Antibacterial Activity of Intact Royal Jelly, Its Lipid Extract and Its Defatted Extract

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Abstract

The study of antibacterial activity of royal jelly produced by honeybees (*Apis mellifera*) in Chiang Mai, Thailand was conducted. Intact royal jelly, the lipid extract and the defatted extract were antibacterial against all gram positive and gram negative bacteria tested with different potency. The intact royal jelly exhibited the highest antibacterial activity. The lipid extract, which contained mostly acidic polar compounds was more effective than the defatted extract when assayed against non-spore forming bacteria, but less effective in spore-forming bacteria such as *B. cereus*. The storage period of royal jelly also influenced the antibacterial activity, which maximally peaked within 24 hr. after collection and then decreased into constant level. Different storage temperature did not significantly affect the antibacterial property. The acidity enhanced but autoclaved condition somewhat reduced the antibacterial activity of intact royal jelly. By bioautography it was shown that the active principles in the lipid extract should be acidic polar compounds. The possibility using royal jelly as a new antibiotic in the future is needed to be developed.

Keywords *Apis mellifera*, royal jelly, agar diffusion assay, agar dilution assay, minimum inhibition concentration (MIC), storage period, storage temperature

1. Introduction

Royal jelly has a thick, milky appearance, with a slightly acid, pungent odor and a somewhat bitter taste. It is a secretion produced by the hypopharyngeal and the mandibular glands in the head of the nurse bees. Their constituents reported elsewhere contain moisture (65-70%), protein (15-20%), carbohydrate (10-15%), lipid (1.7-6%), pollen (trace), and ash (0.5-0.6%) [1]. The presence of antimicrobial activity in the royal jelly has been documented for more than 60 years [2]. The active principles exhibit bacteriostatic and bactericidal properties. They were removed from intact royal jelly by extraction with alcohol and acetone and were not destroyed after autoclaving at 120^{0}C for 20 min but unable to be filtered [3]. However, other investigators have been unable to demonstrate antibacterial activity in royal jelly [4]. This discrepancy may be explained that the different investigators had tested royal jelly samples of different ages and that the antibacterial activity of royal jelly diminishes with age [5]. In this study, we used royal jelly produced in northern Thailand to investigate the efficiency of antibacterial activity. The effect of storage temperature and storage period were also determined to acquire the best storage conditions for royal jelly in maintaining the highest antibacterial activity. In addition, all fractions derived from the extraction of royal jelly by diethyl ether were also analyzed to compare the potency of their antibacterial activity.

2. Materials and Methods

Royal jelly produced by honey bees *(Apis mellifera)* was purchased from an apiary in Chiang Mai, Thailand. It was freshly collected 3 days after grafting larvae into the artificial queen cells. After collection, it was kept in several small brown bottles at various temperatures: room temperature (25-27 ^{0}C), refrigerated temperature (2-4 ^{0}C) or deep-frozen temperature (-18^{0}C). After storage for 12 hr, 24 hr, 3 d, 7 d, 15 d, and 30 d, each royal jelly sample was extracted with diethyl ether in a Soxhlet apparatus for 24 hr giving the lipid fraction and the defatted fraction.

2.1 Determination of antibacterial activity of intact royal jelly and the defatted extract by agar-diffusion method

Intact royal jelly stored at different periods and temperatures was applied in cup-reservoirs (0.2-0.4 g/reservoir) which was placed in the seeded nutrient agar plate containing a type of bacteria with the density equivalent to $3x10^8$ cells/ml. A variety of common bacteria tested were both gram positive bacteria (*Sarcina lutia, Bacillus cereus, Staphylococcus aureus)* and gram negative bacteria (*Shigella flexneri, Salmonell typhi, Escherichia coli, Proteus vulgaris, and Pseudomonas aeruginosa*). Antibiotic solutions (ampicillin or chloramphenicol) were used as a positive control while vaseline was used as a negative control. The plates were incubated at 37^{0}C for 18-24 hr. Then, the diameter of the clear inhibition zone was measured. The antibacterial activity of the defatted extract stored at deep-frozen temperature for different periods with varying amount 0.2-0.4 g/reservoir were also determined by the same procedure.

