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Anthelmintic Effect of the *Embelia ribes* Decoction and Embelin Derivatives on *Trichuris muris* in Mice

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Abstract

In murine trichuriasis, the anthelmintic effects of *Embelia ribes* decoction, embelin, the major constituent of *E. ribes*, and its 6 derivatives were evaluated. Among the samples tested, only mono-potassium embelate was effective against *Trichuris muris*, but the effect was unstable in mice pretreated with prednisolone butylacetate that was necessary for establishing the infection in mice. This compound was again poorly effective on the infected athymic mice, while mebendazole was effective to these mice, thus suggesting that mono-potassium embelate is not a powerful anthelmintic against *T. muris*, or murine trichuriasis in mice is not a suitable model for evaluating embelin derivatives.

Key words: trichuriasis; *Embelia ribes*; embelin; mono-potassium embelate, *Trichuris muris*; anthelmintic effect.

Introduction

Whipworm, *Trichuris trichiura*, infection is one of the important soil transmitted helminthiasis in the world, particularly in tropical and subtropical areas. The number of infected people in developing countries is estimated to be approximately five hundred million. Although mebendazole is the only anthelmintics so far known as effective for trichuriasis, it is too expensive to use for mass treatment in the endemic countries and its anthelmintic effect is not satisfactory. Therefore, anti-whipworm drugs cheep and of stable efficacy are still needed.

Sahu (1988, 1993) reported that fruits of *Embelia ribes*, a well known traditional medicine in Nepal called "bayubidanga", was effective to *Trichuris trichiura, Ascaris lumbricoides* and *Ancylostoma duodenale*. He administrated decoction of *E. ribes* to 48 infected people and observed more than 80% of cure rate in trichuriasis patients after 2 weeks of administration by fecal examination. He also showed that the fruits powder was similarly effective for trichuriasis as the decoction was. Side effects such as nausea observed during treatment were negligible (Sahu, 1988, 1993). In contrast, Ismail *et al.* (1993) reported that *E. ribes* decoction was scarcely effective for *T. trichiura* in their trail with 38 infected patients in Sri Lanka.

Therefore, it was urgently necessary to establish whether the *E. ribes* decoction is actually effective to trichuriasis, and if it is, what constituent is responsible to that activity. In this report, we evaluate the anthelmintic effects of *E. ribes* and some of the extracted products on *Trichuris* infection using murine trichuriasis as an animal model.

Material and Method

Animal

Inbred strain of BALB/c mice were purchased

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from SLC Japan (5-week-old male, Shizuoka, Japan). Athymic nude KSN mice maintained by Institute of Animal Center in Kanazawa University were provided by the courtesy of Drs. O. Nikaido and Y. Ishigaki, Faculty of Pharmaceutical Sciences, Kanazawa University, Japan.

Parasite

Mature eggs of *T. muris* were obtained from Prof. Y. Ito, Department of Parasitology, School of Medicine, Kitasato University, Japan and Associate Prof. M. Niimura, Department of Parasitology, School of Medicine, Chiba University, Japan. These eggs are of the same origin (Kitasato University). The eggs were kept in 4°C until use.

Anthelmintics

Mebendazole (Kyowa Hakko Co. Ltd., Tokyo, Japan) and ivermectin (Ivomec^{R}, Merk & Co. Inc., U.S.A.) were used as common anthelmintics against *Trichuris muris* to compare the efficacy.

E. ribes decoction and embelin derivatives were prepared as follows and their chemical structures are shown in Fig. 1. The fruits of *E. ribes* (of Nepal origin) were provided by Dr. Sahu, Ayurvedic Campus, Institute of Medicine, Tribhuvan University, Nepal. The decoction was prepared following the traditional method in Nepal. Namely, 25 g of fruit powder was mixed with 500 ml of distilled water, and boiled until the volume became one-eighth of the original. The supernatant was then collected and used for treatment as soon as possible. The decoction was freshly prepared each time.

Embelin (Fig. 1-1) was obtained in 2.9–3.2% yield from the powdered fruit of *E. ribes* by extraction with ether at room temperature. It gave orange needles, mp 145–148°C (mp 142–143°C: Merck Index, 1983) by recrystallization from acetone or benzene.

