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Microbial Assay of Hepatotoxic Anthraquinones with *Escherichia coli* F-11[1]

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In order to find out the method for a biological detection of luteoskyrin, a hepatotoxic anthraquinone of *Penicillium islandicum* Sopp, we investigated the inhibitory effect of mycotoxins on the growth rate of *Escherichia coli* F-11 and Q-13. The results were summerized as follows;

1) 0.4 μg/ml of (−)-luteoskyrin or 1.5 μg/ml of (+)-rugulosin, hepatotoxic anthraquinoes of *Penicillium islandicum* Sopp or *Penicillium rugulosum*, caused the 50% inhibition of the growth of *E. coli* F-11, an actinomycin D sensitive mutant derived from *E. coli* Q-13, without affecting the parent type.

2) 2—5 μg/ml of patulin, (−)-rugulosin and penicillic acid inhibited the both bacteria.

3) Sporidesmin, flavoskyrin, catenarin and emodin inhibited slightly *E. coli* F-11.

4) No inhibition was observed with chlorine-containing peptide, islandicin, ascradiol, nivalenol, fusarenon-X, rubratoxin B, aflatoxin B or citrinin.

5) *E. coli* F-11 is considered to be a simple tool for the microbial assay of the hepatotoxic luteoskyrin or rugulosin contaminated in fungi-polluted cereal grains or foodstuffs.

Introduction

Luteoskyrin, a hepatotoxic pigment of *Penicillium islandicum* Sopp,[3] is proved to interfere oxidative phosphorylation of rat liver mitochondria,[4–6] nuclear RNA synthesis in Ehrlich ascites tumor[7] and to bind with thymus DNA[8–10] and deoxyribonucleohistone.[11] Long-term feeding experiments with luteoskyrin revealed that liver diseases such as acute liver atrophy, liver cirrhosis, adenoma and hepatoma were induced in mice and rats, depending upon the administered dose and the stage of intoxication.[3,12,13]

In the present paper, we examined to find out a method for biological detection of the toxin, and the results indicated that *Escherichia coli* F-11, an actinomycin D sensitive mutant derived from 1-methyl-2-nitroso-3-nitroguanidine treated- *E. coli* Q-13, is highly sensitive to luteoskyrin and rugulosin among mycotoxins tested, and that this bacterium is utilizable for the microbial assay of the toxins.

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2) Location: *12, Ichigaya-funagawara-cho, Shinjuku-ku, Tokyo.*
3) K. Uraguchi, T. Tatsuno, F. Sakai, M. Tsukioka, Y. Sakai, O. Yonemitsu, H. Ito, M. Miyake, M. Saito, M. Enomoto, T. Shikata and T. Ishiko, *Japan. J. Exp. Med.*, **31**, 19 (1961).
4) I. Ueno, Y. Ueno, T. Tatsuno and K. Uraguchi, *Japan. J. Exp. Med.*, **34**, 135 (1964).
5) Y. Ueno, I. Ueno, T. Tatsuno and K. Uraguchi, *Japan. J. Exp. Med.*, **34**, 197 (1964).
6) I. Ueno, *Jap. J. Biochem. Soc.*, **38**, 741 (1966).
7) Y. Ueno, I. Ueno, K. Ito and T. Tatsuno, *Experientia*, **23**, 1001 (1967).
8) Y. Ueno, A. Platel and P. Fromageot, *Biochim. Biophys. Acta*, **134**, 27 (1967).
9) Y. Ohba and P. Fromageot, *European J. Biochim.*, **1**, 147 (1967).
10) Y. Ueno, I. Ueno and K. Mizumoto, *Japan. J. Exp. Med.*, **38**, 47 (1968).
11) Y. Ueno, I. Ueno, K. Mizumoto and T. Tatsuno *J. Biochem.* (Tokyo), **63**, 395 (1968).
12) K. Uraguchi, *Excerpta Med. Int. Congr. Series*, No. 87, 465 (1965).
13) K. Uraguchi, *Encyclopedia of Pharmacology*, Pergamon Press, in press, 1969.