2.2 Determination of the mechanism of antibacterial action

A portion of the clear inhibition zone from each sample detected by agar a diffusion assay was subcultured in nutrient broth to determine bactericidal activity.

2.3 Determination of antibacterial activity of the lipid extract by standard loop test

The lipid extract was applied to the seeded nutrient agar plate by using a calibrated inoculating loop 0.01 ml. One loop contained about 12.1$\pm$2.94 mg of the lipid extract. Each plate applied with 4 samples, two for the lipid extract, one for positive control as ampicillin or chloramphenicol and one for negative control as vaseline.

2.4 Determination of minimum inhibition concentation (MIC) of intact royal jelly and the defatted extract by agar dilution test

A working solution containing either 1-25 mg/ml intact royal jelly or 1-25 mg/ml of the defatted extract in molten nutrient agar, in increments of 1, were prepared and poured into petri plates. Then, each type of bacterial colony was streaked on each divided surface of the plates. The inoculated plates were incubated at 37^{0}C for 18-24 hr. Then, the MIC was read at the lowest concentration which completely inhibited growth.

2.5 Determination of MIC of the lipid extract of royal jelly by disc-susceptibilty test

Impregnated blotting discs containing 50μg-5mg of the lipid extract/disc or discs were separately spread in seeded agar plates. The inoculated plates were incubated at 37^{0}C for 18-24 hr in an inverted position. The MIC value was determined graphically as previously described [6].

2.6 Detection of antibacterial agent in the lipid extract by bioautography [7]

The lipid samples were redissolved in diethyl ether and applied on silica gel 60G thin-layered microslide chromatograms. The plates were developed at room temperature when pure chloroform or 29% methanol in chloroform or pure methanol was used as a solvent. Whereas the plates were developed at refrigerated temperature when hexane–diethyl ether 1.5:1 (V/V) was used as a solvent for better separation. After developing, the plates were dried and the spots were located under UV-light and iodine vapor.

The developed chromatograms were placed in petri plates (1 chromatogram/plate) and were spread over with the seeded molten nutrient agar. After leaving to cool down, the plates were placed in a refrigerator at 0-4^{0}C for 1 hr and then to an incubator at 37^{0}C for 18-24 hr allowing the antibacterial agent to diffuse from the chromatogram into the agar. A clear inhibition zone was seen against the confluent growth of the test microorganism at the position of the antibacterial agent.

3. Results and Discussion

3.1 Antibacterial activity and the minimum inhibition concentration (MIC) of intact royal jelly

Intact royal jelly had antibacterial activity against all microorganisms tested. Examples of this antibacterial activity of intact royal jelly are shown in Fig. 1 (a-c). Since royal jelly is semisolid thick and milky in appearance, it is difficult to apply the sample into each reservoir in a constant weight. We have tested royal jelly samples in varying amounts from 0.11 g - 0.75 g in each reservoir to determine the antibacterial activity. The result showed a plateau effect that further elevations in royal jelly content did not increase the size of the inhibition zone as shown in Table 1. Therefore, the change in the bacterial activity was not caused by the varying amounts of of royal jelly applied in the reservoir. Interestingly, the antibacterial activity of intact royal jelly changed during storage periods. Within 24 hr after collection, the maximal antibacterial activity of intact royal jelly were shown and then decreased to a constant level afterwards. Similar findings were also shown according to previous reports [8,9]. This evidence, including our study, suggests that the antibacterial substance(s) in intact royal jelly might be in a preform, then after 24 hr storage, it changes maximally into the active form which gradually decreases the activity to a constant level. On the contrary, the storage temperature did not affect the antibacterial activity of royal jelly as strongly as the storage period though the storage at room temperature deteriorated the activity more rapidly, and within a week it turned a yellowish-brown color and rancid. No deterioration has been shown for intact royal jelly kept at refrigerated temperature or deep-frozen temperature however, such intact royal jelly may become quite granular in consistency when it is kept at refrigerated temperature or deep-frozen temperature. This formation of crystals was a normal phenomenon in stored royal jelly as previous reports stating that there were organic acids present in royal jelly in fairly large amounts which tended to crystalize when held at low temperature [4,9].

