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(54) ANTI-CANCER AGENT AND METHOD FOR THE PRODUCTION THEREOF

(71) We, TOBISHI PHARMACEUTICAL CO., LTD., a Japanese Corporation, of No. 9—9, 1-chome, Yaesu, Chuoh-ku, Tokyo, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a new anti-cancer agent and to a method for the production thereof.

According to one aspect of this invention, there is provided an anti-cancer agent which comprises a substance active against cell growth and obtained by culturing *Staphylococcus epidermidis* STF (Bikoken Deposit No. 3706; ATCC 31310). This anti-cancer agent has been found to be effective in inhibiting leukemia and malignant tumors.

According to a second aspect of this invention there is provided a method for the production of an anti-cancer agent, which comprises cultivating *Staphylococcus epidermidis* STF (Bikoken Deposit No. 3706; ATCC 31310) in a culture medium containing sources of carbon, nitrogen and inorganic salts and isolating the anti-cancer agent from the culture medium.

This invention arose from the discovery that the number of leukemia cells in a leukemia patient underwent a surprisingly large reduction when the patient developed a boil, until eventually the amount of leucocyte cells of the patient became normal. A microorganism strain was then isolated from the boil of this patient and cultivated. A filtrate of the culture solution was administered to animals affected by induced leukemia and it was confirmed that the administration of the filtrate gave rise, in the affected animals, to such effects as reduction in content of leukemia virus, reduction in number of leukemia cells, recovery of leukemia affected organs, prolongation of life and increase in number of surviving animals. Subsequent investigations showed that the filtrate is effective not only for leukemia but also generally for malignant tumors.

The culture solution filtrate was subsequently concentrated and water-soluble substances considered to be possible active ingredients were fractionated into a number of fractions and isolated. These substances were purified and themselves shown to have the above described anti-cancer activity.

Identification of the strain:

The strain of microorganism used in the method of the present invention has been found to be a new strain. It was deposited in "Bikoken" and ATCC as Bikoken Deposit No. 3706 and ATCC 31310, respectively. The strain is freely available to all persons at these institutions. The strain was identified to be a strain of the species *Staphylococcus epidermidis* as follows:

Primary discrimination

(1)	Motility	
	37°C	(–)
	24°C	(–)
(2)	Gram staining	(+)
(3)	Catalase activity	(+)
(4)	Shape	Coccus
(5)	Acid fast property	(–)
(6)	Spore formation	(–)
(7)	Growth in air	(+)
(8)	Oxidase activity	(–)
(9)	Glucose utilisation	(+)
(10)	Oxidation fermentation test (Hugh-Leifson test)	F

From the above test results, it was judged

that the microorganism belongs to *Staphylococcus* or *Aerococcus*.

Secondary discrimination:

(1) Coagulase	(–)
(2) V—P reaction	(+)
(3) Pink reaction	(–)
(4) Phosphatase	(+)
(5) Nitrate reduction	(+)

Thus, the microorganism was finally determined to be *Staphylococcus epidermidis*. The inventors named the strain "STF".

Preparation of active ingredient:

The microorganism propagates well in a common medium, i.e. a nutrient medium containing carbon, nitrogen and inorganic salts. In order that the aforesaid active substance should be produced by the microorganism, aerobic cultivation in a liquid medium under stationary condition or under shaking is desirable. Examples of suitable culture media are as follows:

(1) Peptone	17 g
Bouillon extract	3 g
NaCl	5 g
K_2HPO_4	2.5 g
Glucose	2.5 g
Distilled water	1 litre (pH 7.3)
(2) Corn steep liquor	6 g
$NH_4H_2PO_4$	3 g
Yeast extract	2.5 g
Dextrose	10 g
$CaCO_3$	2.5 g
Distilled water	1 litre (pH 4.5)

In addition to such liquid media, common solid media can also be used.

The microorganisms have been successfully cultivated in the above media at a culture temperature of from –20°C to +40°C for from 24 to 120 hours. Optimum culturing temperature was about 37°C. The colony surface was white and circular.

The resulting culture solution was subjected to centrifugation and the solid matter was separated off to obtain a solution (hereinafter referred to as "culture solution"). The solution was utilised in various experiments to be set out below.

Utilisation of the active ingredient:

A culture solution as aforesaid was administered to animals affected by various induced diseases to obtain the following results:

EXPERIMENT 1.

