JOURNAL OF ADVANCED MEDICAL AND PHARMACEUTICAL RESEARCH

Research Article

Received 30thMarch, 2022
Accepted 27thApril, 2022
Published7thMay, 2022

jampr.journals.ekb.eg

Anti-Biofilm Impact of *Anabasis articulata* (Forssk) Moq. Total Methanol Extract Against *Pseudomonas aeruginosa* Clinical Isolates

Ghada Gamal [1], Kamilia A. Abo-El-Seoud[2], Engy Elekhnawy [3], Ghada Attia [2]*

*[1]Pharmacognosy Department, Faculty of Pharmacy, Kafrelsheikh University, Kafrelsheikh**33511**, Egypt*
[2]Department of Pharmacognosy, Faculty of Pharmacy, Tanta University, Tanta 31527, Egypt
[3]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Tanta University, Tanta 31527, Egypt

ABSTRACT

Anabasis articulata (Forssk) Moq. is a rich bioactive member of Family Amaranthaceae. GC/MS analysis of *Anabasis articulata* methyl esters fraction notified twenty known fatty acids, including *n*-hexadecanoic acid, octadecanoic acid and tetradecanoic acid as major constituents. Chromatography analysis of successive fractions revealed the isolation of *β*-sitosterol **1** from its unsaponifiable fraction**,** caffeine **2** form its methylene chloride fraction**,** and 4-acetoxy phenol **3** from the ethyl acetate one. Structural elucidation of these isolates was performed by IR, EI-MS, ^{1}H NMR and ^{13}C NMR techniques. Compounds **2** and **3** were isolated for the first time from *Anabasis articulata* (Forssk) Moq. Meanwhile, methanol extract of the tested plant showed weak total phenolic content. Environmental adaptation of pathogenic microbes through formation of resistible biofilm is constructing a dramatic health hazard, which demanded the exploration of more microbial resisting treatments. One successful strategy is the use of Phytoextracts as noncytotoxic, microbial biofilm inhibitors. Adopting the broth microdilution method, *Anabasis articulata* (Forssk) Moq. methanol extract (AAME) showed antimicrobial activity against *Pseudomonas aeruginosa* clinical isolates at range of 32 to 256 µg/mL minimum inhibitory concentrations (MIC). The inhibiting efficiency of AAME against *Pseudomonas aeruginosa* biofilm formation was concluded at a concentration of half its MIC value using the crystal violet assay (CVA). Our results illustrate the ability of this extract to cutback the percentage of strong, and moderate biofilm forming *P. aeruginosa* clinical isolates from 41.18 % to 17.65 % and candidate it as futural antibiofilm agent.

Keywords:*Anabasis articulata* (Forssk) Moq.,*Amaranthaceae, Anti-biofilm activity, Pseudomonas aeruginosa, Antivirulent effect.*

**Department of Pharmacognosy, Faculty of Pharmacy, Tanta University, El Giesh Street, Tanta 31527, Egypt, Tel: (202) 040-3336007; (202) 040- 3335466, Email: ghada.attia@pharm.tanta.edu.eg*

1. INTRODUCTION

Microbial biofilms formation on biotic, and abiotic systems are recognized as the major cause of life threating recurrent chronic infections, through resisting both host`s immunity and therapeutic antimicrobial agents. Nowadays, inhibition of microbial biofilm production, and surge by natural antimicrobial Phytoextracts is the target of several research.[1] Variant classes of isolated phytochemical are natural, safe antimicrobials of distinguished quorum sensing-related antivirulent effect over classical synthetic antibiotics.[2]

Pseudomonas aeruginosa is opportunistic bacteria that can cause both acute and chronic devastating infections particularly in patients suffering from compromised immune systems. Its common persistence in different clinical settings is mainly attributed to its various virulence factors. One of its important virulence factors is its ability to form biofilms.[3] Biofilm is an architecture of bacteria built commonly by their extracellular polymeric substances (EPS). EPS function as a scaffold, which encases bacterial cells together on the different surfaces. Biofilm has a role in the protection of bacteria from phagocytosis, environmental stresses, and antimicrobials. Thereby, novel drugs are highly needed to find out antibiofilm agents.[4]