The action of royal jelly was both bactericidal and bacteriostatic. The bactericidal action was shown against *S.lutia, S.typhi, S.aureus* and *Sh.flexneri.* whereas the bacteriostatic action was shown against *B. cereus, E.coli, Pr.vulgaris and Ps.aeruginosa.* The MIC against various microorganisms tested of all intact royal jelly samples was about 10-25 mg/ml as shown in Table 2 and Fig.2, except the MIC value against *S. lutia*, the most sensitive microorganism, was about 0.1-0.5 mg/ml. It should be noted that the antibacterial action of intact royal jelly was more effective against gram positive than gram negative bacteria.

Table 1 Plateau effect in antibacterial assay of intact royal jelly stored at deep-frozen temperature

Wt (g)	ϕ (cm)	Wt (g)	ϕ (cm)
a. against S.lutia		*b. against Sh.flexneri*	
0.20	3.55	0.13	2.25
0.25	3.20	0.27	2.25
0.31	3.38	0.30	2.25
0.37	3.20	0.31	2.25
0.57	3.55	0.40	2.25
c. against S.typhi		*d. against E.coli*	
0.21	2.25	0.11	2.4
0.22	2.25	0.20	2.4
0.31	2.30	0.47	2.4
0.40	2.25	0.52	2.4
0.75	2.20	0.56	2.4

ϕ : diameter of inhibition zone

The MIC values of intact royal jelly, kept in a deep-freezer for 3 d were higher than those of samples kept in a deep-freezer for 24 hr or 12 hr. The mechanism of action of antibacterial activity of this intact royal jelly at the MIC value was bacteriostatic against all microorganisms tested. In an effort to understand more about the nature of the antibacterial potency of intact royal jelly, the effect of the acidity and the temperature were partially determined. The acidity of intact royal jelly (pH 3.5-4.5) though enhanced the antibacterial activity but not in all cases. Some organisms such as *B.cereus, Sh.flexneri, Pr.vulgaris* and *Ps.aeruginosa* were more sensitive to intact jelly but not responsive to this pH range of the citrate-sodium citrate buffer. The effect of the temperature, autoclaving under pressure 15 lb/in^2

Table 2 The minimum inhibition concentration (MIC) of intact royal jelly stored in a deep-freezer

Bacterial strains	MIC of intact royal jelly (mg/ml)		
	12 hr	24 hr	3 d
S.lutia	0.16	0.30	0.45
Sh.flexneri	15.5	14.5	17.5
S.typhi	11.5	14.5	15.5
S.aureus	11.5	12.5	25.5
B.cereus	11.5	12.5	18.5
E.coli	14.5	13.5	15.5
Pr.vulgaris	12.5	15.5	17.5
Ps.aeruginosa	>16	15.5	21.5

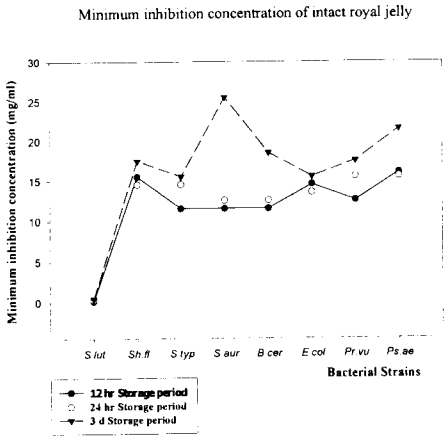

Fig. 2 The minimum inhibition concentration (MIC) of intact royal jelly stored in a deep-freezer for different periods

at 121^{0}C for 15 min did not destroy the antibacterial activity. The antibacterial activities against *S.lutia*, *B.cereus*, *S.aureus*, *Sh.flexneri*, and *E.coli* were only slightly reduced. This result agreed with a previous report that autoclaved royal jelly did not destroy the germicidal principle but somewhat reduced its activity [1]. However, it was different from a report showing that autoclaved royal jelly increased the bactericidal factor fifty fold [8].