Inhibition of blood cancer caused by inoculation of leukemia virus:

30 ICR Mice were divided into three groups. Two groups were inoculated with Friend's leukemia virus. 0.1 ml/10 g/day of the culture solution was intraabdominally administered to one inoculated group for eight days to obtain the following effects in the inhibition of spleen hypertrophy.

TABLE 1

	Weight of spleen (g)	Rate of inhibition (%)
Uninfected group (control)	0.124 ± 0.022	
Infected group (control)	0.724 ± 0.239	
Infected group administered with the culture solution continuously for 8 days	0.219 ± 0.039	84.9

EXPERIMENT 2.

Inhibition of malignant tumor caused by inoculation of leukemia cells:

Mouse leukemia cells L—1210 were inoculated into BDF_1 mice to cause an induced leukemia condition. Normally this would cause to mice to die in a limited period of time. Two groups of mice each comprising 6 mice were inoculated with L—1210 cells and the culture solution was then administered to one of the groups continuously for five days in an amount of 0.1 ml/10 g/day to obtain the following results.

TABLE 2

	Time of death (days after inoculation)
Untreated group	7, 7, 7, 7, 7, 7
Treated group	7, 7, 7, 8, 8, more than 10

Test group/control group ratio. (life prolongation ratio) was higher than 112 (%).

EXPERIMENT 3.

Effect of controlling Ehrlich cancer cells:

Two groups each containing 10 ICR mice were inoculated with Ehrlich ascites tumor cells. The culture solution was administered intraabdominally in an amount of 0.1 ml/10 g/day to one of the groups continuously for five days. The inhibitory effect of this solution was recognised from tumor cell counts on the 10th day which were as shown below. Amount of the culture solution was the same as in (1).

TABLE 3

	Number of tumor cells	Rate of inhibition (%)
Untreated group	7.7×10^8	
Treated group	4.7×10^7	93.7

EXPERIMENT 4.

Effect of controlling Sarcoma 180 cells:

The procedure of the aforesaid Experiment 3 was repeated but with the difference that the batch of 10 mice used were inoculated with Sarcoma 180 cells introabdominally. The cell counts observed on the 10th day were found to be as follows:

TABLE 4

	Number of tumor cells	Rate of inhibition (%)
Untreated group	2.3×10^9	
Treated group	5.3×10^8	76.9

For comparative purposes, the effects of the culture solutions of other well-known microorganisms obtained by the same culturing method were examined. Culture solutions of the following well-known microorganisms were administered to mice inoculated with Friend's leukemia virus. No effect was observed in any of the cases:

Pseudomonas
Klebsiella
Staphylococcus aureus

Isolation and purification of active ingredient:

The active ingredient contained in the culture solution of *Staphylococcus epidermidis* can be isolated and purified by, for example, the following process:

(1) The culture solution is subjected to centrifugation at from 3,000 to 12,000 rpm for from 30 to 10 minutes to divide it into solution and suspended solid matter (precipitates). Then, the precipitates are washed with distilled water and the washings are subjected in turn to the centrifugation. The liquid phases from the various centrifugations are combined. This process is repeated from 1 to 5 times, usually 3 times.

(2) The solution thus collected is concentrated by evaporation at a temperature of from 10—30°C under reduced pressure of from 5 to 30 mmHg for from 1 to 6 hours and the solid matter which precipitates out is separated off, washed with distilled

water and then dissolved in distilled water. The solution obtained is concentrated again by evaporation. This process is repeated from 1 to 5 times, usually 3 times to obtain a white, yellow or brownish white crystalline powder, the colour depending on the state of purity, which is soluble in water.

(3) The crystalline powder eventually obtained is dissolved in distilled water and the solution obtained is fractionated using, for example, a molecular sieve such as Sephadex (Registered Trade Mark for an adsorptive filtration gel produced by Biochemical Co., Sweden) G—25, an adsorptive filtration gel or ion-exchange chromatography. The activities of the respective fractions are examined and the active fractions are selected repeatedly. In this way, a substance having a single activity peak is obtained.

The active ingredient is finally dissolved again in water and purified by, for example ion-exchange chromatography or dialysis.

(4) The active ingredient thus purified can be precipitated from aqueous solution by addition of various organic solvents, change of pH from the acidic region to the alkaline region, salting out followed by reprecipitation and filtration or concentration by evaporation.

The substance has the following properties:

1. Thermal stability — Stable (substance does not lose its activity after being maintained at 100°C for 15 minutes)
2. Ninhydrin reaction (–)
3. Ehrlich reaction (–)
4. Sakaguchi reaction (–)

The cancer activity of the active ingredient thus isolated and purified was confirmed by adding a diluted aqueous solution thereof to a culture solution of L—1210 cells, cultivating the same at 35°C for 48 hours and determining the rate of inhibition of propagation of the cells.

