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ENTEROBACTER SAKAZAKII BE INHIBITED BY *TRIGONA* SPP. PROPOLIS FROM PANDEGLANG INDONESIA

A.E.Z. HASAN*[1], SURYANI[1], S. ESTUNINGSIH[2] AND FITRIANNUR[1]

[1]Department of Biochemistry, Faculty of Mathematics and Sciences, Bogor Agricultural University, Indonesia
[2]Department of Clinic, Reproduction and Pathology, Faculty of Veterinary, Bogor Agricultural University, Indonesia

ABSTRACT

Propolis has many advantages and specific potencies, due to its properties as antibacterial, antivirus, and anticancer. Aim of the study is to determine the propolis potency of *Trigona* spp as antibacterial agent against *E. sakazakii*. The antibacterial activity test was performed by a modified agar well diffusion against three isolates of bacteria *E. sakazakii* IB-19b, IB-29a, and ATCC 35217. Through the initiation test, propolis activity was higher than commercial propolis, and lower than ampicillin. The effect of propolis extract to commercial propolis for each isolate IB-19b, IB -29a, and ATCC 35217 were 143.99%, 169.65%, and 155.76% respectively. The effects of propolis extract to ampicillin for each isolate IB-19b, IB-29a, and ATCC 35217 were 39.37%, 38.94%, and 47.37% respectively. The minimum inhibition concentration (MIC) differed for each isolate, the minimum inhibition concentration for IB-19b, IB-29a, and ATCC 35217 were 12.50%, 25.00%, and 50.00% respectively.

KEY WORDS: **propolis, antibacterial, *Enterobacter sakazakii, Trigona* spp, Indonesia**

A.E.Z. HASAN
Department of Biochemistry, Faculty of Mathematics and Sciences, Bogor Agricultural University, Indonesia

***Corresponding author**

INTRODUCTION

Propolis is a natural product produced by honey bees, and has been widely used as medications or supplements, desserts, anti-inflammation, treatment of diseases, accelerate wound healing, and others. In addition, propolis has many benefits and special potential, as it has anti-bacterial properties, anti-virus, and can inhibit the growth of cancer. Seeing the potential of this propolis, it is necessary to scientifically prove about the potential and use of propolis, especially as antibacterial. Propolis was instrumental in keeping the internal environment aseptic nest. Significant evidence about the antimicrobial properties was derived from the use of this product in the hive itself, which is to wrap the carcass of a dead animal nuisance with propolis, because this animal is too heavy to be removed from the nest (eg, small snakes or rats). The process produces a similar effect embalming, because the animal dead body dries without decay[1]. This is very important to protect the beehive from a bacterial infection that can spread widely. Knowledge effectiveness of antiseptic propolis have been known for a long time. Aristotle suggested the use of propolis to treat abscesses and wounds[1].Propolis can exhibit significant cytotoxic activity and did not affect normal cells[2]. The nature and characteristics of *E. sakazakii* has been widely studied in terms of physiology and resistance to the environment[3]. Research on the properties of these bacteria is an important step in the effort to eliminate these bacteria from food production critical environments such as in the manufacture of powdered milk formula[4, 5]. *E. sakazakii* has properties opportunistic pathogens, the organisms that can cause disease only in certain circumstances. *E. sakazakii* is a contaminant in powdered infant formula that may cause rare diseases bacteremia, necrotizing entherocolitis (NEC), and meningitis after swallowing[5, 6, 7]. Infants are at high risk for E. sakazakii infection .Premature infants were born with low weight, and less than 28 days old. The infective dose ranges from 103 to 108 cells[8]. International Commission on Microbiological Specifications in Food (2002) categorizes these bacteria as' very dangerous for a limited population, endanger lives or chronic substance further in the long term. However, on a natural additive to prevent contamination of milk by bacteria is not yet widely known. One of the natural ingredients that have been tested as a potential antibacterial propolis *Trigona* spp. This study is expected to demonstrate the potential of propolis *Trigona* spp. as antibacterial against *E. sakazakii*, so that propolis can be used as a natural preservative to prevent or reduce bacterial contamination in infant formula. Extract of *Loranthus of Tea* can be inhibited by *E.sakazakii*[9]. The research aims to determine the antibacterial activity of propolis and determining the minimum growth inhibitory concentration (MIC) propolis against *E. sakazakii*.