3.2 Antibacterial activity of the lipid extract

The lipid extract is a pale-yellow oil with a spicy odor. It showed antibacterial activity against various microorganisms tested with some difference from its corresponding intact royal jelly. The lipid extract was tested at an average concentration 12.10±2.94 mg/0.01 ml calibrated loop. Examples of the antibacterial activity of the lipid extract were shown in Fig. 1 (d-f). Similar to the activity of intact royal jelly, the antibacterial activity of the lipid extract increased to the highest within 24 hr after collection of royal jelly and then decreased to a constant level afterwards. Against nearly all microorganisms tested, except *B.cereus*, the lipid extract had antibacterial activity showing the clear inhibition zone. Some resistant colonies were found within the clear inhibition zone against *B.cereus* therefore, the MIC of this microorganism could not be evaluated. The mechanism of action of the antibacterial activity of the lipid extract demonstrated bacteriostatic for nearly all microorganisms tested except showed bactericidal against *S.lutia*. The MIC of the lipid extract against various microorganisms tested were between 50-500 μg/ml depending on the test material and microorganisms as shown in Table 3 and Fig. 3. The MIC value of the lipid extract against *S.lutia* was the lowest, the MIC values against *E.coli*, *S.aureus*, *S.typhi*, *Sh.flexneri*, and *Pr.vulgaris* were increasing respectively, the MIC value against *Ps. aeruginosa* was the highest. The MIC value against *B.cereus* that had shown some resistant colonies on the inhibition zone was not evaluated. Detection of antibacterial agents in the lipid extract by bioautography verified the antibacterial substance in the lipid extract was (were) acidic and polar since the average R_f =0.07 when developed in 29% methanol in chloroform at room temperature while the average R_f value = 0.66 when developed in hexane:diethyl ether 1.5:1 (v/v) at refrigerated temperature.

