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New Assay Method for Surveying Anti-Emetic Compounds from Natural Sources

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Abstract – The new assay method was developed by using young chicks instead of frogs for screening of anti-emetic compounds from natural sources. Comparing with our previous method using leopard and ranid frogs, the advantages of the new method were not only completely parallel results but also decreasing standard errors. Hinesol and eudesmol were isolated from MeOH extract of *Atractylodes lancea* rhizome as the anti-emetic principles.
Key words – Anti-emetic, Hinesol, Eudesmol, *Atractylodes lancea.*

Introduction

We have surveyed Chinese traditional medical preparations with the anti-emetic activity using leopard and ranid frogs (T. Kawai *et al.*, 1994), and shogaols and gingerols from *Zingiber officinale* rhizome, rengyol, phyllrin, and rutin from *Forsythia suspensa* fruits, magnolol and honokinol from *Magnolia obovata* bark, cryptomeridiol, bigelovin, dihydrobigelovin, and taraxastery palmitate and taraxasteryl acetate from *Inula britannica* flower, polyporenic acid C, pachymic acid and dehydropachymic acid from *Poria cocos* sclerotium, were identified respectively as the anti-emetic active principles (K. Kinoshita *et al.*, 1996, T. Tai *et al.*, 1995). The bioassay using frogs is useful in screening anti-emetic activity in a small scale, but it takes a long time for a course of experiment (90 min). On the other hand, the new method using young chicks showed advantages as follows, 1) easy handling, 2) easy counting of retching motion, and 3) short experimental time, within 30 min.

In the present study, anti-emetic principles, isolated earlier in our laboratory, were examined in order to compare the new method with the old one. Meanwhile, new anti-emetic principles were isolated and identified from *Atractylodes lancea* rhizome by using the new method

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Experimental

Reagents – Copper sulfate anhydride (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) was used as the emetic drug. Dimethyl sulfoxide (DMSO), saline and Tween 80 (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) are commercial products. Chloropromazine, metochlopramide and ethyl *p*-aminobenzoate are commercial products for used as reference (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan).

Spectroscopy and chromatography – ^{1}H- and ^{13}C-NMR spectra were recorded using a JEOL GSX-400 spectrometer in $CDCl_3$, CD_3OD or DMSO-d_6 with tetramethylsilane or in D_2O with sodium 3-(trimethylsilyl)-propanesulfonate as an internal standard. Kieselgel 60 F_{256} (Merck) precoated plates were employed for thin-layer chromatography (TLC). Column chromatography was carried out on 70-230 mesh silica gel (Merck). HPLC was performed using an SSC-3100-J pump with an Oyo-Bunko Uvilog 7 UV detector. HR-MS and EI-MS were obtained using a JEOL JMX-DX 302.

Animals – Young male chicks (4 days of age) weighing 25-35 g were purchased from Goto Furanjo Co., Inc. (Saitama, Japan).

Bioassay of anti-emtic activity – The young chicks were divided into 6 groups. The young chicks were set aside for 10 min to stabilize at 38℃. The sample solution (0.1 ml/10 g b.w.) was suspended in 5% DMSO and 1% Tween 80, and injected abdominary at the doses of 10-300 mg/kg. After 10 min copper sulfate anhydride was administered orally at the dose of 200 mg/kg as an emetic agent, and the retching frequency was recorded for 10 min to judge the effect. A significant decrease of the retching frequency is a sign of positive effect of the test compound. The inhibition (%) was calculated as follows:

Inhibition (%) = (A-B) / A × 100

A : control average frequency of retching

B : average frequency of retching after sample treatment

Statistical analysis – All numerical data were expressed as the mean ± S.E. The statistical significance of the difference was determined by an unpaired Student's t-test.