Materials and Methods

E. coli Q-13 (RNase I$^-$) and F-11[14], which were kindly supplied from Dr. Mizuno, University of Tokyo, were inoculated on a nutrient medium (10 g bactopeptone, 3 g yeast extract, 3 g glucose, 1 g NaCl and 1 liter deionized water, pH 7.4) and previously incubated at 37° overnight. After being adjusted the optical density at 650 mμ to 0.6, 1 ml of the cell suspension was mixed with the nutrient medium in a L-type tube to make up a final volume of 10 ml. For experimental purpose, luteoskyrin or rugulosin was first suspended in 0.05 ml of 2M tris and the diluted with distilled water. Final pH was adjusted to 7.0. Other toxins or compounds were dissolved in distilled water.

Mycotoxins were isolated from the following fungal mat or culture broth after the methods reported: Hepatotoxic luteoskyrin and chlorine-containing peptide,[3] *Penicillium islandicum* Sopp; hepatotoxic rugulosin,[15] *Penicillium rugulosum*; nephrotoxic citrinin,[16] *Penicillium citrinum*; carcinogenic patulin,[17,18] *Aspergillus clavatus*; cytotoxic nivalenol[19] and fusarenon-X,[20] *Fusarium nivale*; Penicillic acid, rubratoxin B and ascradiol were kindly gifted from Dr. Kurata (National Institute of Hygienic Sciences, Tokyo). (−)-Rugulosin, aflatoxin B and sporidesmin were supplied by Dr. Shibata (University of Tokyo, Tokyo), Dr. Aibara (National Institute of Health, Tokyo) and Dr. Tayler (National Research Council of Canada), respectively. The inhibitory potency of these toxins were also compared with antibiotics such as actinomycin D and chromomycin A_3. Furthermore, in relation to the chemical structure of luteoskyrin or rugulosin, the inhibitory effect of quinoid pigments such as islandicin, flavoskyrin, catenarin and emodin were examined.

Results and Discussion

As shown in Fig. 1, the growth of *E. coli* F-11 was reduced to 50% of the control by 10 μg/ml of actinomycin D and was completely inhibited by 50 μg/ml. While, the growth of *E. coli* Q-13 was reduced to 50% of the control by 50 μg/ml and the complete inhibition needed 100 μg/ml.

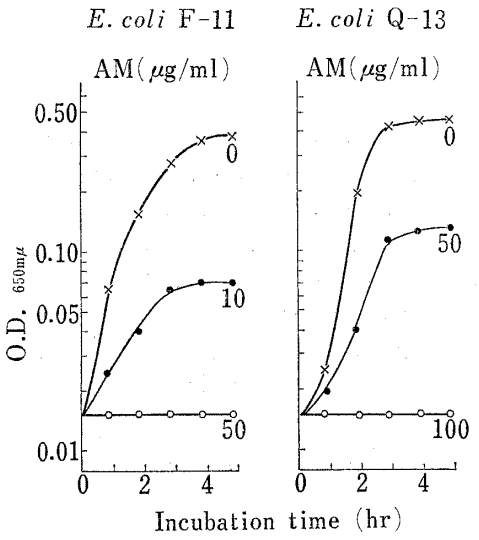

Fig. 1. Effect of Actinomycin D on the Growth of *E. coli* F-11 and Q-13

E. coli was previously grown with shaking on the nutrient medium at 37° for 16—18 hr. 1 ml of the cell suspension was transferred to a L-type tube and mixed with 8 ml of the fresh nutrient medium to continue the cultivation. At desired intervals, the optical density at 650 mμ was measured.