Ammonium embelate was prepared by crystallization of embelin from hot 10% ammonium hydroxide as red-purple needles, mp 153°C. This was proved to be di-ammonium salt (Merck Index, 1983) from the elementary analysis and the UV spectrum (Fig. 1-2).

Potassium embelate was prepared as follows: embelin (500 mg) suspended in acetone (5 ml) was neutralized with 1.0 molar equivalent of 10% KOH (1.12 ml). By this procedure, all crystals came into solution. Dry ether was added until precipitation completed. The precipitates were collected by filtration and washed with ether to yield potassium embelate (0.487 g) as brick-red fine crystals. This was proved to be a mono-potassium salt from the elementary analysis and the UV spectrum (Fig. 1-3).

O-Methylembelin (Fig. 1-4) was prepared as yellow needles, mp 96–97°C (mp 95–96°C; Joshi and Kamat, 1975) in 14% yield on heating embelin in methanol in the presence of acid catalyst such as decanoic acid or short-time treatment of embelin with diazomethane in ether.

Dihydroembelin (Fig. 1-5) was prepared by sodium hydrosulfite reduction of embelin as pale brown unstable crystals.

The compounds so-called "dimer" (Fig. 1-6) and "benzofuran-quinone" (Fig. 1-7) were obtained by heating embelin in boiling water in the presence of acid catalyst such as decanoic acid in 43% and 9% yields, as red fine needles, mp 173–174°C, and black-red crystals, mp 166–167°C, respectively. Details of this chemical transformation and structure determination of the products will be reported separately.

Infection and steroid treatment

In a previous report, we demonstrated that the infection rate and cure rate in mice depended upon dose and a type of steroids used for establishing the infection (Akao *et al.*, 1996). Therefore, the mice used in this experiment were treated with 2.5 mg of hydrocortisone acetate (HC, Fuji Pharmaceutical Co. Ltd., Tokyo) or 1.0 mg of prednisolone butylacetate (PB, Banyu Pharmaceutical Co. Ltd.). HC was injected subcutaneously on day 0, 1, 9 and 10, and PB intramuscularly on the same schedule. They are designated as groups A and B, respectively.

On day 0, mice were orally administered 100 eggs of *T. muris* using a gastric tube. After infection, each mouse was caged separately. Between the 40th and 50th day of infection, fecal examination was carried out. When we found *Trichuris* eggs, the number of eggs per gram (EPG) was determined, and then drugs were orally administered by a gastric tube under light anesthesia for 3 consecutive days. After that, the adult worms expelled in feces were