METHODS

The materials used are stingless beehive *Trigona* spp. from Pandeglang Indonesia, commercial propolis (Propolis brand X), three isolates of bacteria : *E. sakazakii* IB-19b (enterotoxin negative), *E. sakazakii* IB-29a (positive enterotoxin), and *E. sakazakii* ATCC 35217 (*E. sakazakii* American Type Culture Collection) from the culture collection of the Faculty of Veterinary Medicine, Bogor Agricultural University [7,10], media Tryptone Soy Broth (TSB), media Tryptone Soy Agar (TSA), medium Mueller-Hinton Agar (MHA), a standard McFarland, 70% ethanol, propylene glycol technical , amphycillin 500 mg, and distilled water.

(i) Extraction of Propolis

Propolis extracted using methods[11, 12, 13, 14]. Extraction was done by maceration with 70% alcohol solvent. Concentrated extract obtained was weighed to get the value of the yield, then the extract was diluted with propylene glycol in the ratio of propolis: propylene glycol (1:1).

(ii) Antibacterial Activity Test

Antibacterial activity test wells was conducted using a modified agar diffusion method[15]. Standard drug is ampicillin 500 mg tablets with a concentration of 10 mg/mL

(positive control), propolis brand X, distilled water (negative control), and propylene glycol solvent control.

(iii) Stock of Bacteria

Bacteria were derived from primary cultures taken as one inoculating loop and inoculated into TSB new media, followed by incubation at 37 °C for 24 hours. Bacteria from cultures etched into the cup TSA as one inoculating loop, then incubated at 37 °C for 24 hours for obtaining a pure colony. This regeneration culture will be used as working stock that was used in the antibacterial activity test. TSA media composition in 1 liter of distilled water is 15.0 g tryptone, soy peptone 5.0 g, 5.0 g NaCl, and that 15.0 g. TSB media composition in 1 liter of distilled water is 17.0 g tryptone, soy peptone 3.0 g, dextrose 2.5 g,

(iv) Preliminary test

Preliminary test antibacterial activity carried out by well diffusion method as described elsewhere. Bacteria from stock work as one inoculating loop were transferred into 25 mL sterile TSB and incubated at 37 °C for 24 hours . Then the bacterial cultures were centrifuged at a speed of 5000 g for 15 minutes and washed three times with sterile distilled water. Bacterial pellet is suspended with sterile distilled water, and then adjusted to a standard McFarland 1 (3 × 108 CFU/mL). A total of 50 mL of bacterial suspension was distributed to Mueller-Hinton agar. Then so riddled with ± 5 mm diameter using a crock borer sterile. Propolis extract and comparator control (25 µl) were inserted into the hole and then incubated at 37 °C for 24 hours. Clear zone appeared around the hole and measured in units of mm, and this indicates that the sampleshas antibacterial activity.

(v) Determination of Minimum Inhibitory Concentration Growth (MIC)

MIC value determination made after it emerged that propolis extracts have antibacterial activity. The first phase, which propolis dilution with distilled water to obtain several concentrations (100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78%). Each concentration as much as 25 µL propolis extracts tested by inserting hole Mueller-Hinton agar medium which has been inoculated with the test bacteria, incubated at 37 °C for 24 hours. Antibacterial activity was obtained by measuring the clear zone around the sample hole in mm.

(vi) Statistical Analysis

The statistical analysis used in the processing of the data is one factor in experimental design completely randomized design. The following is a model design[16]:Yij = µ + τi + εij; Yij = Observations on the treatment of the i-th and j-th repetition; µ = Influence common averaging τ = Effect of the i-th treatment;ε = random effect on the treatment of the i-th repetition j.

The experimental design was used in determining the value of MIC. Data were analyzed with ANOVA (Analysis of Variance) at level α of 0.05 using SPSS 11 software programs that use advanced test is the test of Duncan.

