ijlpr 2023; doi 10.22376/ijlpr.2023.13.2.P51-P59

International Journal of Life science and Pharma Research

ISSN 2250-0480

Research Article

Antiquorum Sensing Activity of Methanolic Seed (Nutmeg) Extract of *Myristica Fragrans* Against a Gram-Positive Bacterium

Bhavana[1] M and Kodali Vidya Prabhakar [1*]

[1] *Department of Biotechnology, Vikrama Simhapuri University, Nellore-524320*

[1*]*Assistant Professor, Department of Biotechnology, Vikrama Simhapuri University, Nellore, Andhra Pradesh, India*

Abstract: The increase in multidrug-resistant pathogens has created new anti-pathogenic and anti-virulence compounds. This is because of the behavioural changes bacteria acquire, such as increased antibiotic resistance and virulence capacity. This social behaviour is maintained through a signalling transduction pathway called Quorum sensing. The present study aims to evaluate the inhibition of quorum sensing in *Bacillus subtilis* with *Myristica fragrans* seed extract. The seed of *M. fragrans* is called nutmeg. Methanolic nutmeg extract was used to test the anti-quorum sensing activity of *B. subtilis*. Total flavonoid and phenolic concentrations of nutmeg were estimated. 10 µg/ml, 30 µg/ml, 50 µg/ml, 70 µg/ml and 90 µg/ml concentrations of nutmeg extract were used to check against bacterial motilities and biofilm formations. Inhibition of biofilm formation was observed under a fluorescence microscope, and inhibition of swimming and swarming motilities were observed on 0.3 % and 0.5 % agar plates, respectively. These observations suggest that the seed of *M. fragrans* showed anti-quorum sensing activity against *B. subtilis*. This research work helps to study and isolate natural quorum-sensing inhibitors from medicinal plants. These inhibitors can synthesize novel anti-pathogenic or anti-virulence drugs that combat bacterial infections by interrupting with quorum-sensing controlled phenotypes and decreasing bacterial virulence.

Keywords: *B. subtilis;* Motility; *M. fragrans*; Nutmeg; Quorum sensing

***Corresponding Author**

Dr. Vidya Prabhakar Kodali , Assistant Professor, Department of Biotechnology, Vikrama Simhapuri University, Nellore, Andhra Pradesh, India

Received On 21 September, 2022

Revised On 30 November, 2022

Accepted On 13 December, 2022

Published On 1 March, 2023

Funding This work is supported by the National Medicinal Plants Board (NMPB), Department of AYUSH, Ministry of Science and Technology, (F. No.Z.18017/187/CSS/R&D/AP-01/2017-18) and DST-FIST (F. No.DST-FIST-LSI-595 (LSI-026).

Citation Bhavana M and Vidya Prabhakar Kodali , Antiquorum Sensing Activity of Methanolic Seed (Nutmeg) Extract of Myristica Fragrans Against a Gram-Positive Bacterium.(2023).Int. J. Life Sci. Pharma Res.13(2), P51-P59 http://dx.doi.org/10.22376/ijlpr.2023.13.2.P51-P59

1. INTRODUCTION

Quorum sensing is a multicellular and group behaviour expressed by bacteria. The expression of these bacteria's genes and characters depends on their species' cellular density. When the concentration of signalling molecules produced by these bacteria reaches a certain threshold level, bacteria will start expressing quorum-sensing regulatory genes and their respective phenotypes. This multicellular or group behaviour is called quorum sensing [1].

1.1. Mechanism of quorum sensing in Gram-negative and Gram-positive bacteria

The mechanism of QS mainly depends on the synthesis and release of signalling molecules. These signalling molecules are different in Gram-negative and Gram-positive bacteria. These molecules are divided into 3 classes: 1. AHLs (N-acyl homoserine lactones) in Gram-negative bacteria; 2. Autoinducing peptides/oligopeptides in Gram-positive bacteria; 3. AI-2 molecules. These are the signalling molecules both in Gram-negative and Gram-positive bacteria. These signalling molecules have an essential role in the production of infection by bacteria. When the number of signalling molecules if crossed a threshold level, these signals bind to their respective response regulators and express their target genes (Fig. 1). The expression of target genes leads to the expression of various quorum sensing phenotypes like the production of virulence factors, biofilm formation, antibiotic resistance, motility, etc[2].

