*Phuket mar. biol. Cent. Res. Bull.***78:** 69–76 (2021)
DOI: 10.14456/pmbcrb.2021.6

STUDY OF *Anadara ferruginea* (Reeve, 1844) AS A SOURCE OF ANTIBACTERIAL AGENTS AGAINST PATHOGENIC BACTERIA

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ABSTRACT: The development of multi-drug resistant bacteria has increased the demand for new compounds which possess antibacterial characteristics. Among marine invertebrates many of these have various compounds with these traits. This research aims to determine the antibacterial activity of fractions extracted from *Anadara ferruginea*. The extraction method was thin-layer chromatography (TLC). Open Column Chromatography (OCT) was used as a fractionation method and nine fractions were revealed. The antibacterial sensitivity test was carried out by the Kirby-Bauer disk diffusion method. The antibacterial ability was tested by using the bacteria species *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* and the development of the inhibition zone was followed during four days. All nine fractions possessed antibacterial agents against the four pathogenic test species. Fraction III was during the four days found to be the most active on *B. cereus*, *E. coli* and *S. aureus* with each average inhibition zone width of: 7.03 mm; 6.97 mm; 6.13 mm respectively. However, fraction IX showed the highest antibacterial activity on *P. aeruginosa* with an inhibition zone of 7.00 mm.

Keywords: *Anadara ferruginea*, extraction, antibacterial activity, pathogenic bacteria

INTRODUCTION

The resistance of pathogenic microorganisms to antibiotics is increasing. This observation raises significant problems in health care and encourages research on looking for new antibiotic compounds as an alternative option in treating diseases caused by multi-drug resistant pathogenic species. Various approaches have been implemented, and new antibiotics from natural ingredients both animals and plants (Pringgenies 2009) have been found. An overview on various compounds with antibacterial characteristics found in molluscs of marine and estuarine ecosystems is presented by Chakraborty and Joy (2020).

Bivalves are filter feeders which accumulate food, waste, and other contaminants such as bacteria. The type and the way filter feeders are ingesting food cause accumulation of pathogenic bacteria in the organism. Therefore, it is an advantage for the filter feeders to have the potential to produce bioactive compounds and many of them produce secondary metabolites in the form of bioactive compounds, including Paolin I and Paolin II (Soediro and Padmawinata 1993). Extraction from various bivalves has revealed antibacterial activity and among these extract from the bivalve *Anadara granosa* (Ramasamy and Balasubramanian 2014; Dewiningsih *et al*. 2017). The bivalve *Anadara ferruginea* is one of the common species found in Indonesian waters. The species may have the potential as a source of new antibacterial compounds to combat pathogenic microorganisms that have become resistant due to overuse of existing antibiotics.

By these facts, this study aims to determine the antibacterial activity of extract from *A. ferruginea* on *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

MATERIALS AND METHODS

Sample extraction: This study uses a solid-liquid extraction method with rotary evaporator as an extraction tool (Kristanti and Aminah 2008). The extraction process was performed by immersing 200 g of dry bivalve meat in 1000 mL of n-hexane solvent, and the resulting substance was immersed in ethyl acetate, and finally in methanol (Rifai and Trianto 2003).

Positive control and negative control tests: As a positive control ceftizoxime sodium in a concentration of 50 µg /disk was tested on all four pathogenic bacteria. Negative control tests were carried out using n-hexane, ethyl acetate, and methanol solvents on the test bacteria.

Qualitative test on the antibacterial activity of *A. ferruginea* extract: The qualitative test for antibacterial activity of *A. ferruginea* extract on the test bacteria was carried out directly using 0.1 gram of crude extract. The appearance of the inhibition zone was read after 24 hours incubation.

Open-Column Chromatography (OCC): Silica gel was used in the open column chromatography process as absorption material. A mixture of silica gel and extract was inserted in the column according to Pringgenies *et al*. (2015) and Pringgenies *et al*. (2017).

Thin-Layer Chromatography (TLC): Thin-layer chromatography was carried out on the extract to obtain the pattern of the extract according to Pringgenies *et al*. (2017). The detection method was UV light. The samples obtained from the OCC chromatography was analysed by TLC, and the samples with the same spots, *i.e*. having the same retardation factor (R_f value), were pooled as one fraction and evaporated.