RESULTS AND DISCUSSION

The yield of propolis extracts obtained (17.76%) in this study is greater than that obtained in the study[12, 13, 14], which amounted to 8.25% and 8.20% respectively. The solvent used in this study is 70% ethanol. Ethanol is a solvent that has semipolar nature so that the active component with diverse polarity can be extracted more perfect. Advantages of ethanol as a solvent is to have a low boiling point, so as to facilitate separation of active components in propolis, as well as reducing their numbers in the extract. According to[17], class of flavonoid compounds can be extracted by either using 70% ethanol. Flavonoids are the most important active compounds in the extract and propolis[18]. Propolis extraction by maceration using 70% ethanol produces 20% higher yield than using absolute ethanol. Therefore, the use of 70% ethanol can increase the amount of active compounds extracted. In addition, because it uses a crude propolis propolis nest, then there are other constituent components which can be extracted from the honeycomb, especially night (waxe) or wax. Beeswax is mainly composed of fatty acids and monoester simple alcohols with a carbon chain length saturated. Thus, the use of 70%

ethanol is also based on that candles were not extracted because it is not soluble in ethanol 70%. Rough propolis used was taken from a beehive *Trigona* spp. This kind of bees produce propolis in an amount more than the production of honey[19]. All phenol compounds have strong absorption in the ultraviolet region because it has an aromatic ring structure[17]. The color of propolis depends on the composition of the phenolic compounds contained in extracts, namely flavonoids. Propolis extract obtained in this study is brown. Propolis is darker in ethanol, contains flavonoids more, so that the yield is also higher than the younger colored propolis[20]. Based on statistical analysis, in each isolate, extract of propolis has a different activity with propolis X. Mathematically, the effectiveness of propolis extract of against propolis X in each isolate of *E. sakazakii* IB-19b, IB-29a, and ATCC 35217 respectively was 143.99%, 169.65%, and 155.76%. This difference in activity may be affected by different sources of propolis, propolis retrieval time, the ability to extract agar diffusion, and the difference in sensitivity of bacteria. Based on the magnitude of the percentage of the effectiveness of propolis extract of propolis on X, then the activity of propolis extract against bacteria test is bigger and better than propolis propolis X. The diameter of inhibition zone on *E. sakazakii* isolates each IB-19b, IB-29a, and ATCC35217 respectively 16.07, 13.25, and 12.71 mm (Fig 1). While the diameter of inhibition zone propolis X in each isolate of *E. sakazakii* IB-19b, IB-29a, and ATCC 35217 respectively were 11.16, 7.81, and 8.16 mm.

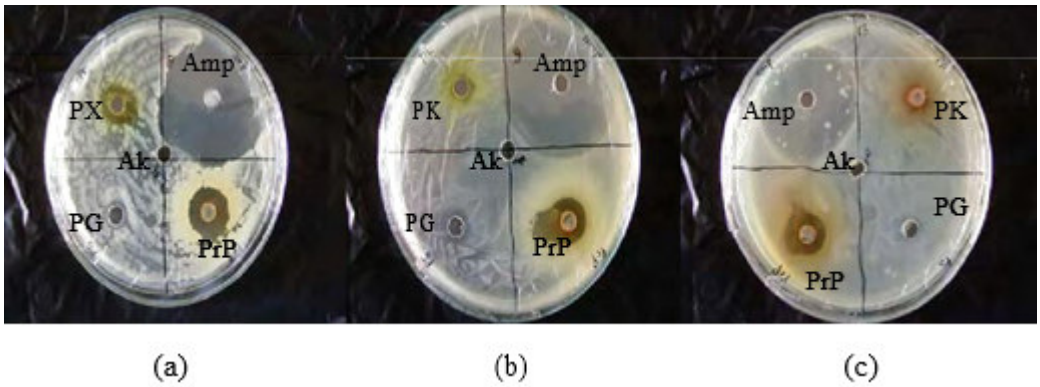


Figure 1
Results of preliminary tests diameter clear zone antibacterial activity of Propolis

Description : *E. sakazakii* (a) IB-19b; (b) IB-29a; (c) ATCC 35217
Amp= Amphicillin;
Ak = Aquadest
PG = Propilen Glicol
PX = Propolis X
PrP = Extract Propolis 100%

