Extractives of kitakobusi *Magnolia kobus* DC. var. *borealis* Sarg. III.: Antibacterial and Antifungal Activity of Extractives

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キタコブシ *Magnolia kobus* DC. var. *borealis* Sarg. の抽出成分(第3報)
—抽出成分の抗菌及び抗カビ活性—

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Abstract

Investigations were carried out on the antibacterial and antifungal activity of fractionated extractives and lignans that were obtained from different organs and tissues (leaves, xylem, bark and flower buds) of kitakobusi *Magnolia kobus* DC. var. *borealis* Sarg. by solvent fractionation. Light petroleum ether-solubles (LPE-sol's) and diethyl ether-solubles (Et_2O-sol's) from different organs of the tree species was found to have relatively high antifungal activity. LPE-sol's from different organs used had the antibacterial activity. The Et_2O-sol and ethyl acetate solubles (EtOAc-sol) obtained from xylem have the highest antibacterial activity, but it was appreciably lower when compared with that of streptomycin.

Phenolic lignans in kitakobusi, especially (−) -syringaresinol, have significant antifungal activity, but a rather weak antibacterial activity.

Key words : antibacterial activity, antifungal activity, extractives, lignan, *Magnolia kobus* DC. var. *borealis* Sarg.

Received August 29, 1997. 1997年8月29日受理

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1. Introduction

Extractives of trees have been utilized widely in many fields as medicine, perfume, agricultural chemicals, dye, antiseptics etc. as well as extractives of herbaceous plant since ancient times. The wide application of tree extractives are conducted customary in spite of understanding functions and chemical structures of extractives insufficiently.

Recently, studies on the extractives of tree have been focused on physiological, pharmacological and antimicrobial activities. Hinokitiol (β-thujaplicin), one of the important component of essential oils or terpenoids in *Cupressaceae*, has not only antibacterial activities[1] but also insecticidal activity against termites and cockroaches. Apigenin, leuteolin and their glucoside, which are flavonoids, inhibit the activity of xantine oxidase that concerns gout[2]. Among phenolic compounds, lignans are most interesting because of their potential application as pharmaceuticals[3,4]. Podophyllotoxin and its glycosides have already been used as anticancer drugs[5]. However, the survey of lignans with respect to the relationship between chemical structure and biological activities of them has been insufficient.

Many parts of *Magnolia* have been utilized as herbal medicines. Bark of *Magnolia obovata* Thunberg is called as " Hou Piao in Chinese (Kouboku in Japanese)" in herbal medicines. The bark contains magnol and honokiol that are known to be effective pharmacological agents for lowering blood pressure, muscle atony and so on, in addition it may act as antioxidants[6]. Flower buds of *Magnolia* which are called as " Shin-i in Chinese " in herbal medicines are also applied as sedative agents as well as analgesic and antiphlogistic[7]. However, since the tree species producing " Shin-i " had not been well defined until three decades ago, the tree species has been confused in a field of herbal medicine. Recently, as a result of morphological and chemical investigations, " Shin-i " is referred to *M. fargesii* Cheng in China, while to *M. salicifolia* Maxim and *M. kobus* DC. in Japan[8,9]. In spite of these, investigations on the extractives of *M. kobus* DC. are rather limited[10,11].

Kitakobusi *Magnolia kobus* DC var. *borealis* Sarg. is a variety of kobusi *M. kobus* DC., and has larger leaves and flowers as compared with those of *M. kobus* DC. The tree species is widely distributed in Hokkaido, northern and middle parts of Japan-sea side in Honshu island in Japan. The flower buds of kitakobusi were also sometimes termed " Shin-i ", but the components have not been clarified. Acetone solubles from the bark of kitakobusi were reported to be effective in inhibiting the growth of some Eumycetes[12], although the study on the extractives of the tree has just started.

