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Antiinflammatory Activity of Americanin A

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Abstract □ Americanin A from the seeds of *Phytolacca americana*, was investigated for the antiinflammatory activity. The compound significantly inhibited edema induction, granuloma formation, arthritis induction and leucocyte emigration in CMC-pouch. But its anticarrageenin activity was not exhibited in adrenalectomized rats. These findings together with decrease in the contents of ascorbic acid and cholesterol in adrenals by administration of the compound suggest that its activity is mediated through stimulation of the pituitary-adrenal axis. The acute toxicity of the compound is very weak and gastric ulceration was not produced by the compound.

Keywords □ Americanin A, *Phytolacca americana*, Phytolaccaceae, Lignan, Antiinflammatory activity.

Americanin A is a lignan derivative isolated from the seeds of *Phytolacca americana* (Phytolaccaceae)[1] which has traditionally been used in rheumatic disease in Korea. The compound is a yellowish white powder, mp 246~7°, and soluble in alcohol, but insoluble in water.

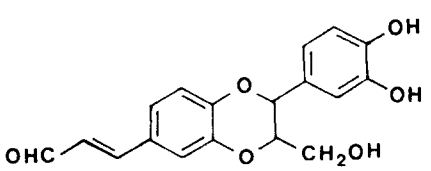

Fig. 1: The chemical structure of americanin A.

In the preliminary communication[2], americanin A has been reported to have anticarrageenin action without any effects on central nervous system in rats.

This paper deals with the antiinflammatory activity of the compound in acute and chronic inflammatory models of rats in detail.

MATERIALS AND METHODS

Materials

Americanin A was isolated from the seeds of *Phytolacca americana*, purified and characterized by spectral analysis. Mefenamic acid, hydrocortisone and aspirin (U.S.P. grade) were used as reference drugs. The drugs was suspended in 0.5% CMC solution for animal experiments. Carrageenin was obtained from Copenhagen Factory, Denmark.

Animals

Male Sprague-Dawley rats and male dd mice were used. The animals were housed in cages at least one week before the experiments. The laboratory chows (Samyang Yuji Co.) and tap water were given *ad libitum*. The room temperature was maintained at 20~23°.

Adrenalectomy was performed bilaterally by removing the adrenals and their adherent adipose tissue through a dorsal midline incision at the level of kidneys of rats. After surgery, the rats were allowed free access to food and 0.9% saline for 7 days before being used in the experiment.

Methods

1) Carrageenin-induced Paw Edema Test

The paw edema was induced in the rats weighing 150~180g by subplantar injection of 0.1ml of 1% carrageenin suspension in

physiological saline into left hind paw according to Winter *et al.*[3] The volume of the injected paw was measured before and every 1 hr after carrageenin injection with modified plethysmometer of Singh *et al.*[4] The drugs were administered orally 0.5 hr before injection of carrageenin.

2) Cotton Pellet Granuloma Test

Two sterile cotton pellets of 10 ±1 mg were implanted subcutaneously in each groin of the rats weighing 185~205g under ether anesthesia by the modified method of Srimal and Dhawan.[5]

The drugs were administered orally once a day for 7 days from the day of pellet implantation. The animals were sacrificed on the 8th day and the pellets and granulation tissues were dissected and dried to a constant weight at 60°. The wet and dry weights of the granulation tissues were obtained. The adrenals and thymus were also excised and weighed.

3) Adjuvant-induced Arthritis Test

Arthritis was induced in rats weighing 130 ~170 g by subplantar injection of 0.05ml of a suspension of heat-killed *Mycobacterium tuberculosis* (5mg/ml) in mineral oil into the left hind paw according to the method of Rosenthale.[6] The paw volume was measured plethysmometrically, immediately after the injection of adjuvant on the day 0 and every 3 or 4 days until the day 21. The drugs was administered orally once a day for 14 days from the day of the adjuvant injction.

The adjuvant-injected hind paw began to be inflamed after the injection, but the edema of the contralateral paw occurred after a delay of 14 days. Thus the non-injected paw volume was simultaneously measured. The adrenals, thymus and spleen were excised and weighed after the test.