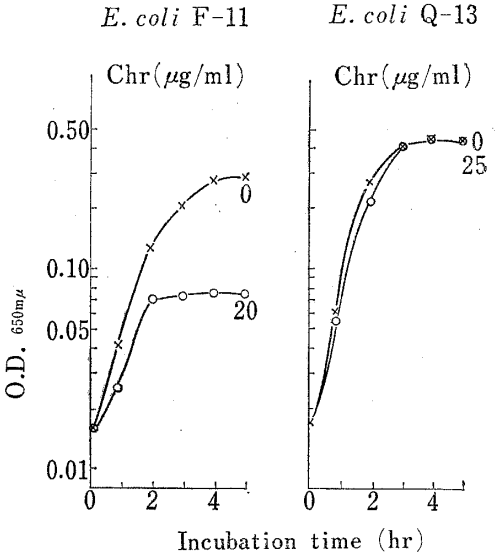

Fig. 2. Effect of Chromomycin A_3

14) R. Nozawa and D. Mizuno, *J. Biochem.* (Tokyo), **63**, 765 (1968).
15) Y. Ueno, Y. Iitoi, H. Yamakawa, N. Sato, M. Saito, M. Enomoto, I. Ueno and H. Tsunoda, *Folia pharmacol. japon.*, in press, 1969.
16) F. Sakai, *Folia pharmacol. Japon.*, **51**, 431 (1955).
17) F. Dichens and H.E.H. Jones, *Brit. J. Cancer*, **15**, 85 (1961).
18) F. Dichens and H.E.H. Jones, *Brit. J. Cancer*, **19**, 392 (1965).
19) T. Tatsuno, M. Saito, M. Enomoto and H. Tsunoda, *Chem. Pharm. Bull.* (Tokyo), **16**, 2519 (1968).
20) Y. Ueno, I. Ueno, T. Tatsuno, K. Ohkubo and H. Tsunoda, *Experientia*, **25**, 1062 (1969).

This agrees with the finding of Nozawa, *et al.*[14] that *E. coli* F-11 is sensitive to actinomycin D.

The effect of chromomycin A_3 on the growth of the bacterium is shown in Fig. 2. *E. coli* F-11 was inhibited by 20 μg/ml, though in the case of *E. coli* Q-13 no inhibition was observed with 25 μg/ml of the antibiotic. Therefore, it is very likely that *E. coli* F-11 was also sensitive to chromomycin A_3.

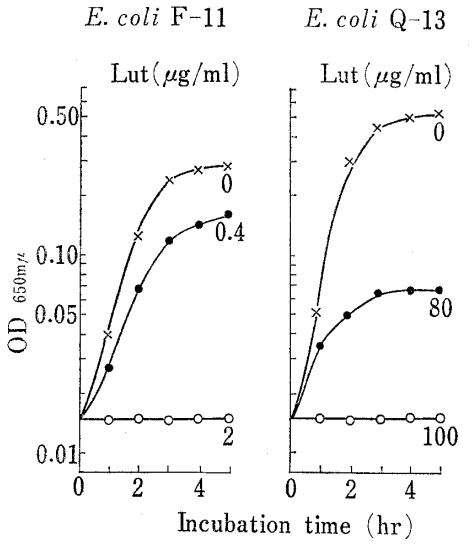

Fig. 3. Effect of Luteoskyrin

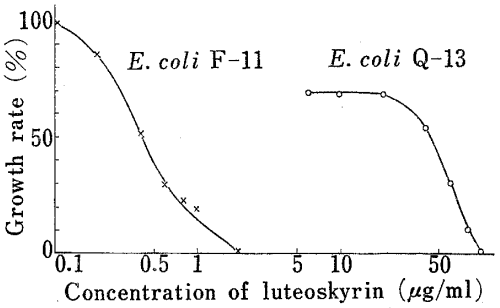

Fig. 4. The Relation between the Concentration of Luteoskyrin and the Growth Rate