In this study, we isolated thirteen lignans from EtOH extractives of kitakobusi as described previously[13]. Twelve lignans were isolated from the leaves, and one was isolated from xylem. Nine among these twelve lignans isolated from the extractives of the leaves were furofuran type with 2,6-diaryl-3,7-dioxabicyco-[3, 3, 0]-octane skeleton, while the others were tetrahydrofuran type compounds. (−) -Syringaresinol was isolated from xylem. Distribution of these compounds in the tree and seasonal variation in the leaves were also reported[14]. However, the biological activity of the extractives in the tree is not clear yet. The biological activity such as antibacterial and antifungal activity

seemed to be closely related to self protection of tree against plant pathogens. This paper deals with the antimicrobial activities of fractionated extractives and isolated lignans from the tree against bacteria and fungi to clarify not only the biological significance but also to identify the active compounds in " Shin-i ". Particularly, as the objectives of this study are applications of the extractives to medical field and sanitary materials, the bacteria used in this study were typical Gram positive and negative bacteria, and the bacteria involved human disease such as food poisoning and inflammation of the lung.

2. Experiment

2.1 Fractionation of ethanol extractives

Ethanol extractives (EtOH-ext) were prepared by extraction of xylem, leaves, bark and flower buds in kitakobusi by EtOH. LPE-sol was prepared from EtOH-ext by liquid -liquid extraction after removal of chlorophyll by filtration. Similarly, Et_2O-sol and EtOAc-sol were obtained from EtOH-ext by successive extraction with Et_2O and EtOAc. EtOAc-insol denotes EtOAc-insoluble part of the EtOH-ext.

Thirteen lignans were isolated from *M. kobus* as reported previously[13]. Fig. 1 shows the trivial name and chemical structure of the lignans. Twelve lignans (I-XII) among them were isolated from leaves, and one (XIII, syringaresinol) was isolated from xylem. In addition, the occurrence of pinoresinol which had not been isolated from kitakobusi was suggested by HPLC. Since (+) -pinoresinol was found in the flower buds of *M. fargesii* Chen. (Shin-i)[15], (+) -pinoresinol (XIV) isolated from *Abies koreana* Wilson[16] was also used in the biological assays.

2.2 Antifungal assay

Antifungal assay of the extractives and the lignans was performed by the thin layer chromatographic (TLC) bioautography[17]. *Cladosporium herbarum* Fr (AHU 9262) was grown on the potato-glucose-agar medium for 7-10 days until the spores were formed sufficiently. The spores were recovered from the medium by washing with 70 ml of liquid medium which consisted of 10 ml of 30% glucose solution and 60 ml of a solution of 7g KH_2PO_4, 3g $Na_2HPO_4 \cdot 2H_2O$, 4g KNO_3, 1g $MgSO_4 \cdot 7H_2O$ and 1g NaCl in 1,000 ml H_2O. The medium was filtered with cheese cloth to give a spore suspension for TLC bioautography.

The extractives (10^5ppm, 10μl) and lignans (10^3ppm, 10μl) were developed on the silica gel plate (silica gel 60F254, thickness 0.5mm, Merck) with n-hexane : acetone (2 : 1, v/v). The spots were recorded using a UV lamp. After removal of developing solvent, the spore suspension was sprayed on the plate. The resulting plate was incubated at 25°C until the spore was spread on the plate (3 or 4 days) in a dark growing cabinet which was saturated with steam. The antifungal activity was evaluated by visible inspection after incubation. If the growth of fungus is inhibited by extractives, the spots of extractives on the plate are white. Basis of the color of the spot, the activity was classified into three inhibition zones of spore growth. A strong inhibition zone was a clear white spot. A weak zone was a pale black spot compared with the color of background, and a medium zone was a white spot but partly colored.

