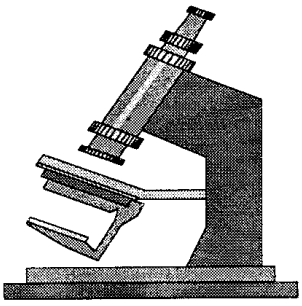

PRESENT STATE OF BASIC STUDIES ON PROPOLIS IN JAPAN

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JAPAN

I. Introduction

In October 1985, the 30th International Apicultural Congress held in Nagoya first introduced propolis into Japan, when the latest reports on basic studies on propolis and the clinical application of propolis to the intractable diseases were presented by the researchers and the medical doctors from various countries abroad. Together with the introduction of a crude propolis by the Brasilian beekeepers, the name of *propolis* became known instantly, as a promising substance. However, during the first five years since 1985, propolis was regarded only as a healthy food and a folk remedy and was not paid much attention by scientists. Propolis studies, made public in 1987, related only to general findings, introduction of overseas literature, and distribution of flavonoid components[1].

In 1991, at the 50th Japan Cancer Society Meeting, MATSUNO, from the National Institute of Health reported three compounds of propolis with tumour killing activities, from his experience in curing terminal-stage uterine cancer with propolis[2]. With this as momentum, many pharmaceutical companies and research institutes have started to study propolis with the prospect of using it in various fields. Among them, Hayashibara Biochemical Laboratories Inc. - in the course of the interferon research - took a great interest in the BRM (biological response modifiers)-like effects of propolis, such as the antiviral and the immune activating effects[3-4], studied propolis for the last few years, and published a number of remarkable research results.

This paper describes representative studies on the antimicrobial effect and on the cytotoxic effect of propolis, from 1985 up to the present, in Japan.

II. Antimicrobial Activity of Propolis

The strong antimicrobial activity of propolis is often compared to a "natural antibiotic". Its antimicrobial characteristics have recently been studied throughout the world.

In Japan, AGA et al.[5][6] MATSUNO[7], ITOH et al.[8], and NAKANO et al.[9] studied and reported the effects of propolis and of the antimicrobial sub-

stances isolated from propolis on various types of fungi, yeasts and bacteria, as well as on specific pathogenic microbes, such as *Helicobacter pylori*, and Methicillin-resistant *Staphylococcus aureus* (MRSA), between 1992 and 1995.

1. Antimicrobial Activity of Brazilian Propolis[5]

In 1992. AGA et al., from the Hayashibara Biochemical Laboratoires Inc., published their research results on the antimicrobial activity of an ethanol aqueous solution extract of Brazilian propolis against 8 strains of fungi, 4 strains of yeast, and 42 strains of bacteria, including *Enterobacter* and *Actinomyces.*

In their study, the propolis extract showed a strong antimicrobial activity against *Micrococcus lysodeikticus* (MIC: 15.6 µg/ml), *Bacillus cereus, Enterobacter aerogenes* (MIC: each 31.3 µg/ml), *Corynebacterium equi, Mycobacterium phlei, Thermoactinomyces intermedius, Arthroderma benhamiae and Microsporum gypseum* (MIC: each 62.5 µg/ml), and a very weak antimicrobial activity against enterobacters, such as *Bifidobacterium, Lactobacillus, Eubacterium,* and *Bacteroides.*

This antimicrobial spectrum agreed with the results obtained in the case of German propolis, studied by J. METZNER et al.[10], and of American propolis, studied by L.A. LINDENFELSER[11], in which the antimicrobial activity, was recognized against 7 strains of bacteria such as *Micrococcus lysodeikticus* and *Bacillus cereus,* and *Arthroderma benhamiae.*

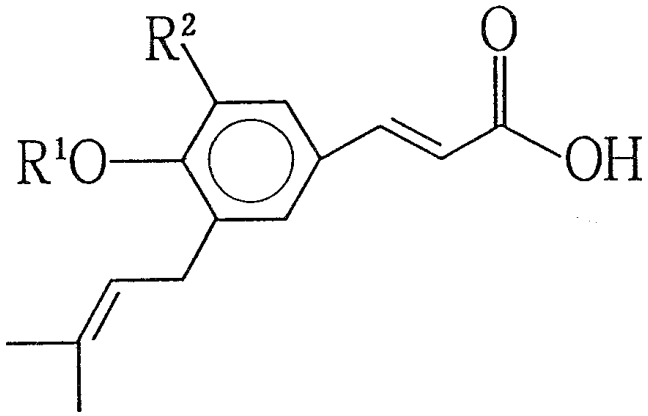
The MIC values of Brazilian propolis showed an antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus* and *Arthroderma benhamiae* which was 3 times higher than that of German propolis. These results suggest a difference in the antimicrobial substance contents and the presence of stronger antimicrobial substances. Accordingly, it is expected that an isolation and identification of these substances and a further elucidation of the mechanism of the antimicrobial action will be conducted.

2. Isolation and Identification of Antimicrobial Compounds in Propolis[6]

In a later study, AGA et al. isolated and identified three antimicrobial compounds from Brazilian propolis. As shown in Fig. 1, they identified these compounds as 3,5-diprenyl-4-hydroxicinnamic acid (as Compound 1), 3-prenyl-4-dihydro-cinnamoloxicinnamic acid (as Compound 2), and 2, 2-dimethyl-6-carboxiethenyl-2H-1 benzopyran (as Compound 3). The results of this study were published in 1994.