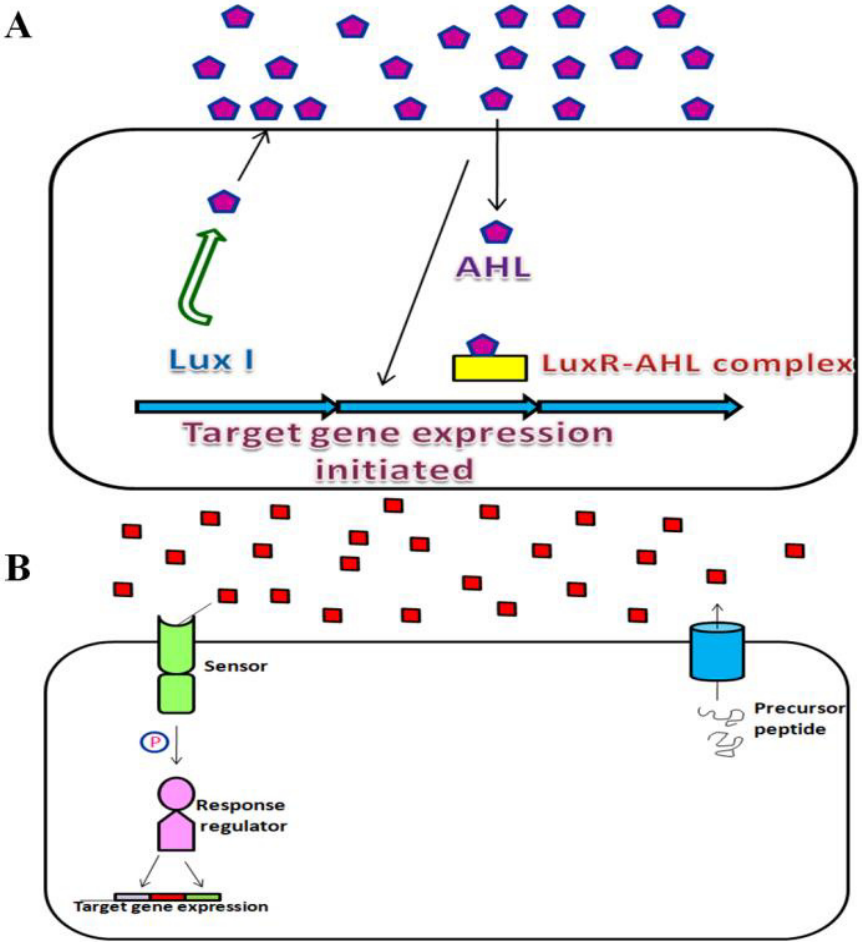

Fig 1: A) Quorum sensing signalling mechanism in Gram-negative bacteria: the signalling molecules (AHLs) are produced from autoinducer synthase (LuxI) and diffused out of the bacterial cell. When the number of signalling molecules reaches to a threshold value, the signalling molecules will bind to regulatory proteins and form the AHL-LuxR complex to initiate target gene expression. B) Quorum sensing signalling mechanism in Gram-positive bacteria: When the number of autoinducing peptides ( ) reaches a particular threshold value, then the sensor kinase protein present in the cell membrane was activated and phosphorylated the response regulator and binds to promoters to express QS target genes.

1.2. Mechanism of quorum quenching

Quorum quenching defines the mechanism of inhibiting quorum sensing signalling by quorum sensing inhibitors. This process is done at different levels: 1) Inhibition at the level of autoinducer synthases, 2) Inhibition at the level of signalling molecule and receptor binding, 3) Inhibition at the level of target gene expression. This inhibition of bacterial communication leads to the attenuation of virulence capacity. The ideal quorum-sensing inhibitor has specific features; it should be chemically stable with low molecular mass and show high specificity towards quorum-sensing regulatory protein without causing side effects in bacteria and its host. Quorum sensing is an important signalling mechanism for antibiotic treatment failure in many bacteria. Many antibiotic-resistant bacteria were not killed by using normal antibiotics. Quorum sensing studies help achieve novel methods for treating many bacterial diseases by reducing their pathogenicity without

affecting their growth [1]. This is a behavioural modification of bacteria without affecting their growth and cellular modifications. Therefore, in recent years, the development of novel quorum quenching drugs has had great benefits [2].

1.3. Plants as natural quorum-sensing antagonists

Recently there has been an increase in interest in the therapeutic role of natural plant extracts in decreasing the interactions between microbes. The use of plants, plant products, and their purified components could open up the possibility of using these compounds as novel anti-quorum sensing agents. Nowadays, plant-derived developments have led to the invention of new drugs to treat central chronic infections caused by microbes. Many essential oils show quorum quenching activity against pathogenic bacteria. For example, Spice oils from cumin, pepper & fennel act against *C. violaceum*, Essential oils of *Thymus daenensis* & *Satureja hortensis* against *Staphylococcus aureus* and *Eucalyptus globules* & *Eucalyptus radiate* against *Acinetobacter baumannii*, etc[3].In this study, *Myristica fragrans* seed extract is used to test against the quorum-sensing activity of Gram-positive *B. subtilis*. *Bacillus subtilis* cause many infectious diseases like pulmonary diseases and endocarditis in drug abusers [4,5]. *M. fragrans* is native to the rainforest of Indonesia [6]. Nutmeg is a seed used as a spice in food recipes produced from the *M. fragran*t tree. This fruit and seed were widely used as a flavouring agent in several dishes. In India and the Middle East, this spice is used as medicine. Nutmeg essential oil has antimalarial activity [7]. Biosynthesized nanoparticles from nutmeg were used to treat multidrug-resistant typhoid-causing bacteria [8]. Mace is used as a spice that covers the nutmeg seed. Nutmeg produces essential oils that treat rheumatism, general aches, inflammations, and limb pains [9]. Nutmeg is used to promote sleep and to treat nervous complaints. The kernel of nutmeg produces essential oils, fats, and starch. The majority of constituents present in the oils control several enzyme activities [10]. *B. subtilis* is a soil microbe expressing quorum-sensing phenotypes such as swimming and swarming motility assays, biofilm formation, exoprotease production, etc. *B. subtilis* regulates the quorum sensing through the ComQXPA quorum sensing system. Biofilm formation and motility behaviour were expressed by *the B. subtilis* bacterium, which increases the infectious nature of bacteria. In this study, we tested nutmeg oil's anti quorum sensing activity against motility and biofilm formation of *B. subtilis* [11]. Nowadays, traditional antibiotics may lead to the development of multidrug-resistant bacteria, which may lead to failure in treatment of several infectious diseases. Therefore, this work mainly focuses on suppressing or decreasing the pathogenicity microbe instead of killing by using *M. fragrans* (nutmeg) seed extract.

