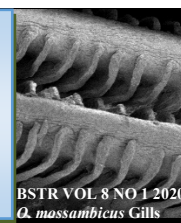




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Inhibition of *Streptococcus pneumoniae* Hyaluronidase by Honeys of Malaysian origins

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ABSTRACT

Streptococcus pneumoniae, a major human pathogen causing meningitis, pneumonia and bacteraemia produces hyaluronidase as its virulence factor. This enzyme increases the bacterial permeability to the host tissue by degrading hyaluronic acids that maintain the viscosity of connective tissues. Limited hyaluronidase inhibitors are available at the moment, thus leading us to identify potential hyaluronidase inhibitors from local products such as honeys. Three local honeys (Tualang, Kelulut and Gelam) have been selected and undergo hyaluronidase inhibition test through hyaluronic acids turbidity reduction assay. Honey that shows the highest and lowest anti-hyaluronidase was analysed for flavonoid and phenolic content, to identify association between the contents and hyaluronidase inhibition rate. All honeys showed various degrees of inhibition against hyaluronidase of *S. pneumoniae* where honey with the highest anti-hyaluronidase activity is Kelulut with 18% inhibition, while honey with the lowest anti-hyaluronidase activity is Gelam with 2% inhibition. Kelulut also demonstrated highest phenolic content, where significant association was found between the inhibition and the content. Therefore, this study supported the claim that higher phenolic contents indicate greater inhibitory activity on hyaluronidase enzyme. The findings suggest that local Malaysian honey have potential as hyaluronidase inhibitor which could be beneficial for future treatment against *S. pneumoniae* infections.

INTRODUCTION

Streptococcus pneumoniae is categorised as a major human pathogen which commonly colonizes human upper respiratory tract. This pathogen can cause life threatening diseases which include meningitis, pneumonia and bacteraemia as well as less dangerous but more common diseases such as sinusitis and otitis media [1-2]. There have been several challenges in combating diseases caused by *S. pneumoniae* such as the antimicrobial resistant by the microbe as well as the existence of non-vaccine serotype strains which call for further investigation for treatment strategies [3]. There are various substances produced by *S. pneumoniae* which have specific function for the purpose of colonisation and invasion to the host tissues which can be spread to organs and eventually affect the immune system of the host [4]. It is mentioned that one of the virulence factors is hyaluronate lyase or hyaluronidase enzyme [5].

Hyaluronidase is an important enzyme produced by both prokaryotes and eukaryotes which degrade hyaluronic acid, or also known as hyaluronan. Hyaluronic acid can be found in extracellular matrix of soft connective tissues and important in many biological processes such as embryogenesis, migration of cell, the healing of wound, malignant transformation as well as tissue turnover [6]. Degradation of hyaluronic acid by hyaluronidase can increase the connective tissues permeability and also decrease the body fluids viscosity. This condition may contribute to invasion of bacteria which permit greater access for microbe to migrate between the host tissues for colonization or invasion. Due to this mechanism of action, hyaluronidase is also called as the "spreading factor" which can enhance several pathological conditions or progression of disease through spreading of venom and toxins as well as other bacterial pathogens into host deeper tissues [7]. The significant impact of hyaluronidase towards human pathophysiological function

triggered attention to seek for anti-hyaluronidase compound either in the synthetic or natural type [8-10].

Anti-hyaluronidase properties were claimed to be played by bioactive molecules in the *Areca catechu* (palm species) and *Glycyrrhiza uralensis* (liquorice plants) extracts including phenolic, flavonoids and tannins [9, 11]. There also report showing the potential anti-hyaluronidase properties in honeys. Kolayli *et al.* 2016 [10] found high anti-hyaluronidase activity observed in honey from heather, oak and chestnut which also highly content of phenolic and flavonoid, thus suggesting the inhibition of hyaluronidase could be contributed by these phytochemical properties.

Medicinal values of honey as antimicrobial agents have been evaluated and evidenced in numerous studies, either as a pure extract or in combination with antibiotics [12,13]. The geographical factor, in the sense of floral origin highly affect the honey content, which later give different antibacterial effectiveness on specific species of pathogen [14, 15]. Malaysian local honey is differed from other honeys due to different source of nectar that highly affected by the climate and weather in the region. At the moment, no study has been conducted to search for anti-hyaluronidase activity in local Malaysia honeys. Thus, this study aims to identify and compare the anti-hyaluronidase activity of three types of local honeys; Tualang, Kelulut and Gelam and to find the association of this inhibition activity with its flavonoid and phenolic contents.