Isolation and purification of anti-emetic principles from the rhizome of *Atlactylodes lancea* De Candle – The crude drug (3.0 kg) was extracted successively with $CHCl_3$, MeOH and H_2O. Each extract was examined by the anti-emetic bioassay using $CuSO_4$. The methanolic extract (287.5 g) showing anti-emetic activity was chromatographed on a silica gel column [$CHCl_3$-MeOH], and five fractions, fr. A - fr. E, were obtained. Each fraction was tested for anti-emetic activity, and using the bioassay, Fr. C showed anti-emetic activity, which was chromatographed on a silica gel column [$CHCl_3$-MeOH] and four fractions, fr. C-1 - fr. C-4, were obtained.

The fr. C-1, which had an anti-emetic activity, was subjected to HPLC using a silica gel column (Develosil 60-5, 1×25 cm, $CHCl_3$-MeOH) and three fractions, Fr. C-1-1 - Fr. C-1-3, were obtained. The active fraction, Fr. C-1-1, was finally subjected to HPLC using a silica gel column [Develosil 60-3 ($AgNO_3$), 1×25 cm, n-hexane-AcOEt] and hinesol (172 mg) and β-eudesmol (167 mg) were obtained as potential anti-emetic principles. The structures of both compounds were identified by MS, ^{1}H and ^{13}C-NMR, DEPT experiment, ^{1}H-^{13}C COSY and ^{1}H-^{13}C long range COSY (COLOC).

Results and Discussion

It is desirable to use a small size animal for the bioassay of biologically active principles of natural sources due to the shortage of the materials. For that reason small frogs weighing under 20 g were used earlier, but it took a long experimental time (90 min). A new method using young chicks showed some advantages. Handling for the operation of bioassay is easy; the emetic motion is observed without any feeding only by counting of the retching frequency; only a short experimental time is required.

The new method was first compared with the old one using three anti-emetic drugs which have already been reported previously. The three drugs including ethyl p-amino benzoate, an inhibitory drug for reflex emesis, chloropromazine for centrally acting emesis, and metochlopramide, an anti-emetic through acceleration of gastrointes- tine tract movement, were examined by the new method using young chicks. As shown in Table 1, by the new method, ethyl *p*-aminobenzoate, chloropromazine and metochlopramide inhibited the emesis induced by copper sulfate anhydride at the dose of 20 mg/kg and 50 mg/kg, and these results were the same as those obtained by the old method by using frogs (K. Kinoshita *et al.*, 1996). On the basis of this result, we demonstrated that the new method is more useful than the old one for the anti-emetic preliminary screening.

The anti-emetic principles of the crude drug materials, which were isolated and assayed by the earlier method, were examined again by the new method. As shown in Tables 2~6, all anti-emetic compounds, which were assay- ed by using frogs, showed significantly smaller number of retches induced by copper sulfate when examined by the new method. Shogaols and gingerols from *Zingiber officinale* rhizome showed the reduced number of eme- sis, 40% and 60%, at the dose of 20 mg/kg and 50 mg/kg, respectively (Tables 2 and 3).

Rengyol and rutin, the anti-emetic prin-

Table 1. Effects of anti-emetic drugs on copper sulfate-induced emesis in young chicks

drugs	dose (mg/kg)	No. of young chicks	No. of retches (mean ± S.E.)	inhibition (%)
control		6	51.3±5.08	
chloropromazine	10	6	32.7±3.22*	36.3
	20	5	27.8±6.20*	45.8
	50	6	19.6±3.93***	61.8
control		6	6.0±5.52	
metochlopramide	10	6	38.2±3.40	17.0
	20	6	23.2±5.71*	49.6
	50	6	11.5±3.04**	75.0
control		6	52.5±3.03	
ethyl-*p*-aminobenzoate	10	6	41.7±5.94	20.6
	20	5	35.2±4.62*	33.0
	50	6	14.7±2.12***	72.0

significantly different from the control value, *p<0.1, **p<0.01, ***p<0.001

Table 2. Effects of anti-emetic compounds of *Zingiber officinale* rhizome on copper sulfate-induced emesis in young chicks