2. MATERIALS AND METHODS

2.1. Bacterial growth and culture conditions

Bacillus subtilis, a Gram-positive bacterium was used in this study. It was maintained at 37°C in Nutrient agar media. The test organism was purchased and obtained from Microbial Type Culture Collection (MTCC).

2.2. Preparation of plant extract

Methanolic extract of nutmeg powder was used to test antiquorum sensing activity. Nutmeg seeds were bought from local Ayurveda shops of Sri Potti Sriramulu Nellore District, Andhra Pradesh, India. Nutmeg is a seed produced from the fruit of the *Myristica fragrans* tree (Fig.2). The following methods were used to make the final extract of nutmeg powder.

Fig 2: Seed of *Myristicafragrans* (Nutmeg)

Methanol (95%) was used for the extraction process. Nutmeg powder (100grams) was weighed and extracted with 400ml of 95% methanol. Soxhlet apparatus (Merk) was used for this extraction method. The total extraction method was run at 4 cycles and 50°C temperatures. The solvent was collected and stored at 4°C until use [12]. The total solvent that was collected after the soxhlet extraction process was concentrated using a rotary evaporator at 50°C. When the required amount of extract was formed it was collected into Petri dishes. The remaining solvent (methanol) was evaporated in a hot air oven at 50°C and finally, the crude extract was stored at 4°C until use [13]. Total nutmeg powder of 100 grams (Ey) was processed into the final crude extract of 7.76 grams (Ex). The percentage of yield of plant extract was 7.76% w/w and the formula calculated it

$$Ex/Eyx100$$ [14]

Ey= Weight of plant powder
Ex= Final crude extract of plant powder

2.3. Estimation of total flavonoid and phenol concentrations

2.3.1. Estimation of total flavonoid content

Total flavonoid concentration was estimated by standard aluminium chloride colorimetric assay [15]. A common solution of Quercetin in concentrations 20, 40, 60, 80, and 100µg/ml was prepared in 50% Methanol. These diluted standard solutions (0.5ml) were mixed separately with 1.5ml of 95% ethanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M

potassium acetate and 2.8ml of distilled water. After incubation at room temperature for 30min, the absorbance of the reaction mixture was measured at 435nm. The amount of 10% aluminium chloride was substituted by the same amount of distilled water in the blank. Similarly, 0.5ml of Methanol extracts were reacted with aluminium chloride to determine flavonoid content.

2.3.2. Estimation of total phenol content

Total phenolic content of each extract was determined by the Folin–Ciocalteu method [16]. In brief, Folin–Ciocalteu reagent was diluted 10-fold with distilled water. First, the methanolic extract of nutmeg (0.1ml) was mixed with 0.75ml of the diluted Folin–Ciocalteu reagent and incubated for 10 minutes at room temperature. Then, 0.75ml of 2% sodium carbonate (w/v) solution was added. The mixture was allowed to stand in the dark for 45 minutes and OD at 600nm was measured against a blank, containing distilled water instead of sample extract. Total phenolic concentration values were determined from a calibration curve prepared with Gallic acid standards (20, 40, 60, 80, 100µg/ml).

2.4. Preparation of standard concentrations

Required concentrations of the methanolic seed (nutmeg) extract and standard to be tested for antiquorum sensing were prepared by dissolving in Dimethyl Sulphoxide (DMSO). Quercetin was used as a standard to test anti quorum sensing activity [17]. All stocks were stored at 4ºC until use.

2.5. Antiquorum sensing assays

Nutmeg powder extract was used to test antiquorum sensing activity against *B. subtilis* bacteria. Quercetin can be used for all antiquorum sensing studies as a standard comparison for quorum sensing inhibition [17]. Therefore, we selected inhibition of quorum sensing phenotypes which are expressed by the desired bacteria.

2.5.1. Motility assays

Gram-positive *B. subtilis* bacterium was selected for motility assays. This organism exhibits swimming and swarming motilities when placed in the plate's centre with 0.5% and 0.3% agar, respectively. These motility assays were observed for the motility of bacteria in semisolid and liquid agar media.

2.5.1.1. Swarming motility

Swarming motility assay for *B. subtilis* was done by pointing a small drop of the organism at the center of agar medium plates containing 0.5% NaCl, 1% peptone, 0.5% filter sterilized D-glucose, and 0.5% agar with increasing concentrations (10µg/ml to 90µg/ml) of test samples. Plates with *M. fragran*t extracts were treated as test controls. Plates with Quercetin were treated as positive control and plate without any test extracts were treated as a negative control. A swarming plate with DMSO was also observed for motility inhibition. After solidification, all plates were incubated in upright positions at 37ºC and for 24h [18].

2.5.1.2. Swimming motility

A swimming motility assay of *B. subtilis* was done by pointing a small drop of an organism at the centre of agar medium plates containing 0.5%Tryptone, 0.5% NaCl and 0.3% agar with increasing concentrations (10µg/ml to 90µg/ml) of test samples. Plates with *M. fragran*t extracts were treated as test controls. Plates with Quercetin were treated as positive control, and plate without any test extracts were treated as negative control. A swimming plate with DMSO was also observed for motility inhibition. After solidification, all plates were incubated in upright positions at 37ºC and for 24h[18].

2.5.2. Antibiofilm activity

2.5.2.1. Microscopic analysis

The microscopic analysis of biofilm confirmed the inhibition of biofilm production by nutmeg extract. Fluorescence microscopic analysis was used for this assay. For all techniques, *B. subtilis* culture was incubated in a static position with broth and test samples in the test tubes. All tubes were incubated with 18X18mm coverslips to form biofilm on the coverslips. All incubated coverslips with test samples were recovered and stained with 0.2% Acridine orange dye for 2 minutes, washed with distilled water, and air dried. All stained coverslips were mounted on slides and observed under fluorescence microscope at 10X magnification [19].

