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(12) (19) (CA) **Brevet-Patent**

(11) (21) (C) **2,106,176**
(22) 1993/09/14
(43) 1994/03/17
(45) 2001/02/13

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(51) Int.Cl.[5] C11B 3/10, A61K 31/23
(30) 1992/09/16 (92110677.7) CN
(30) 1992/09/17 (92110839.7) CN
(30) 1993/01/01 (93100735.6) CN

(54) **LIPIDES NEUTRES A PARTIR DU NOYAU DE LACRYMA-JOBI**
(54) **NEUTRAL LIPIDS FROM KERNEL OF JOB'S TEARS**

(57) The present invention concerns anti-tumor neutral lipids of the kernel of Job's tears (Coix lacryma-jobi), their extraction and refinement from the kernel of Job's tears, and a pharmaceutical composition containing the neutral lipids. The neutral lipids of the kernel of Job's tears and their pharmaceutical compositions are useful as anti-tumor agents, and effective for enhancement of immunity and protection from many diseases.

Abstract of the Disclosure

The present invention concerns anti-tumor neutral lipids of the kernel of Job's tears (Coix lacryma-jobi), their extraction and refinement from the kernel of Job's tears, and a pharmaceutical composition containing the neutral lipids. The neutral lipids of the kernel of Job's tears and their pharmaceutical compositions are useful as anti-tumor agents, and effective for enhancement of immunity and protection from many diseases.

The present invention concerns neutral lipids with anti-tumor effect, their extraction and refinement and pharmaceutical compositions containing them. More specifically, the invention concerns neutral lipids from the kernel of Job's tears, a method to extract and refine the lipids from the kernel and an anti-tumor emulsion of the neutral lipids from the kernel of Job's tears for injection and oral administration.

The kernel of Job's tears is a dry kernel of Coix lacryma-jobi. It has been used as a traditional medicine and nourishment for thousands of years in China.

In 1961, Tyunosin Ukita and Akio Tanimura (Ukita T. et al., Chem. Pharm. Dull, 9(1):43, 1961) reported an extraction method for coixenolide as an anti-tumor element from the kernel of Job's tears and the results of its anti-tumor activity tests. They also reported the formula of coixenolide as $C_{38}H_{70}O_4$. However, a pharmaceutical preparation thereof was not mentioned. The extract was reported to inhibit the growth of mouse Ehrlich ascites tumor. Coixenolide was extracted by the following steps:

Powder of the kernel: extracted with acetone three times; extract: dissolved in petrol ether, filtered and concentrated; brown syrup: dissolved in petrol ether, run through silica gel column by petrol ether; eluate: 0.2N KOH added to separate acidic element; Neutral oil: run through alumina column and silica column to give coixenolide.

Coixenolide was obtained as a pure compound and exhibited strong anti-tumor activity. But there is no report as to its clinical application. Because of the two kinds of column chromatography (alumina and silica) used in its extraction and refinement, the productive rate of coixenolide was quite low. Thus it was very expensive to obtain and no commercial products have been developed.

In the nineteen eighties, Si Pei-hai obtained a crude oil from the kernel of Job's tears directly with petrol ether and produced an intravenous emulsion (Si Pei-hai, The Extraction of the Oil of the Kernel of Job's tears and the Preparation of its Emulsion, Zhejiang Pharmacology, 3(6):18-20, 1986). The extraction and refinement steps of the oil of the kernel of Job's tears were as follows:

Powder of the kernel of Job's tears: extracted by petrol ether three times; extract: absorption, decolouration and evaporation to give rise to the oil of the kernel of Job's tears.

The oil had a relative density of 0.9033-0.9057 (20˚C), diopter 1.4670-1.1708 (20˚C), iodine value 83-90 and acid value <36. The intravenous emulsion comprised the oil extracted from the above method (as main component), and two emulsifiers, span and tween. The composition was:

Oil from the kernel of Job's tears	10 g
Span-80	1 g
Tween-80	1.5g
Water for injection use	q.s. to 100 ml

The intratumor emulsion was just used for the research of a pharmaceutical preparation and its body distribution. There are the following obstacles to applying the intraemulsion for human clinical use:

1. The main component, the oil from the kernel of Job's tears, was not pure or safe enough to meet the requirements of the Chinese Pharmacopoeia, e.g. its acid value was controlled just below 36.

2. Tween and span can pass through cell membranes, and are lysoactive and toxic. Only suitable isoosmoticum can be used to prevent lysogenesis. Actually, most commercial intravenous emulsion products of England, Germany, United States, France, China and Japan do not use tween or span as emulsifier.

3. The above composition does not include the essential element of isoosmoticum, and is therefore ineffective.

An object of the present invention is to provide a method of extraction and refinement to obtain anti-tumor neutral lipids from the kernel of Job's tears (NLKJ). The method comprises simple steps and low cost, but provides standard neutral lipids meeting the needs of intravenous use.

Another object of the present invention is to provide a pharmaceutical composition with NLKJ, in the form of an O/W emulsion for use as anti-tumor drug. The composition is safe and suitable for oral, intravenous or intraarterial administration.

Accordingly, one aspect of the present invention provides a neutral lipid isolated from kernel of Job's tears (Coix lacryma-jobi) (abbreviated as "NLKJ") consisting essentially of glycerides and alkyl fatty acid ester, and wherein said neutral lipid has the following physio-chemical properties: acid value <0.20, iodine value 95.00-107.00, saponification value 185.00-195.00, relative density 0.915-0.918 (20˚C) and diopter 1.470-1.475 (20˚C).

Another aspect of the present invention provides a method for the preparation of NLKJ comprising the steps of: (a) extraction with an organic solvent, absorption by absorbent and decoloration; (b) alkalizing saponification; (c) demulsification by acetone; and (d) extraction by an organic solvent.

Yet another aspect of the present invention provides a pharmaceutical composition comprising NLKJ and a pharmaceutically acceptable carrier.

A further aspect of the present invention provides the use of NLKJ as a medicament for the treatment of tumours.

The NLKJ according to the present invention is a light yellow transparent liquid at room temperature. NLKJ is easily dissolved in petrol ether, ether and benzol.

It is also soluble in acetone. Its solubility decreases slightly in methanol and alcohol, and it is not soluble in water. When treated as an oil, NLKJ displays physical and chemical constants as follows: acid value <0.20, iodine value 95.00-107.00, saponification value 185.00-195.00, relative density 0.915-0.918 (20°C), and diopter 1.470-1.475 (20°C). NLKJ is comprised of triglyceride (91.48 ± 3.43%), diglyceride (1.47 ± 0.63%), monoglyceride (5.75 ± 3.19%) and alkyl fatty acid ester (1.0 ± 0.78%). The lipoclastic fatty acid residues are comprised of hexadecanoic, octodecanoic, octadecenoic and octadecadienoic acids.

The extraction and refinement of NLKJ is as follows:

Extraction by organic solvent: the organic solvent is, e.g. acetone, petrol ether, ether, alcohol or hexane. The extraction method is, e.g. percolation, filtering or seeping through the powder of kernel of Job's tears. A crude oil can be extracted with a large amount of foreign matter, e.g. free fatty acids, pigments;

Absorption and decolouration: a common absorbent is used, e.g. 1% active carbon, 3% white potter's clay, 10% alumina or others. A yellow oil will be obtained after the crude extract is absorbed;

Alkalizing saponification: after a suitable amount of alkaline solution (e.g. NaOH or KOH) is added for saponification, an emulsion is formed;

Demulsification by acetone: a suitable amount of acetone will turn the emulsion transparent;

Liquid-liquid extraction: a suitable amount of a second organic solvent, e.g. petrol ether, ether or hexane, is used for extraction and settlement; the phase containing mainly acetone (containing acidic elements, water) is discarded; the organic solvent is evaporated and a residue consisting mainly of NLKJ of the present invention will remain;

Refinement: between alkalization saponification and demulsification with acetone, a step of washing the emulsion with hot water can be added; and/or after liquid-liquid extraction, one step of decolouration and/or washing by hot water can also be added if necessary, resulting in NLKJ of high purity suitable for intravenous use.