Antibacterial test: The antibacterial test was carried out by the disk diffusion method according to the Kirby-Bauer principles. The principle of the method is described by Volk and Wheeler (1990). Antibacterial test with diffusion agar was used according to the method described by Pringgenies and Renta (2014). Three replicates of each test were performed and the size of the inhibition zone was measured daily for 4 days.

RESULTS

Positive control and negative control tests

Positive control tests were carried out to determine the diameter of the inhibition zone formed by commercial antibiotics. Testing ceftizoxime sodium on test bacteria revealed that this commercial antibiotic was able to form a zone of inhibition on all test bacteria. The biggest inhibition zone, 7.65 mm/96 hours, was found on *P. aeruginosa*, while the smallest zone, 5.51 mm/96 hours, was found on *S. aureus*. Negative control tests were carried out on the three solvents used for extraction and chromatography, and none of these revealed any inhibition zone.

Qualitative antibacterial activity test of *A. ferruginea* extract on test bacteria

The qualitative test of *A. ferruginea* extract on pathogenic species showed that the crude extract had antibacterial activity on *B. cereus*, *E. coli*, *P. aeruginosa*, and *S. aureus*. Based on the solvent test, crude extracts of *A. ferruginea* with n-hexane solvent did not reveal antibacterial activity on *E. coli* and *P. aeruginosa*, but was positive for *B. cereus* and *S. aureus*. The extracts with ethyl acetate and methanol solvents showed antibacterial activity on *B. cereus*, *E. coli*, *P. aeruginosa*, and *S. aureus*.

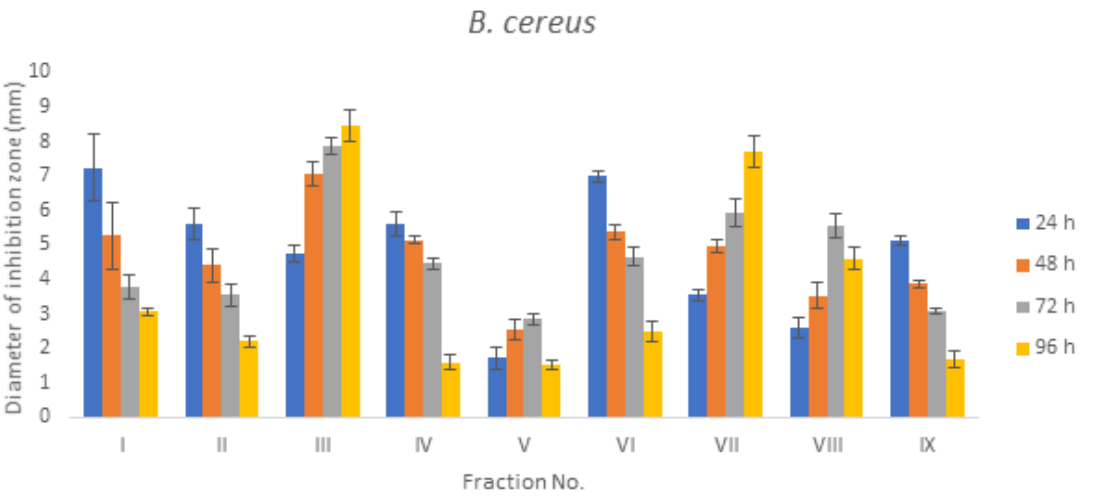
Determination of the eluent used for Thin-Layer Chromatography (TLC)