4) Leucocytes Emigration Test in CMC-pouch

The CMC-pouch method of Ishikawa *et al.*[7] was adopted with the rats weighing 160~18(g in this experiment. The back of the anima was shaved roundly in width of 5 cm and ! ml of air was subcutaneously infused there About 24 hr after air infusion, 5 ml of steril ed 2% CMC-saline suspension of the drug was injected into the pouch.

The animals were killed by ether anesthesi 6 hr after the infusion of the drugs and th fluid in the pouch was collected. The aliquo of 0.05ml of the fluid was mixed with 0.4! ml of Türk solution for staining of the leu cocytes.

5) Measurement of Glucose and Cholesterol Contents in Serum and Ascorbic acid and Cholesterol Contents in Adrenal Cortex.

The drugs were dosed to the rats weighin 250~350 g and 3.5 hr later, the animals wer anesthetized by pentobarbital sodium. Th blood was collected from descending aorta an each adrenal gland was excised. The bloo was centrifuged at 3000 rpm for 20 min an the glucose and cholesterol contents in th serum were determined by enzyme metho using Automatic Blood Analyzer (Abbott Lab oratories, Model ABA-200).

The medulla was eliminated from adrena gland and the cortex zone was weighed an homogenized in distilled water by the metho of Guillement *et al.*[8] The contents of ascorbi acid and cholesterol were measured by th method of Zannoni *et al.*[9] and Abell *et al.*,[10] respectively.

6) Gastric Ulceration in Fasted rats

Rats weighing 220~250 g were deprived o food but allowed free access to water for 2

hr before drug administration. Five hr after the drug administration, the animals were sacrificed by ether anesthesia and the stomach was removed. The stomach was inflated by injecting with 10 ml of saline through esophageal junction and immersed in 5% neutral formalin solution for 10 min to fix the outlayer of the gastric wall. Subsequently, the stomach was incised along the greater curvature and the length and diameter of each lesion in the glandular portion were measured. The sum of area (mm^2) of all lesions in each stomach was expressed in terms of ulcer index.

7) Measurement of LD_{50}

The LD_{50} values of americanin A were determined in oral and intraperitoneal route in male mice weighing 18~22 g and in male rats weighing 145~160g. After administration of the compound, observation was undertaken for 72 hr.

RESULTS

Effect on Carrageenin-induced Paw Edema in Rats

The results are summarized in Table I. Americanin A at doses of 100 and 300 mg/kg exhibited a highly significant inhibition of paw edema throughout the measuring periods of 1, 2, 3 and 4 hr after the drug administration.

At a dose of 100 mg/kg, its maximum inhibitory potency was shown as much as 59.2% 1 hr after dosing and at a dose of 300 mg/kg, 70.9% 3 hr after dosing. Hydrocortisone, a reference drug, showed also a highly significant inhibition at a dose of 100 mg/kg.

Effect on the Formation of Cotton Pellet Granuloma in Rats

Americanin A depressed the formation of granulation tissue induced by implantation of cotton pellets in rats, as shown in Table II. In the formation of wet granuloma, the inhibition rate was 29.1 and 41.3% at the doses of 30 and 100 mg/kg, respectively, and in case of dry granuloma, the rate was 24.3% and 35.4% at the doses of 30 and 100 mg/kg, respectively. Hydrocortisone at a dose of 50 mg/kg, showed a stronger inhibition than the compound. It is, however, noted that the atrophy of adrenals and thymus occurred significantly after treatment of hydrocortisone, whereas not in case of the compound. The treatment of the compound for 7 days did hardly affect the normal growth of the rats, whereas the treatment of hydrocortisone caused no body weight gain.

Table I: The effect of americanin A and hydrocortisone on carrageenin-induced paw edema in rats.