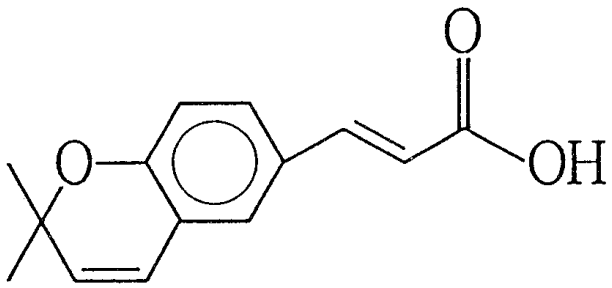
Table 1 shows the antimicrobial activity (MIC values) of these compounds against three types of microbes selected as specimens to be studied.

As shown in Table 2, in comparison to the original propolis extract, the 3,5-diprenyl-4-hidroxicinnamic acid (Artepillin C) had a stronger antimicrobial activity against cutaneous fungi (ex. *Microsporum, Arthroderma),* putrefying bacteria (ex. *Bacillus),* Corynebacterium, and pyogenic bacteria (ex. *Pseudomonas).*



Compound 1: $R^1 = H$, $R^2 = CH_2CH = C(CH_3)_2$
(3,5-diprenyl-4-hydroxicinnamic acid) (Artepillin C)

Compound 2: $R^1 = CO(CH_2)_2Ph$, $R^2 = H$
(3-prenyl-4-dihydrocinnamoloxicinnamic acid)



Compound 3:
(2,2-dimethyl-6-carboxiethenyl-2H-1-benzopyran)

Fig. 1 - Structures of Antimicrobial Compounds Isolated from Brazilian Propolis

Table 1

Antimicrobial Activity of Isolated Compounds 1-3[6)]

	Composition*	MIC (µg/ml)		
		B. cereus	*E. aerogenes*	*A. benhamiae*
Compound 1	5.2%	15.6	31.3	15.6
Compound 2	2.3%	31.3	62.5	> 250
Compound 3	0.8%	125	125	62.5
Crude propolis	-	31.3	31.3	125

** as a percentage relative to the dry solid crude propolis*

It has long been said that propolis is effective for the treatment of dermal disorders and burns. This characteristic of propolis suggests that Artepillin C plays a leading role in the antimicrobial and anti-inflammatory activities of propolis.

Later, KIMOTO et al. disclosed that Artepillin C played a principal role not only in the antimicrobial activity, but

Table 2

Antimicrobial Activity of Artepillin C[6)]

Strains	MIC (μ g/ml)	
	Artepillin C	Propolis
Microsporum gypseum (IFO 8231)	7.8	62.5
Arthroderma benhamiae (JCM 1885)	15.6	62.5
Bacillus cereus (IFO 3466)	15.6	31.3
Bacillus subtilis (ATCC 6633)	31.3	31.3
Corynebacterium equi (IFO 3730)	31.3	62.5
Micrococcus lysodeikticus (IFO 3333)	31.3	15.6
Pseudomonas aeruginosa (IFO 3453)	31.3	125
Enterobacter aerogenes (IFO 3321)	31.3	31.3
Mycobacterium smegmatis (JCM 5866^T)	31.3	500
Mycobacterium phlei (JCM 5865^T)	62.5	62.5
Staphylococcus aureus (ATCC 6538P)	62.5	250
Staphylococcus epidermidis (ATCC 12228)	62.5	500
Thermoactinomyces intermedius (JCM 3312^T)	62.5	62.5
Micrococcus luteus (IAM 1099)	125	250
Propionibacterium acnes (JCM 6425^T)	125	500
Flavobacterium meningosepticum (IFO 12535)	250	250
Kloeckera apiculata (JCM 5947)	500	500
Saccharomyces cerevisiae (IFO 0214)	500	250

also in the "anticancer action" of Brazilian propolis to be introduced below.[12)]

3. *Anti-Helicobacter Pylori Substances in Propolis*[8)]

ITOH et al., at the Zenyaku Kogyo Co. Research Institute, examined the antimicrobial activity of Chinese, Argentinian and Brazilian propolis against *Helicobacter pylori,* which connection to gastritis and gastric ulcer was suspected. Their research results were published in 1994.

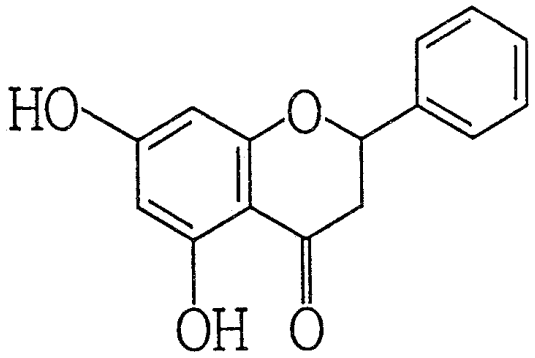
According to these results, the Argentinian propolis showed the highest antimicrobial activity with a MIC value of 50 μg/ml, followed by Chinese propolis at 100 μg/ml, and by Brazilian propolis at 200 μg/ml. They reported that eac propolis showed an anti-*Helicobacter pylori* effect.

They further isolated fractions that had anti-*Helicobacter pylori* activity from propolis by means of column chromatography and identified these fractions as pinocembrin (MIC: 12.5 μg/ml), galangin and chrysin (MIC: each 25 μg/ml). The anti-*Heliocobacter pylori* activity of pinocembrin showed an antimicrobial activity equal to that of Lansoprazole, which was used as a control (Fig. 2).

