Inhibition of *Escherichia coli* biofilm formation by *Streptomyces sdLi* crude extract

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Abstract

Biofilm is a microbial-protecting environment initiated on surfaces that reveals major health problems such as biofilms represented by dental plaques. Fighting biofilm formation is a hugely demanded process. Here, the crude extract of *Streptomyces sdLi* (sediment lake Iraq-sdLi) was used to check the anti-biofilm formation bioactivity (ABFB) against *Escherichia coli* (Orooba Meteab Diwanyah 4, OMD4) isolated from milk samples. Using a cross-streak method, each strain of *Streptomyces* spp. was tested for the best broad-spectrum ABFB. A triplex polymerase chain reaction (TPCR) method targeted specific genes and a fragment (hemin receptor molecule (*chuA*), uncharacterized protein YjaA (*yjaA*), and *chuA* TspE4.C2) was used to categorize 18 isolated OMD4. Using the alcoholic extract of liquid growth of the best strain with ABFB, a crystal violet biofilm assay (CVBA) was employed to test the ABFB against OMD4. The results of the screening test revealed *Streptomyces sdLi* with strongest ABFB; however, ethyl acetate, as one of the *sdLi* extracting solvents, was the most potent in in inhibiting the biofilm formation. The TPCR resulted in 18 isolates categorized into four groups A, B1, B2, and D in which B2 and D are known for their significant pathogenic activities in humans and animals. The results of the CVBA showed that *Streptomyces sdLi* extract was potential for its ABFB. This study recognizes that the *Streptomyces sdLi* extract is potential for deactivating biofilm formation by pathogenic *E. coli* which encourages future studies to consider this microorganism and/or its extract as a cure for the treatment of *E. coli* related illnesses in humans and animals.

Keywords: Biofilm, *E. coli*, *Streptomyces*, TPCR

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تثبيط الغشاء الحياتي لجرثومة الاشريشيا القولونية بالحليب بواسطة المستخلص الخام لستربتومايسيس سدلي

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الخلاصة

تعتبر الأغشية الحيوية بيئة محمية للميكروبات على الأسطح والتي تكشف عن مشاكل صحية كبيرة مثل الأغشية الحيوية التي تمثلها لويحات الأسنان. إن عملية مكافحة تشكيل الأغشية الحيوية هي عملية مطلوبة لإنجاح مكافحة الجراثيم باستخدام المضادات الحيوية. إن هدف هذه الدراسة هو استخدام المستخلص الخام من *Streptomyces sdli* للتحقق من النشاط الحيوي للمضادات الحيوية ضد الإشريشيا القولونية

نوع OMD4 المعزولة من عينات الحليب. تم اختبار أربعون سلالة من *Streptomyces* spp. لأفضل طيف واسع النطاق. استُخدمت طريقة تفاعل سلسلة البلمرة الثلاثية للجينات (*chuA* و *yjaA* و *chuA* TspE4.C2) والتي استخدمت لتصنيف ١٨ سلالة من OMD4. تم تحضير المستخلص الكحولي للنمو المرقي لأفضل سلالة أنتجت مضاد حيوي واسع الطيف لفحص اختبار تحطيم الأغشية الحيوية المكونة من قبل OMD4. كشفت النتائج أفضل عزلات من *Streptomyces sdli* ذات صفات مضادات حيوية قوية. كانت المستخلصات المنتجة بواسطة خلات الإيثيل الأكثر فعالية في تثبيط الأغشية الحيوية. أسفرت نتائج سلسلة البلمرة الثلاثية عن ١٨ سلالة مصنفة إلى أربع مجموعات (A, B1, B2, and D) والتي تُعرف فيها كل من B2 و D بأنشطتها المُمْرِضة المهمة في البشر والحيوانات. تبين هذه الدراسة بأن مستخلص *Streptomyces sdli* يعمل على إيقاف تكوين الأغشية الحيوية المنتجة بواسطة جراثيم الايشيريشيا القولونية الممرضة. تشجع هذه النتائج على إجراء دراسات مستقبلية حول فعالية هذه الكائنات الحية الدقيقة أو مستخلصها كعلاج للأمراض ذات الصلة بالإيشريشيا القولونية في البشر والحيوانات.