Anabasis articulata (Forssk) Moq. (Amaranthaceae) is a shrub spread widely over halic and xeric regions of Egypt.[5]Chemical profile of *Annabsis articulata* (Forssk) Moq. revealed the presence of different secondary metabolites such as triterpenes, saponins, alkaloids and phenolic compounds.[6-8] Traditionally, its used to treat diabetes, kidney infections, fever, headache, and eczema [8]. Recent report referred to the significant anti-ulcer activity of *Anabasis articulata* (Forssk) Moq. ethyl acetate fraction (400 mg/kg b.w.) on indomethacin ulcerated ratsin comparison to standard ranitidine. Moreover, high percentage of antimicrobial index range (86.9- 60.9%) against *P. aeruginosa*, *B. subtilis*, *S. aureus*, and *C. albicans* was observed for its total methanolic extract.[9]

In the current study, we aim to continue separation of *Anabasis articulata*(Forssk) Moq. secondary metabolites from its successive fractions. Exceedingly, we investigate the antimicrobial, and unfamiliar antibiofilm potential of AAME against *P. aeruginosa* clinical isolates using CVA.

2. MATERIALS AND METHODS

2.1. General

Rotary flask evaporator (IKA RV10 D599, Wilmington, North Carolina, USA), Faculty of Pharmacy, Tanta University, Egypt. The following apparatus were provided by Faculty of Science, Kafr El-Sheikh University; Spectro line UV lamp, West Bury, New York, USA; Melting point apparatus "Galenkamp type", England; Sonicated water bath, Branson 3510 E-MTH, Mexico; Bruker High Performance Digital FT-NMR Spectrometer Avance III 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR, Germany.FT/IR-6100, Japan, Faculty of Science, El- Mansoura University. GC/MS finnigan mat SSQ7000, USA, research Institute, Egypt; supplied with DB-5 MS (5% phenyl methysiloxane) capillary column (30 m x 0.25 mm ID) and helium flow rate 1ml/min; adjusted programmed temperature of 150 ◦C for 3 min then increased to 280 ◦C for 5 min at rate of 5 ◦C/min; electron ionization mode (EI, 70 ev. energy) and mass detection range (*m/z*) of 49.97-500.3. ESI-MS positive and negative ion acquisition mode was carried out on XEVO TQD triple quadruple instrument, Waters Corporation, Milford, MA01757 U.S.A, mass spectrometer, Faculty of Pharmacy, Ain Shamse University. Microplate reader (FluoStar Omega, Germany, Nwah centre, Egypt). Vacuum liquid chromatography apparatus(5x15cm). Materials for chromatography; Silica gel (Fluka) 70-230 mesh for column chromatography, Silica gel 60(0.015-0.040mm) for Vacuum Liquid Chromatography (VLC) column (Merck, E. Merck, Darmstadt, Germany), Pre coated TLC sheets silica gel G F254 (E. Merck), Reversed phase octadecylsilyl-silica gel (RP-C18, Merck, Germany). Spray reagents For TLC analysis; anisaldehyde/sulfuric acid spray reagent for sterols and phenolic compounds.[10]

2.2. Plant Material

Dry aerial parts of *Anabasis articulata* (Forssk) Moq. growing wild in Rafah, North of Sinai Peninsula, Egypt, were collected in June 2013. It was authenticated by Prof. Dr. Ahmed Sharaf El-din; Professor of Plant Ecology; Botany Department; Faculty of Science; Tanta University; Egypt. Voucher sample no. Aa-001 were kept at Pharmacognosy Department, Faculty of Pharmacy, Tanta University. The dried coarse powdered aerial parts were stored for further analysis in plastic containers.

2.3. *Chromatographical investigation of Anabasis articulata (Forssk) Moq. successive fractions*

Four kilograms of air-dried *Anabasis articulata* (Forssk) Moq. aerial parts powder, were macerated with methanol till exhaustion. Methanol was evaporated under reduced pressure to give 420g AAME. It was subjected to liquid–liquid fractionation by solvents of different polarities to yield 19g *n*-hexane (AAH) fraction, 22g of methylene chloride (AAMC) fraction and 18g of ethyl acetate (AAEA) fraction.