Compound	Dose (mg/kg, p.o.)	No. of animals	Increase percent in paw volume (M±S.E.)			
			1	2	3	4(hr)
Control	—	6	29.9±0.29	47.6±3.83	48.1±2.54	35.6±4.63
Americanin A	100	6	12.2±3.59* (59.2)	23.0±2.76* (51.7)	25.5±3.99* (47.0)	15.1±5.55* (57.6)
	300	6	15.4±3.78* (48.5)	28.0±4.08* (41.2)	14.0±4.18** (70.9)	16.3±3.25* (54.2)
Hydrocortisone	100	6	18.9±2.24* (36.8)	16.6±6.32* (65.1)	16.7±7.05* (65.3)	16.3±3.25* (54.2)

Significantly different from the control group; *$p<0.01$, **$p<0.001$.
The figures in parentheses indicate inhibition percentage.

Table II: The effect of americanin A and hydrocortisone on the formation of cotton pellet granuloma in rats.

Compound	Dose (mg/kg, p.o.)	No. of animals	Mean body wt. (g)		Granuloma wt. (mg/kg)		Adrenal wt. (mg/100g)	Thymus wt (mg/100g)
			Initial	Gain	Wet wt.	Dry wt.		
Control	—	6	205.0±6.5	38.1±2.7	81.8± 8.31	18.1±1.21	24.1±3.3	156.6± 8.3
Americanin A	30	6	209.2±7.1	36.6±3.1	58.0±10.41 (29.1)	13.7±1.43* (24.3)	26.1±4.9	125.1±11.7
	100	6	190.0±5.7	30.0±2.8	48.0± 6.50** (41.3)	11.7±1.27* (35.4)	29.2±5.3	173.8±14.0
Hydrocortisone	50	6	202.5±6.3	0±2.2	26.5± 5.08**	8.3±1.04**	18.2±3.9*	51.0± 8.6*

Significantly different from the control group; *$p<0.05$, **$p<0.01$.
The figures in parentheses indicate inhibition percentage.

Table III: The effect of americanin A and mefenamic acid on the swelling of the rat left hind paws treated with complete adjuvant.

Treatment	Dose (mg/kg/ day, p.o.)	No. of animals	Increase percent of paw volume (M±S.E.)					
			3	7	10	14	17	21(day)
Arthritic control	—	6	115.2± 9.1	89.4±12.0	69.7±7.3	109.2±9.0	146.3±30.8	211.0±54.
Americanin A	30	6	91.2± 8.5 (20.8)	65.8± 5.4 (26.4)	57.5±9.0 (17.5)	57.7±9.7* (47.2)	85.6±29.0 (41.5)	189.3±48. (10.3)
	100	6	99.6± 7.6 (13.5)	75.0±11.1 (16.1)	52.7±5.1 (24.4)	45.2±5.1* (58.6)	61.5±11.0* (58.0)	154.8±61. (26.6)
Mefenamic acid	15	6	80.6±10.0* (30.0)	53.5±10.4 (40.2)	47.9±6.9 (31.3)	38.1±7.9* (65.1)	44.3±11.6* (69.7)	96.3±50. (54.4)

* Significantly different from the control group ($p<0.05$).
The figures in parentheses indicate inhibition percentage.

Effect on Adjuvant Arthritis in Rats

The result is shown in Table III. In the left hind paw injected with complete adjuvant, the paw was severely swollen on the 3rd day after the injection, followed by decrease in the edema as measured on the 7th and 10th day. However, from the 14th day the edema was much more greatly increased until the 21st day of last measurement. In the treated group with 100mg/kg, the volume was significantly decreased on the 14th and 17th day. Mefenamic acid at 15mg/kg showed a stronger activity than that of the compound.

The adjuvant arthritis is an immunologica inflammatory syndrome. The contralateral, *i.e* right hind paw volume was measured with th resulting data shown in Table IV. The arthriti control group showed a considerable increase i paw volume as compared with normal contro group 14 days after the adjuvant injection.