Introduction

The gram-negative bacterium, *Escherichia coli*, is microscopically known for its rod form. The lower-intestine located microorganism is categorized in the *Enterobacteriaceae* family. *E. coli* are arranged into wide spectra of genotyped groups that induce various human and animal illnesses of intra or extraintestinal diseases such as urinary tract infections (UTI). This pathogenicity is linked to *E. coli* specific virulence genes which ensure the completion of the infection processes including adhesion, colonization, bacterial proliferation, and fighting body defense mechanisms. Modern methodology relying on those virulence factor has improved understanding the groups of *E. coli* which basically are included in enteric/diarrheal illness group, UTI group, and sepsis/meningitis group (1-5). *E. coli* can affect humans via contaminated food such as milk, meat, and vegetables placing a critical public health problem. Whole milk or milk products are considered as an important media for bacterial growth which enhance the transmission of food-borne pathogens such *E. coli* (5).

Biofilm can broadly be defined as a bacterial ecosystem with complex nature initiated by bacterial attachment to a bacterial organic polymer matrix covered surface. The system also contains different microbial components with some substances of non-cellular origins such as mineral crystals. This system allows the microbial communities living within a biofilm to ensure homeostasis with each other and external environment. Biofilm structure is very complex and it strongly depends on the nature of the environmental niches in which a biofilm gets established (6). Biofilm formation enables microorganisms to escape and resist a wide range of substances such as antibiotics (7). It has been recognized that *Streptomyces* generate some metabolites which work against biofilm and the helping prokaryotic machine; quorum-sensing produced by *Proteus mirabilis*, UTI pathogen (8).

In the current work, the crude extract of *Streptomyces sdLi* were used to check the anti-biofilm formation bioactivity (ABFB) against *E. coli* OMD4 isolated from the milk samples.

Materials and methods

Sample collection of *Streptomyces*

Iraqi river sediments and soil from various locations were used to collect *Streptomyces*. The samples were subjected to serial dilution processes using sterile water that reached up to 10^{-6}. The cycloheximide-pretreated *Actinomycetes* agar (Difco) plates were inoculated with 0.1ml of the diluted solutions and incubated for 3-5 days at 29°C. The growth was sub-cultivated by using tryptic soy broth (Oxoid, CM0129B) for purification purposes. The biochemical tests (starch hydrolysis, casein, glucose, sucrose utilization, lipid hydrolysis, citrate utilization, Rhamnose utilization, nitrate reduction, H_2S Production, and ability to grow in 8%) were done to differentiate the microorganisms (9). As a screening test using a cross-streak method, each of 40 isolates of *Streptomyces* spp. was tested for the best broad-spectrum ABFB. The tryptic soy broth was used to sub-cultivate the microorganism to be centrifuged at 13,000 rpm for 15 min and then harvesting the supernatant.

Isolation of *E. coli* OMD4

Eighteen milk samples collected form the healthy cows and cows with mastitis from different locations in Al-Diwaniyah Governorate. Eighteen OMD4 isolates were detected by using the MacConkey agar (overnight incubation at 37ºC) and the Eosin Methylene Blue (EMB) agar. All biochemical tests were performed to identify the characteristics of the isolates.

Extraction of bioactive substances of *Streptomyces sdLi*

International Streptomyces Project (ISP2) media for streptomyces (selective media), yeast extract 4g, malt extract 10g, dextrose 4g, and agar 20g, was used to grow those microorganisms under suitable conditions for 9 days at 29ºC of incubation with continuous shaking. The methods used in the current section were followed from Jensen *et al.* (10). The extract was constituted using 100% of dimethyl sulfoxide (DMSO) and kept at 4°C until further use in the anti-biofilm assay. For the fractioning of the extraction according to solvents used, bio-guided fractionation by using water

solubilization before using diethyl ether, n-hexane, ethyl acetate, chloroform, and water had been done. Solvent-resulted layer was removed in three repeats of dissolving processes. After that, an evaporating step was performed before using100µL DMSO as a carrier.