2.6. Statistical analysis

All results were analyzed as mean±SD. All statistics were analyzed using the Prism software.

3. RESULTS AND DISCUSSION

We have already studied antibacterial study of methanolic extract of nutmeg [20].

3.1. Estimation of total flavonoid and phenol concentrations

The result of total flavonoid and phenolic content in crude extract of nutmeg powder was estimated using the equation of calibration curve of Quercetin standard was y = 0.0057x - 0.0926 and for Gallic acid was y = 0.0002x - 0.001. It is observed that flavonoid concent is more than phenolic concent in the methanolic crude extract of nutmeg powder (Fig. 3).

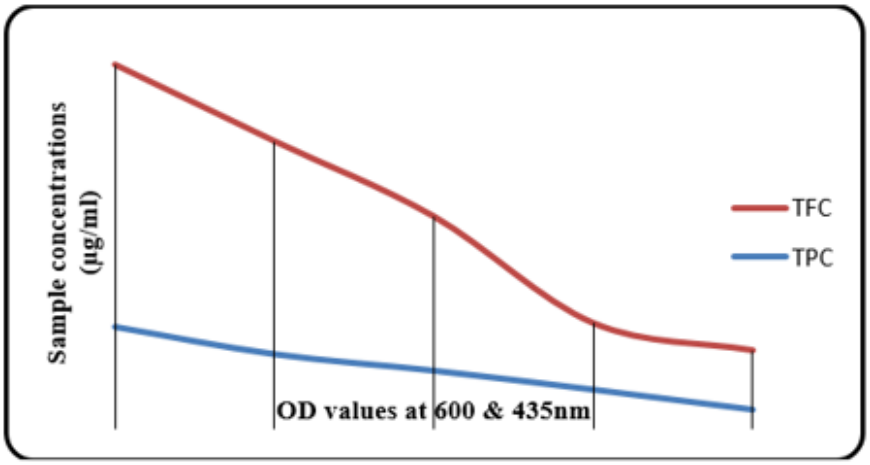

Fig 3: Graph representing Total flavonoid and phenolic concentration in Nutmeg powder; TPC: Total phenolic concentration; TFC: Total flavonoid concentration

3.2. Motility and biofilm formation assays

B. subtilis organism can switch between two mutual lifestyles, motility and biofilm formation. This bistable switch controls bacteria to grow under unfavourable environments [21,22].

3.2.1. Swimming and Swarming motility

Bacteria can't modify the environment because of their tiny size and structure; therefore, they adapt to move towards environments that can increase their prospects for survival [23]. This ability of bacteria to move towards food and away from stressful environments is called motility. Swarming is multicellular motility on semisolid surfaces [24]. Swimming motility is an individual cell movement of bacteria in liquid medium or medium with lower agar [25]. By quorum sensing signalling, swimming and swarming cells are usually differentiated from vegetative cells by forming multicellular groups between the same or different species. These swimming and swarming behaviours create resistance to multiple antibiotics [26]. Generally, bacteria can move in 1 or 3-dimensions. Bacteria moving on surfaces without the help of flagella is called gliding motility and movement with the help of flagella is called swarming motility. Bacterial movement in all three dimensions is called swimming motility [27]. Motility inhibition tests were done because bacterial motility was associated with its virulence capacity (Fig. 4 (i) & (ii) and Fig. 5 (i) & (ii)) [28]. We examined the swimming motility of *B. subtilis* in 0.3% agar medium (Fig. 5 (i) & (ii) and the swarming motility of *B. subtilis* in 0.5% agar medium in Petri plates (Fig. 4 (i) & (ii). Distance travelled by the organism from the center of the plate towards the edge of the plate is considered as swimming/swarming motility and this distance was measured. A concentration dependent decrease in the motilities was observed with test extracts (Fig. 4 & 5). Nutmeg extract showed an observable inhibition in the motility of *B. subtilis.*