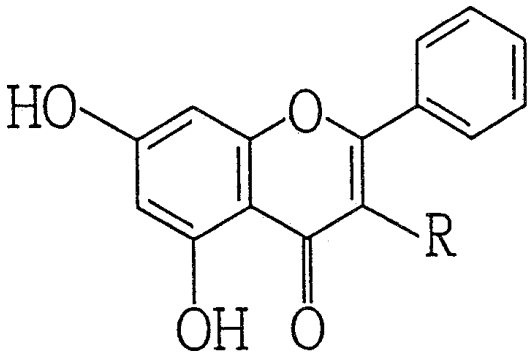
However, pinocembrin showed a lower antimicrobial activity against other microbes than *Helicobacter pylori* (MIC: 50->200 μg/ml). This suggested that one of the factors contributing to the propolis anti-ulcer effect was a specific anti-*Helicobacter pylori* activity owed to flavonoids, such as pinocembrin.

4. *Anti-MRSA Compound Isolated from Brazilian Propolis*[9)]

In 1995, NAKANO et al., from the Hayashibara Biochemical Laboratories Inc., studied the active substances



pinocembrin



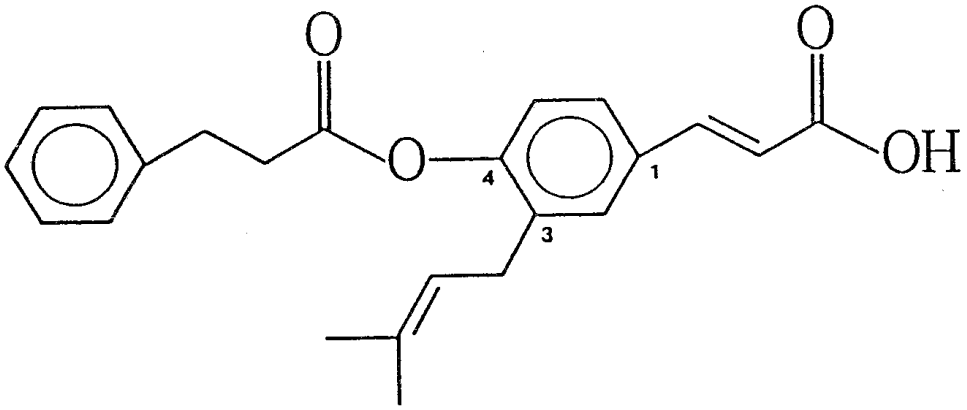
galangin (R = OH), crysin (R = H)

compound	MIC (µg/ml)
pinocembrin	12.5
galangin	25
chrysin	25

*Fig. 2 - Anti-*Helicobacter pylori *Effect of Flavonoids in Propolis*[8]

in Brazilian propolis to determine whether Brazilian propolis exhibits anti-MRSA activity.

This substance was 3-prenyl-4-dihydrocinnamoloxicinnamic acid, having the chemical structure shown in Fig. 3. As illustrated in Fig. 3, only 2 µg/ml of this substance, evaluated by the MIC method, had an anti-MRSA activity, which was 100 to 400 times higher than that of each component of the propolis ethanol extract and of propolis.



3-prenyl-4-dihydrocinnamoloxicinnamic acid

compound	MIC (µg.ml)
propolis extract	200
caffeic acid	>800
coumaric acid	>800
cinnamic acid	>800

compound	MIC (µg.ml)
galangin	>800
Kaemferol	>800
Artepillin C	200
anti-MRSA compound	2

Fig. 3 - Anti-MRSA Compound Isolated from Brazilian Propolis[9]

In 1994, this substance was isolated from Brazilian propolis by AGA et al.[6] (Hayashibara Biochemical Laboratories), as an antimicrobial substance, together with Artepillin C. At the time, however, no study was made on the anti-MRSA activity.

As regards the studies on the anti-MRSA activity of propolis made so far, GRANGE et al.[13] reported a relatively strong anti-MRSA activity of the extract of French propolis in 1990. An activity of the same degree as that in this report was recognized in Brazilian propolis.

The anti-MRSA activity of propolis is considered to be due to the synergetic effect of complex components in propolis. During their work of isolation, the fraction containing this 3-prenyl-4-dihydrocinnamoloxicinnamic acid was the most active, so NAKANO et al. estimated that this substance was the main component of the anti-MRSA activity of Brazilian propolis.

Findings on the anti-microbial activity of propolis and of isolated substances against specific microbes in Japan have been described herein. It is interesting that, in addition to the known effect of flavonoids, both Artepillin C, reported by AGA et al., and New Clerodane Diterpenoid found by MATSUNO as an antitumor substance have strong antimicrobial activities.

We expect that new substances with antimicrobial activity against specific pathogenic microbes will be discovered in propolis, and through basic research, application studies for clinical use will follow soon.

III. Immune Activating and Cytotoxic Effect of Propolis

Commercially available books and European papers[14] describe that propolis and its components are effective in inhibiting human malignant tumors and cancer cells. However, there were no papers objectively evaluating the mechanism or degree of the antitumor effects of propolis before 1990.

In Japan, MATSUNO[2)7)] from the National Health Institute, ARAI et al.[15], KIMOTO et al.[12] from the Hayashibara Biochemical Laboratoires Inc., and SUZUKI et al.[16] from the Suzuka College of Technology published the results of their studies on propolis and on some of its isolated or fractionated active components, between 1991 and 1996.

1. Cytotoxic Effect of New Clerodane Diterpenoid Isolated from Brazilian Propolis[7]

In 1990, MATSUNO from the National Health Institute found that the ethanol extract of Brazilian propolis transformed human hepatic carcinoma cells and uterine carcinoma cells cultured *in vitro,* and that it inhibited their growth.

Afterwards, he orally administered a large quantity of propolis drink to one of his relatives, who had uterine cervix cancer and was unable to undergo surgery because of her poor physical strength. He also continuously applied the propolis ethanol extract directly on the affected part. As a result, the lesion became a burn-like scar several weeks later, and her uterine cervix cancer disappeared[2].