drugs	dose (mg/kg)	No. of young chicks	No. of retches (mean ± S.E.)	inhibition (%)
control		5	39.0±1.52	
[6]-shogaol	10	6	38.8±3.34	-
	20	6	26.0±1.88***	33.3
	50	6	19.8±2.34***	49.2
control		6	41.0±1.67	
[8]-shogaol	10	6	38.3±2.76	-
	20	6	25.7±2.43***	37.3
	50	6	19.0±2.13***	53.7
control		6	50.3±2.20	
[10]-shogaol	10	6	41.5±1.48	-
	20	6	25.2±2.06***	49.9
	50	6	15.6±3.17***	69.0

significantly different from the control value, ***p<0.001

Table 3. Effects of anti-emetic compounds of *Zingiber officinale* rhizoma on copper sulfate-induced emesis in young chicks

drugs	dose (mg/kg)	No. of young chicks	No. of retches (mean ± S.E.)	inhibition (%)
control		5	45.7±2.79	
[6]-gingerol	10	6	41.8±1.78	-
	20	6	26.5±3.25**	42.0
	50	6	19.2±1.74***	58.0
control		6	45.5±2.28	
[8]-gingerol	10	6	43.0±2.03	-
	20	6	27.2±2.21***	40.2
	50	6	21.5±2.25***	52.7
control		6	40.2±1.30	
[10]-gingerol	10	6	39.8±2.10	-
	20	6	35.7±4.00	11.1
	50	6	1.6±3.61***	46.3

significantly different from the control value, $^{**}p<0.01$, $^{***}p<0.001$

ciples from *Forsythia suspensa* fruits, showed the activity (Table 4).

The anti-emetic compounds, magnolol, honokiol and cryptomeridiol, from *Magnolia obovata* bark showed the 40% inhibition (Table 5).

The compounds, taraxasteryl palmitate, taraxasteryl acetate and bigelovin, isolated from *Inula britannica* flower, showed the anti-emetic activity (Table 6).

The principles from *Poria cocos* sclerotium, polyporenic acid C, pachymic acid, dehydropachymic acid, showed the anti-emetic activity (Table 7).

Among the anti-emetic principles, shogaols, gingerols, magnolol and honokiol, which have the suppressive effects on central nervous system, showed relatively stronger activity than the others. In addition, young chick is a warm-blood animal with well developed central and peripheral nervous systems. There are only a small individual difference among young chicks so that this method shows lower standard errors than the method using frogs, amphibian. By the present experimental results,

Table 4. Effects of anti-emetic compounds of *Forsythia suspensa* fruits on copper sulfate-induced emesis in young chicks

drugs	dose (mg/kg)	No. of young chicks	No. of retches (mean ± S.E.)	inhibition (%)
control		6	44.7±3.22	
rengyol	10	6	40.7±3.22	-
	20	6	26.5±3.85**	40.7
	50	5	30.2±1.02	32.4
control		6	48.0±2.72	
rutin	10	6	45.8±9.32	-
	20	6	23.5±4.69**	51.0
	50	6	30.5±2.78**	36.5

significantly different from the control value, $^{**}p<0.01$

Table 5. Effects of anti-emetic compounds of *Magnolia obovata* bark fruits on copper sulfate-induced emesis in young chicks

drugs	dose (mg/kg)	No. of young chicks	No. of retches (mean ± S.E.)	inhibition (%)
control		6	44.0±2.89	
magnolol	10	6	33.3±2.25	24.3
	20	6	25.5±4.04***	42.0
	50	6	24.0±3.33***	45.5
control		6	44.7±3.62	
honokiol	10	6	38.8±1.66	13.2
	20	6	34.2±3.00	23.5
	50	6	28.3±1.80**	36.7
control		6	46.7±2.58	
crypto-	10	6	40.3±1.71	13.7
meridiol	20	6	34.7±2.59	25.7
	50	6	26.2±3.02***	43.9

significantly different from the control value, $^{**}p<0.01$, $^{***}p<0.001$

Table 6. Anti-emetic effects of *Inula britannica* flower on copper sulfate-induced emesis in young chicks