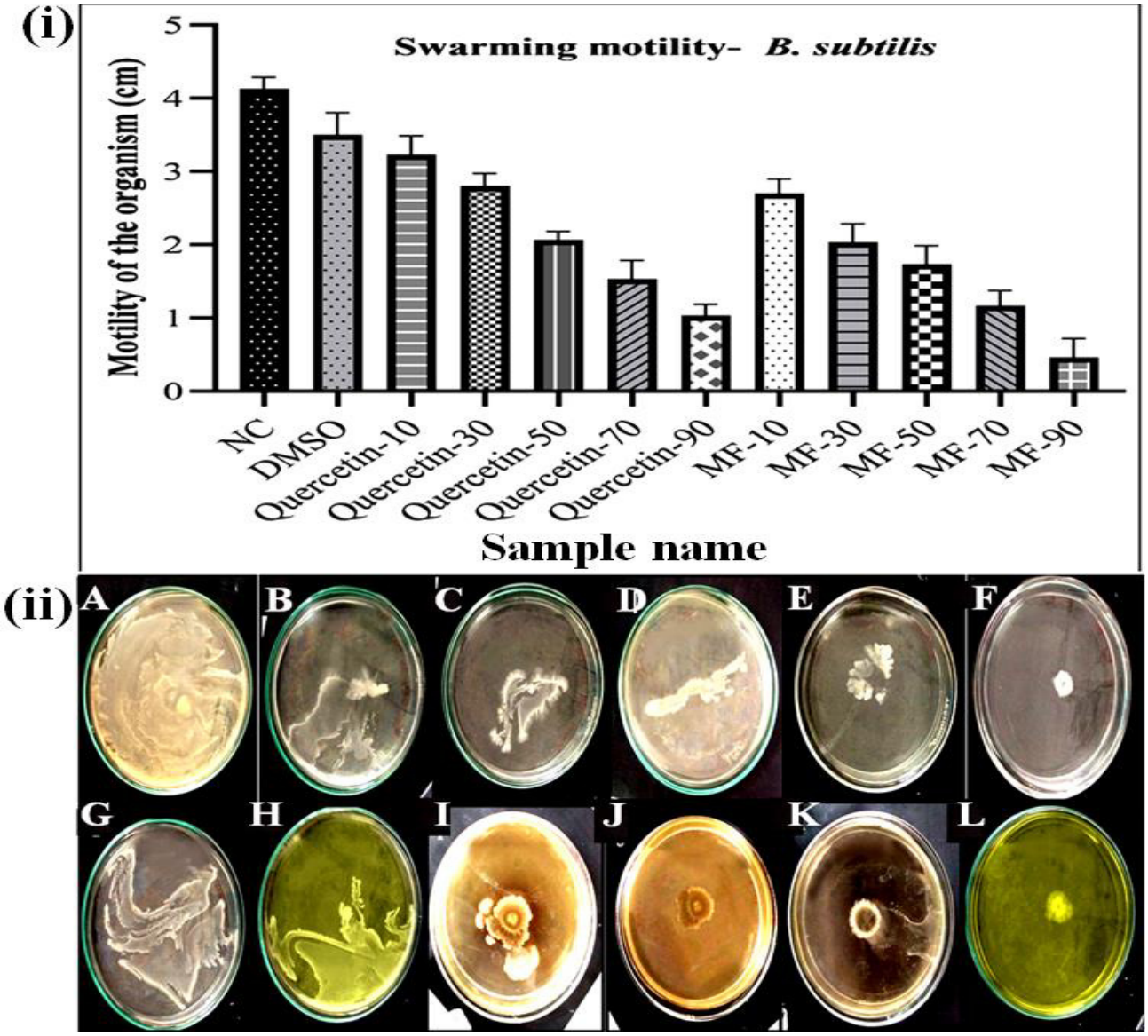

Fig 4: Effect of *M. fragrans* seed extract on swarming motility of *B. subtilis*: (i) Quantitative analysis of motilities of *B. subtilis* by *M. fragrans* seed (10-90 µg/ml) extract were measured by distance travelled by bacteria in cm. Values are the average of triplicate independent experiments and SDs are shown. Bars with** represent P<0.0001. (ii)A) Negative control, 'B' to 'F') Plates seeded with *M. fragrans* (10-90 µg/ml), G) Plate Seeded with DMSO, 'H' to 'L') Plates seeded with Quercetin (10-90 µg/ml).**