Since then, he started to study the isolation and purification of the antitumor active substances contained in propolis, and observed the cytotoxic effect of the isolated fractions substances on human hepatocellular carci-

noma, HuH13. As a result, he found this effect in the following three substances two of which were known as quercetin and caffeic acid phenethylester, the third being a new compound of the clerodane diterpenoid type. He reported his findings at the 50th Japanese Cancer Association Congress in 1991.

Clerodane diterpenoid, in particular, was very active in destroying tumor cells, especially human cervical carcinoma cells (HeLa cells) and Burkitt's Lymphoma cells, in addition to HuH13.

His findings suggested that this substance showed selective toxicity to tumor cells, stopped the cell growth cycle in the gene synthesis phase (S phase), changed the properties of the cell membranes and finally killed these cells, by disturbing their ionic permeability.

Although the details of the mechanism are still being analysed, this substance acts on tumor cells in phase S, when the tumor cells are growing more actively than the normal cells and the genes are synthesized rapidly. Therefore, MATSUNO assumes that tumor cells are ultimately destroyed, because they are growing at a different speed.

The effects of clerodane diterpenoid on HuH13 and on two normal cells (non-transformed primary rabbit kidney cells and human diploid fibroblast cells) were examined. As a result - as shown in Fig. 4 - a large difference between the effects of different concentrations of this substance was found, and it was proved that an appropriate concentration of this substance could kill tumor cells without affecting the normal cells.

These results emphasized the possibility that a new treatment, which could kill tumor cells exclusively, without damaging normal cells, could be developed by determining an appropriate concentration and administration method for this substance. MATSUNO confirmed the effects of this treatment on patients suffering from cancer and made public the details of his experience at the 51st Japanese Cancer Association Congress.

2. Biological Effects of Propolis on the Macrophage Function and the Tumor Metastasis[15)17)]

ARAI et al. from the Hayashibara Biochemical Laboratoires Inc. has begun to study the biological activity of Brazilian propolis from various perspectives since 1990. In order to make the propolis easy to use, they powdered the propolis extract, by using anhydrous maltose and tried to confirm the effectiveness and mechanism of a BRM-like substance, aiming at isolating and identifying this substance.

This propolis powder was dispersive in water, was free of endotoxin that activates the macrophages and contained 13.8% propolis-derived solids, so that the concentrations during the experiment were all expressed in terms of propolis powder.

They discovered a macrophage activation phenomenon related to the immune function of living organisms, in 1993, published the results of their minute studies on the effects of propolis on macrophage spreading, phagocytosis, motility, and cytokine production[17)].

In 1994, they made public its inhibitory effect on lung metastasis in mice[15].

After adding a culture medium containing propolis powder solution to the abdominal macrophage obtained from BALB/c mice, a stretching was observed (Fig. 5). This phenomenon, correlated to the propolis concentration and the time, led to the determination of a dose reaction.

Similarly, the effects of this propolis on phagocytosis and motility, on the TNF production in the presence of LPS (lipopolysaccharide; pyrogen) on the inhibition of the cytotoxic factor NO (nitrogen oxides) production were studied. As a result, it was revealed, *in vitro,* that the effects depended on the concentration and the time, or on the presence or absence of an LPS stimulus. Moreover, *in vivo,* though the TNF production in the mice blood was increased by an LPS stimulus, by administering 0.2 mg and 2.0 mg propolis/mouse, 3 hours before an LPS stimulus, ten times more TNF could be produced.

Propolis, as a BRM-like substance activated macrophages, but propolis by itself did not induce the cytokine production *in vivo.* However the cytokine production was sharply accelerated by an LPS stimulus. These results suggest the activation effect of propolis on the immune cells which produce cytokines.

Prior to a test on the inhibition of tumor metastasis by propolis, its effects on the growth of mouse colon carcinoma cell (Colon 26) were studied. As shown in Fig. 6, this propolis inhibited the growth of the cells in a concentration-dependent way and had a direct effect.

Next, 40 µg of propolis were administered to BALB/c mice (7 weeks - old females), to which Colon 26 cells were then transplanted. Various concentrations of propolis were continuously administered for 6 days and the metastatic lesions in the lung were counted 14 days later. As shown in Table 3, the optimal dose of propolis was determined. Lung metastases were reduced to 80% in the group receiving 0.1 mg propolis powder, to 57% in the 0.2 mg group, and to 70% in the group receiving 0.4 mg.

Table 3

Inhibition of the Lung Metastasis of Mouse Colon Carcinoma (Colon 26) under the Influence of Propolis[16]

Group	Dose i.v./mouse	Number	Colonies Average ± SE
Propolis powder	0.1 mg/0.2 ml	13	94.7 ± 13.7
	0.2 mg/0.2 ml	13	66.1*±9.6
	0.4 mg/0.2 ml	13	81.0±13.3
Anhydrous maltose	0.4 mg/0.2 ml	13	134.0 ± 6.1
Physiological saline	0.2 ml	13	115.9 ± 15.4

*grafted cells: $5x10^3$/mouse, *; $p < 0.05$*

Based on these results, they assumed the following. The dose of propolis administered to the mice was too small to directly inhibit cell growth *in vivo.* The administration of propolis activated the immune cells, mainly macrophages, and inhibited and reduced the implantation of metastatic