MATERIALS AND METHOD

Extraction of honey samples

Honey samples such as Tualang, Kelulut and Gelam were purchased from beekeepers or honey collector from Pahang, Kelantan and Kedah, respectively. Commercialized honey was bought from local supermarket in Kuantan, Pahang. All the honeys were purchased within one month before the laboratory test was started. The extraction of honeys was carried out according to the method described by Ibrahim *et al.* 2010 [16] with slight modifications. Sixty gram of each honey samples were extracted with 100 ml of 80% methanol at pH 2. Both solutions were mixed well with stirrer in an aluminum foil-sealed beaker. Then, the honey mixture was shaken in the water bath (Memmert, Germany) for 1 hour at 55°C with 150 strokes per minute. The extracts were filtered to remove any particles and the final volume was adjusted with distilled water [17] for final concentration needed (0.1 g/ml, 0.18 g/ml and 0.3 g/ml).

Preparation of pneumococcal hyaluronidase culture

Streptococcus pneumoniae strain was selected from previous study where the strain showed high hyaluronidase activity [18]. The presence of hyaluronidase of this selected strain was re-confirmed through hyaluronic acid turbidity reduction assay prior to test with honey samples. Initial inoculum was prepared by inoculate glycerol stock *Streptococcus pneumoniae* into Brain Heart Infusion (BHI) (Oxoid Ltd, Hampshire) broth till reach optical density (OD) OD₆₀₀ 0.010 (in range 0.008 – 0.012) in bijou bottle. The culture was incubated within 5% CO₂ at 37 °C for 24 hours. Then, the grown bacterial culture was centrifuged for 30 minutes (10000 rpm at 4 °C). The crude supernatant which contain hyaluronidase (crude supernatant is referred as pneumococcal hyaluronidase afterwards) were kept in the cold chain for enzymatic activity test, while the pellet was preserved as glycerol stock.

Determination of anti-hyaluronidase activity through hyaluronic acid turbidity assay

Determination of hyaluronidase inhibition was done through hyaluronic acids turbidity reduction assays. In this process, the reaction mixture which consist of 100 ml of pneumococcal hyaluronidase, 100 ml of phosphate buffer (200 mM, pH 7, 37C) with 77 mM sodium chloride and 0.01% Bovine Serum Albumin (BSA) (Fisher Scientific, USA) were mixed with 25 ml of honey sample extracts. The mixture was incubated at 37°C for 10 minutes prior to hyaluronic acid turbidity reduction assay.

The turbidity reduction assays were started with the addition of 100 ml of hyaluronic acid (substrate) (Acros Organics, Belgium) (0.03% in 300 mM sodium phosphate, pH 5.35) into the honey-pneumococcal hyaluronidase mixture. The assay mixture then was incubated at 37°C for 45 minutes, before the mixture was precipitated with 1 ml of albumin acid solution made up of 0.1% BSA (in 24 mM sodium acetate and 79 mM acetic acid with pH of 3.75). The mixture was left at room temperature for 10 minutes before the absorbance was measured at 600 nm by using spectrophotometer (Secomm, France) [17]. The negative control sample was prepared by adding all solution except the honey, while positive control was prepared by adding ascorbic acids instead of honeys into the mixture [19]. All tests were prepared in triplicate. The inhibition percentage of the sample was calculated based on the following equation (*Asb* is referred to absorption, where mean of control *Asb* is referring to negative control readings):

$$\text{Inhibition percentage} = \frac{\text{mean of sample } Abs^*}{\text{mean of control } Abs} \times 100$$