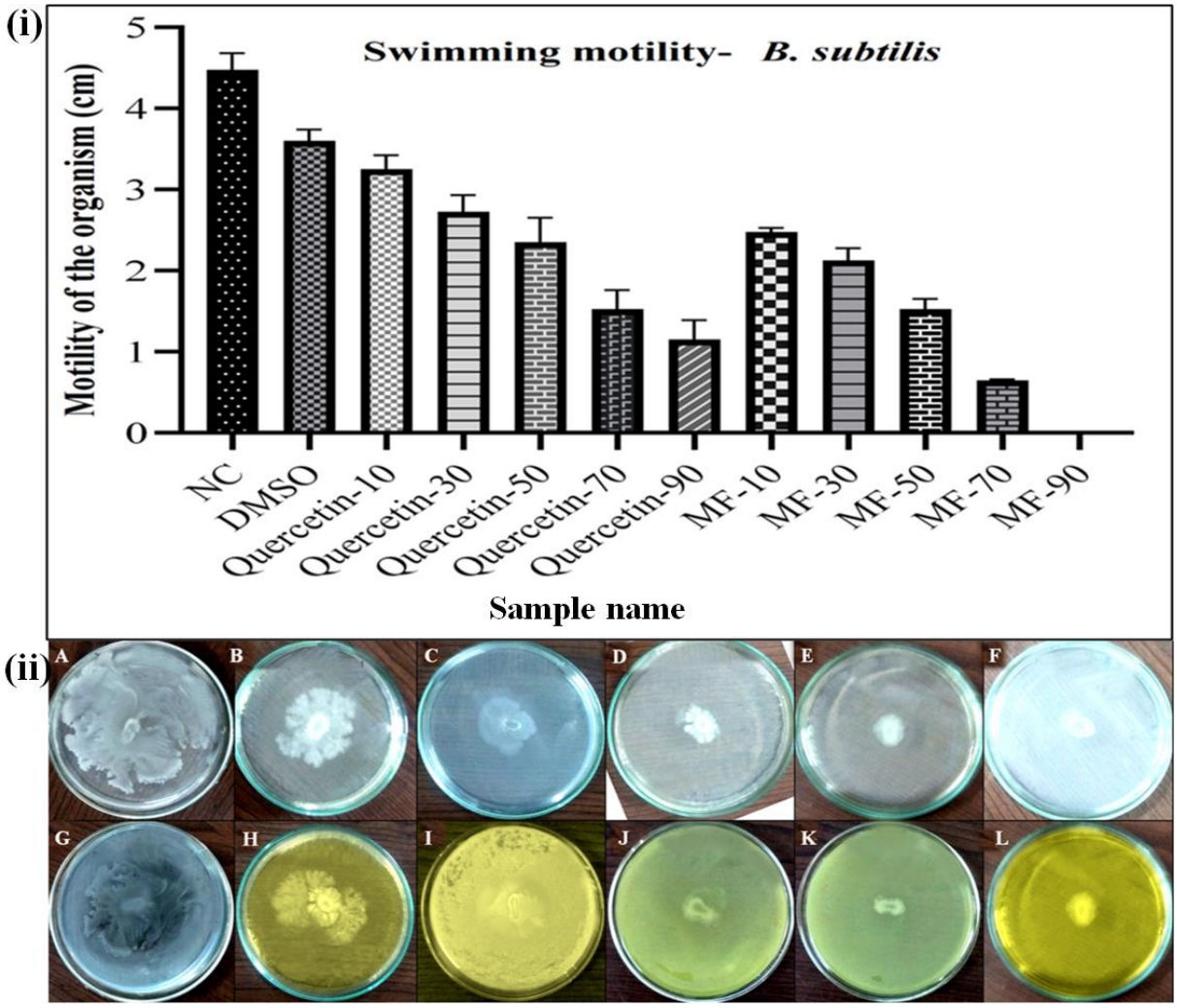

Fig 5: Effect of *M. fragrans* seed extract on swimming motility of *B. subtilis*: (i) Quantitative analysis of motilities of *B. subtilis* by *M. fragrans* seed (10-90 µg/ml) extract were measured by distance travelled by bacteria from the centre of the plate in cm. Values are the average of triplicate independent experiments and SDs are shown. Bars with ** represent P<0.0001. (ii)A) Negative control, 'B' to 'F') Plates seeded with *M. fragrans* (10-90 µg/ml), G) Plate seeded with DMSO, 'H' to 'L') Plates seeded with Quercetin (10-90 µg/ml).**

Bacteria increase pathogenicity because of their multicellular motility. This multicellular behaviour will depend on extracellular signals and intracellular physiological parameters—bacterial swimming and swarming motility influence many pathogen and host interactions to make healthier infections in hosts [29]. The motility of bacteria depends on the presence of sufficient potassium ions. Since potassium ions will stimulate the secretion of surfactin [30]. Lipopeptide Surfactin enhances the surface motility of *B. subtilis* in liquid and semiliquid mediums. *B. subtilis* uses this compound to colonize plant roots [26]. Ifm is the gene locus of flagella of *B. subtilis* that requires for the motility of this peritrichous organism. SwrA is the operon in *B. subtilis* that controls flagella assembly and regulates swarming motility [31]. Bacterial motility is a proficient adaptive strategy that allows bacteria to overcome nutrient limitations or stressful environments and move towards nutrient-rich environments.

3.2.2. Antibiofilm activity

Communities of *B. subtilis* bacteria form biofilms [32]. Biofilm formation is a process in which bacterial cells accumulate and placed in a matrix formed with an extracellular polysaccharide substance (EPS). For many decades, *B. subtilis* is a model organism to study Gram-positive bacteria. *B. subtilis* forms 3-dimensional biofilms invitro: pellicle biofilm, colony biofilm and surface-attached biofilm. Populations of *B. subtilis* form biofilms in intestinal tracts and skin of humans and roots of plants [33]. Antibiofilm studies were done with nutmeg extract against *B. subtilis* biofilm.

3.2.2.1. Quantification of biofilms

MTT and MBEC assays are done in microtiter plates to quantify the biofilm present in wells after treating the bacteria with test extract. MTT assay is a Micro titer plate assay that quantifies the biofilm after incubating the *B. subtilis* culture for 24 hrs with and without test extracts. MBEC is a minimum biofilm eradication assay to quantify the biofilm that is eradicated after 16-18 hrs of incubating the *B. subtilis* culture with and without test extracts. We have already done these studies earlier [20].

3.2.2.2. Fluorescence microscopic analysis

All coverslips incubated in *B. subtilis* bacterial culture with nutmeg and Quercetin samples were observed under a fluorescence microscope. A concentration-dependent decrease in the biofilm formation was observed on all coverslips. Less biofilm formation was observed on coverslips incubated with extract than Quercetin (Fig. 6) [20].

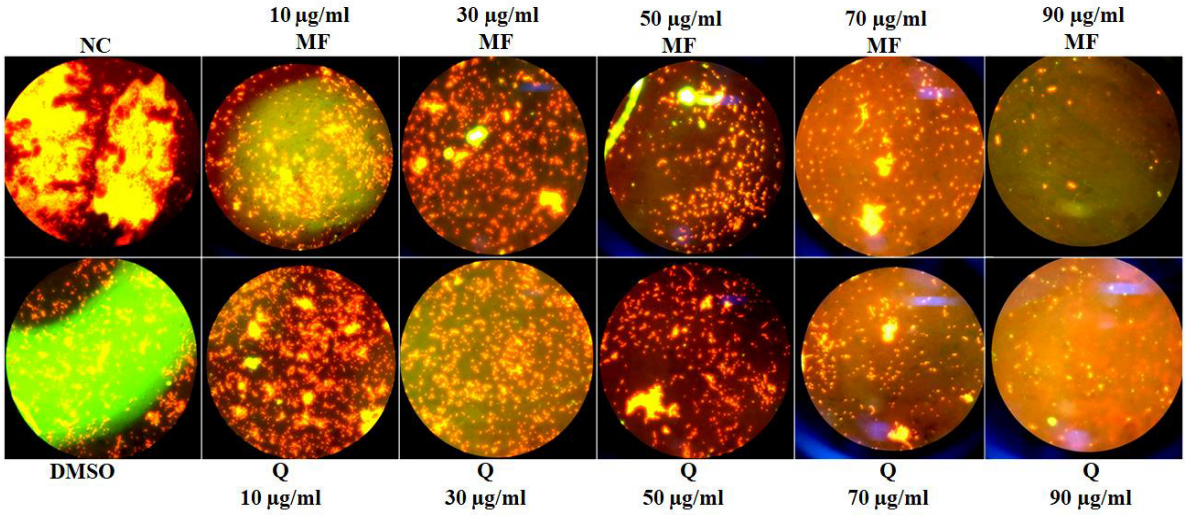

Fig 6: Fluorescence microscopic analysis of *B. subtilis* biofilm: NC: Negative control; MF: *M. fragrans*; Q: Quercetin.