Crystal violet biofilm assay

The alcoholic extract was used as a liquid growth of the best strain with ABFB, a crystal violet biofilm assay (CVBA) was performed to test the ABFB against OMD4 biofilm formation in urinary catheters. The cell density used was 1.5×10^7 CFU/ml treated with two concentrations of the extract (10 and 15mg/ml) plus using a no-treatment control. The mixture was placed in a 96-well plate pre-introduced with a 1cm-steralized piece of Foley catheter in each well. Then, the plate was incubated at 37°C for 24h. After that, a washing step with distilled water was performed, and a staining step by using 1% crystal violet was done followed by another washing step. Then, a re-solubilizing step utilizing 97% ethanol at 200µl was generated. After removing the liquid to another 96-well plate, the optical density (OD) was read at 595nm. A triplicate-based experiment was followed in the current study. Minimum inhibitory concentration (MIC) was tested. The same test procedures were followed to examine the activity of each solution extracted using different solvents.

Extraction of DNA

The DNA was extracted using a boiling method from Abdallah *et al.* (11). As an initiating process, 250µl of sterile distilled water was used to dissolve a few colonies of the OMD4 followed by a boiling method contained inoculated of *E.* an Eppendorf tube including a specific medium followed by an 18hours incubation at 37°C. The collected cells, then, centrifuged for 10mins at 6000r/min, which they were used in a heat-based lysis method. After removing the debris of the cells, the supernatant was used in a phenol/chloroform method.

Triplex polymerase chain reaction

Triplex polymerase chain reaction (TPCR) methods were followed from Clermont *et al.* and Gordon *et al.* (12,13). The primers employed in the current study were used to detect the genes coding current as a *chuA*, *yjaA*, and TspE4.C2, are for *ChuA* (279bp); F: 5`-GAC GAA CCA ACG GTC AGG AT-3` and R: 5`-TGC CGC CAG TAC CAA AGA CA-3`, for *yjaA* (211bp); F: 5`-TGA AGT GTC AGG AGA CGC TG-3` and R: 5`-ATG GAG AAT GCG TTC CTC AAC-3`), and for TspE4.C2 (152bp); F: 5`-GAG TAA TGT CGG GGC ATT CA-3` and R: 5`-CGC GCC AAC AAA GTA TTA CG3`. The total volume used for the PCR reaction was 25µl that contained PCR water at 11.25µl, buffer at 2.5µl 10X (CinnaGen Co., Iran), $MgCl_2$ at 0.75µl (CinnaGen Co., Iran), dNTPs at 1µl (CinnaGen Co., Iran), each primer at 1µl (20 pmol) (CinnaGen Co., Iran), Taq polymerase at 2.5U (CinnaGen Co., Iran), and DNA template at 3µl. The conditions of the thermal cycler (MJ Mini, BIO-RAD-USA) were: 94°C for 4min initial denaturation, 30 cycles of (denaturing for 5sec at 94 °C, annealing for 10sec at 57°C), and 5min at 72°C of final extension. PCR products were separated through a 2% agarose gel using electrophoresis. UV light imager was used to screen the results on the gel.

Results

Primary screening test

The results of the strain identification revealed 18 *Streptomyces* strains. However, only *Streptomyces sdLi* crude broth showed the strongest ABFB against biofilm formation.

Minimum inhibitory concentration of the *Streptomyces sdLi* extract against biofilm formation by *E. coli* OMD4

The MIC of the *Streptomyces sdLi* extract against biofilm formation by OMD4 was recorded to be 20mg/ml (Figure 1).

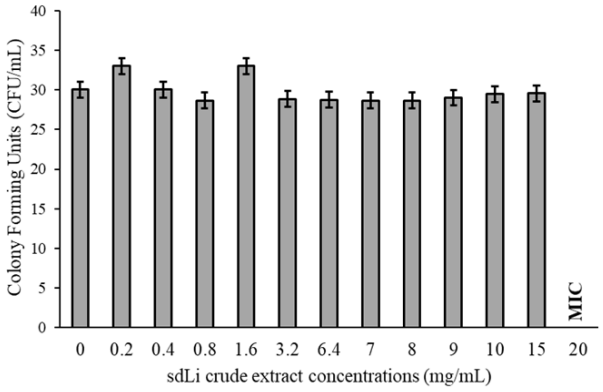

Figure 1: Colony forming unit on LB agar with the use of the spread plate method. Expression of each value was by mean of triplicates ± standard deviation. Error bars (calculated from three repetitions) are also presented.