Six grams of AAH fraction was saponified[11] to produce 3.5 g saponifiable part (AAH-SAP) and 1.9 g unsaponified part (AAH-USM). The (AAH-SAP) was methylated[11], where 1µL of the prepared aliquot was GC/MS analysed. The AAH-

USM residue (1.9 g) was chromatographed on a silica gel column. Gradient elution method was adopted, starting with *n*-hexane, and followed by increasement of solvent polarity by methylene chloride. Five pooled fractions (I-V) were obtained. Group III (245mg; eluted with *n*-hexane: methylene chloride (50:50)) was chromatographed on silica gel column, where it was gradually eluted with *n*-hexane: ethyl acetate to yield 20 mg of compound **1**.

AAMC fraction (7g) was chromatographed on a silica gel vacuum liquid column and eluted gradually with *n*-hexane: ethyl acetate mixtures of increasing polarity to give six fractions I-VI. Group VI (780mg; eluted with 60% ethylacetate in hexane) was redissolved in methanol, where a white material was precipitated and filtered. The resultant precipitant (200mg) purification was achieved with gradient elution by variant grads of polarity of the solvent system methylene chloride: methanol on silica gel column to yield 50 mg of compound **2.**

AAEA fraction (8g) was chromatographed on a VLC silica gel column by gradient elution method, starting with methylene chloride then increasing polarity with methanol. Five fractions from I-V were produced. Group III (1.2g; eluted with 5% methanol in methylene chloride) was subjected to recrystallization by methanol. Further separation of the formed crystals (250mg) was done on reversed phase column chromatography using solvent system methanol : water 35% to obtain three sub fractions III_{a-b}. Sixty mg of Group III_a was chromatographed on silica gel column and isocraticly eluted using solvent system 2% methanol in methylene chloride to yeild 16 mg of pure compound **3**.

2.3.1. Determination of AAME Total Phenolic Content

Total phenolic content of AAME was determined using the Folin–Ciocalteu method as described by Attard.[12] Gallic acid stock solution of 1mg/ml in methanol was prepared, from which six-fold dilutions of 50, 100, 400, 600, 800 and 1000µg/ml were made for calibration curve achievement. AAME sample was prepared at a concentration of 5mg/mL in methanol. Briefly, the procedure consisted of mixing 10µL of sample/standard with 100µL of Folin-Ciocalteu reagent (Diluted 1: 10) in a 96-well microplate. Then, 80µL of 1M Na2CO3 was added and incubated at room temperature (25 ◦C) for 20 min in the dark. At the end of incubation time the resulting blue complex colour was measured at 630 nm. The results were recorded using microplate reader FluoStar Omega, where Data are represented as means ± SD.

2.4. Antibiofilm Activity of AAME Against P. aeruginosa Clinical Isolates

2.4.1. Bacterial isolates

Seventeen *P. aeruginosa* clinical isolates were isolated from clinical specimens of patients admitted to Tanta university Hospitals. These isolates were identified by biochemical tests.[13]

2.4.2. Antibacterial activity

The susceptibility of *P. aeruginosa* isolates to AAME was tested using Kirby-Bauer method. [14] Sterile filter paper discs were loaded with AAME (1000µg/mL) and put on the surface of Muller-Hinton agar plates after spreading the bacteria on the surfaces of the plates. Positive control (ciprofloxacin 5µg) and negative control (dimethyl sulfoxide, 10 % v/v) were used.

2.4.3. Determination of minimum inhibitory concentrations (MICs)

The MIC values were determined using broth microdilution method[14] in microtitration plates (96 wells). AAME was serially two-fold diluted using Muller-Hinton broth. After bacterial inoculation into the wells, the plates were incubated overnight at 37 ◦C. Each plate contained a positive and negative control. The lowest concentration which inhibited the visual growth of bacteria was recorded as MIC value (no. of trials= 3).