E. coli F-11 or Q-13 was cultured for 3 hr in the presence of varied amount of luteoskyrin.

The effect of luteoskyrin on the growth of *E. coli* was investigated (Fig. 3). Luteoskyrin of 0.4 μg/ml reduced the growth of F-11 cells 50% of the control, and 2 μg/ml was enough to cause the complete inhibition. While, the growth of Q-13 cells was reduced to 80% of the control by 80 μg/ml and completely inhibited by 100 μg/ml. The relation between the concentration of luteoskyrin and the growth rate of *E. coli* F-11 and Q-13 is shown in Fig. 4. The decreased growth rate of *E. coli* F-11 was parallel to the concentration of added luteoskyrin ranging from 0.1 μg/ml to 2.0 μg/ml. In case of *E. coli* Q-13, however, the dose response curve was attained when 20 μg/ml or more was added to the culture medium. From the above results, the concentration of luteoskyrin causing 50% inhibition was estimated to 0.4 μg/ml for F-11 cells and 40 μg/ml for Q-13 cells. This indicates that F-11 is 100 times sensitive to

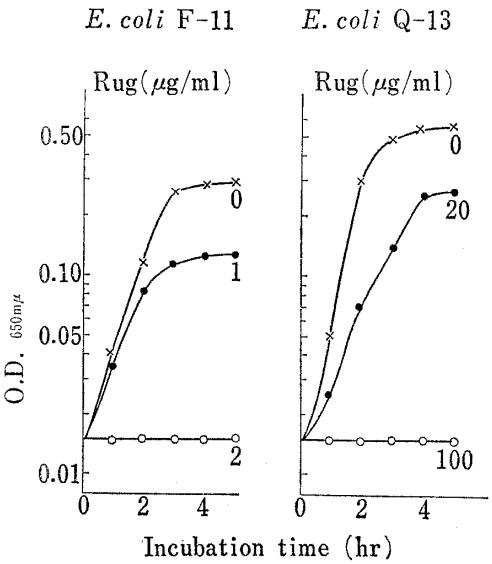

Fig. 5. Effect of (+)-Rugulosin

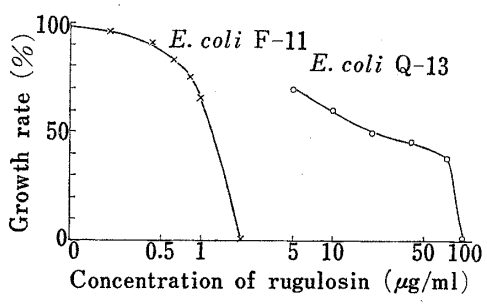

Fig. 6. The Relation between the Concentration of (+)-Rugulosin and Growth Rate of *E. coli*

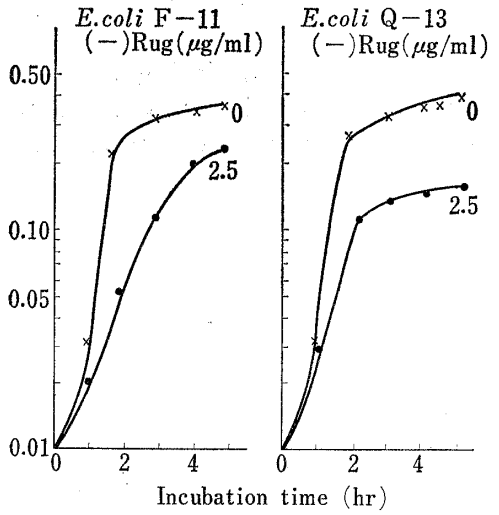

Fig. 7. Effect of (−)-Rugulosin

luteoskyrin than the parent type Q-13, and that the inhibitory concentration of luteoskyrin is remarkably lower than the antibiotics noted in the above.

The similar experiment was conducted with (+)-rugulosin, as shown in Fig. 5 and 6. The decreased growth rate of *E. coli* F-11 was parallel to the concentration of added rugulosin ranging from 0.2 μg/ml to 2 μg/ml. The concentration of 50% inhibition was estimated to 1.5 μg/ml for F-11 cells and 20 μg/ml for Q-13 cells. Therefore, F-11 was also sensitive to rugulosin as well as luteoskyrin, though the sensitivity was about one forth of luteoskyrin.

The inhibitory effect of (−)-rugulosin is shown in Fig. 7. The both strains were inhibited to the same degree by 2.5 μg/ml. The similar result was obtained with 5 μg/ml of patulin and penicillic acid. Noticeable findings are that the inhibitory concentration of these three toxins was lower than that of the antibiotics.