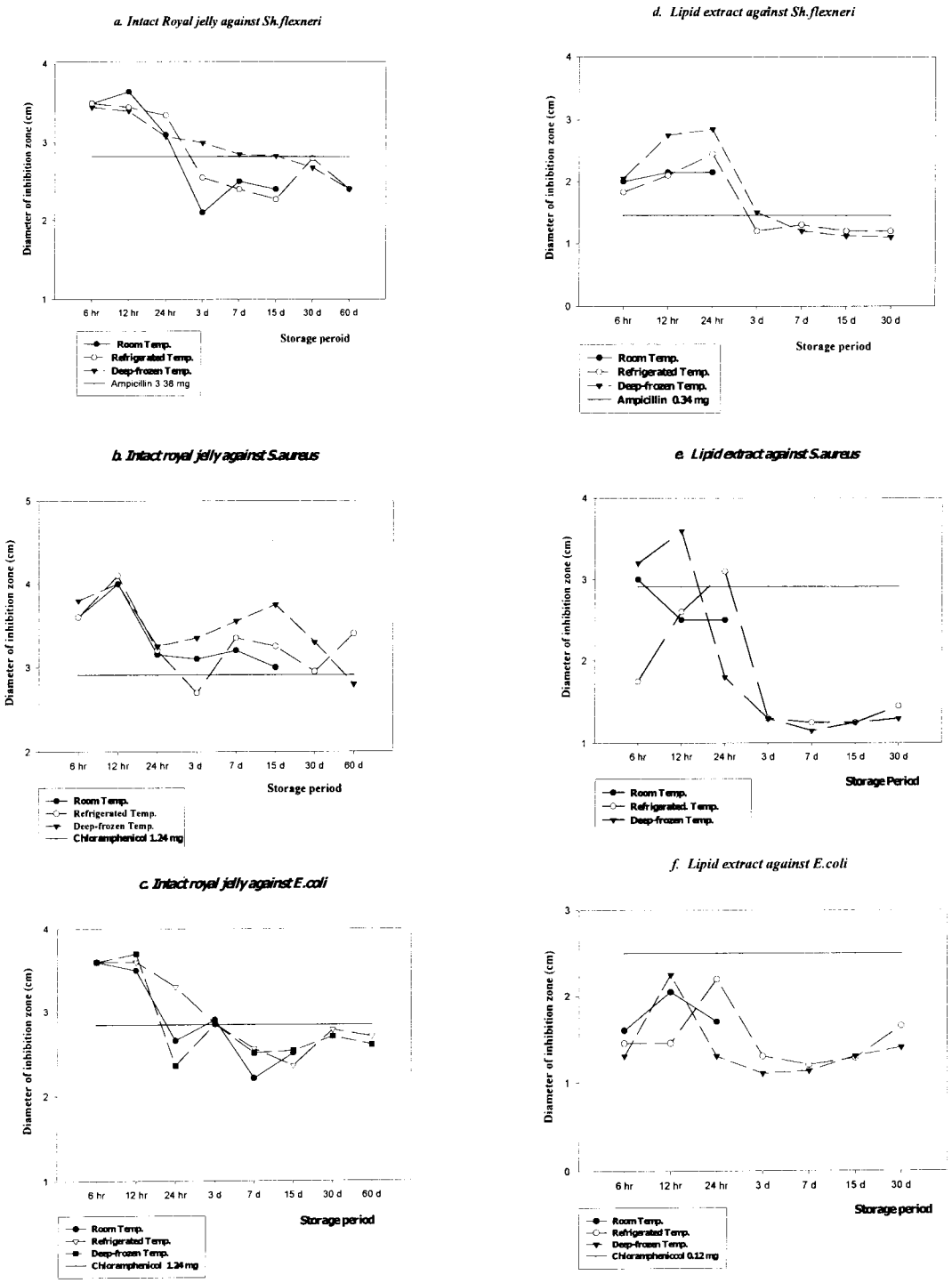

Fig.1 Antibacterial activity of intact royal jelly (a-c) and the lipid extract (d-f)

Table 3 The minimum inhibition concentration (MIC) of the lipid extract

Bacterial strains	MIC of the lipid extract (ug/ml)			
	12 hr	24 hr	3 d	7 d
S.lutia	51.1	78.8	110.3	109.6
Sh.flex	213.6	224.2	287.0	259.6
S.typhi	159.6	185.0	207.0	274.0
S.aur	190.0	148.3	201.6	179.6
B.cer	85.1	88.4	129.9	175.0
E.coli	78.0	97.5	138.4	170.3
Pr.vul	299.6	156.0	310.0	301.2
Ps.aeru	319.7	213.2	418.3	504.4

N.B. *Sh.flex:Sh.flexneri; S.aur:S.aureus; B.cer: B.cereus(with resistant colonies); Pr.vul:Pr.vulgaris; Ps.aeru: Ps.aeruginosa*

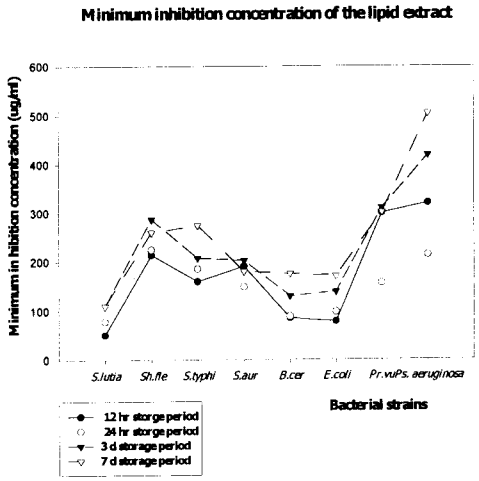

Fig. 3 Minimum inhibition concentration of the lipid fraction extracted from intact royal jelly at different storage periods