The emulsion of the present invention can be formed as a pharmaceutical composition with NLKJ as the main component, soy (or egg) lecithin as the emulsifier and water. It may also contain glycerol, sorbitolum or the like as isoosmoticum. It can also contain other conventional anti-tumor drugs. The method of emulsification includes two conventional steps: homogenization and dispersion. Within the pharmaceutical composition the content of NLKJ can vary over a wide range. It is an O/W type emulsion. Within 100 ml of the emulsion, NLKJ ranges from 2.5 to 25 g, preferably from 5 to 25 g, more preferably 10 g; soy or egg lecithin ranges from 0.3 to 3.0 g, and glycerol ranges from 1.25 to 6 g.

The NLKJ and its pharmaceutical composition of the present invention have a high anti-tumor activity. The latter can be used especially in the therapeutic treatment of lung cancer, carcinoma hepatic and other metastasizing cancers of middle or advanced stage. It can enhance body immunity and protect the body from many diseases, thus can also be used in treating disease involving immune deficiency. Combination of NLKJ emulsion with a small dosage of chemotherapy can strengthen the anti-tumor activity and reduce the toxicity of chemotherapy.

Testing of anti-tumor activity of NLKJ and its pharmaceutical composition on human tumors will be illustrated by Example 9 and Example 10. The effects of this pharmaceutical composition on immunity, the enhancement of the combination with a small dosage of chemotherapy, and the protective effect on the decrease of leucocytes induced by chemotherapy will be illustrated in Example 11, Example 12 and Example

13, respectively. The clinical effects of this pharmaceutical composition are described in Example 14.

The compounds of this invention were administrated at a therapeutically effective dosage, i.e. the amount which, when administrated to a mammal in need thereof, is therapeutically sufficient to effectively treat tumor diseases. Administration of the active compounds herein can be by the intravenous, intraarterial or oral route.

Embodiments of the invention will be described with reference to the accompanying drawings, in which:

Fig. 1 shows graphically the effect of NLKJ on proliferation of Leukaemia P388, L1210;

Fig. 2 shows graphically the effect of NLKJ on formation of cell colony of colon tumour M7609;

Fig. 3 shows graphically the therapeutic effect of NLKJ emulsion on metastasis of melanoma (B16) in lung in vivo (every third day);

Fig. 4 shows graphically the therapeutic effect of NLKJ emulsion on metastasis of melanoma (B16) in lung in vivo (iv, every day); and

Fig. 5 shows graphically the therapeutic effect of NLKJ emulsion on metastasis of melanoma (B16) in lung in vivo (ip, every day).

The following Examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. They should not be considered as limiting the scope of the invention, but merely as being illustrative and representative thereof.

Example 1

Extraction and Refinement of NLKJ

100 Kg of powder of the kernel of Job's tears was extracted with acetone. A 5 Kg extract was obtained after the evaporation of acetone, absorbtion and decolouration. 152 g NaOH (or equivalent amount of KOH) dissolved as 5% hot alkaline solution was added for alkalizing saponification. The weight of alkali added depended on the acid value of the extract. The high temperature of the alkali made the saponification more rapid and thorough. The emulsion was separated after it had

settled, and was washed 2-3 times until the emulsion turned to neutral (pH 6-7). Then 1:1 (v/v) acetone was added for demulsification. After settlement the acetone phase was extracted with enough petrol ether, the mixture was settled and the acetone was discarded. NLKJ remained in the petrol ether. Absorbent was added for pigments and pyrogen, neutral oil was obtained after filtration and petrol ether evaporation, then boiled for 0.5 hour with hot distilled water under vacuum, settled and the neutral oil was heated to 100°C to evaporate off water. The absorbent was added again and filtered out. 2 Kg of light yellow NLKJ of the present invention was obtained. It was subsequently heated to 160°C for 2 hours for sterilization, and was then packaged and ready for intravenous and oral emulsion.

The absorbent mentioned above can be active carbon, white pottery clay alumina or others.

Examples 2 - 8

Composition of NLKJ emulsion

The compositions for oral use, intravenous use and intraarterial use will be illustrated in detail in Examples 2-8.

Example 2

NLKJ	10.0 g
Soy lecithin for injection use	1.5 g
Glycerol for injection use	2.5 g
Water for injection use	q.s. to 100 ml

The emulsion was suitable for oral, intravenous or intraarterial administration.

Example 3

NLKJ	10.0 g
Lecithin for injection use	1.2 g
Glycerol for injection use	2.5 g
Water for injection use	q.s. to 100 ml

The emulsion was used for intravenous or intraarterial administration.

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Example 4

NLKJ	15.0 g
Pluronic F88	2.0 g
Glycerol	2.5 g
Distilled water	q.s. to 100 ml

The emulsion was oral emulsion.

Example 5

NLKJ	15.0 g
Soy lecithin for injection use	2.0 g
Glycerol for injection use	2.5 g
Water for injection use	q.s. to 100 ml

The emulsion was intravenous or intraartery emulsion.

Example 6

NLKJ	5.0 g
Phospholipid	0.75 g
Glycerol	1.25 g
Distilled water	q.s. to 100 ml

The emulsion was oral emulsion.

Example 7

NLKJ	2.5 g
Phospholipid	2.5 g
Glycerol	3.0 g
Distilled water	q.s. to 100 ml

The emulsion was oral emulsion.

Example 8

NLKJ	1000 g
Soy lecithin for injection use	120 g
Glycerol for injection use	250 g
Water for injection use	q.s. to 10000 ml

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The emulsion was intravenous or intraartery emulsion.

Example 9
Anti-tumor Activity of NLKJ in vitro

(1) Effect of NLKJ on Inhibition of Proliferation Leukaemia P388 and L1210 Cultured Cells

Methods

20 ul NLKJ was added in different concentration into the 96 wells microplate of cultured leukaemia cell lines in duplicate, 20 ul pbs was added into the wells of control, $9x10^5$ cells was in each well, the highest concentration of NLKJ was 100 ul/ml, cells were incubated for 48 hr at 37°C and under 5% CO_2, cells were counted with Coulter Counter, the inhibition ratio and the concentration of drug which inhibited 50% of the colony proliferation of the tumor cells(IC_{50}) was calculated,

Inhibition ratio= ((cell number of control- cell number of treatment)/(cell number of control- cell number at the beginning of test))x 100%

Results

NLKJ showed evident anti-tumor activity in leukaemia P388 and L1210 cells in vitro. The dosage above 50 ul/ml inhibited almost all cell's proliferation. The IC_{50} of NLKJ on P388 and L1210 cells were 15.2 ul/ml and 28.8 ul/ml respectively. Their coefficients of correlation were 0.9136 and 0.9454 respectively(Fig. 1 and Table 1).

Table 1. Effect of NLKJ on Proliferation Leukaemia P388, L1210 and Colon Tumor M7609 Cells
IC_{50} of NLEJ (ul/ml)

P388	L1210	M7609
15.1	28.8	11.9

(2) Test on Formation Inhibition of Colon Tumor M7609 Cell Colony

Methods

0.1 ml suspension of colon tumor(M7609) cells (2000/ml) at

logarithmic stage prepared after digested by 0.15% trypsin was added in 3.8 ml RPMI 1640 medium containing 20% bovine serum in d-4 cm round cell container for incubation, 0.1 ml NLKJ of different concentration was added in the container after 24 hr incubation at 37°C and under 5% CO_2, the cells incubated 9 days more, after dyeing with 0.1% crystal violet in alcohol the colony was counted, colony formation inhibition ratio and IC_{50} was calculated.

Results

NLKJ markedly inhibited the formation of cell colony of colon tumor M7609. The concentration of 50 ul/ml inhibited all the formation. The IC_{50} of NLKJ on the formation of cell colony of colon tumor M7609 was 11.88 ul/ml. Their coefficient of correlation was 0.9445(Fig. 2 and Table 1).

Example 10

Therapeutic Effect-of NLKJ Emulsion on Metastasizing Tumor in vivo

(1) Therapeutic Effect of NLKJ Emulsion on Growth of Human Hepatoma(QGY) in vivo

Methods

Hepatoma(QGY) tissues growing luxuriantly were homogenized in physical saline(1:4) and prepared as suspension, each nude mouse was inoculated subcutaneously 0.2 ml of the suspension at its armpit, nude mice were divided into groups at random the next day of inoculating, the doses of NLKJ emulsion were 25 ml/kg, 12.5ml/kg and 6.25 ml/kg, the emulsion administrated intravenously at the tail of nude mouse every day within 10 days, one group of mice were administered with cyclophosphamide(CTX) taking a dose of 30 mg/kg intraperitoneal every day within 7 days. the nude mice of control group were administered with relative emulsion just absent NLKJ (blank emulsion) taking a dose of 25 ml/kg intravenously at the tail of nude mouse every day within 10 days. Tumor tissues of all nude mice were sampled and weighted 30 days after inoculating.