*Absorbance at 600nm wavelength

Total phenolic content assay

Total phenolic content of honey extract was measured with spectrophotometric method by using Folin-Ciocalteu reagent (Merck KGaA, Germany) following method described by Alam *et al.* 2017 [20]. It is a process where phosphomolybdic-phosphotungstic acid (folin) reagent was reduced to blue-colored complex in an alkaline solution due to the presence of phenolic compound [21]. Folin-Ciocalteu reagent was prepared by dissolved 1 ml reagent in 9 ml of distilled water. Then, 100 µl of the Folin-Ciocalteu dissolved reagent was mixed with 20 µl of selected honey extracts (which showed highest and lowest anti-hyaluronidase percentage) and 80 µl of 7.5% sodium carbonate (Na₂CO₃) (Merck KGaA, Germany). The mixture was incubated for 30 minutes in a dark condition at 26.8°C. The absorbance was taken at 765 nm after the incubation. The total phenolic contents were determined using gallic acid (GA) calibration curve. The graph curve was prepared by plotting the absorbance readings against GA concentration (10 – 100mg/L); $y = 0.0005x + 0.0779$, $R^2 = 0.9943$ where y is the absorbance reading and x is the sample concentration. The total phenolic contents of the extracts were calculated using equation below:

$$TPC = (CV/M)$$

C is the concentration of the samples from the GA calibration curve (mg/ml), V is the volume of the extraction solvent (ml) and M represents the weight (g) of the honey. Ethanol was used as blank and the analyses were done in triplicate.

Total flavonoid content assay

The total flavonoid content in the selected honeys (with highest and lowest anti-hyaluronidase percentage) were determined through method described by Alara *et al.* 2017 [22]. Briefly, 2% aluminium chloride (AlCl_3) (R&M, UK) was dissolved in ethanol before 50 μl of AlCl_3 solution was mixed together with the honey samples. The mixture was incubated for 1 hour at room temperature. The incubation process which will produce flavonoid-aluminium complex was measured at 420 nm. The flavonoid content was determine using Quercetin (QE) calibration curve which was plotted with concentration of 10-100 mg/L; $y = 0.112x + 0.178$, $R^2 = 0.9945$ where y is the absorbance reading and x is the sample concentration. The total flavonoid contents of the extracts were calculated using equation below:

$$\text{TFC} = (\text{CV}/M)$$

C is the concentration of the samples from the QE calibration curve (mg/ml), V is the volume of the extraction solvent (ml) and M represents the weight (g) of the honey. Ethanol was used as blank and the analyses were done in triplicate.

Statistical analysis

All tests were done in triplicate and the data were expressed as mean \pm standard deviation. Mann-Whitney test was used to compare honey with the highest and lowest anti-hyaluronidase activity, as well as to compare total phenolic compound and total flavonoid content between the two selected honeys. Spearman's correlation analysis was used to find association between the phenolic and flavonoid contents with anti-hyaluronidase activity in both selected honeys. All tests were analyzed by using SPSS software. $p \leq 0.05$ was considered as significant value.

RESULTS AND DISCUSSION

Anti-hyaluronidase activity

This study determined the anti-hyaluronidase activity of three types of Malaysian honeys through hyaluronic acid turbidity reduction assay by using hyaluronidase isolated from *Streptococcus pneumoniae*.

From Fig. 1, it can be seen that all of the honeys tested had the ability to inhibit hyaluronidase enzyme, with different inhibition percentage for different type and concentration of honeys. In this study, the highest inhibition or anti-hyaluronidase activity among the honeys was exhibited by Kelulut honey at concentration of 0.3 g/ml with 18% inhibition value while the lowest inhibition was Gelam with 2% inhibition, also at 0.3 g/ml, with significant difference at $p = 0.050$ (Table 1).

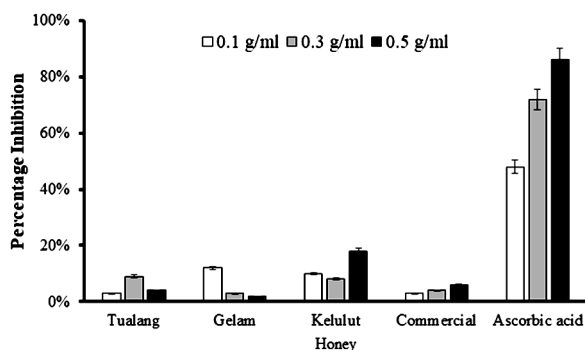


Fig. 1. Anti-hyaluronidase activity of the honey samples.

Table 1. Comparison of anti-hyaluronidase activity between Kelulut and Gelam.