3.3 Antibacterial activity and the minimum inhibition concentration (MIC) of the defatted extract

The defatted extract was sticky, yellow and semisolid in appearance with pH of 5.0 and if stored more than 3 days became thicker and harder. It also exhibited the antibacterial activity though their efficiency was the lowest compared to the lipid extract or intact royal jelly as shown in Fig. 4. The MIC value against various tested microorganisms except *S.lutia* were generally 12.5-25.0 mg/ml as shown in Table 4 and Fig. 5.The MIC against *S. lutia* was the lowest of 0.8-1.6 mg/ml while the MIC against *Ps.aeruginosa* was the highest of 27 mg/ml. Storage periods also affected the antibacterial activity of the defatted extract. After 3 d storage, the MIC values declined about two fold.

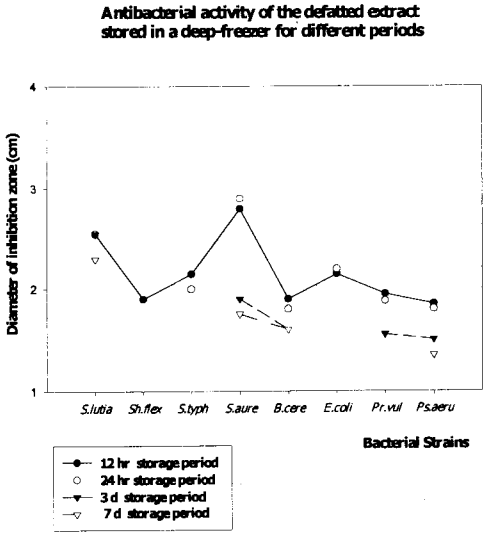

Fig. 4 Antibacterial activity of the defatted extract stored at deep-frozen temperature for different periods

The activity of the defatted fraction at its MIC was bacteriostatic against all microorganisms tested. Autoclaving under pressure 15 lb/in^2 at 121^{0}C for 15 min did greatly reduce the antibacterial activity of the defatted extract compared to intact royal jelly which partially reduced its activity depending on types of microorganisms tested. Only the antibacterial activity against *S.lutia,* and *Sh.flexneri* were still active whereas those against the other microorganisms such as *S.aureus*, *S.typhi*, *Pr.vulgaris*, *B.cereus, E.coli,* and *Ps.aeruginosa* were inactive.

Our finding that the defatted extract exhibited the antibacterial activity against both gram positive and gram negative bacteria tested is quite different from others who reported that the antibacterial component in royal jelly exhibited only in lipid fraction while a defatted component did not have antibacterial activity [2,5]. Royalisin, a novel protein purified from

Table 4 The minimum inhibition concentration (MIC) of the defatted extract stored at deep-frozen temperature for different periods

Bacterial strains	The MIC of the defatted extract (mg/ml)			
	12 hr	24 hr	3 d	7 d
S.lutia	1.6	1.6	1.4	0.8
Sh.flexneri	20.5	21.5	14.5	15.5
S.typhi	24.5	23.0	14.5	16.5
S.aureus	17.5	17.5	12.5	13.5
B.cereus	14.5	15.5	12.5	13.5
E.coli	18.5	18.5	14.5	146.5
Pr.vulgaris	24.5	23.0	14.5	14.5
Ps.aeruginosa	>25.0	>25.0	>25.0	27.5

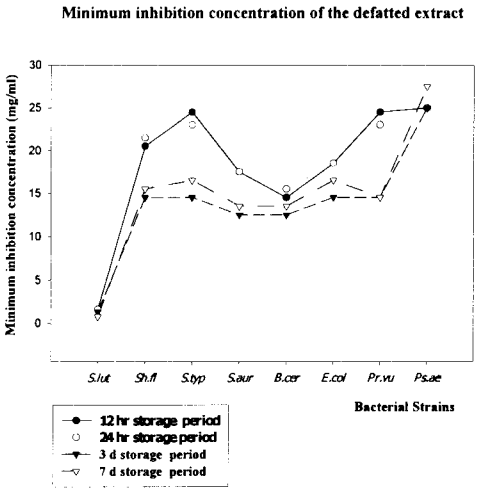

Fig. 5 Minimum inhibition concentration of the defatted extract stored at deep-frozen temperature for different periods

royal jelly though, exhibited potent antibacterial activity but showed activity only against gram positive bacteria such as *Lactobacillus, Bifidobacterium,* and *Leuconostoc,* but not against gram-negative bacteria such as *E.coli, Klebsiella,* and *Salmonella* [9].