Results

All doses of NLKJ emulsion, 25 ml/kg, 12.5 ml/kg and 6.25 ml/kg, showed good therapeutic effect on the growth of human hepatoma(QGY), and good dose-activity correlation relationship, the highest dose (25 ml/kg) got inhibition ratio as almost high as the treatment of CTX(Table 2).

Table 2. Therapeutic Effect of NLKJ Emulsion on Growth of Human Hepatoma(QGY) in vivo

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Sample	Dose	Number	Tumor (g)	inhibition (%)	P value
NLKJ	6.25ml/kg	5/5	1.68 ± 0.52	49.70	<0.01
NLKJ	12.5 ml/kg	5/5	1.02 ± 0.31	61.08	<0.01
NLKJ	25.0 ml/kg	5/5	0.54 ± 0.21	83.83	<0.01
CTX	30.0 mg/kg	5/5	0.28 ± 0.08	91.00	<0.01
Control	25.0 ml/kg	5/5	3.34 ± 0.86	-	-

(2) Therapeutic Effect of NLKJ Emulsion on Growth of Human Small Cell Lung Cancer(SPC) in vivo

Methods

SPC tumor tissues growing luxuriantly were cut into pieces of 2 mm, one of the pieces were inoculated under the skin of armpit of each nude mouse, nude mice were divided into groups at random the next day of inoculating and administered, the dose of NLKJ emulsion was 25 ml/kg iv x10(qd), one group of mice were administered with dacarbazine(DTIC) taking a dose of 40 mg/kg iv x 10(qd), the nude mice of control group were administered with blank emulsion taking a dose of 25 ml/kg iv x 10(qd), tumor tissues of all nude mice were sampled and weighted 30 days after inoculating.

Results

The dose of 25 ml/kg of NLKJ showed good therapeutic effect on human small cell lung cancer. The inhibition ratio was as high as 62.35%(Table 3).

Table 3. Therapeutic Effect of NLKJ Emulsion on Growth of Human Small Cell Lung Cancer(SPC) in vivo -

Sample	Dose	Number	Tumor (g)	inhibition (%)	P value
NLKJ	25 ml/kg	5/5	1.34 ± 0.32	62.35	<0.01
DTIC	40 mg/kg	5/5	0.18 ± 0.04	94.94	<0.01
Control	25 ml/kg	5/5	3.56 ± 0.83	-	-

(3) Therapeutic Effect of NLKJ Emulsion on Metastasis of Melanoma(B16) in Lung in vivo

Methods

Melanoma B16 tissues growing at logarithmic stage were digested with 0.15% trypsin, 0.2 ml suspension of 2.5 x 10^5 cells/ml in RPMI 1640 medium from the tissues inoculated intra-

venously into the tail of each C57BL/6 mouse, mice were divided into groups at random 3 days after inoculating and administered differently, mice were killed the 21 days after inoculating, metastasized colonies of melanoma in mouse's lung were counted to calculate the inhibition ratio and compare the different effects of different administrations of NLKJ emulsion on metastasis of melanoma in lung, 100mg/kg cyclophosphamide(CTX) was administered in the same way to compare with NLKJ emulsion.

Results

The different effect of different administrations on metastasis of melanoma

(i) All the combination of the dose(6.25ml/kg, 12.5ml/kg and 25ml/kg) and the administration (every third day, 4 times and intravenously at tail) of NLKJ emulsion showed evident inhibition effect on metastasis of melanoma B16(Table 4, Fig. 3).

Table 4. Therapeutic Effect of NLKJ Emulsion on Metastasis of Melanoma(B16) in Lung in vivo(every third day)

Sample	Dose (ml/kg)	Administration	Number	Colony No. ($\bar{X} \pm SD$)	Inhibition (%)	P value
NLKJ	6.25	iv × 4(3,5,7,9)	30/30	93.43±15.74	36.88	<0.01
NLKJ	12.5	iv × 4(3,5,7,9)	30/30	79.87±19.02	46.08	<0.01
NLKJ	25.0	iv × 4(3,5,7,9)	30/30	47.83±15.53	67.76	<0.01
CTX	100mg/kg	ip × 2(3,5)	30/30	18.80± 8.50	95.90	<0.01
CK−	25.0	iv × 4(3,5,7,9)	30/30	>147	-	-

(ii)All the combination of the dose(6.25ml/kg, 12.5ml/kg and 25 ml/kg and the administration (every day, 7 times and intravenously at tail) of NLKJ emulsion showed evident inhibition effect on metastasis of melanoma B16, too(Table 5, Fig. 4).

Table 5. Therapeutic Effect of NLKJ Emulsion on Metastasis of Melanoma(B16) in Lung in vivo(iv, every day)

Sample	Dose (ml/kg)	Administration	Number	Colony No. ($\bar{X} \pm SD$)	Inhibition (%)	P value
NLKJ	6.25	iv × 7	30/30	83.23±22.82	44.87	<0.01
NLKJ	12.5	iv × 7	30/30	51.87±13.28	63.31	<0.01
NLKJ	25.0	iv × 7	30/30	32.83±11.80	78.77	<0.01
CTX	100mg/kg	ip × 2(3,5)	30/30	8.0 ± 6.22	98.67	<0.01
CK	25.0	iv × 7	30/30	>155	-	-

(iii)All the combination of the dose(6.25ml/kg, 12.5 ml/kg and 25 ml/kg and the administration (every day, 7 times, and intraperitoneal) of NLKJ emulsion showed also evident inhibition effect on metastasis of melanoma B16(Table 6, Fig.5).

Table 6. Therapeutic Effect of NLKJ Emulsion on Metastasis of Melanoma(B16) in Lung in vivo(ip, every day)

Sample	Dose (ml/kg)	Administration	Number	Colony No. ($\overline{X} \pm SD$)	Inhibition (%)	P value
NLKJ	6.25	ip × 7	30/30	80.47±18.18	29.25	<0.01
NLKJ	12.5	ip × 7	30/30	68.50±16.77	39.64	<0.01
NLKJ	25.0	ip × 7	30/30	47.07±13.72	58.28	<0.01
CTX	100mg/kg	ip × 2(3,5)	30/30	0	100	<0.01
CK	25.0	ip × 7	30/30	>114	-	-

Example 11

Enhancement of Mouse Immunity from NLKJ Emulsion

(1) Inducement of Proliferation of Mouse Spleen Lymphatic Cell from NLKJ Emulsion in vitro

Methods

Spleen cells were obtained aseptically from C57BL/6 mouse, cell density was regulated to be 1 x 10^7/ml with RPMI-1640 medium, each cell hole contained 100ul medium, 100ul drugs and 50ul ConA, here mentioned drugs were NLKJ emulsion (4 concentrations), lentinanin (4 concentrations), blank emulsion and water, 4 duplications for each treatment, cells were incubated for 48 hr at 37°C and under 5% CO_2, then ^{3}H-Tdr was added to the wells(0.5uci/well), incubated 20 hr more, cells were collected then and CPM value were measured with liquid scintillation counter.

Results

It can be seen in Table 7 that the cells incubated with both NLKJ and lentinanin got higher CPM value, i.e. both NLKJ and lentinanin induced the proliferation of mouse spleen lymphatic cell in vitro. The correlation relationship between dose and CPM value was also showed.