Propolis has lower activity against Gram-negative bacteria than Gram-positive bacteria[21]. Propolis extracts for antibacterial activity test carried out by the same[12] against two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) showed lower activity than the two tested Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*). This difference is caused by differences in compilers and structure of the bacterial cell wall of Gram negative and Gram positive. The outer membrane of Gram positive have a high content of peptidoglycan and single-layered and does not have a polysaccharide layer. Outer membrane of Gram-negative bacteria containing peptidoglycan is rich in lipids and form a layer of lipopolysaccharide. This layer is semipermeable, can not be by passed by large molecules, but can be by passed by small molecules such as nucleosides, oligosaccharides, and amino acids. Propolis or nanopropolis can inhibit Gram negative and Gram positive bacteria[13]. Based on

statistical analysis, in each isolate, extract of propolis has a significantly different activity against ampicillin. Mathematically, the effectiveness of propolis extracts to ampicillin on *E. sakazakii* isolates IB-19b, IB-29a, and ATCC 35217 were 39.37%, 38.94%, and 47.37% respectively. This percentage shows the activity of ampicillin were significantly different and larger than propolis extracts. The effectiveness of different values and ampicillin greater activity than the extracts of propolis because of differences in the sensitivity of bacteria to antibacterial. But with antibacterial properties owned by the propolis, become a consideration for the use of propolis as an antibacterial active ingredient when the resistance of bacteria to antibiotics. Under this standard, the activity of propolis against all isolates were strong category and higher than the activity of propolis X, which is in the range of 10-20 mm. Propolis X activities against *E. sakazakii* IB-19b includes strong (10-20 mm), then for *E. sakazakii* IB-29a and *E. sakazakii* ATCC 35217 were moderate (5-10 mm). Ampicillin activity demonstrated by very strong activity category (> 20 mm), even on *E. sakazakii* isolates IB-19b showed ampicillin activity of these bacteria and were very susceptible to ampicillin with a diameter of 40.82 mm clear zone. On the *E. sakazakii* IB-29a and *E. sakazakii* ATCC 35217 the ampicillin activity were very strong showing zone of inhibition of 34.02 mm and 26.82 mm respectively. Based on[22], which test the vulnerability of 6 strains of *E. sakazakii* bacteria, suggests that these bacteria were susceptible to ampicillin and these results are consistent with the results tested on 195 isolates of *E. sakazakii* [6], and the mechanism of action of propolis extract cannot be known for sure to the research conducted. However, the antimicrobial properties of propolis owned allegedly associated with the synergistic effect of the compounds present in propolis. Five propolis from Indonesia content flavonoids and inhibited cancer breast cell MCF-7[14]. Propolis can damage the cytoplasmic membrane, inhibit the motility of bacteria and enzyme activity[21]. According to the literature[21] class of phenolic compounds (flavonoids, flavones)were contained in propolis antibacterial activity. Flavonoids have the ability to bind to the extracellular proteins and protein integral to join the bacterial cell wall and disrupt the permeability of cell walls[23]. In addition, the content of triterpenoids contained in propolis also plays a role in the synergistic effects possessed antibacterial propolis. Tannin compounds in propolis extract are thought to have antimicrobial properties because of its ability inactivation of the enzyme protein, and protein transport layer[23]. Saponins forming foam soap in water and a surface active ingredient saponins, which can reduce the permeability of cell walls, enabling other active ingredients to damage the cell wall and cell lysis[17, 23]. Minerals like Zn or Mn from coconut fruits can inhibited damaged cell and triggers cell from stress[24]. Ampicillin is a β-lactam antibiotics and belongs to the class of semisynthetic penicillin. Mechanism of action of ampicillin which inhibits bacterial cell wall formation by preventing the merger of N-acetyl muramic acid into the structure of peptidoglycan. Inhibition of peptidoglycan biosynthesis cause a weakened cell wall and cannot withstand the pressure from the cytoplasm to the cell rupture. Mechanism of action of specific causes owned ampicillin has a great antibacterial power and is bactericidal with a broad spectrum, can inhibit the Gram positive and Gram negative. Determination of MIC were conducted to determine the lowest concentration of propolis extracts which can still inhibit bacterial growth of *E. sakazakii* (Fig 2). Parameter for growth inhibition of *E. sakazakii* was by measuring the clear zone of inhibition at various concentrations of 100, 50, 25, 12.5, 6.25, and 3.125%. These concentrations were selected based on research [12, 13, 14]in addition ,there is a decrease in activity with the smaller decline in concentration. At MIC determination, *E. sakazakii* IB-29a and ATCC 35217 the extract showed activity in the solvent control, but based on statistical analysis, the influence of solvent propylene glycol were not significant, because of the activity shown by the significantly different propolis extracts with propylene glycol (P <0, 05). Activities of propolis extract itself on each test bacteria showed a different trend. This suggests that each bacterium has a different sensitivity. Antibacterial activity obtained showed activity variation depending on the concentration and