	Kelulut	Gelam	<i>p</i> value
Absorbance reading for samples at OD ₆₀₀ (Mean \pm standard deviation)	0.089 \pm 0.038	0.014 \pm 0.011	*0.050
Mann-Whitney Test * <i>p</i> value \leq 0.05			

Previous study suggests that the inhibition of hyaluronidase from honey are likely to correspond the floral sources which indirectly reflect the phytochemical contents in the honey [10]. To the best of our knowledge, this is the first study reports on anti-hyaluronidase activities in honeys of Malaysian origin. Malaysian honeys such as Tualang, Kelulut and Gelam have been extensively tested for various potential contribution in inhibiting various diseases including as antibacterial properties. Its antibacterial activities were proven for a number of pathogenic bacteria including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes* [23-24]. Similarly, Mohd Aspar *et al.* 2109 [25] had reported antibacterial activities of local honeys including Kelulut, Tualang and Acacia honey on would-infecting Gram positive bacteria; *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis* and Gram negative bacteria; *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella typhimurim*, where the highest activity was demonstrated by Kelulut honey. In a study done by Mshelia *et al.* 2018 [26], *Streptococcus pneumoniae* and *Streptococcus pyogenes* highly sensitive to honey, where higher sensitivity was observed in honey mixture with lemon juice. One of the possible reasons for that findings is the acidic pH in the honey and lemon mixture. Although a number of antimicrobial studies have been done, very less are looking on the inhibition mechanism on the bacterial virulence factors. Thus, this study is looking on potential of honey in inhibiting one of pneumococcal virulence factor; hyaluronidase.

In this study, Kelulut was found to be the most potential local honey exhibit highest inhibition activity towards hyaluronidase, after the positive control ascorbic acids. Li *et al.* 2001 [8] firstly reported the inhibition of pneumococcal hyaluronidase by L-ascorbic acids or vitamin C. The finding was supported by Botzki *et al.* 2004 [27] which demonstrated the capability of L-ascorbic acid 6-hexadecanoate (Vcpal) to inhibit streptococcal and bovine testicular hyaluronidase (BTH), where highest inhibition was observed for *Streptococcus agalactiae*. Structural analysis showed that the inhibition occurs due to hydrophobic interaction of the compound with conserved amino acids Ala-84, Leu-91, Tyr-93, Tyr-220 and Leu-344 in BTH model [27]. It was suggested that the inhibition may enhanced by acidic condition. The present findings may support the suggestion since Kelulut possess low pH. Similarly, Gelam and Tualang also acidic in nature, albeit a bit higher of pH was recorded for the latter [28, 29]. High acidity in Kelulut are due to fermentation process which produce high organic acids and hydrogen peroxide [30]. Fermentation in honey was suggested to be influenced by high moisture content, as supported by proximate analysis done by Zae *et al.* 2020 [31], where higher moisture content increase fermentation process. Interestingly, that proximate analysis showed few differences content between Tualang, Gelam and Kelulut honey. By highlighting the differences between Gelam and Kelulut, it was observed that, higher moisture, ash, protein and fat content in the former, while higher total available carbohydrate and energy in the latter. These differences require further investigation to find any direct involvement in hyaluronidase inhibition. In other explanation,

inhibition of hyaluronidase was suggested to be contributed by the phytochemical contents of the inhibitor substance, including the high content of flavonoid and phenolic compounds which is discussed in the details below. The present result showed a rule of concentration-dependent manner in the commercial honey and ascorbic acids, where the anti-hyaluronidase activity is increased with the increasing concentration of the compound. While in contrast to Gelam honey, the inhibition is decreased with the increase of the honey concentration.

There are several studies showed inhibition of hyaluronidase in concentration-dependant rules, where various kind of inhibitors was used such as *Prismatomeris tetrandra*, L-Ascorbic Acid 6-Hexadecanoate, *Trametes lactinea* extracts, *Vitis rotundifolia* (Muscadine) berry seeds and skins and also with sodium copper chlorophyllin complex and chlorophyllin analogs [27,32-35]. Inhibition mechanism which follow concentration-dependent manner is supported by the law of mass action by which the higher the concentration of the inhibitor may prevent the substrate binding, where the rate of reaction for the inhibition is directly proportional to enzyme-inhibitor formation. In this study, Kelulut, Gelam and Tualang honey showed that their anti-hyaluronidase activity did not follow the concentration-dependent manner. At the moment, there is no clear explanation of honey as hyaluronidase inhibitor to justify the reasons and factors which may cause this non concentration-dependent result. However, there are several studies of hyaluronidase inhibition which result to non-concentration dependent manner. This was demonstrated by a study which used apigenin as hyaluronidase inhibitor where the inhibition did not always follow concentration-dependent manner.