Table 7. Inducement of Proliferation of Mouse Spleen

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Lymphatic Cell from NLKJ Emulsion and Lentinanin in vitro

Treatment	Sample(Concentration)	CPM ($\bar{X}\pm SD$)
NLKJ	100(1:4)	7765 ± 1008**
NLKJ	100(1:2)	7165 ± 1163**
NLKJ	100(1:1)	6540 ± 946**
NLKJ	100(mother)	6230 ± 1130**
Lentinanin	100(1:4)	11555 ± 1279**
Leutinanin	100(1:2)	11025 ± 1133**
Leutinanin	100(1:1)	7700 ± 747**
Leutinanin	100(mother)	7420 ± 957**
Blank	100(mother)	4612 ± 719
Control	100(mother)	4795 ± 487

** P<0.01 compared with Blank and Control

(2) Influence of NLKJ Emulsion on the Activity of Natural Killer Cell of Mouse in vitro

Methods

Spleen cells were obtained aseptically from C57BL/6 mouse as effective cells, cell density was regulated to be 1 x 10^6/ml, the density of aim cells, YAC-1 cells, were regulated to be 1 x 10^4, the two suspensions of cells were mixed in 1:1 (v/v), i.e. the ratio between effective and aim cell was 1:100, ^{3}H-Tdr were added to the wells(0.5uci/well), cells were incubated for 48hr at 37°C and under 5% CO_2, cells were collected then and CPM value were measured with liquid scintillation counter, specific inhibition ratio(Pi) was indicated as the activity of natural killer cell, Pi of NLKJ emulsion was compared with the Pi of lentinanin, blank emulsion and the medium were taken as control.

$$Pi = (1-(\text{CPM value of treatment}/\text{CPM value of control})) \times 100\%$$

Results

The results showed that NLKJ stimulated the activity of natural killer cell of mouse in vitro as like as lentinanin did(Table 8). The negative correlation relationship between dose and CPM value can be seen also.

Table 8. Influence of NLKJ Emulsion on the Activity of

Natural Killer Cell of Mouse in vitro

Treatment	Sample(Concentration)	CPM	($\bar{X}$±SD)	Pi %
NLKJ	100(1:4)	5840 ±	1045**	52.2
NLKJ	100(1:2)	6830 ±	1085**	44.1
NLKJ	100(1:1)	8355 ±	1250**	31.7
NLKJ	100(mother)	10925 ±	790	12.7
Lentinanin	100(1:4)	5544 ±	855**	54.5
Leutinanin	100(1:2)	9395 ±	995**	35.5
Leutinanin	100(1:1)	8130 ±	930**	33.5
Leutinanin	100(mother)	8625 ±	1135	29.5
Blank	100(mother)	12710 ±	1125	
Control	100(mother)	12235 ±	725	

** P<0.01 compared with Blank and Control

(3)Effect of NLKJ Emulsion on Proliferation of Spleen Lymphatic Cell of Mouse Bearing Tumor in vivo

Methods

1 x 10^4 leukaemia cells L1210 were inoculated subcutaneously at each mouse's armpit, mice were divided into 6 groups(10 mice/group) at random the next day of inoculating, the 6 treatments were 6.25, 12.5, 25ml/kg of NLKJ, 10ml/kg of lentinanin, blank emulsion and physiological saline, killed the mice after administered 7 times every day, spleen cell suspension obtained aseptically from the mice was regulated to be the cell density of 1 x 10^7/ml, 100ul cell suspension and 100ul medium was added into each well, duplicated 3 wells, after incubated at 37°C and under 5% CO_2 for 48hr, 0.5uci/well ^{3}H-Tdr was added, incubated 20hr more, measured the CPM value of collected cells to compare with the control cells.

Results

It can be seen from Table 9 that NLKJ emulsion could increase the proliferation of spleen lymphatic cell of the mouse bearing leukaemia. The enhancement of NLKJ emulsion on mouse immunity was also increased as the dose increased. The enhancement on mouse immunity was observed in the treatment of lentinanin.

Table 9. Effect of NLKJ Emulsion on Proliferation of Spleen Lymphatic Cell of Mouse Bearing Tumor in vivo

Treatment	Dose and Administration	CPM	($\bar{X}$±SD)
Lentinanin	10ml/kg iv x 7	7410 ±	770**
NLKJ	6.25ml/kg iv x 7	8970 ±	415**
NLKJ	12.5ml/kg iv x 7	10720 ±	565**
NLKJ	25.0ml/kg iv x 7	14330 ±	360**
Blank	10ml/kg iv x 7	5690 ±	1180△
Control	10ml/kg iv x 7	5230 ±	455

** P <0.01 compared with blank or control

△ P <0.01 compared with control

(4)Effect of NLKJ Emulsion on Activity of NK Cell of Mouse Bearing Leukaemia in vivo

Methods

1 x 10^4 leukaemia cells L1210 were inoculated subcutaneously at each mouse's armpit, mice were divided into 6 groups(10 mice/group) at random the next day of inoculating, the 6 treatments were 6.25, 12.5, 25ml/kg of NLKJ , 10ml/kg of lentinanin, blank emulsion and physiological saline, killed the mice after administered 7 times every day, spleen cell suspension obtained aseptically from the mice was regulated to be the cell density of 1 x 10^6/ml, 100ul the cell suspension(effective cell) and 100ul YAC cells(target cell, with the cell density of 1 x 10^4), and 0.5uci/well 3H-Tdr was added into each well, duplicated 3 wells, after incubated at 37°C and under 5% CO_2 for 24hr, measured the CPM value of collected cells, specific inhibition ratio(Pi) was indicated as the activity of natural killer cell.

Pi = (1 - (CPM value of tested group/CPM value of control group)) x 100%

Results

Table 10 shows the stimulation of NLKJ emulsion and lentinanin to the activity of natural killer cell of mouse bearing leukaemia cells, indicating the promotion of mouse immunity.

Table 10. Effect of NLKJ Emulsion on Activity of NK Cell of Mouse Bearing Leukaemia in vivo

Treatment	Dose and Administration	CPM ($\bar{X}\pm SD$)	Pi %
Lentinanin	10ml/kg iv x 7	5660 ± 260**	54.5**
NLKJ	6.25ml/kg iv x 7	10350 ± 1600	16.8
NLKJ	12.5ml/kg iv x 7	8000 ± 960**	34.2**
NLKJ	25ml/kg iv x 7	6210 ± 890**	50.1**
Blank	10ml/kg iv x 7	12510 ± 430△	
Control	10ml/kg iv x 7	12450 ± 340	

** P <0.01 compared with blank or control
△ P <0.01 compared with control

(5) Influence of NLKJ Emulsion on Induction of Interleukin-2 of Mouse Bearing Leukaemia in vivo

Methods

1 x 10-E4 leukaemia cells L1210 were inoculated subcutaneously at each mouse's armpit, mice were divided into 6 groups(10 mice/group) at random the next day of inoculating, the 6 treatments were 6.25, 12.5, 25ml/kg of NLKJ , 10ml/kg of lentinanin, blank emulsion and physiological saline, killed the mice after administered 7 times every day, spleen cell suspension obtained

B

aseptically from the mice was regulated to be the cell density of 1 x 10^7/ml, 2ml the cell suspension containing ConA 5ug/ml, duplicated 3 wells, after incubated at 37°C and under 5% CO_2 for 24hr, took the supernatant, measured the activity of interleukin-2(IL-2) with the Interleukin-2 depended cell clone CTLL and the method of 3H-Tdr incorporation.

Results

Table 11 shows that all treatment of NLKJ stimulated the induction of interleukin-2 of the mouse bearing tumor.

Table 11. Influence of NLKJ Emulsion on Induction of Interleukin-2 of Mouse Bearing Leukaemia in vivo

Treatment	Dose and Administration		CPM ($\bar{X}\pm SD$)
Lentinanin	10ml/kg	iv × 7	2750 ± 123**
NLKJ	6.25ml/kg	iv × 7	1460 ± 184
NLKJ	12.5ml/kg	iv × 7	2080 ± 386
NLKJ	25ml/kg	iv × 7	6020 ± 910**
Blank	10ml/kg	iv × 7	1750 ± 487ΔΔ
Control	10ml/kg	iv × 7	1830 ± 95

** P <0.01 compared with blank or control

ΔΔ P >0.01 compared with control

(6) Influence of NLKJ Emulsion on Phagocytosis Activity of Mouse Phagocyte in vivo

Methods

Swiss mice were divided into two groups at random, one group administered NLKJ emulsion intraperitoneally 7 times every day, the other administered blank emulsion in the same way, 0.5 ml hydrolyzed lactalbumin administered intraperitoneally after last administration, 1 ml erythrocyte suspension(2%) of chicken was given the next day in the same way, 30 min later killed the Swiss mice by the dislocation of cervical vertebra, fix the Swiss mice face up, open the peritoneum, washed the abdominal cavity with 2 ml physiological saline for 1 min, took out 1 ml of the water and drip on the surface of glass, incubated at 37°C for 30 min keeping moisture, washed down the unstuck cells after then, fixed the cells on glass with acetone:methanol 1:1(v/v) after drying, dyed the cells with 4% Giemsa-phosphate buffer for 3 min, washed with distilled water and dried, counted the phagocyte(more than 100) under microscope, calculated the phagocytosis percentage(PP) and phagocytosis index(PI) as the following:

Phagocytosis percentage = (number of phagocyte phagocytosising erythrocyte / number of the all phagocyte) x 100%

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Phagocytosis index = (number of erythrocytes being phagocytosised / number of phagocyte) x 100%

Results

It can be seen in Table 12 that NLKJ promoted the phagocytosis activity of the mouse phagocyte.