Crystal violet biofilm assay using sdLi crude extract

This study established a close relationship between inter bacterial communication and biofilm formation by OMD4 isolates. The untreated OMD4 isolate optical density (OD) reading on 595nm wavelength was found out to be 2.900 (Figure 2.a). Based on figure (2.b), the OD reading of untreated OMD4 would form colonies above 30. It is therefore any wells that give 30 colonies or more can be considered to have no effect on growth with sdLi crude extract. Biofilm formation was reduced by 63% at 15mg/mL sdLi extract.

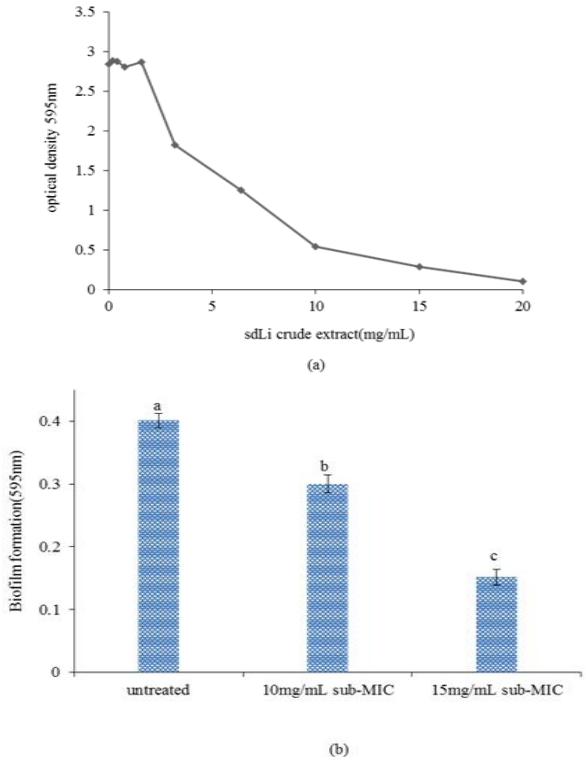

Figure 2: Effect of sdLi crude extract. (a) Different concentrations of sdLi extract effect on ECO4 read by optical density at 595nm. (b) Biofilm formation reduction. Significant difference ($p< 0.05$) was indicated by varying letters; a- b- c.

The effects of the sdLi extracted on the biofilm formation according to the extracting solvents

Ethyl acetate, as one of the sdLi extracting solvents, was the most potent in inhibiting the biofilm formation (Figure 3).

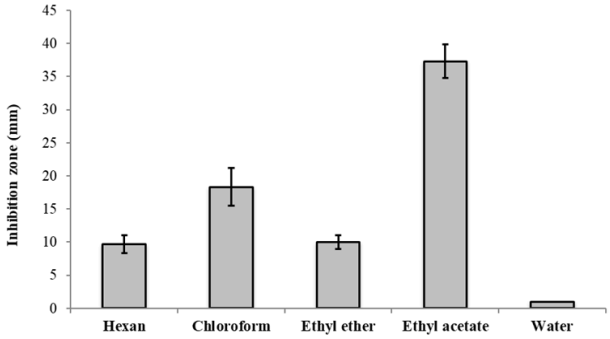

Figure 3: Inhibition zone according to the extracting solvents. Each value is expressed as the mean of triplicates ± standard deviation and error bars (calculated from three repetitions)

TPCR results

The TPCR resulted in 18 strains categorized into four groups (A, B1, B2, and D) in which B2 and D are known for their significant pathogenic activities in humans and animals, figure 4.

