63.11 ± .25
DW	Methanol	70.20 ± .84	84.18 ± .38	77.34 ± .88	66.53 ± .51
	Water	74.79 ± .53	37.55 ± .78	60.6 ± .88	72.81 ± .84
DP	Methanol	89.55 ± .13	88.39 ± .28	74.55 ± .22	84.49 ± .47
	Water	36.77 ± .54	40.82 ± .28	51.45 ± .27	89.82 ± .31
Ampicillin	—	80.20 ± .94	85.18 ± .78	87.34 ± .28	76.53 ± .41

CW; China white, CP; China pink, DW; Desi white, and DP; Desi pink.

effective extracts. The results of antibacterial activity were strongly correlated with minimum inhibitory concentration. The extracts exhibiting significant antibacterial activity showed lowest MIC values (Table 2). The least MIC value was shown by methanolic extract of Desi pink (DP) (2.10 μg/mL) and China pink (CP) against *E. faecalis* and *S. aureus,* respectively. Among aqueous extracts of different varieties of *A. sativum*, both the Chinese varieties have greater MIC value as compared to Desi varieties. Presence of outer membrane and overexpressed efflux pump in Gram negative bacteria makes them resistant to most of the available antibiotics, but methanolic extracts of *A. sativum* significantly inhibit both the Gram-negative sinusitis isolates, that is, *K. pneumonia* and *P. mirabilis*. Previous studies have shown that *A. sativum* contain variety of phytochemicals which are responsible for its biological activities. Organosulfur compounds and phenolic compounds are reported to be the main antibacterial compounds in *A. sativum.*[18] Allicin is the chief ingredient of *A. sativum* and mainly responsible for more of its biological activities including antimicrobial activity.[19]

Anti-Biofilm Potential of Extracts

Development of microbial biofilm is a key factor involved in the virulence of microbial infections, which ensures protection and superior survival of microbial community from environmental fluctuations, antibiotics, and host defensive mechanisms.[20] It has been suggested that challenges of antibiotics treatments in eradication of pathogenic microbes are due to existence of biofilm, also known as microbial communities, adhered to host cell surfaces and covered by extra polymeric substances (EPS). Extracellular polysaccharides, DNA, and proteins make up the EPS matrix that surrounds living bacterial cells.[21,22] Results of biofilm inhibition by different varieties of *A. sativum* extracts are presented in Table 4, and the findings of current study indicated that all the varieties of *A. sativum* extracts may have interfered the biofilm formation of sinusitis isolates and effective for the elimination of microbial infection by these bacteria in the sinuses. Results showed that all varieties of *A. sativum* have significant potential to inhibit the biofilm formation of sinusitis isolates. The maximum biofilm inhibition was shown by methanolic extract of Desi pink (DP) against *P. mirabilis* while the least inhibition was exhibited by water extract of China white (CW) against *K. pneumonia*. Results indicated that methanolic extracts of different varieties of *A. sativum* were more effective as compared to water extracts. Against *E. faecalis*, the highest inhibition (89.82%) was shown by methanolic extract of Desi pink (DP) while the least inhibition (38.67%) was observed by water extract of China white (CW).

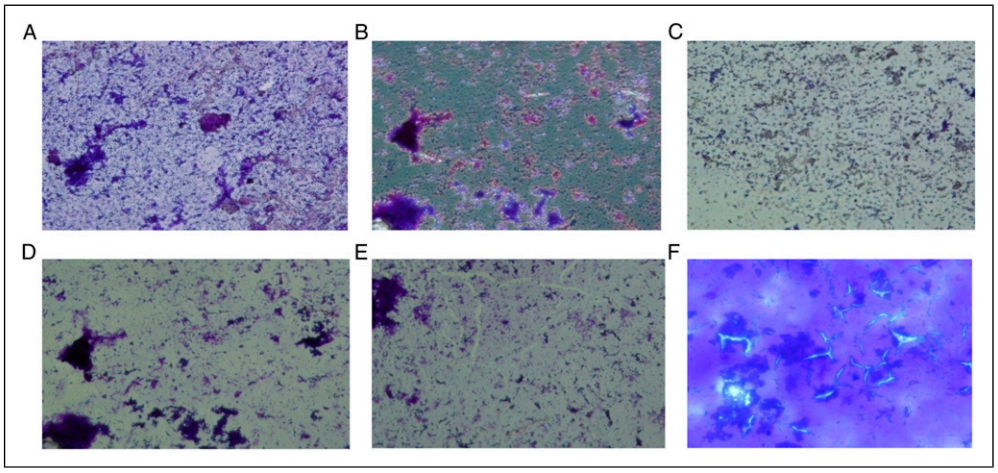

Figure 2. Phase contrast microscopic images of biofilm inhibition of negative control (A), methanolic extracts of Desi white (B), Desi pink (C), China white (D), China pink (E), and Ampicillin (F) against *Staphylococcus aureus*.

Similarly, against *K. pneumonia* and *P. mirabilis*, maximum biofilm inhibition (89.55 and 88.39%) was shown by methanolic extract of Desi pink (DP) while the least inhibition (19.08 and 37.37%) was exhibited by water extract of China white (CW). Against *S. aureus*, methanolic extract of Desi white (DW) and water extract of China white showed maximum and lowest inhibition of biofilm. It is also indicated that both the Desi varieties (DP, DW) of *A. sativum* were more effective against eradication of sinusitis isolates biofilm as compared to Chinese varieties (CP, CW). Previous studies reported that phytochemicals present in medicinal plants decrease the expression of genes involved in pathogenicity of bacteria by inhibiting the biofilm formation.[23] Observations of phase contrast and SEM results of the cell density and morphology of biofilms further supported the destructive abilities of different varieties of *A. sativum* extracts on biofilms of sinusitis isolates (Figure 2).

Conclusion

From the current study, it was concluded that all selected varieties of *A. sativum* have significant antibacterial and antibiofilm activities against sinusitis isolates. Further, it can also be concluded that both Desi varieties of *A. sativum* are more effective against sinusitis isolates as compared to Chinese varieties. From antimicrobial and antibiofilm potential of *A. sativum*, we can conclude that this plant has significant potential to be utilized against sinusitis infection and this should be fully investigated prior to its clinical application. We suggest further studies directed on the bioassay-guided fractionation of methanolic extract of *A. sativum* to determine if different isolated compounds or a refined fraction can reach an MIC at a therapeutically appropriate concentration.

Acknowledgments

The authors are thankful to Higher Education Commission (HEC) of Pakistan for providing fund for this research under the project of NRPU # 4927.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

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