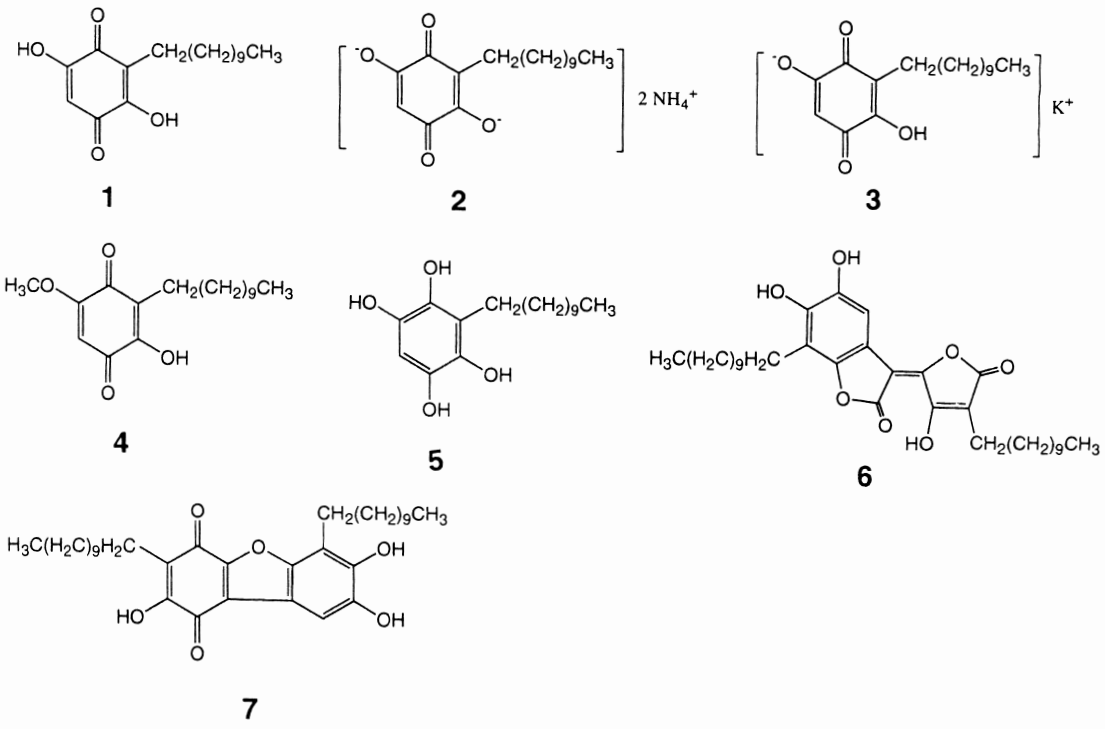

Fig. 1 Structures of Embelin and its Derivatives.

monitored everyday for additional 14 days. After observation, all mice were sacrified to count the worms remaining in coecum and colon.

The infection rate, intensity of infection, and worm reduction rate (WRR) were calculated by the following equations:

$$\text{Infection rate (\%)} = \frac{\text{No. of mice infected}}{\text{No. of mice inoculated}} \times 100.$$

$$\text{Intensity (\%)} = \frac{\text{No. of adult worms recovered from a mouse}}{\text{No. of eggs inoculated}} \times 100.$$

$$\text{\% WRR} = \frac{\text{No. of worms expelled within 2 wks}}{\text{No. of worms expelled within 2 wks + No. of worms remained}} \times 100.$$

Statistics

Statistical analyses were carried out by Mann-Whitney U-test.

Results

Firstly anthelmintic constituent in *E. ribes* was examined by *in vitro* examination using *T. muris*. The activity of embelin itself was not determined, because it was completely insoluble in saline. However, its salts, ammonium embelate and potassium embelate, were soluble in water and showed strong activity in killing the worms with minimum lethal concentrations (MLC) of 0.1 μg/ml (after 4 hr) and 0.1 mg/ml (after 12 hr), respectively. Therefore, we continued to evaluate the anthelmintic effect of *E. ribes* decoction and embelin derivatives *in vivo*.

In our previous experiments, we have observed that the effect of anthelmintics such as mebendazole diminished for the mice which were profoundly pretreated with steroids to acquire high infection rate and high infection intensity (Akao *et al.*, 1996). Thus, two groups of mice (group A: pretreated with HC, 2.5 mg × 4 and group B: pretreated with PB, 1.0 mg × 4) were used for evaluation of the efficacy of drugs, because susceptible ICR mice pretreated with

Table 1 Anthelmintic effect of *Embelia ribes* and embelin derivatives on BALB/c mice pretreated with hydrocortisone acetate (Group A*)

Drug	Dose†	Eggs in feces EPG/1000	No of adult worms appeared in feces			Adult worms in intestine	WRR‡ (%)
			1st wk	2nd wk	Total		
E. ribes decoction	0.5 ml	35	1	ND	1	58	2
		38	0	ND	0	26	0
Embelin	2 mg	2	0	ND	0	6	0
		3	3	ND	3	2	60
		14	12	6	18	17	51
		15	0	ND	0	24	0
		21	0	ND	1	78	1
		61	1	ND	1	43	2
		68	1	3	4	6	40
		85	36	ND	36	37	50
Ammonium embelate	1 mg	3	9	8	17	8	68
		15	0	1	1	39	3
Potassium embelate	1 mg	9	12	31	43	7	86
		14	12	6	18	0	100
		15	43	4	47	3	94
		15	3	20	23	9	72
		18	10	0	10	0	100
Dimer	2 mg	3	7	ND	7	15	32
		32	0	ND	0	50	0
Embelin+Dimer (1mg+1mg)	2 mg	12	2	0	2	18	10
		16	6	6	12	8	60
O-Methylembelin	2 mg	22	0	0	0	99	0
		83	26	23	49	18	73
Dihydroembelin	2 mg	67	1	0	1	66	1
		143	7	22	29	69	30
Control (10% ethanol)	0.5 ml	45	1	0	1	24	4
Mebendazole	2 mg	1	7	ND	7	0	100
		1	7	ND	7	0	100
		8	13	ND	13	0	100
		19	31	ND	31	0	100
		20	6	0	6	0	100
		35	53	0	53	0	100
		59	11	ND	11	34	25
Ivermectin	0.03 mg	50	42	ND	42	1	99