drugs	dose (mg/kg)	No. of young chicks	No. of retches (mean ± S.E.)	inhibition (%)
control		6	45.3±2.53	
taraxasteryl palmitate	10	6	38.2±1.99	15.7
	20	6	32.3±3.34	28.9
	50	6	28.2±3.80**	37.7
control		6	46.0±3.19	
taraxasteryl acetate	10	6	40.8±2.54	11.3
	20	6	28.5±2.39**	38.0
	50	6	28.0±2.00***	39.1
control		6	50.8±3.33	
bigelovin	10	6	44.5±5.12	12.4
	20	6	33.3±3.08**	34.5
	50	6	33.2±0.73**	34.6

significantly different from the control value, **p<0.01, ***p<0.001

Table 7. Effects of anti-emetic compounds of *Poria cocos* sclerotium on copper sulfate-induced emesis in young chicks

drugs	dose (mg/kg)	No. of young chicks	No. of retches (mean ± S.E.)	inhibition (%)
control		6	48.8±3.00	
polyporenic acid C	10	6	27.5±2.06***	43.6
	20	6	38.6±2.50	20.9
	50	6	46.0±1.95	-
control		6	48.7±3.32	
pachymic acid	10	6	46.3±2.79	-
	20	6	33.0±3.06*	32.2
	50	6	29.0±2.14**	40.5
control		6	44.8±1.66	
dehydropachymic acid	10	5	40.4±1.17	9.8
	20	6	31.0±2.43**	30.6
	50	6	27.8±2.92**	37.9

significantly different from the control value, *p<0.05, **p<0.01, ***p<0.001

the new method has been established as standard assay method for anti-emetic effects. Young chicks also have some advantages for breeding and lower cost in comparison with pigeons.

We have developed the new method for screening the anti-emetic principles in *Atractylodes lancea* rhizome.

Atractylodes lancea rhizome contains 5-9% of volatile oil which is mainly composed of β-eudesmol, hinesol and atractylon. The herb is used therapeutically in rheumatism, abdominal distention, poor appetite with nausea, retention of fluid and phlegm, edema, and mild diarrhea in Chinese medicine. However, there was no report for the anti-emetic action of the principles.

The decreasing numbers of retching action by the MeOH extract were 52.0% at a dose of 300 mg/kg as shown in Table 8. Hinesol and β-eudesmol were obtained as potential anti-emetic principles of the extract as shown in Table 8.

On the basis of these results, the new method was demonstrated as the conventional primary screening method for the survey of anti-emetic principles from the natural sources.

Table 8. Effects of the extracts, the fractions and anti-emetic principles from *Atractylodes lancea* rhizome on copper sulfate-induced emesis in young chicks

drugs	dose (mg/kg)	No. of young chicks	No. of retches (mean ± S.E.)	inhibition (%)
control		6	45.8±2.06	
$CHCl_3$ ext.	300	6	39.2±1.06	14.4
MeOH ext.	300	5	22.0±1.82***	52.0
H_2O ext.	300	6	46.7±2.62	-
control		6	48.7±2.12	
Fr. A	20	6	49.3±1.94	-
Fr. B	150	5	29.0±0.97***	40.5
Fr. C	150	6	23.5±1.73***	51.7
control		6	45.3±2.33	
Fr. D	20	6	46.3±1.98	-
Fr. E	150	5	45.0±3.28	
control		6	46.7±2.20	
Fr. C-1	100	6	26.5±2.23***	43.3
Fr. C-2	30	6	43.2±3.31	-
Fr. C-3	30	6	45.2±2.48	-
control		6	43.7±2.04	
Fr. C-4	30	6	47.2±1.54	-
control		6	40.8±2.04	
Fr. C-1-1	80	6	22.3±3.62**	44.9
Fr. C-1-2	20	6	34.8±3.71	14.7
Fr. C-1-3	20	6	36.0±3.65	11.8
control		6	45.8±1.74	
hinesol	30	6	32.2±1.45***	29.7
control		6	46.5±1.02	
β-eudesmol	30	6	29.7±1.28***	36.2

*significantly different from the control value, **p<0.01, ***p<0.001*

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