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2.4.4. Antibiofilm activity estimation by crystal violet assay (CVA)

The impact of AAME (at 0.5 MIC values) on the biofilm formation by *P. aeruginosa* isolates was assessed by CVA.[15] Optical density (OD) values were detected by ELISA reader at 490 nm, where the cut-off OD (ODc) was determined by addition of the mean OD to 3 standard deviations (SD) of the negative control. The ability of *P. aeruginosa* isolates to form biofilm was classified into four categories: No-biofilm formation (ODc < OD < 2 ODc); weak biofilm formation (2 ODc < OD < 4 ODc); moderate biofilm formation (4 ODc < OD < 6 ODc); strong biofilm formation (6 ODc < OD).

2.5. Statistical Analysis

Data were obtained as means ± SD. Statistical analysis was carried out using GraphPad Prism 8 (USA). Results were analysed by one-way analysis of variance (ANOVA).

3. RESULTS and DISCUSSION

3.1.Phytochemical Investigation

3.1.1. *GC/MS analysis of the methylated AAH-SAP fraction*

GC/MS analysis of the AAH-SAP methyl ester fraction encountered twenty known fatty acids, among which 88.58 % were saturated and 4.57 % were unsaturated one. *n*-Hexadecanoic acid, octadecanoic acid and tetradecanoic acid were detected with peak area 42.44 %, 14.62% and 5.89%, respectively (Table1 & Figure 1).

Table (1): GC /MS analysis of AAH-SAP methyl ester fraction.

Peak No.	R t (min.)	Name	[M]+ m/z	Molecular formula	Peak area%
1	7.12	Nonanal	142	$C_9H_{18}O$	0.90
2	10.60	2-Decenal (E)	154	$C_{10}H_{18}O$	1.46
3	14.99	Dodecanoic acid methylester	214	$C_{13}H_{26}O_2$	0.38
4	16.62	Dodecanoic acid	200	$C_{12}H_{24}O_2$	4.56
5	16.62	N, N-Bis (2-Hydroxy ethyl) dodecanamide	287	$C_{16}H_{33}NO_3$	4.56
6	17.22	9-Oxononanoic acid	172	$C_9H_{16}O_3$	1.29
7	17.22	12-Hydroxydodecanoic acid	216	$C_{12}H_{24}O_3$	1.29
8	18.32	Methyl tetradecanoate	242	$C_{15}H_{30}O_2$	1.50
9	18.32	Octadecanoic acid methyl ester	298	$C_{19}H_{38}O_2$	1.50
10	18.62	Azelaic acid methyl ester	202	$C_{10}H_{18}O_4$	0.75
11	18.62	9-Octadecenoic acid (Z)-methyl ester	296	$C_{19}H_{36}O_2$	0.75
12	19.94	Tetradecanoic acid	228	$C_{14}H_{28}O_2$	5.89
14	21.24	Pentadecanoic acid	242	$C_{15}H_{30}O_2$	1.23
15	21.47	Hexadecanoic acid methyl ester	270	$C_{17}H_{34}O_2$	3.34
16	23.73	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	42.44
17	24.61	9,12-Octadecadienoic acid (Z, Z)	280	$C_{18}H_{32}O_2$	1.13
18	26.09	Octadecanoic acid	284	$C_{18}H_{36}O_2$	14.62
19	28.24	Eicosanoic acid	312	$C_{20}H_{40}O_2$	0.93
20	28.24	4,8,12,16-Tetramethylheptadecane -4-olide	324	$C_{21}H_{40}O_2$	3.40