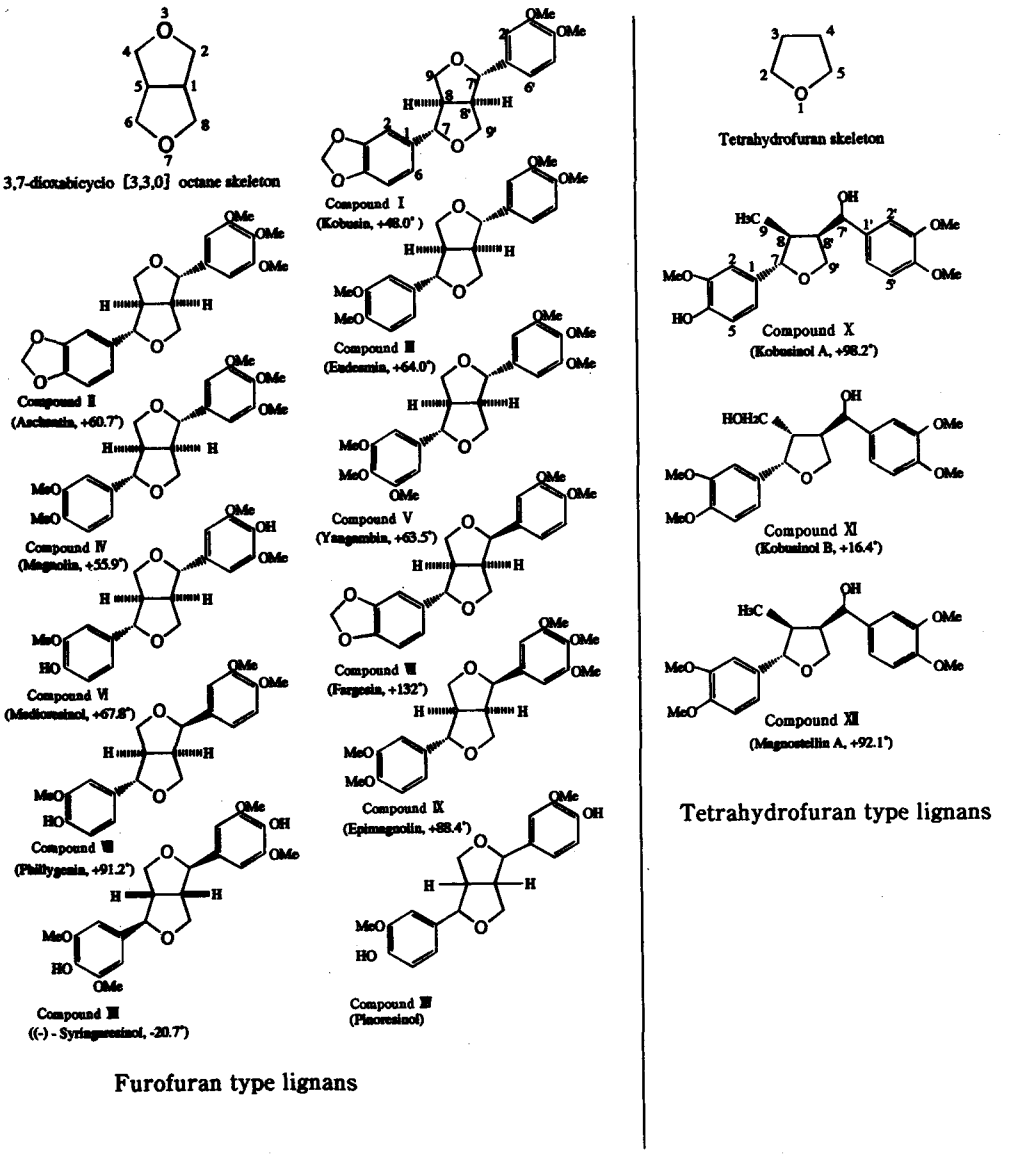

Fig. 1 Chemical structures of lignans isolated from *M. kobus* DC. var. *borealis* Sarg. The values in parentheses are $[\alpha]_D^{25}$.

2. 3 Antibacterial assay

Four bacteria, *Bacillus subtilis* Cohn. (IFO 3009), *Pseudomonas syringae* subsp. syringae van Hall. (IFO 3508), *Staphylococcus aureus* Rosenbach (IFO 12732) and *Klebsiella pneumoniae* Trevisan. (IFO 31277) were used for the antibacterial assay of the extractives.

These bacteria were preincubated in the fluid nutrient broth medium for 1 day at 30°C.

One-half ml of the preincubated culture was mixed with 15ml of sterilized nutrient agar at 46°C, then the mixture was coagulated by cooling. Fifty $\mu\ell$ of the extractives and lignans solution at 10^3, 10^4 and 10^5ppm were immersed into paper discs (8mm in diameter, 1.3mm in thickness). After removal of solvent in the disc, the disc was placed on the agar plates. After incubation for 24h, transparent region in the plate were measured by a micro caliper to evaluate the degree of the growth inhibition of bacteria. The growth inhibition was estimated by the following equation.