Generally, medicinal plants, various species of marine algae, higher plants, dietary fruits, herbs, and spices were used to treat bacterial infections. When we tested the methanolic extract of nutmeg powder against quorum sensing, there was a decrease in swimming and swarming motilities in *B. subtilis* (Fig. 4(i)&(ii) and Fig.5(i)&(ii)). Quercetin is used as a standard against quorum sense because there are studies that Quercetin has anti-quorum sensing activity [17]. Quercetin is a pure flavonoid having antiquorum sensing and antibacterial activity. In this study, compared to Quercetin, our test extract has higher anti quorum sensing activity than the positive control.

4. CONCLUSION

Nutmeg oil has a long history of use in India and worldwide for a wide range of medicinal uses as well as cosmetic purposes. The present study is to identify the anti-quorum sensing activity of the methanolic extract of nutmeg. The significant advantage of using nutmeg extract it specifically intrudes into an expression of *B. subtilis* quorum sensing phenotypes (biofilm formation, swimming and swarming motilities) rather than being bactericidal. Inhibiting quorum sensing phenotypes may offer new hope in combating multidrug-resistant and high pathogenic bacteria with future applications in different fields, including agriculture, aquaculture, veterinary, medicine, and food industries. Future work helps identify and synthesize anti-pathogenic drugs against bacterial quorum sensing.

5. FUNDING ACKNOWLEDGMENTS

Dr. KVP Acknowledges the National Medicinal Plants Board (NMPB), Department of AYUSH, Ministry of Science and Technology, New Delhi for financial support (F. No.Z.18017/187/CSS/R&D/AP-01/2017-18) and DST-FIST (F. No.DST-FIST-LSI-595 (LSI-026).

6. AUTHORS CONTRIBUTION STATEMENT

Dr Kodali Vidya Prabhakar designed the study, including sample collection, anti-quorum sensing assays, and the estimation of flavonoid and phenolic concentrations. Mrs. Bhavana conducted the total experimental studies, analyzed results and prepared literature for the total manuscript. All authors read and approve the final version of the manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

8. REFERENCES

1. Hentzer M, Givskov M. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. J Clin Invest. 2003;112(9):1300-7. doi: 10.1172/JCI20074, PMID 14597754.
2. Asfour HZ. Anti-quorum sensing natural compounds. J Microsc Ultrastruct. 2018;6(1):1-10. doi: 10.4103/JMAU.JMAU_10_18, PMID 30023261.
3. Koh CL, Sam CK, Yin WF, Tan LY, Krishnan T, Chong YM et al. Plant-derived Natural Products as Sources of Anti-Quorum sensing compounds. Sensors (Basel). 2013;13(5):6217-28. doi: 10.3390/s130506217, PMID 23669710.
4. Flindt MLH. Pulmonary disease due to inhalation of derivatives of *Bacillus subtilis* containing proteolytic enzyme. Lancet. 1969;1(7607):1177-81. doi: 10.1016/s0140-6736(69)92165-5, PMID 4181838.
5. Reller LB. Endocarditis caused by *Bacillus subtilis*. Am J Clin Pathol. 1973;60(5):714-8. doi: 10.1093/ajcp/60.5.714, PMID 4201628.
6. Agbogidi OM, Azagbaekwe OP. Health and nutritional benefits of nutmeg (*Mysticafragrans*houtt.). Sci Agric. 2013;1(2):40-4.
7. Ibrahim MA, Cantrell CL, Jeliazkova EA, Astatkie T, Zheljazkov VD. Utilization of Nutmeg (Myristica fragrans Houtt.) Seed Hydrodistillation Time to Produce Essential Oil Fractions with Varied Compositions and Pharmacological Effects. Molecules. 2020;25(3):565. doi: 10.3390/molecules25030565, PMID 32012955.
8. Balakrishnan S, Sivaji I, Kandasamy S, Duraisamy S, Kumar NS, Gurusubramanian G. Biosynthesis of silver nanoparticles using Myristica fragrans seed (nutmeg) extract and its antibacterial activity against multidrug-