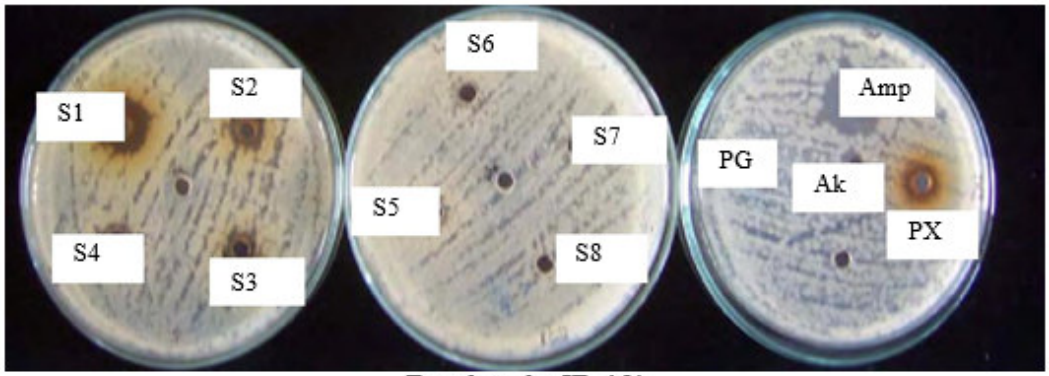
type of bacteria, propolis activity decreased with decreasing concentration. Similarly, the different isolates, obtained significant differences in the activity of propolis against each isolate. Third isolates showed different results can be caused by different vulnerabilities. Because, basically, three isolates were Gram-negative bacteria The results of different tests using extracts of propolis showed distinct differences in reaction to the antibacterial ingredient in propolis extracts. Based on the results of determination of MIC, propolis showed high effectiveness in isolates IB-19b to the minimum effective concentration of 12.5%, and less effective against bacteria ATCC 35217. This may be due to the inhibition zones that occur and vary by several factors such as the toxicity of materials test, diffusion ability of test materials to the media, and the micro-environmental conditions in vitro[25].In addition to these factors, *E. sakazakii* has one of the characteristics of the bacteria that are pathogenic and can increase the pathogenicity, for example, it can produce capsular adhesive material so that bacteria can form bio-films[7]. Because some strains have capsules and form biofilms, then penetrate into bacterial cells this active ingredient propolis becomes slower. MIC determination results also showed a concentration-dependent activity of propolis. The highest propolis antibacterial activity at a concentration of 100%, and a decline in activity with decreasing concentration. Through statistical analysis, the effect of concentration showed no significant difference on each isolate at a concentration of 3.125%. However, at this concentration value it is not significantly different from the control solvent propylene glycol. Therefore in MIC determination, the concentration through statistical tests differ significantly with propylene glycol with higher activity than propylene glycol. MIC value on each of the different isolates, isolates IB-19b, IB-29a, and ATCC 35217 was 12.50% (7.33 mm), 25.00% (6.69 mm), and 50.00% (6.62 mm) respectively (Table 1).