Growth inhibition=(Diameter of inhibition zone−Disc diameter)/2

The growth inhibition of the bacteria by streptomycin sulfate was used as reference in the evaluation of antibacterial activity assay. Streptomycin sulfate was purchased from Wako Pure Chemicals Co. Ltd.

3. Results and Discussion

3.1 Antifungal activity

Antifungal activity of extractives was evaluated by growth inhibition of *C. herbarum* using TLC bioautography.

Table 1 shows the antifungal activity of extractives fractionated with organic solvents. EtOH-ext of leaves with Rf value of 0.41, 0.48 and 0.54 had relatively high activity. These high active antifungal components were also found in the corresponding LPE-sol and Et_2O-sol with Rf value in the range of 0.40−0.57. In addition, the LPE-sol with RF value of 0.28 and 0.33 have strong antifungal activity. Other polar components of the EtOH-ext, that were fractionated as EtOAc-sol and EtOAc-insol, had a rather low antifungal activity. The EtOAc-sol from xylem showed a moderate antifungal activity. By contrast, corresponding LPE-sol and Et_2O-sol exhibit a strong antifungal activity. Except for EtOAc-insol the extractives from bark and flower buds contained components with significant activity. These components had low Rf values in the range of 0.10−0.37. These results suggest that the components of extractives with antifungal activity in the LPE-sol and Et_2O-sol of leaves differed from those of the extractives from the other organs.

In general, LPE-sol seemed to be mainly essential oils containing monoterpenoids, sesquiterpenoids and aromatic compounds. Hence, an attempt to identify active compounds in LPE-sol's was conducted by means of TLC using authentic monoterpenoids as reference. Fig. 2 shows the thin layer chromatograms of LPE-sol from the bark and the reference monoterpenoids, where small spots in the chromatograms of compound nos. 2−6 were attributable to contaminants due to the long storage period of authentic compounds. Hydrocarbons in the monoterpenoids had Rf values in the range of 0.80−0.95, while monoterpenes with hydroxyl and carbonyl groups in the range of 0.60−0.70 and 0.45−0.50, respectively. Since the extractives with strong antifungal activity in LPE sol's from Kitakobusi had Rf values in the range of 0.26−0.48, it was assumed that most of the active extractives were not monoterpenoids except for the monoterpene with carbonyl group.

Lignans are present in Et_2O-sol. Hence, antifungal activity of fourteen lignans was surveyed to identify the compounds with the activity in Et_2O-sol. As shown in Table 2,

Table 1 Antifungal activity of ethanolic extracts against *Cladosporium herbarum*.

Fraction	Leaves		Xylem		Bark		Flower buds	
	Rf	Inhibition	Rf	Inhibition	Rf	Inhibition	Rf	Inhibition
EtOH-ext	0.54	++	0.44	++	0.57	+		
	0.48	+++	0.34	+	0.44	+		
	0.41	++	0.25	++	0.38	+		
	0.37	+	0.18	+	0.33	+++	0.33	++
	0.32	+	0.10	+	0.32	+	0.23	++
	0.19	+	0.05	+	0.25	++	0.14	++
	~0.07	+	~0.02	+	0.21	++	0.09	+
					0.17	+	~0.05	+
					0.13	+		
					~0.05	+		
LPE-sol	0.57	++	0.82	+	0.74	+	0.88	+
	0.48	+++	0.53	+	0.59	+	0.83	+
	0.40	+++	0.43	++	0.52	+	0.54	+
	0.33	+++	0.31	++	0.36	+	0.46	+
	0.28	+++	0.26	+++	0.31	+++	0.41	+
	0.19	+	0.18	+	0.28	++	0.37	+++
	0.13	++	0.13	+	0.22	++	0.32	++
	~0.04	+	~0.05	+	0.18	+	0.22	++
					~0.07	+	~0.07	+
Et_2O-sol	0.53	++	0.45	+	0.46	+		
	0.50	++	0.40	+	0.41	+		
	0.44	+++	0.47	+	0.36	+	0.34	+++
	0.40	++	0.42	++	0.32	+++	0.28	++
	0.36	+	0.30	+	0.28	+	0.21	++
	0.32	+	0.25	+++	0.24	+++	0.14	++
	0.28	+	0.13	+	0.20	++	0.10	++
	0.21	+	0.06	+	0.17	++	~0.04	+
	0.13	++	~0.02	++	0.14	+		
	~0.03	+			~0.07	+		
EtOAc-sol	0.50	+	0.44	+	0.30	++	0.33	++
	0.45	+	0.30	+	0.27	+	0.24	++
	0.40	+	0.26	++	0.20	++	0.15	+
	0.17	+	0.14	+	0.15	+	~0.06	+
	~0.04	+	0.06	+	~0.08	+		
			~0.03	+				
EtOAc insol	0.29	+					0.33	+
	~0.05	+	~0.06	+	~0.07	+	~0.08	+