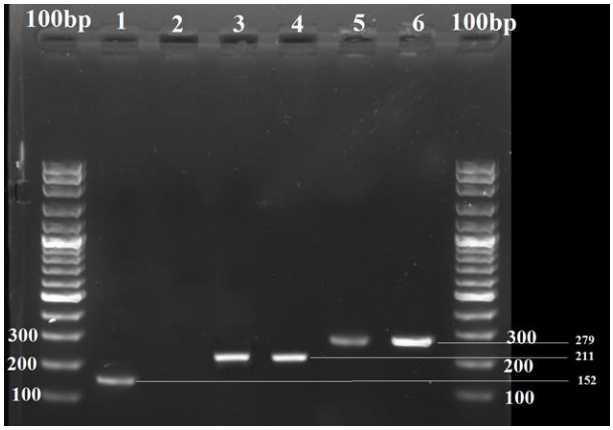

Figure 4: Agarose gel electrophoresis of lane 1: TspE4.C2 (152bp), lane 4: *yjaA* (211bp), and lane 6: *chuA* (279bp) genes.

Discussion

The TPCR resulted in 18 strains categorized into four groups A, B1, B2, and D. The groups B2 and D are known for their significant pathogenic activities in humans and animals. The extract of the sdLi was highly effective against OMD4 biofilm formation. This agrees with the fact that this extract was found to cause deactivation of virulence factors and significantly reduce forming of biofilm. The lowest concentration should be in the range of 10 and 15 mg/ml. Further investigations should be launched in the future testing those concentrations for a deeper knowledge. The result suggests that the OD reading becomes lower when concentrations of the secondary metabolite extract are increased. Based on this, it can be inferred that the OMD4 biofilm formation has been inhibited. It has been proven that extracts can perform their beneficial duties, destruction of biofilm, without harming cells and thus agrees with our results; however, the pathogenicity and pathogen evolution can be strengthened via the formation of biofilm (14).

In quorum sensing of microorganisms, binding of a specific acyl homoserine lactone (AHL) signal molecules to a LuxR, regulatory proteins, is the first step in the quorum sensing induction. Disrupting this system via the inhibition of this step can provide vital solution in deactivating the biofilm formation (15). Compared to native AHL ligands, some cognate receptors have the capability to analogue-bind at higher rated affinity. However, the analogues then inactivate gene expression and therefore become antagonists

(16,17). Therefore, the results in this study suggest that the effect of the extract in a concentration-dependent manner to inhibit biofilm formation without effect on growth development is probably due to interference with native AHLs signals of cells and therefore become antagonists.

Marine microorganisms such as *Streptomyces* sp. OUCMDZ-3436 have been detected to have secondary products that can be highly effective against various bacterial activities and components such as quorum sensing after transforming the skeleton of α-pyrone into pyridine-2(1H)-one (18-21).

Conclusions

The presented work here informs that *Streptomyces sdLi* extracts have strong destruction activities against pathogenic E. coli isolated from milk samples. Furthermore, these actions are more dominant especially when using ethyl acetate as the main solvent. The observed functions against the biofilm structure may reveal promising medical interventions against a wide range of pathogens that use biofilm formation as a virulent tool to escape antimicrobials.

Acknowledgments

This study recognizes that the *Streptomyces sdLi* extract is potential for deactivating biofilm formation by pathogenic *E. coli* which encourages future studies to consider this microorganism and/or its extract as a cure for treatment of *E. coli* related illnesses in humans and animals.

Conflict of interest

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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References