Table 1
MIC of propolis extract

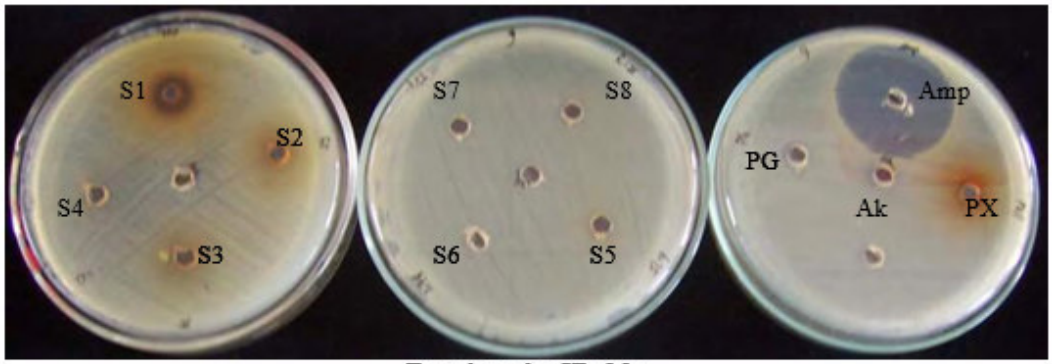
Treatment	Diameter Inhibition Zone (mm)		
	***E. sakazakii* ATCC 35217**	***E. sakazakii* IB-29a**	***E. sakazakii* IB-19b**
100 %	10,8233 ± 0,3669	10,7133 ± 0,4302	14,0400 ± 1.2978
50 %	6,6167 ± 0,1050	7,6500 ± 0,4051	10.6867 ± 0,4051
25 %	6,2667 ± 0,4818	6,6867 ± 0,2055	9.2433 ± 0,2055
12,5 %	5,8333 ± 0,2139	6,1833 ± 1,5552	7.3300 ± 0.0700
6,25 %	5,9867 ± 0,1193	5,8833 ± 0,8799	6.5233 ± 0.5590
3,125 %	5,8000 ± 0,1311	5,6533 ± 0,6091	6.3000 ± 0,1114
Propolis X	7,0267 ± 0,7214	7,2433 ± 1,0659	9.9033 ± 0.7850
Propilen glycol	6,6000 ± 0,4371	6,4500 ± 0,7594	5.7000 ± 0,0200
Amphicillin	28,4033 ± 0,6536	35,6450 ± 0,1344	25,6400 ±1.9968
100 %	5,4333 ± 0,2811	5,4633 ± 0,0737	5,4300 ± 0,1229

CONCLUSION

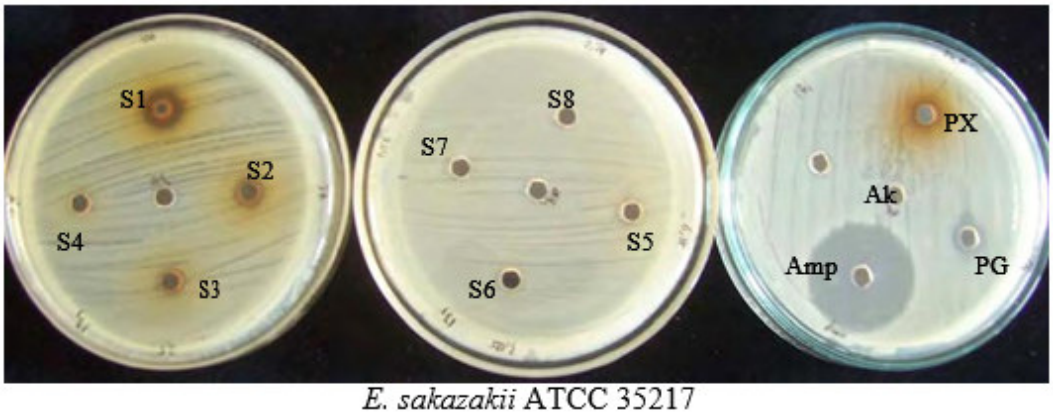
Propolis were extracted from beehives *Trigona* spp from Pandeglang Indonesia with maceration using alcohol 70% yield of 17.76%. Through the preliminary test propolis extract has higher effect than propolis X, and lower than ampicillin. The effectiveness of propolis extract against propolis X in each isolate IB-19b, IB-29a, and ATCC 35217 was 143.99%, 169.65%, and 155.76% respectively. The effectiveness of propolis extracts to ampicillin in each isolate IB-19b, IB-29a, and ATCC 35217 were 39.37%, 38.94%, and 47.37% respectively. The minimum growth inhibitory concentration obtained on each of the different isolates, isolates IB-19b, IB-29a, and ATCC 35217 were 12.50%, 25.00%, and 50.00% respectively.



E. sakazakii IB-19b



E. sakazakii IB-29a



E. sakazakii ATCC 35217

Figure 2
Result test of MIC

Description: S1= Propolis Extract 100%
S2 = Propolis Extract 50%
S3 = Propolis Extract 25%
S4 = Propolis Extract 12,5%
S5 = Propolis Extract 6.25%
S6 = Propolis Extract 3.12%
S7 = Propolis Extract 1.56%
S8 = Propolis Extract 0.78%

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