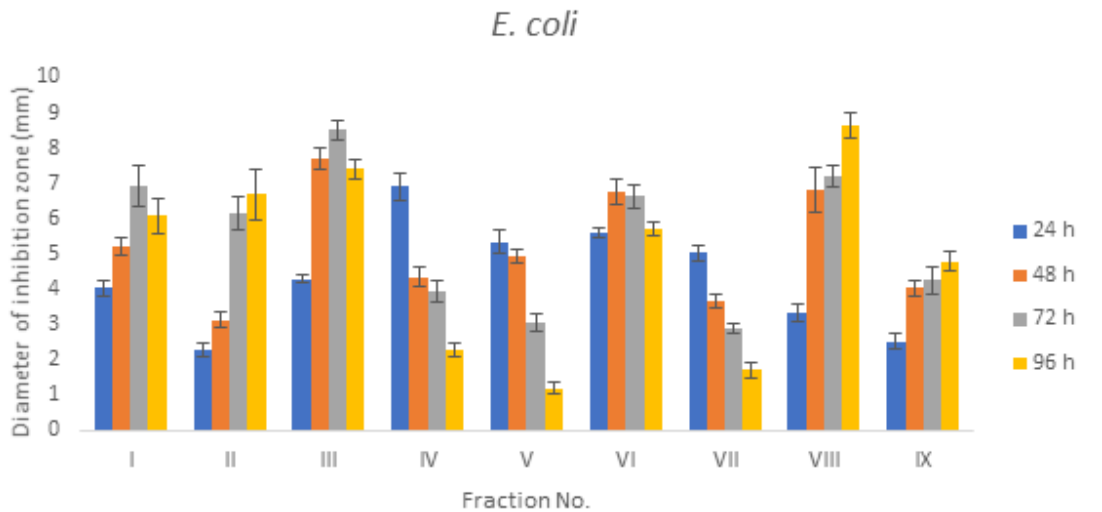
The optimal solvent for separation of the compounds by TLC was ethyl acetate and methanol in the ratio 10 : 1.

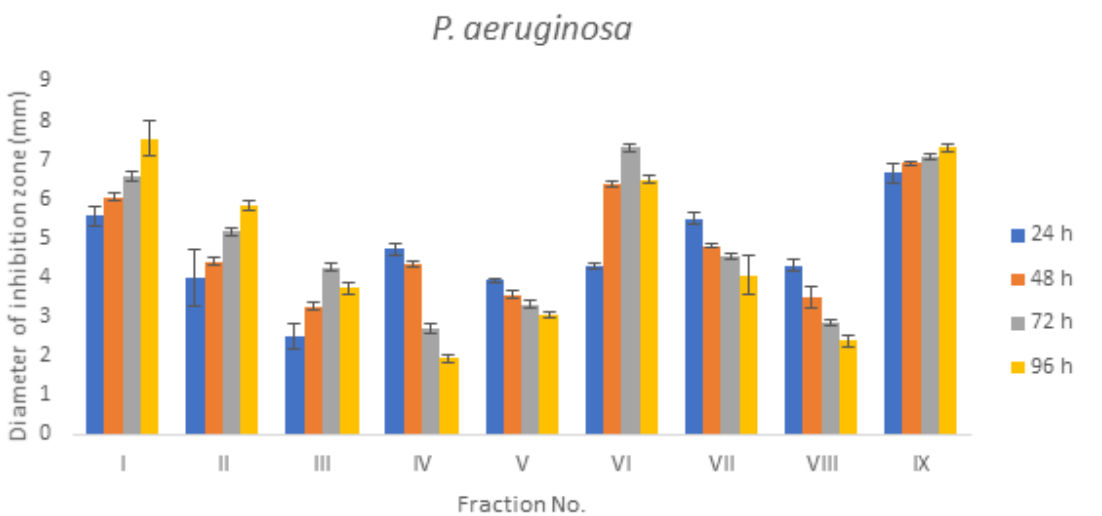
Fractionation by Open-Column Chromatography (OCC)

Two grams of *A. ferruginea* extract was used for OCC and the separation resulted in 58 vials with a volume of 20 mL each. The 58 samples were analysed by TLC and R_f value were calculated. The data is not shown. The results of the chromatography analysis were grouped according to their R_f values, which produced 9 fractions. All the fractions were then processed using an evaporator.

The results of fractionation with OCC showed that fraction IX was the heaviest fraction, weighing 0.5238 g, while the lightest fraction was fraction VI with a weight of 0.0847 g.