4. Conclusions

All intact royal jelly, the lipid extract and the defatted extract had antibacterial activity while the intact royal jelly exhibited the broadest in antibacterial activity. The lipid extract was more effective than the defatted extract when assayed against non-spore forming bacteria but less effective in spore-forming bacteria. The storage period of intact royal jelly also influenced the antibacterial activity whereas storage temperature had no significantl affect. The maximal peak of antibacterial activity of intact royal jelly and the lipid extract were shown within 24 hr after collection and then decreased to a constant level. After storage of intact royal jelly more than 3 d, the antibacterial activity of both intact royal jelly and the lipid fraction generally declined. The acidity also enhanced the antibacterial activity but autoclaving somewhat reduced the antibacterial activity of intact royal jelly. The active principle (s) contained in the lipid extract should exist in the acidic fraction and may be polar aromatic or unsaturated compounds. The defatted extract however, improving its antibacterial activity after 3 d storage in contrast to the intact form whose activity deteriorated after 3 d storage. Therefore, the best condition for royal jelly storage in acquirire the best antibacterial activity should be storage for 12 hr - 3 d in deep frozen temperature compromising the best activity for both principles in the lipid and defatted fraction.

5. Acknowledgements

Authors gratefully appreciate Ms Jitra Chaitep, Ms Patcharin Pramoonjukgo, Assoc. Prof. Dr. Parimond Kanjanasthiti and Prof. Dr. Maitree Suttajit for their helpful criticism and valuable advice. We are also grateful to Prof. Dr. Floyd W. Dunn for careful reading of the manuscript. This work was partially supported by Vajira Medical Research Foundation.

6. References

[1] Townsend, G.F., and Lucas, C.C., Chemical Examination of Lipid Fraction of Royal Jelly, Science, Vol.92, p. 43, 1940.

[2] McClesky, C.S., and Melampy, R.M., Bactericidal Properties of Royal Jelly of the Honeybee, J.Econ.Entomol., Vol.32, pp.581-587, 1939.

[3] Willson, R.B., Royal Jelly : A Review, Amer. Bee.J., January, pp. 15-21; February, pp. 55-59, 1955. And September: p. 356 ; October, p. 397, 1957.

[4] Dayan, A.D.. A Note on Royal Jelly, A Critical Evaluation, J.Pharm.Pharmacol., Vol.12, pp. 377-383, 1960.

[5] Blum, M.S., Novak, A.F., and Taber, S., 10-Hydroxy-2-Decenoic Acid, An Antibiotic Found in Royal Jelly, Science, Volume 140, pp. 452-453, 1959.

[6] Levine, H.B., Stevens, D.A., Cobb, J.M., and Gebhardt, A.E., Miconazole in Coccidio do Mycosis I, Assays of Activity in Mice and in vitro, J.Inst.Dis., Vol.132, No.4, pp. 407-414, 1975.

[7] Hamilton, P.B., and Cook, C.E., Some Techniques for Bioautography of Antimicrobial Substances on Thin-layer Chromatography, J.Chromatog., Vol.35, pp. 295-296.

[8] Dixon, S.E., and Shuel, R.W., Study in the Mode of Action of Royal Jelly in Honeybee Development I, Can.J.Zool., Vol.36, pp. 197-204, 1958.

[9] Fujiwara, S., Imai, J., Fujiwara, M., Yaeshima, T., Kawashima, T., and Kobayashi, K. A Potent Antibacterial Protein in Royal Jelly, J.Biol..Chem., Vol.265, No.19, pp. 11333-11337, 1990.