The result demonstrated that the inhibitory activity on bovine testicular hyaluronidase was decreased as the concentration of apigenin increased [36]. In another study, tannin as one of the investigated flavonoids showed active and complete inhibition towards the bovine testicular hyaluronidase at 50 μ M concentration, while the other flavonoids showed inhibition range within 29% to 76% at the concentration of 250 μ M [9]. Variability of hyaluronidase inhibition action not only observed for the different concentration of the inhibitor, but towards different source of hyaluronidase. This was shown in a study which used heparin as hyaluronidase inhibitor against hyaluronidases of different sources. It was found that the inhibition reactions toward venom hyaluronidase are more efficient at lower concentration compared to bovine testes hyaluronidase, while there was no inhibition reaction occurred on hyaluronidases originated from leech and *Streptomyces*. From these findings, it can be concluded that the inhibition of hyaluronidase enzyme may vary according to several factors such as the types of inhibitors used, the concentration of the inhibitors and also the sources of the hyaluronidase enzyme. This is proven in a study by Isoyama *et al.* 2006 [37] which claimed that different hyaluronidase inhibitors may result to different selectivity towards hyaluronidases. However, to prove this claim, further investigations must be made on the local honeys as inhibitor for pneumococcal hyaluronidase.

Correlation of total phenolic and total flavonoid on anti-hyaluronidase activity

Table 2 showed total of phenolic and flavonoid contents in different concentration of honeys and their anti-hyaluronidase activity percentage results. For total phenolic content, all honeys except Gelam, present with the compound at various amounts. For total flavonoid content, the presence of the substance can only be seen on Kelulut and Tualang honey at concentration of 0.18 and 0.3 g/ml. However, there are several honeys which resulted in negative results. The negative results were due to the low level of the substance which hardly detected in the assay. Comparison of phenolic and flavonoid content in Kelulut and Gelam showed a significant value, $p = 0.046$ for both comparison (**Table 3**).

Table 2. Total phenolic and flavonoid substances of honeys at different concentration and its anti-hyaluronidase percentage.

Honey samples	Concentration (g/ml)	Total phenolic (mg GAE/100g) ^a	Total flavonoid (mg QE/100g) ^a	Anti-hyaluronidase (%)
Kelulut	0.1	2499.33 \pm 0.040	-357.14 \pm 0.001	10
	0.18	1307.56 \pm 0.022	148.81 \pm 0.006	8
	0.3	2756.33 \pm 0.012	297.62 \pm 0.014	18
Tualang	0.1	1049.33 \pm 0.015	-357.14 \pm 0.006	3
	0.18	486.44 \pm 0.031	17.86 \pm 0.043	9
	0.3	869.67 \pm 0.049	1035.71 \pm 0.058	4
Commercial	0.1	792.67 \pm 0.004	-571.43 \pm 0.002	3
	0.18	589.67 \pm 0.026	-217.26 \pm 0.014	4
	0.3	364.53 \pm 0.032	-146.83 \pm 0.003	6
Gelam	0.1	-314.00 \pm 0.002	-696.43 \pm 0.001	12
	0.18	-95.33 \pm 0.002	-315.48 \pm 0.007	3
	0.3	-6.80 \pm 0.002	-220.24 \pm 0.001	2
Ascorbic acid	0.1	34882.67 \pm 0.068	-178.57 \pm 0.002	48
	0.18	9653.11 \pm 1.568	210.32 \pm 0.004	72
	0.3	7318.53 \pm 0.096	103.57 \pm 0.001	86

^aThe values are mean \pm standard deviation

Table 3. Comparison of p value of total phenolic and total flavonoid between Kelulut and Gelam honey.