B. cereus
Diameter of inhibition zone (mm)
0
1
2
3
4
5
6
7
8
9
10
I
II
III
IV
V
VI
VII
VIII
IX
Fraction No.
24 h
48 h
72 h
96 h


E. coli
Diameter of inhibition zone (mm)
0
1
2
3
4
5
6
7
8
9
10
I
II
III
IV
V
VI
VII
VIII
IX
Fraction No.
24 h
48 h
72 h
96 h


P. aeruginosa
Diameter of inhibition zone (mm)
0
1
2
3
4
5
6
7
8
9
I
II
III
IV
V
VI
VII
VIII
IX
Fraction No.
24 h
48 h
72 h
96 h

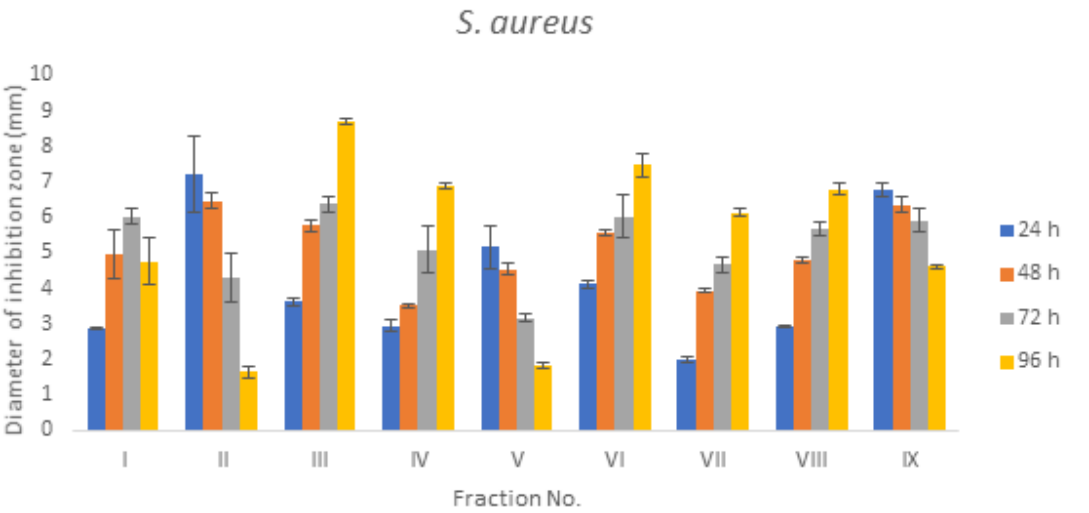


Figure 1. Inhibition zones of fractions from *A. ferruginea* on *B. cereus*, *E. coli*, *P. aeruginosa*, and *S. aureus* during four days. The bars are standard errors.

Antibacterial Sensitivity Test for The Fractions Obtained by Open Column Chromatography (OCC)

The results of the antibacterial tests on the four pathogenic bacteria *B. cereus*, *E. coli*, *P. aeruginosa*, and *S. aureus* are shown in Fig. 1 and Appendixes 1–4.

All fractions have antibacterial activity on the pathogenic bacteria. For *B. cereus* the fractions are separated into three groups, increasing activity: III and VII, decreasing activity: I, II, IV and IX, and increasing and decreasing activity: V and VIII. For *E. coli* the results are for increasing activity: II, VIII and IX, decreasing activity: IV, V, and VII, and increasing and decreasing activity: I, III and VI. The three groups for *P. aeruginosa* are for the first mentioned group I, II and IX, the second group IV, V, VII and VIII and the third group III and VI. Finally, for *S. aureus* the first group consists of III, IV, VI, VII and VIII, the second group II, V, and IX and the third group has only one member, *i.e.* I. None of the fractions reacted on the pathogenic bacteria in the same way.

DISCUSSION

The findings in this research confirmed that *A. ferruginea* extract has the potential as an antibacterial agent on all the tested pathogenic species. The crude extract swabbed on the paper disk was able to form a zone of inhibition around the paper disk. *A. ferruginea* extract with ethyl acetate and methanol solvent was determined to be active on the four test bacteria, while the extract with n-hexane solvent was only active on *E. coli*, and *P. aeruginosa*. This finding showed that the non-polar compounds in *A. ferruginea* extract did not form antibacterial activity on *E. coli* and *P. aeruginosa*, but showed antibacterial activity on *B. cereus* and *S. aureus*. The presence of antibacterial and antiviral activity in *A. ferruginea* was stated by Soediro and Padmawinata (1993) who explained that various bivalves, including *A. ferruginea*, produce the compounds of Paolin I and Paolin II which can be used as antibacterial and antiviral agents.

All fractions from the OCC were able to cause inhibition of the pathogenic bacteria during the incubation time 4 x 24 hours. According to Wattimena *et al*. (1991), antibacterial agents are bacteriostatic when the inhibition zone narrows and exhibits high turbidity after 24 hours incubation, and vice-versa if the inhibition zone remains clear up to 48 hours incubation time, then the antibacterial agent is bactericidal.