As for the other compounds, 5 μg/ml of sporidesmin, 48.5 μg/ml of flavoskyrin, 136 μg/ml of catenarin and 50 μg/ml of emodin inhibited partially the growth of F-11 without affecting the parent type. No inhibition was observed with chlorine-containing peptide, islandicin, ascradiol, nivalenol, fusarenon-X, rubratoxin-B, aflatoxin B or citrinin.

Table I. Inhibitory Effect of Mycotoxins and Antibiotics on *E. coli* F-11 and Q-13

Type	Compounds	ID_{50} (μg/ml) F-11	ID_{50} (μg/ml) Q-13	Ratio of F-11/Q-13
I	(−)-Luteoskyrin	0.4	40	1/100
	(+)-Rugulosin	1.5	20	1/13
II	Patulin	5.0	5	1/1
	(−)-Rugulosin	2.5	2.5	1/1
	Penicillic acid	5.0	5	1/1
III	Sporidesmin	5+	(5)	
	Flavoskyrin	48+	(48)	
	Catenarin	136+	(136)	
	Emodin	50+	(50)	
IV	Chlorine-containing peptide	(100)	(100)	
	Islandicin	(125)	(125)	
	Ascradiol	(100)	(100)	
	Nivalenol	(25)	(25)	
	Fusarenon-X	(40)	(40)	
	Rubratoxin B	(100)	(100)	
	Aflatoxin B	(10)	(10)	
	Citrinin	(20)	(20)	
	Actinomycin D	10	50	
	Chromomycin A_3	20	(25)	

\+ partial inhibition ( ) no inhibition

From these results, as summarized in Table I, the mycotoxins and related compounds were classified into four types depending upon the inhibitory effect on the bacteria as follows; type I, inhibitory only to F-11: type II, inhibitory to the both bacteria: type III, inhibitory to F-11 but slight in degree: type IV, no inhibitory to the both bacteria.

Table II. Comparative Toxicity of Luteoskyrin and Rugulosin on Biological Systems

		(−)-Luteoskyrin	(+)-Rugulosin
ID_{50} (μg/ml)	*E. coil* F-11	0.4	1.5
	Tetrahymena	1—2.5[22]	5—10
	HeLa cell	0.1—0.3[23]	
LD_{50} (mg/kg)	mice (male, *i.p.*)	40.8[13]	83.0[15]

Table II shows the comparative ID_{50} of luteoskyrin and (+)-rugulosin to several biological systems. In all cases, luteoskyrin is about 2 to 3 times toxic than rugulosin. Chemically speaking, luteoskyrin and rugulosin have nearly the same structure, and the only difference is the lack of one OH group in rugulosin.[21] This lack might be responsible for the less toxicity of rugulosin.

As to the bioassay, *E. coil* F-11 is more sensitive to the toxic anthraquinones than the protozoon[22] and less sensitive than HeLa cell.[23] However, the cultivation of the bacterium is so easy and the time required for the assay is only a few hour. Therefore, the practical application of this microbial assay is expected to give a tool for the detection of luteoskyrin or rugulosin in fungi-contaminated cereal grains or foodstuffs.

According to Nozawa, *et al.*[14] *E. coli* F-11 was presumed to be defficient in polysaccharides composing the cell wall of the bacterium, and this lack is presumably one of the reason why the mutant is so highly sensitive to the toxic anthraquinones. Impermeability of luteoskyrin to cellulose membrane or Millipore filter (unpublished data) supports the above mentioned assumption, though a biochemical resolution of the inhibitory action is remained to be dissolved.

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21) U. Sankawa, S. Seo, N. Kobayashi, Y. Ogihara and S. Shibata, *Tetrahedron Letters*, **53**, 5557 (1968).
22) Y. Ueno and M. Saheki, *Japan. J. Exp. Med.*, **38**, 157 (1968).
23) M. Umeda, *Acta Path. Jap.*, **14**, 373 (1964).