ND: not determined

*Pretreated with HC subcutaneously 2.5 mg × 4 times (on day 0, 1, 9 and 10)

†Indicated doses were administered orally for 3 consecutive days.

‡Worm reduction rate

steroids (HC, 2.5 mg × 4, s. c. or PB, 2 mg × 4, i. m.) very poorly responded to anthelmintics including mebendazole and embelin derivatives (Tsuda and Kondo, 1993). For the mice of groups A and B, the rate of infection and intensity of infection were 50%, 10.4% and 100%, 5.4%, respectively, and the WRR of mebendazole was scarcely affected (89% and 100%, respectively). It must be noticed that for the group B mice, the natural expell of worms during 2 weeks was 30%.

Table 1 shows the anthelmintic effects of the *E. ribes* decoction, embelin, and its derivatives on *T. muris* for the group A mice. The decoction of *E. ribes* did not show any anthelmintic effect. Anthelmintic effects of embelin, ammonium embelate, the dimer, *O*-methylembelin, and dihydroembelin were unstable. In contrast, potassium embelate was constantly effective. The average WRR of potassium embelate (90.4%) was almost the same as that of mebendazole (89%).

Table 2 shows the results for group B mice. Previously, we demonstrated that PB pretreatment of mice markedly reduce the effect of anthelmintics on murine trichuriasis (Akao *et al.*, 1996). In accord with this, two of the three mice responded only weakly to potassium embelate and the average WRR was 57%. Again, the decoction was poorly effective (28%) and the efficacy of the other compounds were

Table 2 Anthelmintic effect of *Embelia ribes* and embelin derivatives on BALB/c mice pretreated with prednisolone butylactate (Group B*)

Drug	Dose†	Eggs in feces EPG/1000	No. of adult worms appeared in feces			Adult worms in intestine	WRR‡ (%)
			1st wk	2nd wk	Total		
E.. ribes decoction	0.5 ml	12	2	1	3	8	27
		29	1	1	2	5	29
Embelin	2 mg	12	0	0	0	8	0
		16	5	8	13	0	100
		58	1	1	2	7	2
Potassium embelate	2 mg	20	9	0	9	15	38
		78	2	0	2	10	17
		136	5	3	8	0	100
Dimer	2 mg	16	0	0	0	8	0
		17	0	0	0	7	0
		18	1	0	1	5	17
Benzofuran-quinone	2 mg	13	1	0	1	6	14
		28	0	4	4	6	40
		2	1	1	2	8	40
Control (10% ethanol)	0.5 ml						30(11)§
Mebendazole	2 mg						100(4)‖
Ivermectin	0.03 mg	8	5	0	5	0	100
		30	13	0	13	0	100

*Pretreated with PB intramusculary 1.0 mg × 4 times (on day 0, 1, 9 and 10)
†Indicated doses were administered orally for 3 consecutive days.
‡Worm reduction rate
§Average natural expel of worms for 11 mice.
‖Average worm reduction rate for 4 mice.