TABLE 5

% dilution	Rate of inhibition (%)	
	Batch A	Batch B
50	100	100
100	100	95.3
200	83.0	83.5
400	72.7	78.6
800	69.0	61.4
1600	59.1	56.4
3200	49.9	–

Thus, it is apparent that the substance produced by *Staphylococcus epidermidis* is capable of controlling various malignant cell growth as in leukemia or tumor formation (liquid cancers and solid cancers). The substance does not exhibit acute toxicity.

Administration of the active ingredient:

The active ingredient will generally be administered in an amount of from 3 to 300 mg/kg/day, the amount employed being affected by the administration route. The culture solution from which solid matter has been removed can be used directly. However, as a matter of course, it is better to use the isolated and purified active ingredient to enable the dosage to be controlled precisely. Various modes of administration are possible such as intravenous, subcutaneous, intracutaneous or intramuscular injection, peroral administration, suppositories, troches and emulsions. Accordingly, the form of preparations can be selected suitably and can be a powder, tablet (monolayer tablet, double layer tablet, enteric tablet, etc.), capsule, solution or suspension for

injections packed in ampoules or vials, sublingual tablets, troches, suppositories or fatty emulsions, generally of dosage unit form.

The following examples further illustrate the present invention.

EXAMPLE 1.

Staphylococcus epidermidis STF was cultivated at 37°C for 48 hours in a medium of the following composition:

Corn steep liquor	6 g
$NH_4H_2PO_4$	3 g
Yeast extract	2.5 g
Dextrose	10 g
$CaCO_3$	2.5 g
Distilled water	to 1 litre (pH 4.5)

The culture solution obtained after 48 hours was subjected to centrifugation at 10,000 rpm. for 30 minutes to divide the same into a supernatant liquid and precipitate. The precipitate was re-dispersed in one litre of distilled water and the dispersion was allowed to stand at 37°C for 18 hours and was then subjected again to centrifugation at 10,000 rpm. to divide the same into a supernatant liquid and precipitate. This process was repeated five times. The resulting supernatant liquids were combined and concentrated by evaporation under a reduced pressure of 10 mmHg at ambient temperature for about two hours. After about 1/30 concentration was attained, the combined liquid was passed through a layer of Sephadex G—25 and subjected to U.V. spectroanalysis using an indicator of OD (optical density)$_{265}$. The separation of fractions involved a method of detecting polysaccharides (Molisch reaction). A chromatogram of L—1210 cell propagation-inhibition activity was surveyed and active fractions were separated out. This process was repeated three times to obtain the fraction of highest activity. An aqueous solution of this fraction was concentrated by evaporation to obtain a white powder. The powder was washed with water, dissolved again and concentrated by evaporation. This process was repeated five times to obtain white crystalline powders. The yield of active substance from one litre of the culture solution was about 0.3 g.

EXAMPLE 2.

25 litres of a liquid medium having the same composition as that used in Example 1 were prepared. Culturing was effected at 37°C for two days. The culture solution was subjected to centrifugation (6,000 rpm, 20 mins.) to remove the microbial cells. The resulting supernatant liquid (about 23 litres) was concentrated to a volume of about 500 c.c. at 40°C under reduced pressure.

The precipitate thus formed was washed with 100 c.c. of distilled water and the washing procedure was repeated three times. The washings were combined with the concentrate and the whole volume concentrated to a volume of 500 c.c. at 40°C under reduced pressure. The solute in the concentration was divided into two fractions (one having molecular weights of more than 10,000 and the other having molecular weights of less than 10,000) using Diaflon No. 10,000 which is a molecular sieve membrane produced by Amicon Far East Ltd. The solution is produced containing solutes of molecular weight of less than 10,000 was passed through a diethylaminoethyl cellulose column (OH-type) to divide it into acidic substances and neutral and basic substances. Thereafter, the neutral and basic substances were further sub-divided into two fractions (one having molecular weights of 5,000—10,000 (original solution) and the other having molecular weights of less than 5,000) with Diaflon No. 5000. The fraction containing solutes of molecular weight of less than 5,000 was further divided into two fractions (one having molecular weights of 1,000—5,000 and the other having molecular weights of less than 1,000) with Diaflon No. 1000. The U.V. and IR spectra of the above fractions of molecular weights 5000 to 10,000 and 1000 and 5000 were determined and are recorded in the accompanying drawings, wherein:

Figure 1 shows the U.V. spectrum of an active ingredient of molecular weight 5,000—10,000 (determined after dilution of original solution with 10 times its own volume of water);

Figure 2 shows the U.V. spectrum of an active ingredient of molecular weight of 1,000—5,000 (original solution from fractionation);

Figure 3 shows the IR spectrum of an active ingredient of molecular weight of 5,000—10,000; and

Figure 4 shows the IR spectrum of an active ingredient of molecular weight of 1,000—5,000.