+++ : strong inhibition zone, ++ : medium inhibition zone,
+ : weak inhibition zone.

the Rf values of lignans were from 0.03 to 0.34. Syringaresinol (XIII, Rf=0.20) showed the highest activity among the lignans. Kobusin (I, Rf=0.34), medioresinol (VI, Rf=0.09), phillygenin (VIII, Rf=0.06), kobusinol A (X, Rf=0.27) and pinoresinol (XIV, Rf=0.16) revealed moderate activity. Since kobusin occupied 6.1% of total lignans from the flower buds and 1.4% from the bark, Et_2O sol with Rf values of 0.34 from the buds and EtOH-ext with Rf value of 0.33 might be assigned to kobusin. Similarly, Et_2O sol with Rf values of 0.20 from the bark might be syringaresinol. Thus, it is likely that a part of lignans in Kitakobusi involves in the antifungal activity.

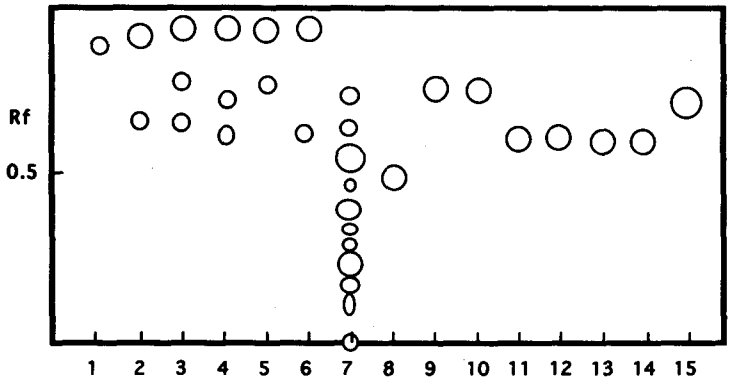

Fig. 2 Thin layer chromatograms of monoterpenes and LPE-sol from the bark of *M. kobus* DC. var. *borealis* Sarg. 1: camphene, 2: α-pinene, 3: *dl*-limonene, 4: 3-carene, 5: β-pinene, 6: *d*-limonene, 7: LPE-sol, 8: fenchone, 9: *d*-camphor, 10: isobornyl acetate, 11: α-terpineol, 12: (+)-terpinen-4-ol, 13: citronellol, 14: nerol, 15: α-tocopherol. Developing solvent was n-hexane: acetone (2 : 1, v/v).

Table 2 Antifungal activity of lignan against *Cladosporium herbarum*.

Lignans		Rf	Inhibition
Kobusin	I	0.34	++
Aschantin	II	0.34	+
Eudesmin	III	0.25	+
Magnolin	IV	0.22	+
Yangambin	V	0.24	+
Medioresiol	VI	0.09	++
Fargesin	VII	0.07	+
Phillygenin	VIII	0.06	++
Epimagnolin	IX	0.34	+
Kobusinol A	X	0.27	++
Kobusinol B	XI	0.11	+
Magnostellin A	XII	0.03	+
Syringaresinol	XIII	0.20	+++
Pinoresinol	XIV	0.16	++

+++ : strong inhibition zone, ++ : medium inhibition zone,
+ : weak inhibition zone.

Compounds VI, VIII, X, XIII and XIV with a significant antifungal activity had free phenolic hydroxyl groups. Thus, the functional group seemed to be involved in the antifungal activity. However, kobusin (I) that did not have the group also possessed moderate activity. It is necessary to investigate further relationship between the chemical structure and the antifungal activity.