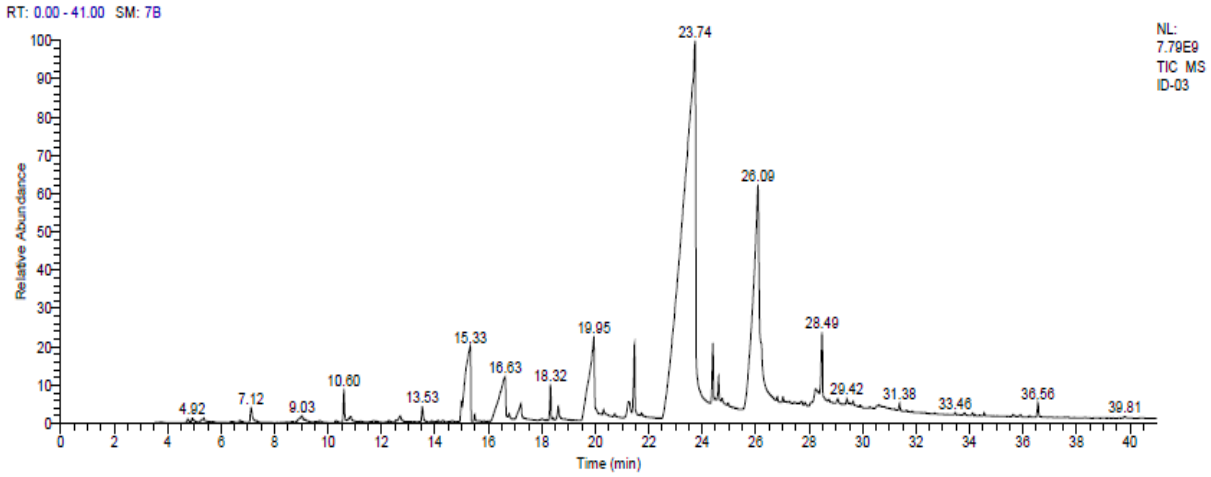

Fig.1. GC/MS total ion chromatogram of methylated AAH- SAP fraction

3.1.2. *Isolation of secondary metabolites from Anabasis articulata (Forssk) Moq successive fractions*

Chromatographic isolation and purification of phytochemicals from USM, AAMC and AAEA fractions output compounds **1-3,** respectively. These compounds were structurally assigned as *β*-sitosterol **1,** caffeine **2**and 4-acetoxy phenol **3**by IR, ^{1}H NMR, ^{13}C NMR, and MS spectroscopical analysis in comparison to the related analogies.[16-20] Both compounds **2**, and **3** are first time isolates from *Anabasis articulata* (Forssk) Moq. (Figure 2). Moreover, our findings confirmed the chemo similarity of Family Amaranthaceae with purine type alkaloid containing Tripe *Salsoleae* of Family Chenopodiaceae.[21]

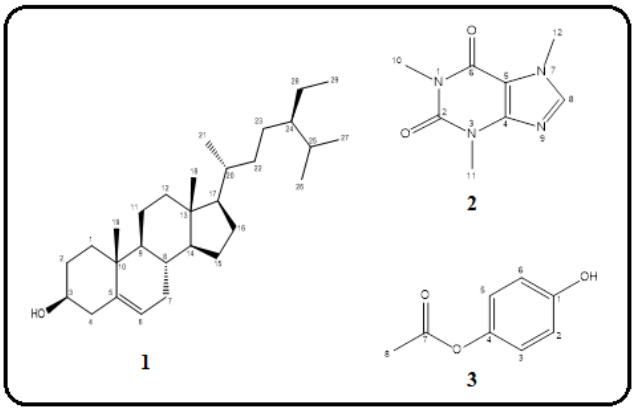

Fig.2. Compounds isolated from Anabasis articulata (Forssk) Moq. successive fractions