Table 12. Influence of NLKJ Emulsion on Phagocytosis Activity of Mouse Phagocyte in vivo

Dose/day	PP %	P value	PI %	P value
6.25ml/kg	31.05 ± 1.96	<0.01	0.68 ± 0.025	<0.01
12.5ml/kg	51.45 ± 3.28	<0.01	1.75 ± 0.14	<0.01
Control	14.00 ± 1.08	-	0.34 ± 0.024	-

Example 12

Anti-tumor Activity of NLKJ Emulsion Combined with Small Dose Chemotherapy

(1) Anti-tumor Therapeutical Effect on Walaer Carcinosarcoma (Solid) W256 in vivo

Methods

Ascites Walaer carcinosarcoma(solid) W256 tissues growing luxuriantly were digested to be cell suspension, regulated the cell suspension to be 1-2 x 10^7/ml cell density, 0.2 ml the suspension was inoculated under the skin of armpit of each rat, the rats were divided into groups at random the next day after inoculating and administered, the doses of NLKJ emulsion were 20ml/kg, 10ml/kg, 5ml/kg iv x 10(qd), the doses of cyclophosphamide were 30mg/kg iv x 7(qd), 10mg/kg iv x 2(third and fifth), tumor tissues of all treatment were sampled and weighted to calculate tumor inhibition ratio on the 14 days after inoculating.

Results

Anti-tumor therapeutical effect of NLKJ emulsion on ascites Walaer carcinosarcoma(solid) W256 was promoted by small dose chemotherapy of cyclophosphamide(Table 13).

Table 13. Anti-tumor Therapeutical Effect on Walaer Carcinosarcoma (Solid) W256 in vivo

Sample	Dose (ml/kg)	Administration	Number	Weight (g)	Inhibition (%)	P value
NLKJ	20	iv × 10	10/10	1.41±0.78	58.16	<0.01
NLKJ	10	iv × 10	10/10	1.83±0.77	45.69	<0.01
NLKJ	5	iv × 10	10/9	2.078±1.1	38.34	<0.05
CTX	10mg	iv × 2	10/10	2.07±1.11	38.58	<0.05
CTX	30mg	iv × 7	10/8	0.4±0.15	88.13	<0.01
NLKJ+CTX	20ml+10mg	iv × 2	10/10	0.93±0.34	72.40	<0.01
NLKJ+CTX	10ml+10mg	iv × 2	10/10	1.09±0.5	67.66	<0.01
NLKJ+CTX	5ml+10mg	iv × 2	10/10	1.72±0.91	48.96	<0.01
Blank		iv × 10	10/10	3.37±1.1	-	-

(2) Anti-tumor Therapeutical Effect on Sarcoma (S-180)

Methods

Sarcoma (S-180) tissues growing luxuriantly were prepared to be cell suspension with physical saline 1:3(w/v), mice were divided into groups at random the next day after inoculating and administered, the dose of NLKJ emulsion was 25 ml/kg iv x 7(qd), the dose of cyclophosphamide was 30mg/kg ip x 2(second and fourth), the dose of mitoxantrone(DHAD) was 2mg/kg ip x2(second and fourth), the dose of mitomycin was 1.5mg/kg ip x2(second and fourth), tumor tissues of all treatment were sampled and weighted to calculate tumor inhibition ratio on the 14 days after inoculating.

Results

Results showed that anti-tumor therapeutical effect of NLKJ emulsion on sarcoma was prompted by small dose of cyclophosphamide, DHAD and mitomycin(Table 14).

Table 14. Anti-tumor Therapeutical Effect on Sarcoma (S-180) in vivo

Treatment	Dose and Administration	No.	Inhibition %	Pi %
NLKJ	25ml/kg iv × 7	10/10	23	<0.05
NLKJ+CTX	25ml/kg+30mg/kg ip × 2(1,3)	10/10	44	<0.01
CTX	30mg/kg ip × 2(1,3)	10/10	39.4	<0.05
NLKJ+DHAD	25ml/kg+2mg/kg ip × 2(1,3)	10/10	67	<0.01

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DHAD	2mg/kg	ip × 2(1,3)	10/10	50	<0.01
CLEJ+ mitomycin	25ml/kg+1.5mg/kg	ip × 2(1,3)	10/10	67	<0.01
Mitomycin	1.5mg/kg	ip × 2(1,3)	10/10	39.4	<0.05
Control	25mg/kg	ip × 2(1,3)	30/10		

Example 13

Protection of NLKJ Emulsion on Decrease of Leucocyte Induced by Chemotherapy

Methods

80 Swiss mice were divided into eight groups at random, administered with cyclophosphamide 45mg/kg ip x 3(second, sixth and tenth), harringtonine(HRTN) 1mg/kg ip x 3(second, sixth and tenth), each two of the medicine groups were administered with NLKJ emulsion the same time with the doses 2.4ml/kg and 6.25 ml/kg iv x 7(every day), at the beginning and on the fourth day of each administration blood were sampled from the angle of each mouse's eye, leucocyte of the blood were counted.

Results

Table 15 and Table 16 show that NLKJ emulsion can protect mouse from leucocyte decrease induced either from cyclophosphamide chemotherapy or from harringtonine chemotherapy.

Table 15. Protection of NLKJ Emulsion on Decrease of Leucocyte Induced by Cyclophosphamide Chemotherapy

Medi.	Dose	Administration	No.	Leucocyte number x 100/mm³ (X±SD)								
				0	3	6	9	12	15	18	21	24
CTX+ NLKJ	45mg/kg+ 12.5ml/kg	ip ×1,5,9+ iv ×7	58	62 ±10	41 ±12	39 ±6	43 ±10	76** ±6	66** ±11	61* ±14	66** ±17	±12
CTX+ NLKJ	45mg/kg+ 6.25ml/kg	ip × 1,5,9+ iv ×7	65	60 ±11	50 ±9	39 ±8	62 ±9	81** ±9	69** ±13	65** ±10	64* ±10	±17
CTX	45mg/kg	ip ×1,5,9	62	49▲ ±8	45▲ ±8	41▲ ±8	41▲ ±6	42▲ ±11	45▲ ±8	47▲ ±9	49▲ ±9	±8

Control	58 ±7	69 ±2	69 ±2	68 ±2	88 ±3	82 ±2	81 ±4	86 ±2	72▲ ±2

▲ P<0.001 compared with Control
** and * P<0.01 and <0.05 compared with CTX

Table 16. Protection of NLKJ Emulsion on Decrease of Leucocyte Induced by Harringtonine Chemotherapy

Medi.	Dose	Administration	Leucocyte number x 100/mm³ (X̄±SD) 0	3	6	9	12	15	18	21	24
HRTN+ NLKJ	1mg/kg+ 12.5ml/kg	ip ×1,5,9+ iv ×7	59 ±8	73 ±10	61 ±6	56 ±11	81** ±5	89** ±9	75** ±16	79** ±20	97** ±20
HRTN+ NLKJ	1mg/kg+ 6.25ml/kg	ip ×1,5,9+ iv ×7	65 ±13	67 ±4	75 ±9	70 ±17	96** ±18	98** ±10	89** ±17	86** ±22	89** ±27
HRTN	1mg/kg	ip ×1,5,9	60 ±9	75 ±5	65 ±10	50▲ ±10	44▲ ±10	43▲ ±7	49▲ ±8	53▲ ±9	56▲ ±9
Control			58 ±7	69 ±2	69 ±2	68 ±2	88 ±3	82 ±2	81 ±4	86 ±2	72▲ ±2

▲ P<0.001 compared with Control
** P<0.01 compared with HRTN

Example 14

Clinical Therapeutic Effect of NLKJ Emulsion

Methods

NLKJ Emulsion was administered intravenously or intraarterial in various human cancer therapies. Detail therapeutic methods are omitted.