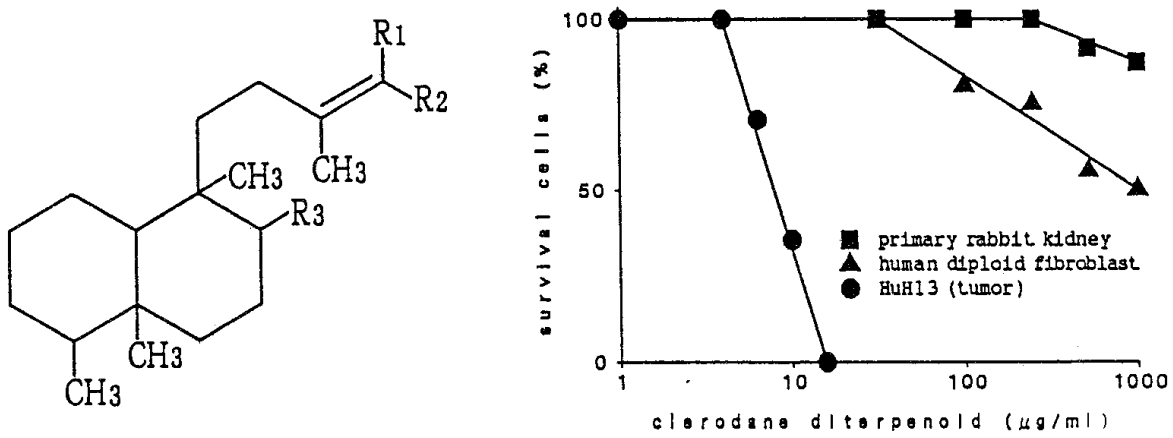


Fig. 4 - Structure and Cytotoxic Effects of New Clerodane Diterpenoid Isolated from Brazilian Propolis

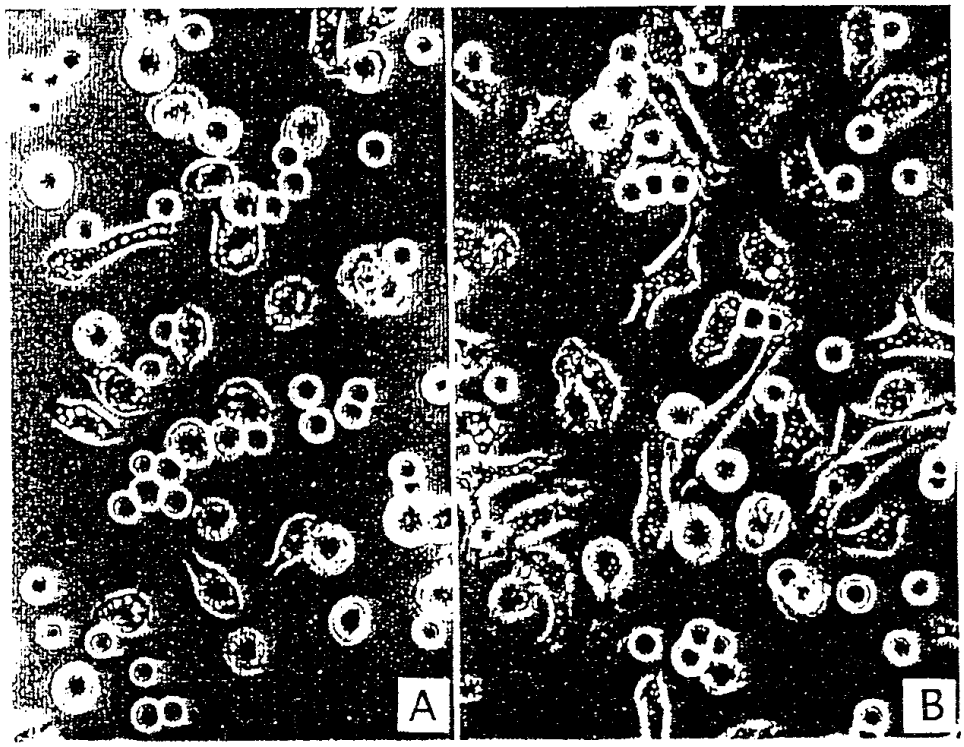


Fig. 5 - Effect of Propolis on Macrophage (BALB/c mice)[15]
A: control; B: cells treated with 0.25 mg/ml propolis powder after 3 hours of contact