3. 3 Antibacterial activity

Antibacterial activity of the extractives and lignans from kitakobusi was investigated against four bacteria, among which two bacteria, *B. subtilis* and *S. aureus*, were Gram positive bacteria, and the others, *P. syringae* and *K. pneumoniae*, were Gram negative

bacteria. Streptomycin sulfate was used as the standard for determining inhibitory activity against all the bacteria.

As shown in Table 3, the EtOH-ext's from leaves, xylem and bark indisputably showed antibacterial activity against the bacteria except for *K. pneumoniae*. The antibacterial activities of the Et_2O-sol and EtOAc-sol from xylem were remarkably high, and those of LPE-sol and EtOAc-sol from leaves relatively high. Among EtOAc-insol's, by contrast, only the one from xylem showed appreciable antibacterial activity, but not the others. The LPE-sol from flower buds also had a rather low antibacterial activity. Thus, certain fractionated extractives indicated the antibacterial activity, but the levels were significantly lower than that of streptomycin.

Table 3 Antibacterical activity of ethanolic extracts.

		Bacillus subtilis	Pseudomonas syringae	Staphylococcus aureus	Klebsiella pneumoniae
Leaves	EtOH-ext	0.4	0.4	0.3	0.2
	LPE-sol	1.5	2.6	2.0	1.3
	Et_2O-sol	0.5	0.7	0.5	×
	EtOAc-sol	1.2	2.0	1.2	×
	EtOAc-insol	×	×	1.3	×
Xylem	EtOH-ext	2.6	2.3	2.1	×
	LPE-sol	1.5	1.0	×	×
	Et_2O-sol	2.2	1.9	1.9	×
	EtOAc-sol	2.9	2.8	2.5	×
	EtOAc-insol	1.8	2.0	1.8	×
Bark	EtOH-ext	1.7	1.0	1.0	×
	LPE-sol	2.1	2.4	2.0	×
	Et_2O-sol	0.8	0.8	0.5	×
	EtOAc-sol	0.3	0.4	0.3	×
	EtOAc-insol	×	×	×	×
Flower buds	EtOH-ext	×	×	×	×
	LPE-sol	0.3	0.2	0.3	×
	Et_2O-sol	×	×	×	×
	EtOAc-sol	×	×	×	×
	EtOAc-insol	×	×	×	×
	Streptomycin	16.0	15.5	16.0	9.0

The assay was performed by the paper disc method. The disc contained 50 μL of 10^5ppm extractives. The values reveal radii of the growth inhibition area of bacteria and the unit is mm. x: no inhibition area

Since the antibacterial activity of streptomycin against *K. pneumoniae* was appreciable lower than those against the other bacteria, *K. pneumoniae* seemed to be a strong resistant bacterium against the antibiotics. LPE-sol from leaves had indisputable antibacterial activity against *K. pneumoniae*. Hence, LPE-sol's, especially LPE-sol from leaves, may be usable as a weak antibiotics.

Although the lignans showed appreciable antifungal activity evenly at 10μg (10^3ppm, 10μl) in a spot, they did not show any antibacterial activity at 50μg (10^3ppm, 50μl) in a disc. The activity appeared at 500μg (10^4ppm, 50μl) in a disc. The difference in the amount of lignans for detectable activity may be attributable to the difference in the interaction of

lignans with microorganism in addition to the methods of assay. Table 4 shows the antibacterial activity of the lignans investigated. Kobusinol B (XI) and syringaresinol (XIII) had rather low antibacterial activity against the bacteria except *K. pneumoniae*. Yangambin (V) had antibacterial activity only against *B. subtilis*, and fargesin (VII) against only *K. pneumoniae*. Pinoresinol (XIV) had low antibacterial activities against *P. syringae* and *K. pneumoniae*. However, their antibacterial activities were much lower than that of streptomycin. Thus, the resistance of lignans against the bacteria is likely to be very low.

Table 4 Antibacterial activity of lignans.

Lignans		*Bacillus subtilis*	Pseudomonas syringae	Staphylococcus aureus	Klebsiella pneumoniae
Eudesmin	I	×	×	×	×
Magnolin	II	×	×	×	×
Yangambin	III	0.3	×	×	×
Fargesin	IV	×	×	×	0.4
Kobusinol B	V	0.2	0.6	0.2	×
Syringaresinol	VI	0.5	0.4	0.2	×
Pinoresinol	VII	×	0.1	×	0.2
Streptomycin		7.0	6.5	6.7	6.4

The assay was performed by the paper disc method. The disc contained 50 μL of 10^4ppm lignans. The values reveal radii of the growth inhibition area of bacteria and teh unit is mm. x : no inhibition area.