Results

Table 17. Therapeutic Effect of NLKJ Emulsion

Patient No.	CR	PR	NC	PD	Effective ratio %
51	0	26	19	6	50.98

Table 18. Improvement of Symptom after Administration

Symptom	No.	Ease	Vanish	Unchange	Worse	Retarded %

Pain	45	31	10	4	0	91.1
Fever	29	11	8	7	3	65.5
Jaded appetite	84	57	16	10	1	73.0
Abdominal distension	38	19	2	12	5	55.3
Insonomnia	41	21	11	9	0	78.0
Powerless	70	37	16	9	8	75.7
Cough	28	14	6	8	0	71.4
Breath with difficulty	11	5	1	5	0	54.5
Irregular Stool	17	3	8	6	8	64.7

Table 19. Enhancement of Human Immunity from NLKJ Emulsion

Immunity	Administ.	No.	Before	After	t value	P value
ANAE (%)	NLKJ	40	39.38±7.92	44.46±13.41	2.477	<0.05
	Chemotherapy	19	50.05±7.69	44.79± 6.64	2.580	<0.05
OKT_1	NLKJ	40	53.47±6.77	56.83± 7.07	3.670	<0.001
	Chemotherapy	19	67.45±4.60	62.91± 6.46	2.424	<0.05
OKT_4/OKT_8	NLKJ	40	1.09±0.20	1.16± 0.33	2.098	<0.05
	Chemotherapy	19	1.39±0.25	1.26± 0.28	2.424	<0.05
NK Cell (%)	NLKJ	40	34.82±12.41	43.91±16.97	3.222	<0.01

Table 20. Protection of Marrow Function from NLKJ Emulsion

Value	Therapy	No.	HB	WBC	PC
Increase	After	102	80	85	79
Unchange	After	102	2	2	4
Decrease	After	102	20	15	19
Effective%			78.43	83.33	77.45
Average	Before	102	10.35±1.77	4.82±1.47	9.31±4.27
	After	102	10.64±1.96	5.78±1.70	10.70±4.51
t value			2.343	6.302	5.070
P value			<0.05	<0.001	<0.001

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Table 21. Improvement of Function of Liver and Spleen from NLKJ

Index	No.	Before		After			Retarded(%)
		Normal	Abnormal	Normal	Retard	Unchange	
Bilirubin	70	52	18	52±4	6	8	55.56
G.P.T.	70	58	12	58±4	5	3	75.00

Table 22. Strength Improvement from NLKJ Emulsion

Patient No.	K P S Evaluation				-	Increasing
	+30	+20	+10	Stable	decrease	%
70	16	22	15	9	8	75.71

Therapy	No.	K P S Evaluation			X^2 value	P value
		>70	50-60	<50		
Before	70	15	28	27		
After	70	39	18	13	21.159	<0.001

	KPS Evaluation	NLKJ (n=51)	Chemotherapy (n=49)	X^2 value	P value
Before	>70	13	40		
	50-60	18	7		
	<50	20	2	29.904	<0.001
After	>70	31	20		
	50-60	10	21		
	<50	10	8	6.459	<0.05
	X^2 value	12.934	17.266		
	P value	<0.001	<0.001		

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A neutral lipid isolated from kernel of Job's tears (Coix lacryma-jobi) (abbreviated as "NLKJ") consisting essentially of glycerides and alkyl fatty acid ester, and wherein said neutral lipid has the following physio-chemical properties: acid value <0.20, iodine value 95.00-107.00, saponification value 185.00-195.00, relative density 0.915-0.918 (20°C) and diopter 1.470-1.475 (20°C).

2. A NLKJ according to claim 1, wherein the glycerides comprise of triglyceride, diglyceride and monoglyceride.

3. A NLKJ according to claim 1, wherein the lipolysis of said NLKJ produces hexadecanoic acid, octodecanoic acid, octadecenoic acid and octadecadienoic acid.

4. A method for the preparation of NLKJ according to claim 1, comprising the steps of:

(a) extraction with an organic solvent, absorption by absorbent and decoloration;

(b) alkalizing saponification;

(c) demulsification by acetone; and

(d) extraction by an organic solvent.

5. The method according to claim 4, wherein said organic solvent of step (a) is acetone.

6. The method according to claim 4, wherein said step (b) comprises the use of NaOH or KOH.

7. The method according to claim 4, wherein said organic solvent of step (d) is petrol ether.

8. The method according to claim 4, wherein said method further comprises, after one or more of said extraction of step (a), step (b), and step (d), the step of washing the emulsion with hot water.

9. A pharmaceutical composition comprising a neutral lipid isolated from kernel of Job's tears (Coix lacryma-jobi) according to claim 1 and a pharmaceutically acceptable carrier.

10. A pharmaceutical composition according to claim 9 in the form of an anti-tumour oil in water emulsion.

C

11. A pharmaceutical composition according to claim 10, further comprising an emulsifier and isoosmoticum.

12. A pharmaceutical composition according to any one of claims 9 to 11, wherein the composition comprises:

from 2.5 to 25 g of NLKJ,

from 0.3 to 3.0 g of soy or egg lecithin,

from 1.25 to 6 g of glycerol, and

distilled water q.s. to 100 ml.

13. A pharmaceutical composition according to claim 12, wherein the composition comprises:

10 g of NLKJ,

1.5 g of soy lecithin for injection use,

2.5 g of glycerol for injection use, and

water for injection use q.s. to 100 ml.

14. A pharmaceutical composition according to claim 9, 10 or 11, further comprising one or more further anti-tumour drugs.

15. The use of NLKJ according to claim 1 as a medicament for the treatment of tumours.

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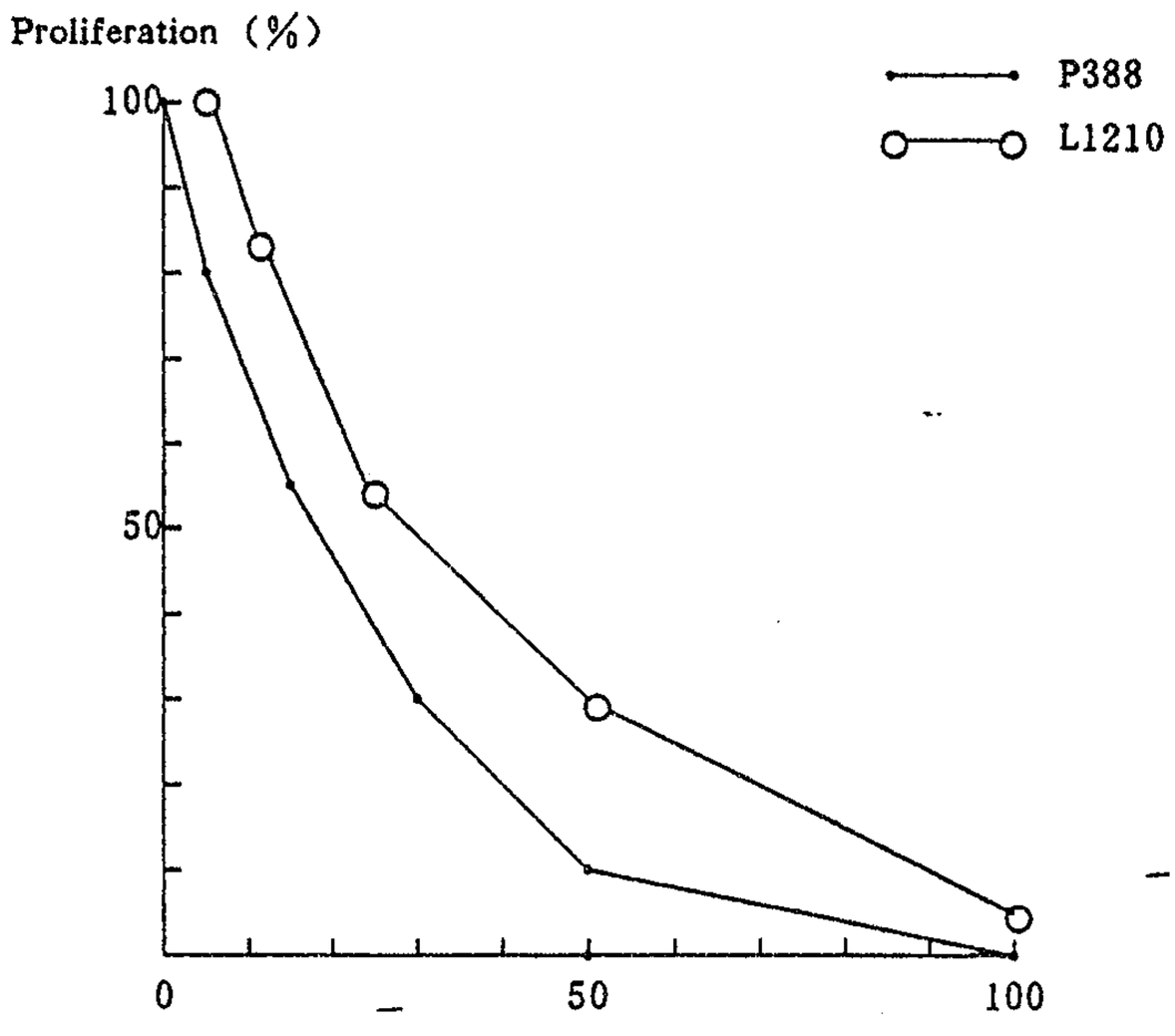