resistant (MDR) Salmonella enterica serovar Typhi isolates. Environ Sci Pollut Res Int. 2017;24(17):14758-69. doi: 10.1007/s11356-017-9065-7, PMID 28470497.
9. Nagano I. *Myristica fragrans*: an Exploration of the Narcotic Spice. Vernal Equinox. 2008;16(1):15-24.
10. Sonavane G, Sarveiya V, Kasture V, Kasture SB. Behavioural actions of *Myristica fragrans* Seeds. Indian J Pharmacol. 2001;33(6):417-24.
11. Kalamara M, Spacapan M, Mandic-Mulec IM, Stanley-Wall NR. Social behaviours by Bacillus subtilis: quorum sensing, kin discrimination and beyond. Mol Microbiol. 2018;110(6):863-78. doi: 10.1111/mmi.14127, PMID 30218468.
12. Ramluckan K, Moodley KG, Bux F. An evaluation of the efficacy of using selected solvents for the extraction of lipids from algal biomass by the Soxhlet extraction method. Fuel. 2014;116:103-8. doi: 10.1016/j.fuel.2013.07.118.
13. Wong Y, Ahmad-Mudzaqqir M, Wan-Nurdiyana W. Extraction of Essential Oil from Cinnamon (Cinnamomum zeylanicum). Orient J Chem. 2014;30(1):37-47. doi: 10.13005/ojc/300105.
14. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med. 2011;8(1):1-10. doi: 10.4314/ajtcam.v8i1.60483, PMID 22238476.
15. Chang C-C, Yang M-H, Wen H-M, Chern J-C. Estimation of total flavonoid content in propolis by two complementary colometric methods. J Food Drug Anal. 2002;10(3):178-82. doi: 10.38212/2224-6614.2748.
16. Lu X, Wang J, Al-Qadiri HM, Ross CF, Powers JR, Tang J et al. Determination of total phenolic content and antioxidant capacity of onion (*Allium cepa*) and shallot (*Allium oschaninii*) using infrared spectroscopy. Food Chem. 2011;129(2):637-44. doi: 10.1016/j.foodchem.2011.04.105, PMID 30634280.
17. Erdönmez D, Rad AY, Aksöz N. Anti-quorum sensing potential of antioxidant quercetin and resveratrol. Braz arch biol technol. 2018;61. doi: 10.1590/1678-4324-2017160756.
18. Issac Abraham SVPI, Palani A, Ramaswamy BR, Shunmugiah KP, Arumugam VR. Antiquorum sensing and antibiofilm potential of *Capparis spinosa*. Arch Med Res. 2011;42(8):658-68. doi: 10.1016/j.arcmed.2011.12.002, PMID 22222491.
19. Sankar Ganesh P, Ravishankar Rai V. Attenuation of quorum-sensing-dependent virulence factors and biofilm formation by medicinal plants against antibiotic resistant Pseudomonas aeruginosa. J Tradit Complement Med. 2018;8(1):170-7. doi: 10.1016/j.jtcme.2017.05.008. PMID 29322006.
20. Bhavana M, VidyaPrabhakar K. Antibiofilm activity of the methanolic extract of nutmeg oil against *Bacillus subtilis*. Asian Pac J Health Sci. 2022;9(4):6-12. ;doi: 10.21276.
21. Veening JW, Smits WK, Kuipers OP. Bistability, epigenetics, and bet-hedging in bacteria. Annu Rev Microbiol. 2008;62:193-210. doi: 10.1146/annurev.micro.62.081307.163002, PMID 18537474.
22. Dubnau D, Losick R. Bistability in bacteria. Mol Microbiol. 2006;61(3):564-72. doi: 10.1111/j.1365-2958.2006.05249.x, PMID 16879639.
23. Macnab RM, Aizawa SI. Bacterial motility and the bacterial flagellar motor. Annu Rev Biophys Bioeng. 1984;13:51-83. doi: 10.1146/annurev.bb.13.060184.000411, PMID 6378075.
24. Berg HC. Swarming motility: it better be wet. Curr Biol. 2005;15(15):R599-600. doi: 10.1016/j.cub.2005.07.042, PMID 16085482.
25. Pearson MM. Methods for studying Swarming and Swimming Motility. Methods Mol Biol. 2019:15-25. doi: 10.1007/978-1-4939-9601-8_3.
26. Ghelardi E, Salvetti S, Ceragioli M, Gueye SA, Celandroni F, Senesi S. Contribution of surfactin and SwrA to flagellin expression, swimming, and surface motility in *Bacillus subtilis*. Appl Environ Microbiol. 2012;78(18):6540-4. doi: 10.1128/AEM.01341-12, PMID 22773650.
27. Manson MD. Bacterial motility and chemotaxis. Adv Microb Physiol. 1992;33:277-346. doi: 10.1016/S0065-2911(08)60219-2.
28. Harshey RM. Bacterial motility on a surface: many ways to a common goal. Annu Rev Microbiol. 2003;57:249-73. doi: 10.1146/annurev.micro.57.030502.091014, PMID 14527279.
29. Fraser GM, Hughes C. Swarming motility. Curr Opin Microbiol. 1999;2(6):630-5. doi: 10.1016/s1369-5274(99)00033-8, PMID 10607626.
30. Kinsinger RF, Shirk MC, Fall R. Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. J Bacteriol. 2003;185(18):5627-31. doi: 10.1128/JB.185.18.5627-5631.2003, PMID 12949115.
31. Calvio C, Celandroni F, Ghelardi E, Amati G, Salvetti S, Ceciliani F et al. Swarming differentiation and swimming motility in *Bacillus subtilis* are controlled by swrA, a newly identified dicistronic operon. J Bacteriol. 2005;187(15):5356-66. doi: 10.1128/JB.187.15.5356-5366.2005, PMID 16030230.
32. Ryan-Payseur BK, Freitag NE. *Bacillus subtilis* biofilms: a matter of individual choice. mBio. 2018;9(6):e02339-18. doi: 10.1128/mBio.02339-18, PMID 30482826.
33. Arnaouteli S, Bamford NC, Stanley-Wall NR, Kovács ÁT. *Bacillus subtilis* biofilm formation and social interactions. Nat Rev Microbiol. 2021;19(9):600-14. doi: 10.1038/s41579-021-00540-9, PMID 33824496.