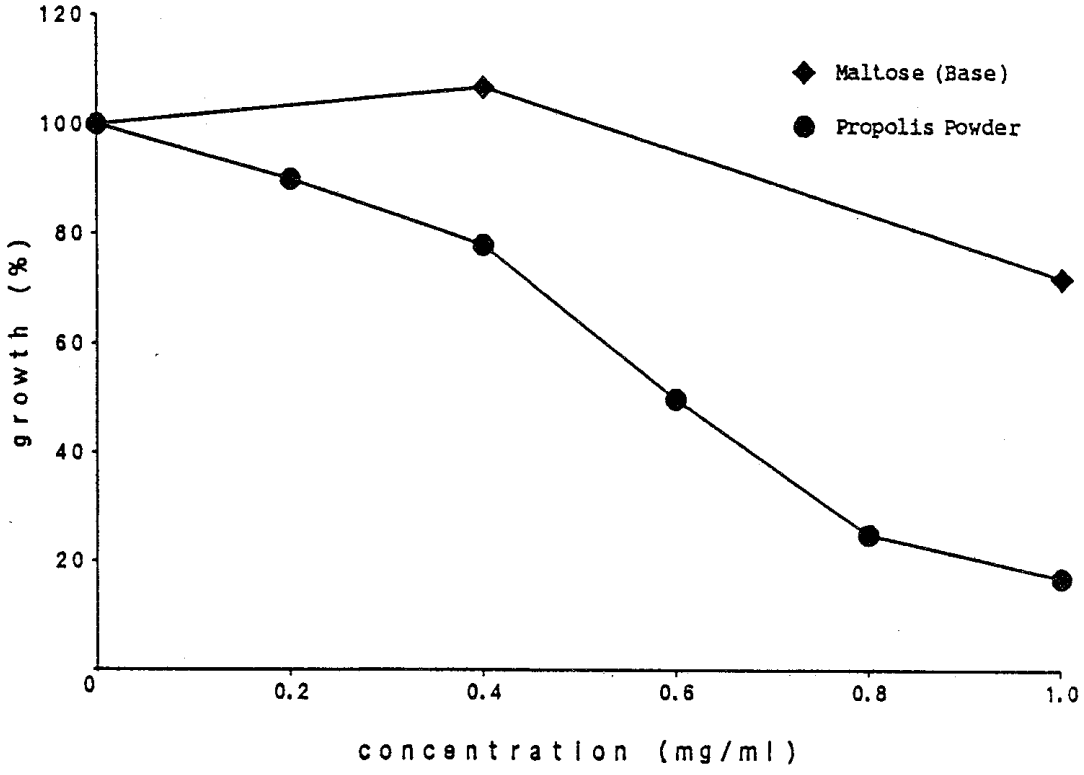


Fig. 6 - Effect of Propolis Powder on the Growth of Colon Carcinoma (Colon 26) Cells (in Vitro)[15] *in Mice*

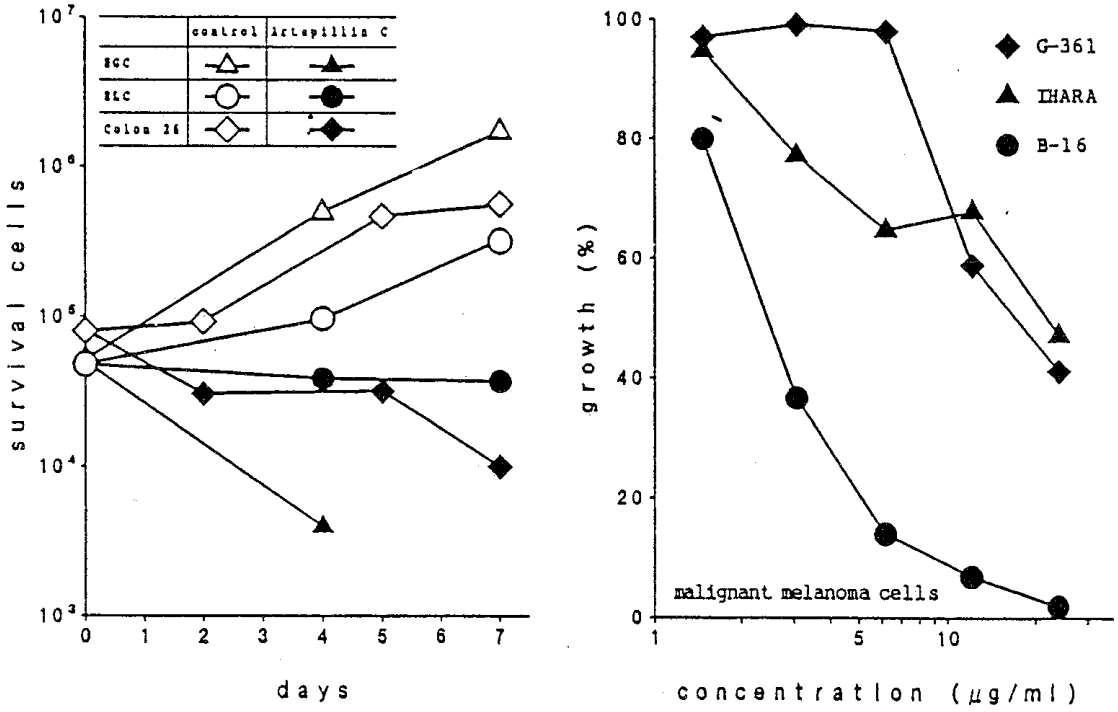


Fig. 7 - Effect of Artepillin C on the Growth of Various Carcinoma Cells (in Vitro)[12]

tumor cells as foreign matter to lung tissues, leading to a reduction in the number of metastatic lesions.

3. *Cytotoxic Effect of Artepillin C Isolated from Brazilian Propolis*[12]

Three substances isolated from Brazilian propolis and identified by AGA et al[6]., especially Artepillin C, have a stronger antimicrobial activity than the crude propolis.

The above-mentioned study on the antitumor effect of propolis, made by ARAI et al., suggested the presence of a substance in propolis that was effective in killing tumor cells. This study also suggested that clerodane diterpenoid, an antitumor substance in propolis discovered by MATSUNO, also had a relatively strong antimicrobial activity[7].Stimulated by these facts, KIMOTO et al. examined the antitumor effect of these three antimicrobial substances, and found that only Artepillin C had a strong cytotoxic effect on various cultured tumor cells and transplanted carcinoma cells. They published their results in 1995[12].

Artepillin C showed a noticeable inhibitory effect on the growth of the 18 types of cultured tumor cells tested, with a dose of only 10 - 100 µg/ml. Its cytotoxic effect was especially eminent in rapidly growing cells.

Fig. 7 shows the effects of Artepillin C on human gastric carcinoma cells (HGC), human lung cancer cells (HLC), and mouse colon carcinoma cells (Colon 26), when 100 µg/ml of Artepillin C were added to cultures. Fig. 7 also presents the effects of Artepillin C, at different concentrations, on malignant melanoma cultured cells (G-361, IHARA and B-16). Aartepillin C demonstrated a conspicuous effect in inhibiting the growth of these cells.