Table 3 Anthelmintic effect of *Embelia ribes* and embelin derivatives on KSN nude mice

Drug	Dose*	Eggs in feces EPG/1000	No. of adult worms appeared in feces			Adult worms in intestine	WRR† (%)
			1st wk	2nd wk	Total		
E. ribes decoction	0.5 ml	16	0	0	0	5	0
Embelin	2 mg	10	0	0	0	5	0
Potassium embelate	2 mg	18	0	0	0	15	0
		230	2	0	2	47	4
Control (10% ethanol)	0.5 ml	14	0	0	0	8	0
		190	0	0	0	24	0
Mebendazole	2 mg	160	40	0	40	0	100
		200	40	0	40	0	100

*Indicated doses were administered orally for 3 consecutive days.
†Worm reduction rate.

unstable. However, mebendazole was constantly effective (100%) for this group.

To ascertain the anthelmintic effect of potassium embelate, further examination was carried out using KSN nude mice which were infected with *T. muris* without pretreatment of steroids. The results are shown in Table 3. Athymic nude mice are unable to eliminate *Trichuris* worm from their intestine (Ito, 1991). In fact, natural elimination of adult worms was not observed for this group during the observation period. In these mice, administrations of the decoction and embelin produced no anthelmintic effect on *T. muris*. Although one of the two athymic mice expelled 2 adult worms within 1 week after administration of potassium embelate, the WRR was only 4%. On the other hand, mebendazole showed 100% WRR for these mice within 1 week after administration.

Discussion

In vitro examinations showed that embelin, as its salt form, had high anthelmintic effect on *T. muris*. This was also indicated by Niimura *et al.* (1993). Embelin is contained in approximately 3% in the fruits of *E. ribes*, and known as the active constituent for cestode infection (Thompson, 1971). Recently, Sahu (1988, 1993) reported that the decoction of *E. ribes* was also effective for *T. trichiura* infection in Nepal. In contrast, Ismail *et al.* (1993) noticed that trichuriasis patients in Sri Lanka did not respond to the decoction of *E. ribes*.

In this study we observed that the *E. ribes* decoction was not or only slightly effective both for prednisolone treated (group B) and hydrocortisone treated (group A) BALB/c mice infected with *T. muris*. Furthermore, our preliminary study revealed that the dedoction was again ineffective to dog whipworm, *Trichuris vulpis* (Data not shown).

In chemical investigations, we have recently found that the most of embelin is changed by boiling with water to produce the dimer (42%), the benzofuran-quinone (9%), and several other compounds. Hence, embelin remained in the decoction of *E. ribes* was less than 10% of the original content in the fruits as evidenced from the UV analysis (unpublished data by the authors). On the other hand, the salt, mono-potassium embelate, was found to be stable on heating with water. Thus, we suspected that, if Sahu's results are correct, embelin derivatives of different chemical forms or some of the transformation products in the decoction possibly have the activity to *T. muris*. Therefore, we tested *in vivo* effect of several embelin derivatives together with embelin and its salts, di-ammonium embelate and mono-potassium embelate, using

murine trichuriasis as a model. The salts will regenerate embelin on contact with an acid. The dimer and the bezofuran-quinone are the transformation products in the decoction. *O*-Methylembelin and dihydroembelin are the compounds expected to produce embelin *in vivo* by hydrolysis and oxidation, respectively.

The *in vivo* experiments on administration of the above derivatives showed that none of them was constantly effective, except mono-potassium embelate, which seemed to be the promising drug. Two out of the five BALB/c mice pretreated with hydrocortisone (group A) completely expelled the worms by the administration of 1 mg × 3 times of mono-potassium embelate, and the worm reduction of the other 3 mice were almost the same as that of mice administered with mebendazole (2 mg × 3 days). However, in the mice pretreated with prednisolone (group B), the worm reduction of mono-potassium embelate was inferior than that of mebendazole. Moreover, potassium embelate was almost ineffective in eliminating the worms in congenitally athymic nude mice.

Although the discrepancy between the mice pretreated with a different kind of steroids and immuno-deficiency mice was not clearly understood, we conclude that the *E. ribes* decoction and embelin are ineffective to murine trichuriasis. Mono-potassium embelate is likely effective to the murine trichuriasis, but its efficacy is largely dependent on the strain of mice, steroid pretreatment, and the kind of *Trichuris* species. Thus, *T. muris* on mice may not be a suitable model for human trichuriasis.

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