The respective fractions were then administered to animals affected by various induced diseases and the results set out in the following Tables 6 to 9 were obtained:

TABLE 6

Anti-cancer effects of fractions of 5,000 <MW <10,000 and 1,000 <MW <5,000 against Leukemia cells L—1210 cultivated *in vitro:*

Fraction	Conc. (mg/ml)	Average cell number ± Standard deviation ($\times 10^4$/ml)	Rate of inhibition (%)	50% cell propagation inhibition conc. IC_{50} (mg/ml)
Cell control group	0	14.1 ± 0.49	–	–
1,000<MW<5,000	0.25	9.9 ± 0.97	29.8	
	0.50	8.5 ± 0.19	39.7	
	1.0	4.9 ± 0.13	65.2	
	2.0	3.7 ± 0.33	73.8	0.70
5,000<MW<10,000	0.25	2.6 ± 0.93	81.6	
	0.50	1.8 ± 0.15	87.2	
	1.0	0.8 ± 0.001	94.3	<0.25

TABLE 7

Anti-cancer effects of fractions of 5,000<MW<10,000 and 1,000<MW<5,000 against Ehrlich tumor cells cultivated *in vitro:*

Fraction	Conc. (mg/ml)	Average cell number ± Standard deviation ($\times 10^4$/ml)	Rate of inhibition (%)	50% cell propagation inhibition conc. IC_{50} (mg/ml)
Tumor cell control group	0	80.4 ± 3.3	–	–
1,000<MW<5,000	0.25	58.7 ± 3.7	39.9	
	0.50	56.6 ± 3.1	43.8	
	1.00	49.7 ± 2.9	56.4	0.72
	2.00	46.1 ± 2.3	63.1	
5,000<MW<10,000	0.25	63.2 ± 1.5	31.6	
	0.50	52.4 ± 2.5	51.5	0.48
	1.00	39.4 ± 2.5	75.4	
	2.00	37.7 ± 1.8	78.5	

TABLE 8

Anti-cancer effects of styeen fractions 5,000<MW<10,000 and 1,000<MW<5,000 against ascites hepatoma cells AH 41C cultivated *in vitro:*

Fraction	Conc. (mg/ml)	Average cell number ± Standard deviation ($\times 10^4$/ml)	Rate of inhibition (%)	50% cell propagation inhibition conc. IC_{50} (mg/ml)
Tumor cell control group	0	115.9 ± 1.52	—	—
1,000<MW<5,000	0.1	93.4 ± 1.85	19.8	
	0.5	93.5 ± 6.60	19.0	
	1.00	90.3 ± 2.74	22.4	>2.00
	2.00	87.7 ± 7.94	24.1	
5,000<MW<10,000	0.1	118.9 ± 2.59	0	
	0.5	62.9 ± 3.35	45.7	
	1.00	27.3 ± 2.61	76.7	0.60
	2.00	23.4 ± 1.95	80.2	

TABLE 9

Anti-cancer affects of styeen fractions 5,000<MW<10,000 and 1,000<MW<5,000 for mice inoculated with Ehrlich ascites tumor cells (influence on number of the whole tumor cells on the 11th day after the inoculation of the cells):

Fraction	Amount (mg/kg)	Average whole tumor cells ± standard deviation ($\times 10^7$)	Rate of inhibition of cell propagation (%)
Control group	0	115.4 ± 12.6	—
1,000<MW<5,000	200	28.4 ± 6.4	75.3
	400	31.2 ± 12.5	72.9
	600	29.0 ± 3.3	74.5
5,000<MW<10,000	200	47.1 ± 20.4	59.1
		55.1 ± 17.1	52.2
		42.2 ± 5.1	63.2

WHAT WE CLAIM IS:—

1. An anti-cancer agent which comprises a substance active against cancer cell growth and obtained by cultivating *Staphylococcus epidermidis* STF (Bikoken Deposit No. 3706; ATCC 31310).