Applying this definition to the measured inhibition zones found for *B. cereus* fractions No. I, II, IV, VI and IX were bacteriostatic, fractions No. III and VII were bactericidal as well as fractions

No. V and VIII by the fact that the growth of the inhibition zone remained clear for more than 48 hours. Furthermore, the zone produced by fraction No. III was wider than the one found for ceftizoxime sodium.

For *E. coli* the fractions No. IV, V and VI were considered to be bacteriostatic, whereas fractions No. II, VIII and IX were bactericidal, and the same was the case for fractions No. I, II and VI, as the zone was clear for more than 48 hours of incubation. Fractions No. III and VIII produced wider inhibition zone than the commercial antibiotics used for the study.

When testing the fractions on *P. aeruginosa* fractions No. IV, V, VII and VIII reacted as bacteriostatic agents, while fractions No. I, II, and IX behaved as bactericidal agents. Fractions No. III and VI were considered as bactericidal as the inhibition was keeping transparent for more than 72 hours.

Finally, fractions No. II, V and IX behaved as bacteriostatic compounds on *S. aureus*, whereas fractions No. III, IV, VI, VII and VIII were identified as bactericidal and the same was found for fraction No. I as the zone remained transparent for at least 72 hours.

The antibacterial sensitivity test of the nine fractions on test bacteria indicated that fraction No. III was most active on *B. cereus*, *E. coli*, and *S. aureus*, even more efficient than ceftizoxime sodium. Fraction No. IX was most active on *P. aeruginosa*. This meant that fraction No. III had antibacterial compounds most effective in combating infestations by *B. cereus*, *E. coli*, and *S. aureus*. Fraction IX was shown to have the most effective antibacterial compounds to inhibit the growth of *P. aeruginosa.*

The difference in the ability of antibacterial activity found in each fraction indicates the variation of the compounds in all of these fractions. Brock and Madigan (1991) stated that the area of inhibitory zones formed around paper disks is influenced by the chemical properties of antibacterial compounds produced by a microorganism. The diffusion rate of antibacterial compounds molecules in agar media is influenced by the molecule and its action on that medium. Substances with heavier molecular masses have a greater diffusion rate than those with lighter molecular masses. In addition, the diffusion rate of some antibacterial agents may be inhibited by the used agar media.

Different species of bacteria defend themselves in different ways. Bacteria evolve self-defense mechanisms to deal with threats to their survival. Such a threat is a change in environmental conditions due to the presence of foreign substances or compounds, which can interfere with cellular activity. To overcome this threat, the bacteria will attempt to neutralize the foreign compound. Some species of bacteria are able to survive by being able to neutralize invasive compounds, but there are also bacteria that are unable to survive and eventually die because they are not well-equipped to neutralize these foreign compounds (Miajlovic and Smith 2014). Some other factors that influence the inhibition of microorganisms are the concentration of antimicrobial substances, temperature, duration of application of antimicrobial substances in microorganisms, sensitivity of microorganisms to antimicrobial substances, and population density of microorganisms.

CONCLUSIONS

Based on the research and investigation of antibacterial activity in *A. ferruginea* extract, it was concluded that the fractionation produced nine fractions which possessed antibacterial activity on the four test bacteria. Fraction No. III was found to be more effective on *B. cereus*, *E. coli*, and *S. aureus*. Fraction No. IX was obviously most active on *P. aeruginosa*.

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Manuscript received: 6 October 2020
Accepted: 8 January 2021

Appendix 1. Bacteria *B. cereus* against Fraction I − IX.