3.1.3. Spectroscopic data

Compound 1 (*β*-sitosterol)**:** white needle crystals, 20 mg, m.p (137-139 ◦C). IR (KBr) *vmax*: 3422, 2936, 2868, 1657, 1464, 1378, 965, 839 cm^{-1}. ESI-MS displayed significant peaks at *m/z* (% relative intensity): 469$[M-H]^-$ (100), 430(5), 407(55), 393(60), 239(20), 73(7). ^{1}H-NMR (400 MHz, CD_3OD-d_4,δ_H, ppm): 3.49 (1H-3, m),5.36 (1H-6, d, *J* = 4.8 Hz), 0.67 (3H-18, s), 0.95 (3H-19, s), 0.85 (3H-21, d, *J* = 7.6 Hz), 0.81 (3H-26, d, *J* = 6.8 Hz), 0.70 (3H-27, d, *J* = 6.8 Hz), 0.82(3H-29, t, *J* = 7.2 Hz), 0.67-2.3 (nH-1, 2 ,4 ,7-9, 11, 12, 14-17, 20, 22-25, 28, m). ^{13}CNMR (100MHz, CD_3OD-d_4,δ_C ,ppm):37.27(C-1; CH_2), 31.66 (C-2; CH_2), 71.80 (C-3; CH), 42.30 (C-4; CH_2), 140.77 (C-5; C), 121.71 (C-6; CH), 31.92 (C-7; CH_2), 31.93 (C-8; CH), 50.16 (C-9; CH), 36.15 (C-10; C), 20.10 (C-11; CH_2), 39.79 (C-12; CH_2), 42.33 (C-13; C), 56.78 (C-14; CH), 24.31 (C-15; CH_2), 28.25 (C-16; CH_2) , 56.08 (C-17; CH), 11.86 (C-18; CH_3), 19.40 (C-19; CH_3),36.52 (C-20; CH),18.79 (C-21, CH_3), 33.96 (C-22; CH_2) , 26.11 (C-23; CH_2), 45.86 (C-24; CH), 29.18 (C-25; CH), 19.82 (C-26; CH_3), 19.05 (C-27; CH_3), 23.09 (C-28; CH_2), 11.99 (C-29; CH_3).

Compound 2 (1,3,7-Trimethyl xanthine or caffeine): white needle- like crystals ,50 mg, m. p = 235-237 ◦C. IR (KBr)*vmax*: 3112, 2954,1702, 1658, 1548, 1485, 1358, 1285, 1237,1025cm^{-1}. EI-MS displayed significant peaks at*m/z* (%relative intensity): 194 $[M]^+$(23) ,82(41), 67(100),55(47).^{1}H-NMR (400 MHz, $CDCL_3$-d_4, δ_H, ppm): 7.49 (1H-8, s),3.34 (3H-10, s), 3.52 (3H-11, s), 3.95 (3H-12, s). ^{13}CNMR (100 MHz, $CDCL_3$-d_4, δ_H, ppm): 151.64 (C-2; C), 148.64 (C-4; C), 107.51 (C-5; C), 155.33 (C-6; C), 141.40 (C-8; CH), 27.85 (C-10; CH_3), 29.67 (C-11; CH_3), 33.52 (C-12; CH_3).

Compound 3 (4-acetoxy phenol): white crystalline powder (16 mg) of m.p. (154-156 ◦C). EI-MS displayed significant peaks at *m/z* (%relative intensity): 152 $[M]^+$(60) ,109(100), 80(55),53(68). IR (KBr)*vmax*:3227, 3117, 3040, 1715, 1662, 1559, 1510,1453, 1375,1324,1243,836cm $^{1.1}$H-NMR (400 MHz, CD_3OD-d_4, δ_H, ppm): 6.736(1H-2, d, *J* = 8.8 Hz),7.307 (1H-3, d, *J* = 8.8 Hz), 7.329 (1H-5, d, *J* = 8.8 Hz), 6.758 (1H-6, d, *J* = 8.8 Hz), 2.095 (3H-8, s). ^{13}CNMR (100 MHz, CD_3OD-d_4, δ_H, ppm): 153.97 (C-1; C), 114.79 (C-2; CH), 122.10 (C-3; CH), 130.29 (C-4; C), 122.10 (C-5; CH), 114.79 (C-6; CH), 169.97 (C-7; C), 22.09 (C-8; CH_3).

3.1.4. AAME Total Phenolic Content Assay

Using Folin–Ciocalteu method AAME conceded low Total phenolic content (17.86± 0.61µg GAE/mg extract).

3.2. Antibiofilm Activity of AAME against *Pseudomonas aeruginosa* clinical isolates

3.2.1. Susceptibility of P. aeruginosa clinical isolates to AAME

AAME exhibited antibacterial activity against the tested *P. aeruginosa* isolates by Kirby-Bauer method. The MIC values of AAME ranged from 32 to 256 µg/mL as shown in Table 2.