Fig. 1

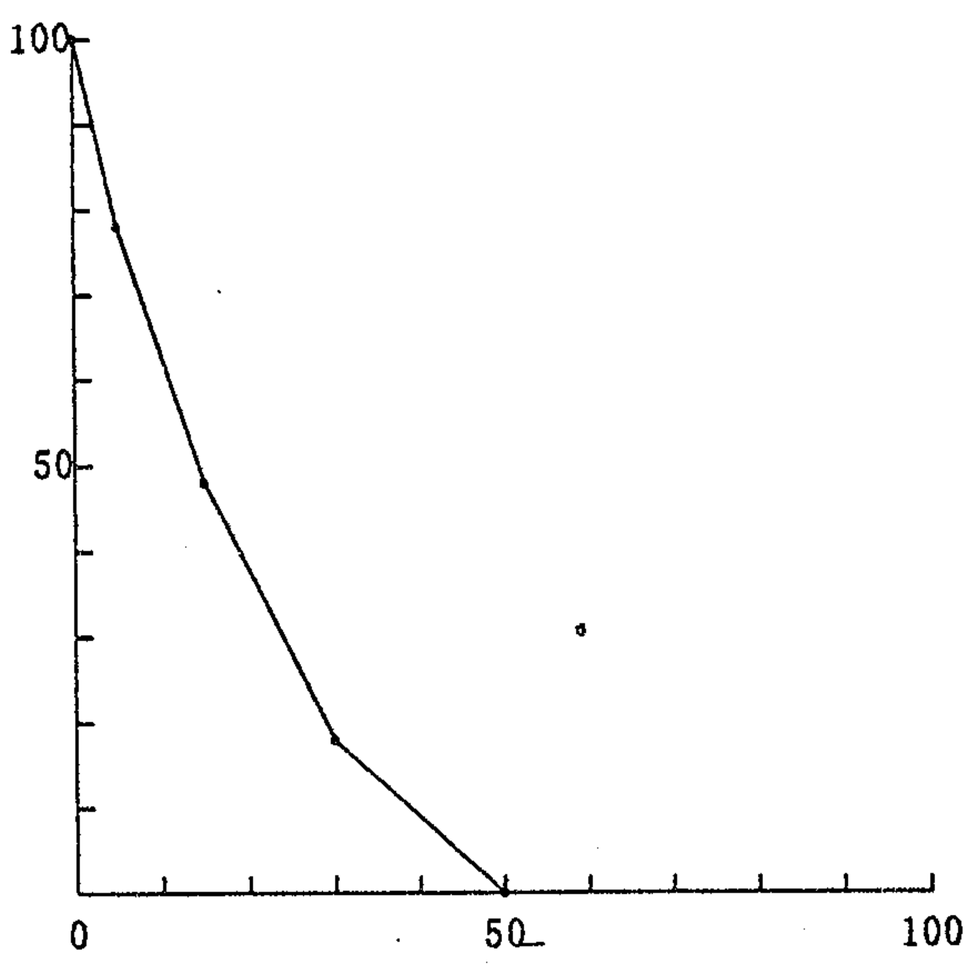
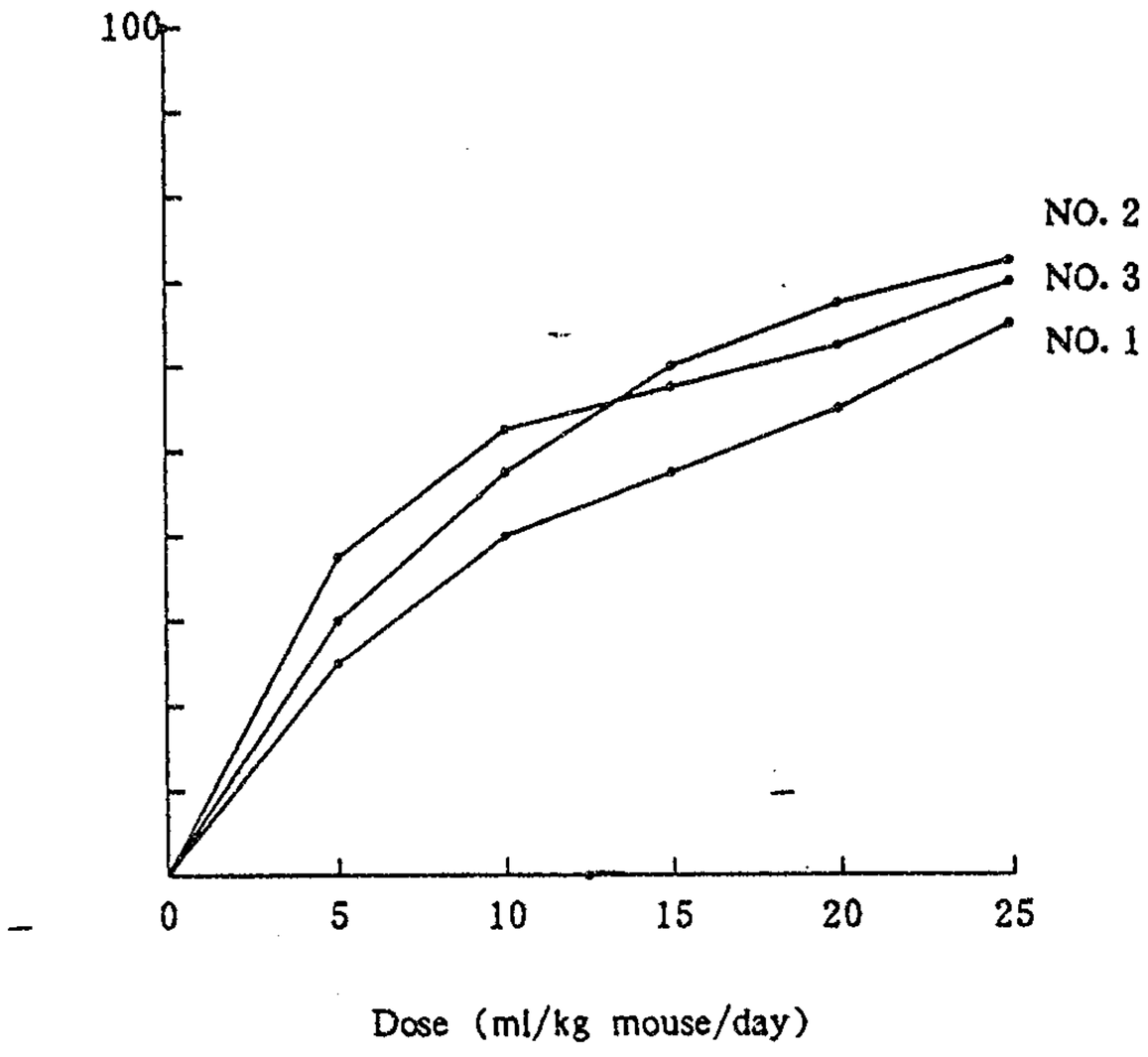