Fig. 8 sets out the effects of 100 µg/ml of Artepillin C on HLC (Fig. 8-a and -b) and HTSA (Fig. 8-c and -d). Fig. 8-a and -c are microscopic photos showing cells cultured without treatment. Fig. 8-b and -d show cells on which Artepillin C was administered and which were cultured for 24 - 48 hours. Severe cell injury (Fig. 8-b) and cell necrosis (Fig. 8-d) can be observed.

Though Artepillin C is not soluble in water, newly developed water-soluble [Artepillin C]-Na was used to examine its antitumor effect. The results of this examination are shown in Fig. 9 and Fig. 10.

[Artepillin C]-Na was more effective than Artepillin C in its anticancer effect, resulting from the DNA synthesis inhibition of human leukemia cells (HL-60 and THP-1) and malignant lymphoma (U937) (Fig. 9). Also [Artepillin C]-Na was more effective than Artepillin C as regards the antitumor effects, causing cell death and growth inhibition of hepatocellular carcinoma cells (rat-derived; HTSA and RL-34, human-derived; PLC/PRF/5), and human larynx carcinoma cells (KB) (Fig. 10).

Next, *in vivo,* 500 µg of Artepillin C were intravenously administered for 4 weeks every other day to nude mice transplanted with HLC, HGC and PLC/PRF/5 as xenografts, and Colon 26 and HTSA as allografts. Fig. 11 shows the growth inhibition of the transplanted carcinoma cells, and the histopathologic findings.

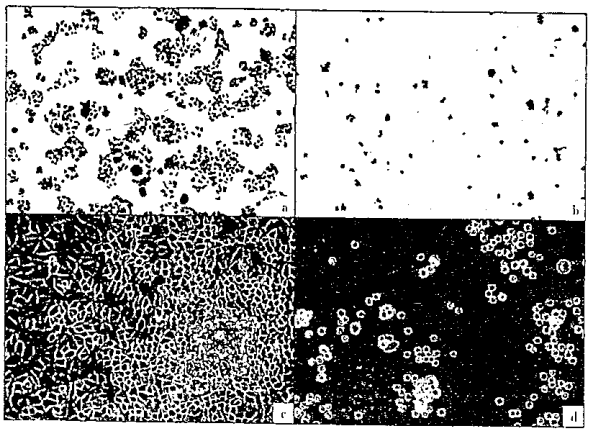


Fig. 8 - Growth Inhibition of Carcinoma Cells by Artepillin C (100 µg/ml) (in Vitro)[12]
a, b: human lung cancer cells (HLC) (24 hours) [a: control; b: added];
c, d: rat hepatocellular carcinoma cells (HTSA) (2 days) [c: control; d: added]

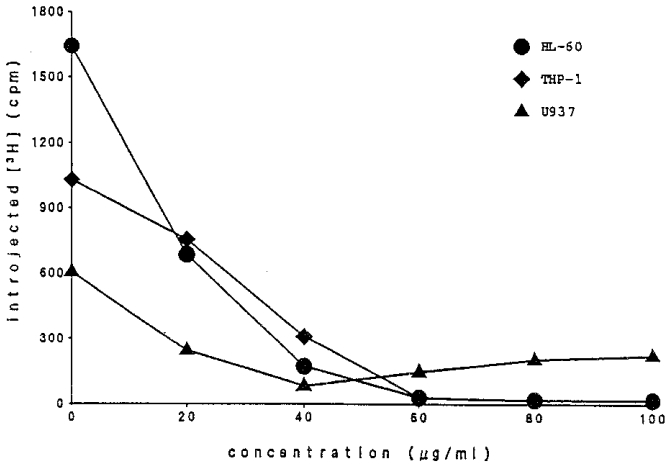


Fig. 9 - Inhibition of DNA Synthesis in Leukemia Cells by [Artepillin C]-Na[12]

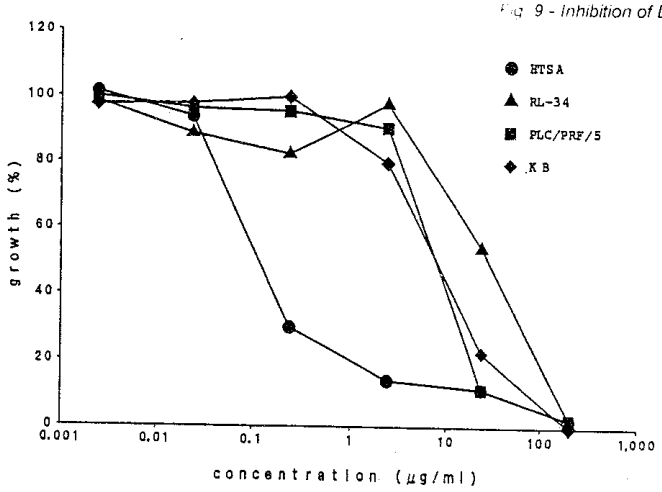


Fig. 10 - Effect of [Artepillin C]-Na on the Growth of Various Carcinoma Cells (in Vitro)[12]
a, b: after 200 days grafted human lung cancer (HLC) cells [a: control; b: 500 µg of Artepillin C injected for 4 weeks]
c-g: pathologic observations in specimen b [c: after 155 days; d-g: after 132 days]