Fraction	Inhibition zone (mm)			
	24 hours	48 hours	72 hours	96 hours
I	7.24 ± 0.98	5.27 ± 0.97	3.77 ± 0.36	3.07 ± 0.10
II	5.61 ± 0.46	4.41 ± 0.49	3.54 ± 0.34	2.20 ± 0.17
III	4.76 ± 0.24	7.05 ± 0.36	7.88 ± 0.23	8.44 ± 0.46
IV	5.62 ± 0.36	5.14 ± 0.10	4.46 ± 0.17	1.58 ± 0.22
V	1.71 ± 0.31	2.56 ± 0.30	2.84 ± 0.15	1.50 ± 0.14
VI	7.00 ± 0.17	5.37 ± 0.21	4.66 ± 0.25	2.49 ± 0.28
VII	3.54 ± 0.14	4.96 ± 0.20	5.93 ± 0.39	7.72 ± 0.46
VIII	2.59 ± 0.30	3.53 ± 0.37	5.57 ± 0.35	4.60 ± 0.31
IX	5.12 ± 0.14	3.86 ± 0.09	3.08 ± 0.08	1.70 ± 0.24

Appendix 2. Bacteria *E. coli* against Fraction I − IX.

Fraction	Inhibition zone (mm)			
	24 hours	48 hours	72 hours	96 hours
I	4.03 ± 0.23	5.21 ± 0.24	6.93 ± 0.58	6,09 ± 0.49
II	2.29 ± 0.17	3.12 ± 0.22	6.14 ± 0.48	6,69 ± 0.72
III	4.28 ± 0.11	7.70 ± 0.31	8.51 ± 0.26	7.40 ± 0.30
IV	6.92 ± 0.39	4.35 ± 0.28	3.94 ± 0.32	2.27 ± 0.19
V	5.35 ± 0.31	4.94 ± 0.22	3.05 ± 0.26	1.21 ± 0.17
VI	5.59 ± 0.15	6.77 ± 0.37	6.64 ± 0.34	5.20 ± 0.21
VII	5.04 ± 0.22	3.67 ± 0.20	2.89 ± 0.16	1.71 ± 0.21
VIII	3.35 ± 0.26	6.81 ± 0.62	7.19 ± 0.30	8.63 ± 0.35
IX	2.53 ± 0.23	4.03 ± 0.24	4.25 ± 0.39	4.78 ± 0.28

Appendix 3. Bacteria *P. aeruginosa* against Fraction I − IX.

Fraction	Inhibition zone (mm)			
	24 hours	48 hours	72 hours	96 hours
I	5.57 ± 0.23	6.06 ± 0.08	6.59 ± 0.12	7.54 ± 0.45
II	4.01 ± 0.73	4.42 ± 0.09	5.17 ± 0.08	5.84 ± 0.12
III	2.49 ± 0.32	3.27 ± 0.10	4.25 ± 0.10	3.73 ± 0.13
IV	4.73 ± 0.15	4.36 ± 0.07	2.71 ± 0.13	1.95 ± 0.10
V	3.93 ± 0.06	3.57 ± 0.09	3.32 ± 0.11	3.06 ± 0.07
VI	4.28 ± 0.07	6.39 ± 0.08	7.32 ± 0.11	6.51 ± 0.12
VII	5.50 ± 0.15	4.81 ± 0.04	4.54 ± 0.07	4.07 ± 0.51
VIII	4.32 ± 0.13	3.51 ± 0.26	2.84 ± 0.07	2.39 ± 0.16
IX	6.68 ± 0.25	6.92 ± 0.06	7.07 ± 0.07	7.32 ± 0.10

Appendix 4. Bacteria *S. aureus* against Fraction I – IX.

Fraction	Inhibition zone (mm)			
	24 hours	48 hours	72 hours	96 hours
I	2.86 ± 0.04	4.96 ± 0.70	6.03 ± 0.20	4.77 ± 0.66
II	7.21 ± 1.06	6.47 ± 0.20	4.31 ± 0.68	1.64 ± 0.15
III	3.62 ± 0.11	5.78 ± 0.16	6.38 ± 0.22	8.72 ± 0.09
IV	2.94 ± 0.16	3.51 ± 0.05	5.09 ± 0.65	6.90 ± 0.07
V	5.16 ± 0.62	4.53 ± 0.17	3.17 ± 0.11	1.82 ± 0.10
VI	4.11 ± 0.11	5.57 ± 0.07	6.02 ± 0.60	7.49 ± 0.33
VII	1.98 ± 0.07	3.94 ± 0.05	4.67 ± 0.23	6.14 ± 0.09
VIII	2.93 ± 0.04	4.78 ± 0.09	5.66 ± 0.19	6.80 ± 0.18
IX	6.77 ± 0.19	6.36 ± 0.22	5.91 ± 0.32	4.61 ± 0.07