Honey	Kelulut	Gelam	p value
Total phenolic ^a	0.656 \pm 0.012	0.101 \pm 0.002	*0.046
Total flavonoid ^a	0.135 \pm 0.014	0.048 \pm 0.001	*0.046

^aThe values are mean \pm standard deviation

* p value ≤ 0.05 through Mann-Whitney Test

Finding from this study support the study by Kolayli *et al.* 2016 [10] who had found higher phenolic contents in the honey which possess greater anti-hyaluronidase activity. In this study, the total phenolic content for Kelulut honey is the highest out of all the honey tested. A possible correlation between anti-hyaluronidase activity and the phenolic and/or flavonoid contents was determined through Spearman's correlation test which showed a significant correlation of anti-hyaluronidase and phenolic content (**Table 4**).

Table 4. Correlation between anti-hyaluronidase and total phenolics content (TPC) and total flavonoids content (TFC).

		Anti-hyaluronidase	TPC	TFC
Spearman's rho	Anti-hyaluronidase	<i>r</i>	1.000	0.639*
		<i>p</i> value	.	0.010
	TPC	<i>r</i>	0.639*	1.000
		<i>p</i> value	0.010	0.071
	TFC	<i>r</i>	0.404	0.479
		<i>p</i> value	0.135	0.071

*Correlation is significant at the $p \leq 0.05$ level.

Based on a previous study, it is expected to observe an increase of TPC and TFC values when there is increase of anti-hyaluronidase activity by the honey. Based on Table 4, a significant correlation was found between total phenolic contents and anti-hyaluronidase activity ($r=0.639$, $p=0.010$), while non-significant correlation observed between total flavonoid and anti-hyaluronidase activity in the honey ($r=0.404$, $p=0.135$). Correlation of phenolic content with the increase of hyaluronidase inhibition has been observed in other study [38]. Kolayli *et al.* 2016 [10] had reported highest phenolic contents was found in oak honey which also demonstrating the highest inhibition towards hyaluronidase. However, the mechanism of actions of phenolic contents in inhibiting hyaluronidase is still unclear and need further investigation.

Therapeutic properties of two major polyphenol contents in honey; phenolic acid and flavonoids was reported elsewhere [39]. Both compounds have been identified in different types of honey, varies in term of quantity. These two bioactive compounds had demonstrated various medicinal properties including as antimicrobial, antioxidant, anti-inflammatory, antineoplastic, antiulcer and cardiovascular-diseases treatment agent [39]. Composition of phenolic contents in Malaysian honeys has been reported elsewhere, where that phytochemical compounds are highly found in Tualang, Gelam, Pineapple and Kelulut honey as compared to Gelam honey [40]. Variation of the phytochemical compounds such as phenolic and flavonoid contents most likely are due to the floral sources [41]. Honey often named based on the geographical location where they are produced, regardless of species of the bees. For example, Gelam honey wildly produced by similar bees of Tualang honey, *Apis dorsata*, where the major nectar and pollen are collected from *Melaleuca cajuputi powell* or known as “Gelam” tree by local people. While Tualang honey was produced by the same bees which collect nectar from *Koompassia excelsia* or “Tualang” tree [42]. Since the differences of each honey are majorly contributed by the nectar sources, it was suggested that the phytochemical contents are highly related to the source of the floral plants.

In addition to phenolic contents, another phytochemical contents that previously describe to act as hyaluronidase inhibitor is flavonoid. Although no significant correlation of flavonoid with hyaluronidase inhibition was observed in this study, flavonoids of various sources have been found to act as hyaluronidase inhibitor [43-44]. This is also supported by several other studies which claimed that flavonoids possess anti-hyaluronidase activity [7, 15, 40].

CONCLUSION

In conclusion, honey of Malaysia origin can be a potential inhibitor against pneumococcal hyaluronidase enzyme. The most potential honey is Kelulut and the least is Gelam honey. Significant correlation of anti-hyaluronidase and phenolic contents support the previous claim of this phytochemical compound as inhibitor for this enzyme. Indirectly, these findings

also suggest potential antimicrobial properties of Kelulut against *S. pneumoniae* through inhibition of virulence factor, hyaluronidase. It is hope that the findings could be beneficial for the future study targeting treatment or prevention against *S. pneumoniae* infections.

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List of abbreviations

Asb	Absorption
BHI	Brain Heart Infusion
BSA	Bovine serum albumin
BTH	Bovine testicular hyaluronidase
GA	Gallic acid
HA	Hyaluronic acids
OD	Optical density
PBS	Phosphate buffer solution
QE	Quercetin
<i>S. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
TFC	Total flavonoid content
TPC	Total phenolic content

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