With respect to chemical structures of lignans, pinoresinol (XIV) and syringaresinol (XIII) have free phenolic hydroxyl groups, while other active lignans, yangambin (V), fargasin (VII) and kobusinol B (XI) do not have any free phenolic hydroxyl groups. Therefore, free phenolic hydroxyl groups do not necessarily involve in the antimicrobial activity.

In this study, the extractives and lignans from kitakobusi showed indisputable antimicrobial activities, but very low antibacterial activity. LPE-sol among the extractives of the flower buds indicated weak antibacterial activity. The fraction was consistent with the active components (essential oil) of " Shin-i " described previously[18]. Taking the low antibacterial activity and pharmacological activity of " Shin-i " in consideration, however, " Shin-i " does not seem to attack microorganisms that cause inflammation but might affect the neural system.

Phenolic lignans indicated the antifungal activity, whereas relationship between antibacterial activity and the lignans could not be clarified. The lignans with pharmacological activity were reported to have no free phenolic hydroxyl group[19,20]. The hydroxyl groups were substituted by methoxyl groups. Therefore, it is difficult to define the biological significance of lignans in connection with phenolic hydroxyl groups. In this conclusion with respect to the lignans, they must not be antibiotics because of lower antibacterial activities compared with streptomycin. Now, further investigations are conducted to clarify the relationship between antimicrobial activity and other tree

extractives.

Acknowledgement.

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 06556026) from Ministry of Education, Science and Culture, Japan. The authors are grateful to Dr. Chen-Loung Chen of Department of Wood and Paper Science, North Carolina State University, for his valuable advice. The authors are also thankful to the Laboratory of Applied Microbiology, Faculty of Agriculture, Hokkaido University, for the donation of the fungus, *C. herbarum*.

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要　　約

コブシ *Magnolia kobus* の花蕾は，漢方薬の辛夷として鼻炎などの処方に用いらていれる。コブシの変種であるキタコブシ *Magnolia kobus* DC var. *borealis* Sarg. は，北海道や本州の日本海側の中部・北部に分布して，その花蕾もまた辛夷といわれている。しかし，キタコブシの抽出成分に関する研究は少なかった。我々は前報で，キタコブシの葉から12種及び木部から1種のリグナンを単離して，各部位のリグナンの季節変動を報告した。

本研究では，キタコブシの各部位（木部，樹皮，葉，花蕾）の連続溶媒抽出物及びリグナンの生理活性を解明するため，特にこの報告では医薬品としての有用性を明らかにするために，病原菌やカビ等に対して抗菌・抗カビ活性を検討した。供試抽出成分は上記の抽出物の他にHPLCで存在が確認されたpinoresinolを用いて，抗菌試験は，ペーパーディスク法により，抗カビ試験はバイオオートグラフィー法により行った。

各器官とも石油エーテル可溶部とジエチルエーテル可溶部に抗カビ活性が見出された。抗菌活性は，各器官の石油エーテル可溶部に見られた。木部ではジエチルエーテル可溶部と酢酸エチル可溶部が最も高い抗菌活性を示したが，抗生物質ストレプトマイシンの抗菌活性に比べかなり低かった。

キタコブシ中のリグナンは，いずれも抗カビ活性を示したが，特に（−）-syringaresinolに強い活性が認められた。抗菌活性では，kobusinol Bとsyringaresinolが枯草菌，緑膿菌，黄色ブドウ球菌に活性を示したが，ストレプトマイシンよりかなり低かった。fargesinやpinoresinolは，肺炎桿菌に対して弱い活性を示した。以上から，キタコブシ中のリグナンには抗菌活性を示すものがあるが，その活性は抗生物質ほど高くないことが示された。

リグナンの化学構造と抗菌活性との関係では，フェノール性水酸基を有するpinoresinolとsyringaresinolが抗菌活性を示したが，フェノール性水酸基を有しないyangambin，fargasinとkobusinol Bも抗菌活性を示した。このことから，抗菌活性発現には必ずしもフェノール性水酸基が必要でないことが示唆された。