1. Kaper JB, Nataro JP, Mobley HLT. Pathogenic *Escherichia coli*. Nat Rev Microbiol. 2004;2(2):123-40. doi: 10.1038/nrmicro818.
2. Allocati N, Masulli M, Alexeyev MF, Di IC. *Escherichia coli* in Europe: an overview. Int J Environ Res Public Health. 2013;10(12):6235-54. doi: 10.3390/ijerph10126235.
3. McClure PJ, Hall S. Survival of *Escherichia coli* in foods. Symp Ser Soc Appl Microbiol. 2000;14(29):61S-70S. doi: 10.1111/j.1365-2672.2000.tb05333.x.
4. Rahal EA, Kazzi N, Nassar FJ, Matar GM. *Escherichia coli* O157:H7-Clinical aspects and novel treatment approaches. Front Cell Infect Microbiol. 2012;2:(138):1-7. doi: 10.3389/fcimb.2012.00138.
5. Donkor E, Aning K, Quaye J. Bacterial contaminations of informally marketed raw milk in Ghana. Ghana Med J. 2007;41(2):58-61. doi: 10.4314/gmj.v41i2.55302.
6. López D, Vlamakis H, Kolter R. Biofilms. Cold Spring Harb Perspect Biol. 2010;2(7):1-11. doi: 10.1101/cshperspect.a000398.
7. Koo H, Allan RN, Howlin RP, Stoodley P, Hall SL. Targeting microbial biofilms: Current and prospective therapeutic strategies. Nat Rev Microbiol. 2017;15(12):740-55. doi: 10.1038/nrmicro.2017.99.
8. Younis KM, Usup G, Ahmad A. Secondary metabolites produced by marine streptomyces as antibiofilm and quorum-sensing inhibitor of uropathogen *Proteus mirabilis*. Environ Sci Pollut Res Int. 2016;23(5):4756-67. doi:10.1007/s11356-015-5687-9.
9. Bergey DH, David H, Holt JG. Bergey's manual of determinative bacteriology. New York: Lippincott Williams & Wilkins; 2000. 787 p. ISBN: 9780683006032 0683006037
10. Jensen PR, Williams PG, Oh DC, Zeigler L, Fenical W. Species-specific secondary metabolite production in marine Actinomycetes of the genus Salinispora. Appl Environ Microbiol. 2007;73(4):1146-52. doi: 10.1128/AEM.01891-06.
11. Abdallah KS, Cao Y, Wei DJ. Epidemiologic investigation of extra-intestinal pathogenic *Escherichia coli* (ExPEC) based on PCR phylogenetic group and fimH single nucleotide polymorphisms (SNPs) in China. Int J Mol Epidemiol Genet. 2011;2(4):339-53. PMCID: PMC3243450.
12. Gordon DM, Clermont O, Tolley H, Denamur E. Assigning *Escherichia coli* strains to phylogenetic groups: Multi-locus sequence typing versus the PCR triplex method. Environ Microbiol. 2008;10(10):2484-96. doi: 10.1111/j.1462-2920.2008.01669.x.
13. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol. 2000;66(10):4555-8. doi: 10.1128/aem.66.10.4555-4558.2000.
14. Jamal M, Ahmad W, Andleeb S, Jalil F, Imran M, Nawaz MA. Bacterial biofilm and associated infections. J Chinese Med Assoc. 2018;81(1):7-11. doi: 10.1016/j.jcma.2017.07.012.
15. Hirakawa H, Tomita H. Interference of bacterial cell-to-cell communication: A new concept of antimicrobial chemotherapy breaks antibiotic resistance. Front Microbiol. 2013;4(114):1-14. doi: 10.3389/fmicb.2013.00114.
16. Hawver LA, Jung SA, Ng WL. Specificity and complexity in bacterial quorum-sensing systems. FEMS Microbiol Rev. 2016;40(5):738-52. doi: 10.1093/femsre/fuw014.

17. Zheng Y, Sintim HO. Molecular insights into how ligands activate or inactivate LasR. Chem Biol. 2014;21(10):1261-3. doi.org/10.1016/j.chembiol.2014.10.001.
18. Storck P, Aubertin AM, Grierson DS. Tosylation/mesylation of 4-hydroxy-3-nitro-2-pyridinones as an activation step in the construction of dihydropyrido[3,4-b] benzo[f][1,4]thiazepin-1-one based anti-HIV agents. Tetrahedron Lett. 2005;46(16):2919-2922. doi: 10.1016/J.TETLET.2005.02.128.
19. López SN. Chemically engineered extracts as an alternative source of bioactive natural product-like compounds. Nat Acad Sci. 2007;104(2):441-4. doi: 10.1073/pnas.0608438104.
20. Ramallo IA. Chemically engineered extracts: Source of bioactive compounds. Chem Res. 2011;44(4):241-250. doi: 10.1021/ar100106n.
21. Chen J. Quorum sensing inhibitors from marine microorganisms and their synthetic derivatives. Marine Drugs. 2019;17(2):80. doi: 10.3390/md17020080.