2. An agent as claimed in claim 1, which is active against leukemia.

3. An agent as claimed in claim 1, which is active against Ehrlich tumor cells.

4. An agent as claimed in claim 1, which has a molecular weight in the range of from 1000 to 5000.

5. An agent as claimed in claim 1, which has a molecular weight in the range of from 5000 to 10000.

TABLE 8

Anti-cancer effects of styeen fractions 5,000<MW<10,000 and 1,000<MW<5,000 against ascites hepatoma cells AH 41C cultivated *in vitro*:

Fraction	Conc. (mg/ml)	Average cell number ± Standard deviation ($\times 10^4$/ml)	Rate of inhibition (%)	50% cell propagation inhibition conc. IC_{50} (mg/ml)
Tumor cell control group	0	115.9 ± 1.52	–	–
1,000<MW<5,000	0.1	93.4 ± 1.85	19.8	
	0.5	93.5 ± 6.60	19.0	
	1.00	90.3 ± 2.74	22.4	>2.00
	2.00	87.7 ± 7.94	24.1	
5,000<MW<10,000	0.1	118.9 ± 2.59	0	
	0.5	62.9 ± 3.35	45.7	
	1.00	27.3 ± 2.61	76.7	0.60
	2.00	23.4 ± 1.95	80.2	

TABLE 9

Anti-cancer affects of styeen fractions 5,000<MW<10,000 and 1,000<MW<5,000 for mice inoculated with Ehrlich ascites tumor cells (influence on number of the whole tumor cells on the 11th day after the inoculation of the cells):

Fraction	Amount (mg/kg)	Average whole tumor cells ± standard deviation ($\times 10^7$)	Rate of inhibition of cell propagation (%)
Control group	0	115.4 ± 12.6	–
1,000<MW<5,000	200	28.4 ± 6.4	75.3
	400	31.2 ± 12.5	72.9
	600	29.0 ± 3.3	74.5
5,000<MW<10,000	200	47.1 ± 20.4	59.1
		55.1 ± 17.1	52.2
		42.2 ± 5.1	63.2

WHAT WE CLAIM IS:—

1. An anti-cancer agent which comprises a substance active against cancer cell growth and obtained by cultivating *Staphylococcus epidermidis* STF (Bikoken Deposit No. 3706; ATCC 31310).

2. An agent as claimed in claim 1, which is active against leukemia.

3. An agent as claimed in claim 1, which is active against Ehrlich tumor cells.

4. An agent as claimed in claim 1, which has a molecular weight in the range of from 1000 to 5000.

5. An agent as claimed in claim 1, which has a molecular weight in the range of from 5000 to 10000.

6. An agent as claimed in any one of the preceding claims, which is stable to a temperature of 100°C for 15 minutes and exhibits negative Ninhydrin, Ehrlich and Sakaguchi reactions.

7. A method for the production of an anti-cancer agent, which comprises cultivating *Staphylococcus epidermidis* STF (Bikoken Deposit No. 3706; ATCC 31310) in a culture medium containing sources of carbon, nitrogen and inorganic salts and isolating the anti-cancer agent from the culture medium.

8. A method as claimed in claim 7, wherein the cultivation is effected aerobically in a liquid culture medium and wherein at completion of cultivation, solid matter is separated from culture solution, the culture solution is concentrated to yield precipitated matter, the precipitated matter is washed with water and redissolved in water, the solution obtained is fractionated, a fraction having anti-cancer activity is selected and purified and then the desired active ingredient is isolated therefrom.

9. A method as claimed in claim 8, wherein cultivation is effected at from −20°C to −40°C for from 24 to 120 hours.

10. A method as claimed in claim 9, wherein cultivation is effected at 37°C.

11. A method as claimed in any one of claims 8 to 10, wherein the fractionation is effected using an adsorptive filtration gel.

12. A method as claimed in any one of claims 8 to 10, wherein the fractionation is effected using a molecular sieve.

13. A method as claimed in any one of claims 8 to 10, wherein the fractionation is effected by ion-exchange chromatography.

14. A method as claimed in any one of claims 8 to 13, wherein in the fractionation, a fraction exhibiting anti-leukemia activity is selected and purified and its active ingredient is isolated therefrom.

15. A method for the production of an anti-cancer agent, substantially as described in either of the foregoing Examples.

16. An anti-cancer agent, whenever produced by the method claimed in any one of claims 7 to 15.

17. A pharmaceutical composition, which comprises an anti-cancer agent as claimed in any one of claims 1 to 6 and 16 in association with a pharmacologically acceptable carrier.

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