Fig. 2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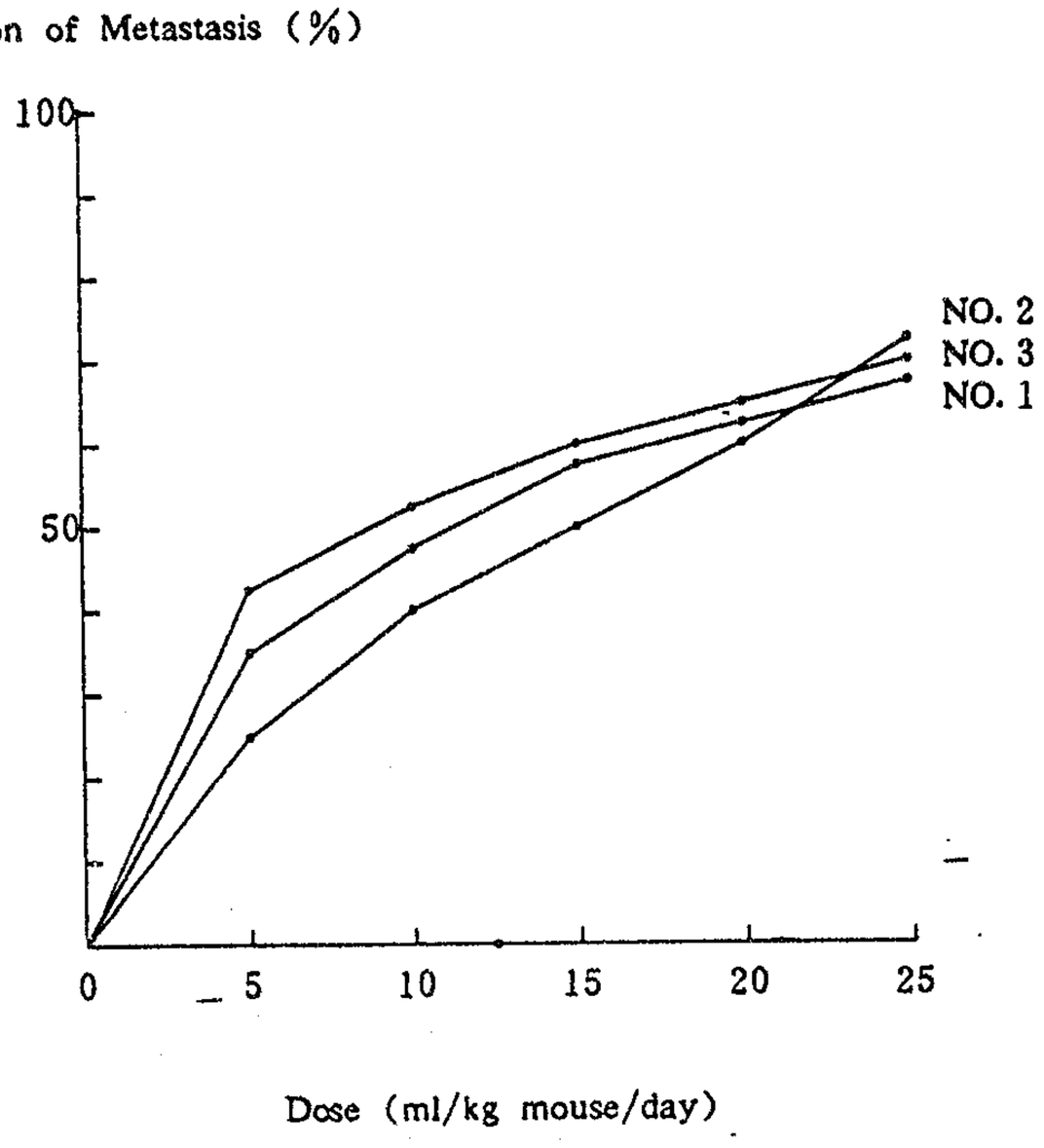
NO. 2
NO. 3
NO. 1
0
5
10
15
20
25
Dose (ml/kg mouse/day)

Fig. 3


Inhibition of Metastasis (%)
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50
0
5
10
15
20
25
NO. 2
NO. 3
NO. 1
Dose (ml/kg mouse/day)

Fig. 4

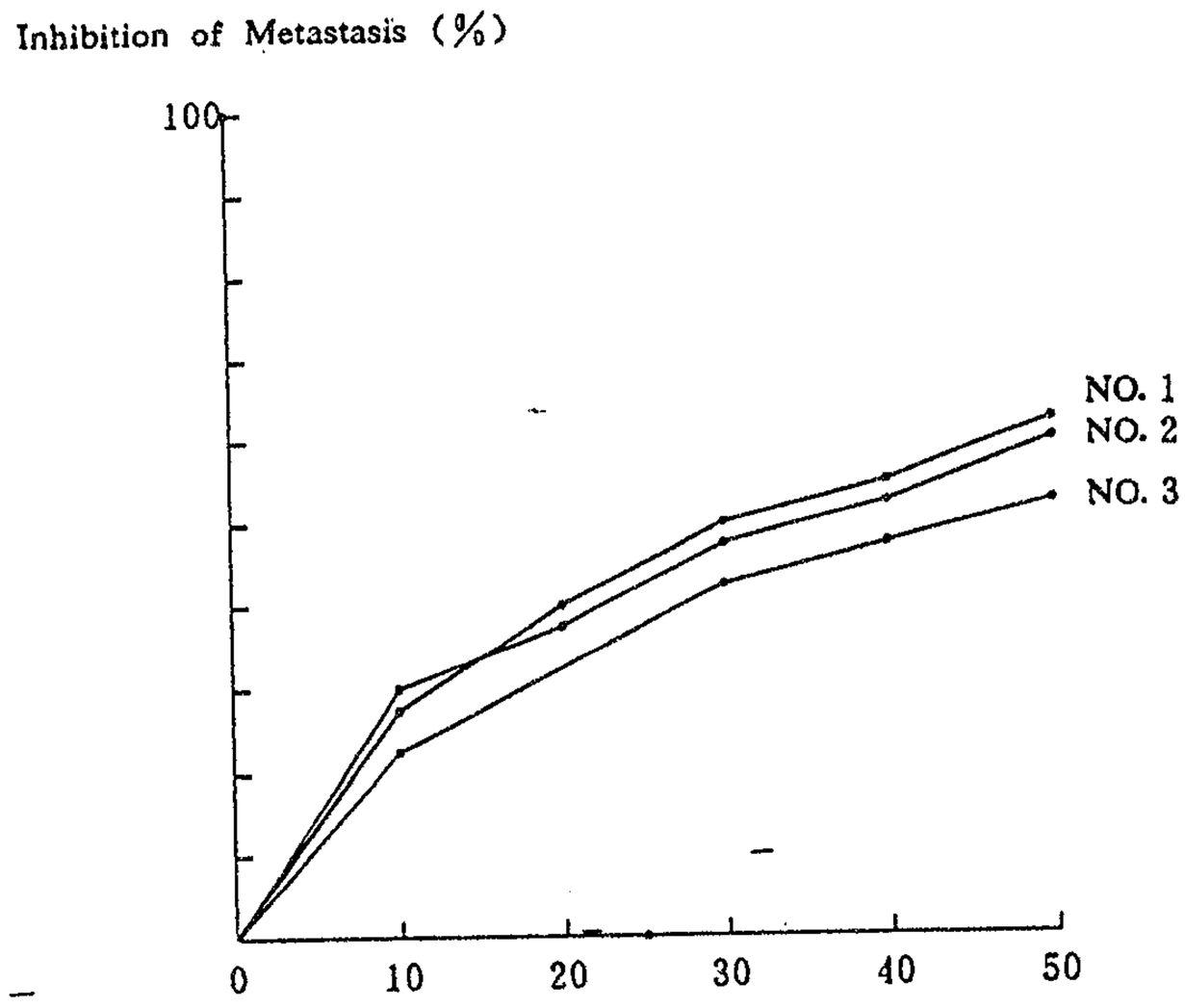
Inhibition of Metastasis (%)
100
NO. 1
NO. 2
NO. 3
0
10
20
30
40
50
Dose (ml/kg mouse/day)

Fig. 5