Table 2. MIC values of AAME against *P. aeruginosa* clinical isolates.

Isolate code	* MIC values (µg/mL)	Isolate code	*MIC values (µg/mL)
P1	32	P10	128
P2	32	P11	256
P3	64	P12	256
P4	32	P13	32
P5	128	P14	128
P6	64	P15	64
P7	32	P16	32
P8	32	P17	64
P9	64		

* No. of trials= 3

3.2.2. Antibiofilm activity of AAME against P. aeruginosa clinical isolates

AAME exhibited antibiofilm activity against the tested *P. aeruginosa* clinical isolates (at half its MIC values) as it resulted in a decrease in the percentage of strong and moderate biofilm forming isolates from 41.18 % to 17.65 % as shown in Table 3.

Table 3. Impact of AAME on biofilm formation by *P. aeruginosa* isolates.

Biofilm Formation capacity	Number of isolates before treatment	Number of isolates after treatment
No formation	6	8
Weak formation	4	6
Moderate formation	4	2
Strong formation	3	1

Biofilm is an important virulence factor of the pathogenic *P. aeruginosa* isolates. Researchers have focused their work to find out new antibiofilm agents. Plants are natural source of structurally diverse bioactive compounds which have antibiofilm activities via the related quorum sensing- antivirulent mechanism of action.[22]

Further chromatographic analysis of *Anabasis articulata* (Forssk) Moq. was done. GC/MS detection of twenty common fatty acids from its AAH-SAP methyl ester fraction, especially high content of *n*-Hexadecanoic acid was established. Moreover, three compounds were isolated and structurally identified as *β*-sitosterol **1,** caffeine **2** from and 4-acetoxy phenol **3** from AAME succsessive fractions**.**

Guzzo et al.[2] demonstrated the anti-biofilm and anti-swarming efficacy of oleanolic acid derivatives and caffeine against clinical isolates of *P. aeruginosa*. However, other report illustrated the anti-*Pseudomonas aeruginosa* action of Hexadecanoic acid methyl ester.[23] Our previous study on *Anabasis articulata* (Forssk) Moq. successive fractions concluded its high content of triterpene type compounds.[9] Herein, we found that AAME had low total phenolic content and high caffeine content of its AAMC fraction. Interestingly, AAME antibacterial and antibiofilm activities against *P. aeruginosa* clinical isolates, may be contributed to its *n*-hexadecanoic acid, caffeine, and triterpene derivatives contents. Yet, inquiry of farther preclinical and clinical investigations is essential.

4. CONCLUSION

Continue phytochemical screening of the Egyptian species *Anabasis articulata* (Forssk) Moq. successive fractions revealed the isolation and spectroscopical identification of caffeine **2,** and 4-acetoxy phenol **3** for the first time, in addition to *β*-sitosterol **1**. Twenty fatty acids were recognized from the GC/MS examination of *Anabasis articulata* SAP fraction, among which were *n*-hexadecanoic acid, octadecanoic acid and tetradecanoic acid. Unfortunately, AAME record low total phenolic content of 17.86± 0.61µg Gallic acid equivalent per mg plant extract. Considering broth microdilution method followed by CVA, Anti-*Pseudomonas aeruginosa* biofilm formation capacity of AAME was accomplished at concentration of half its MIC value. The resultant reduction in strength of strong and moderate biofilm producing tested clinical isolates from 41.18 % to 17.65 %, may intensified its futural anti-*Pseudomonas aeruginosa* use.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Author contributions:

Conceptualization and supervision: Ghada Attia; Investigation: Ghada Attia, Engy Elekhnawy and Ghada Gamal; Methodology: Ghada Attia, Engy Elekhnawy and Ghada Gamal; Writing—original: Ghada Attia and Engy Elekhnawy; Writing—review and editing: Kamilia A. Abo-El-Seoud, Ghada Attia and Engy Elekhnawy; All authors have read and agreed to the published version of the manuscript.

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