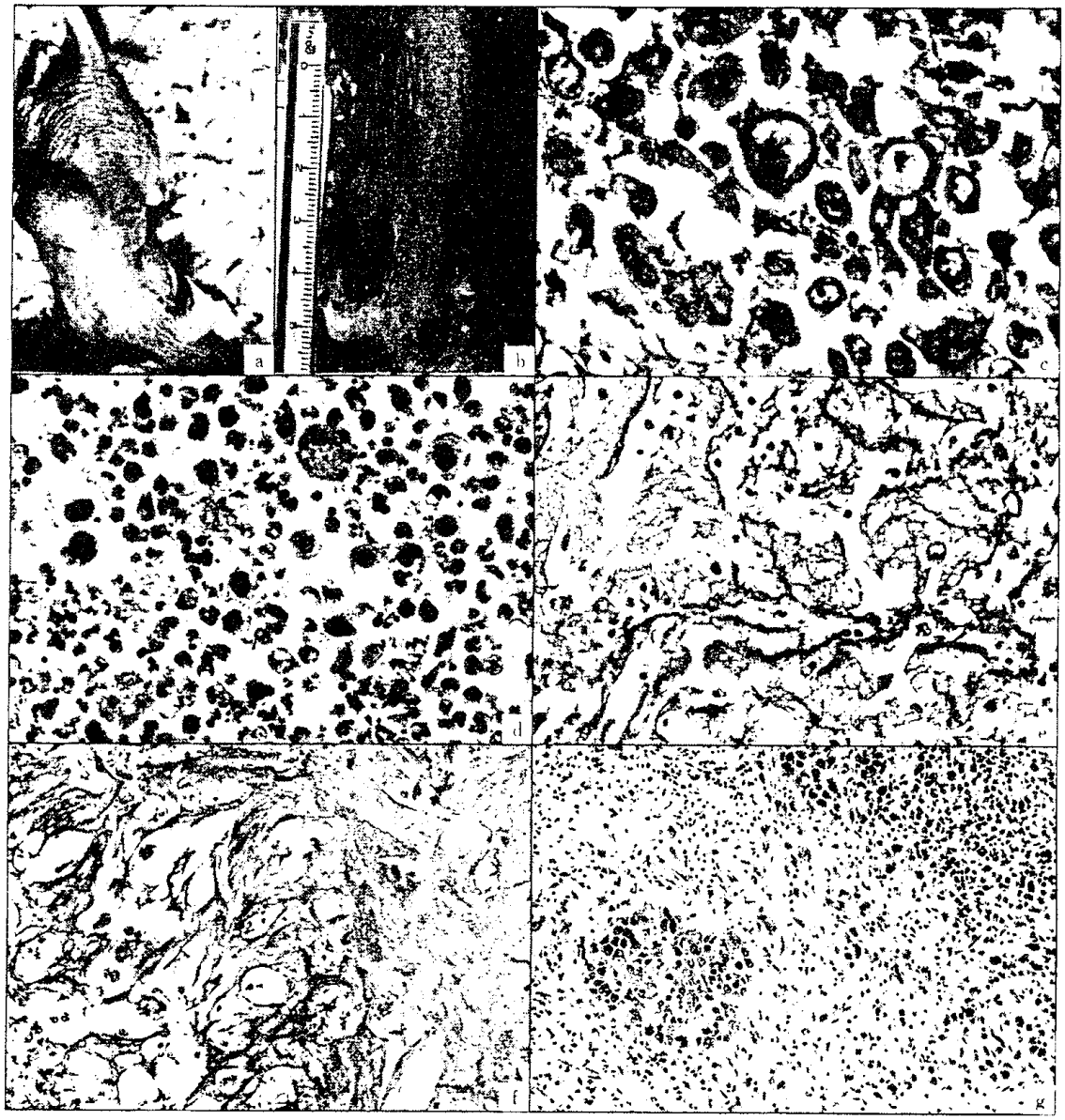


Fig. 11 - Cytotoxic Effect of Artepillin C on Grafted Carcinoma in Nude Mice[12]

As a result, nuclear degeneration (caryolysis and pycnosis) and apotosis-like population death were observed in these carcinoma cells (Fig. 11-c and -d). Small-population physalis turned to large-population degeneration and necrosis, which inhibited the cell growth (Fig. 11-e and -f). Afterwards, collagen, macrophages and helper cells increased around the carcinoma cells which resembled solitary islands (Fig. 11-f).

Conversely, in Fig. 11-a and -b, HLC in the mice which were administered Artepillin C separated into two small tumors, and no further swelling was observed.

These results suggest that the cytotoxicity of Artepillin C to many carcinoma cells inhibits cancer cell growth by inhibiting the DNA synthesis of carcinoma cells (Fig. 9) and by damaging the respiratory enzyme system of intracytoplasm glomerular intima (Fig. 10).

In particular, Artepillin C has a strong antitumor effect on leukemia cells and its future application as an adjuvant drug for intravenous injection chemotherapy is expected.

Several research reports in Japan on the antitumor effects of propolis have been described here. However, there is no complete agreement between propolis and the components isolated from propolis in terms of the effect on cancer cells and on the mechanism. This fact suggests that new antitumor substances and cytotoxic substances (antimicrobial substances) may be discovered in propolis in the future.

Concluding this paper, we would like to say that we expect Japanese researchers to discover new substances which are more effective in dealing with tumor cells without damaging normal cells in other phases than in the gene synthesis period (S period); for instance, in the mitotic period (M period).

Acknowledgment

I wish to thank Prof. Avshalon MIZRAHI from the Israel College of Complementary Medicine and Prof. Mitsuo MATSUKA from the Tamagawa Univ. for this opportunity to introduce "the present status of propolis in Japan"' and Mr. Satoshi IRITANI from Hayashibara for his valuable comments and support to complete this paper.

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