Much inhibition rate was shown on the da 17 and 21 at a dose of 100mg/kg, but statis tical differences between the treated group an the control group could not be obtained. Mefe namic acid also possessed the inhibitory activity

The organ weights as dissected on the 21s

Table IV: The effect of americanin A and mefenamic acid on the swelling of the rat right hind paws untreated with complete adjuvant.

eatment	Dose (mg/kg/day, p.o.)	No. of animals	Increase percent of paw volume (M±S.E.)		
			14	17	21 (day)
thritic control	—	6	19.2±2.2	26.0±10.5	55.0±10.4
nericanin A	30	6	15.4±7.1 (19.8)	31.3± 9.7 (−20.4)	60.4±10.4 (−9.8)
	100	6	14.4±3.9 (25.0)	10.7± 3.0 (58.8)	23.3±10.2 (57.6)
efenamic acid	15	6	5.0±1.4* (74.0)	5.6± 2.5* (78.5)	10.3± 4.7* (81.2)

Significantly different from the control group; *$p<0.01$.
The figures in parentheses indicate inhibition percentage.

Table V: The effect of americanin A and mefenamic acid on the weights of various organs.

Treatment	Dose (mg/kg/day, p.o.)	No. of animals	Organ weights (mg/100g, M±S.E.)		
			Thymus	Spleen	Adrenal
Normal control	—	6	156.6±19.9	486.9±41.1	25.0±3.7
Arthritic control	—	6	151.5±24.4	495.3±24.3	22.3±3.1
Americanin A	30	6	110.2±11.1	542.4±38.2	23.0±2.0
	100	6	134.1± 8.8	495.8±35.2	21.7±1.6
Mefenamic acid	15	6	149.0±17.5	499.7±32.1	21.6±1.5

ay after inducing arthritis is shown in Table . The weights of thymus, spleen and adrenals f the treated rats could not be observed in any ifferences from those of control rats. Further- ıore, the treatment of the compound and me- :namic acid for 14 days did not influence on ıe weights of the organs.

ffect on Leucocyte Emigration

This compound showed an inhibition of leuco- yte emigration into CMC-pouch as shown in able VI. As injected in the pouches at the oses of 5 and 14 mg, the compound showed a gnificant inhibition of emigration as 22.0 and 4.8%, respectively. Whereas aspirin at a dose f 5mg did 44.9% inhibition.

ffect on Carrageenin-induced Paw Edema in Adrenalectomized Rats

The procedure of this experiment was duplicated for confirmation of the result. The compound at the doses of 100 and 300mg/kg did not exhibit any inhibitory potency against the edema in adrenalectomized rats in both exper-

Table VI: The effect of americanin A and aspirin on emigration of leucocyte in CMC-pouch of rats.

Treatment	Dose[a] (mg)	No. of animals	No. of leucocytes (M±S.E.)	Inhibition (%)
Control	—	6	6950±210.8	—
Americanin A	5	8	5420±193.3*	22.0
	15	6	3834±386.7*	44.8
Aspirin	3	7	3827±345.7*	44.9

a) Amount injected into the pouch.
* Significantly different from the control group ($p<0.001$)

Table VII: The effect of americanin A and hydrocortisone on carrageenin-induced paw edema in adrenalectomized rats.

Treatment	Dose (mg/kg, p.o.)	No. of animals	Increase percent of paw volume (M±S.E.)					
			First experiment			Second experiment		
			2	3	4	2	3	4(hr)
Control	—	6	23.5±4.6	40.3±6.4	49.2± 6.6	33.4±5.3	39.0± 8.4	46.7± 9.5
Americanin A	100	6	38.2±9.0	54.9±8.6	63.0± 7.9	31.5±6.9	43.5±10.1	52.6±13.6
	300	6	30.8±6.9	46.5±5.3	61.3±12.1	28.7±6.8	34.8± 5.1	42.8± 5.6
Hydrocortisone	50	6	9.6±1.9 (59.1)	12.6±3.1** (68.7)	13.2± 2.5** (73.2)	15.0±3.3* (55.1)	13.0± 2.5** (66.7)	15.5± 3.1 (66.8)

Significantly different from the control group; *p<0.05, **p<0.01.
The figures in parentheses indicate inhibition percentage.

Table VIII: The effect of americanin A and aspirin on the ascorbic acid and cholesterol contents in the adrenal cortex of rats.

Treatment	Dose (mg/kg, p.o.)	No. of animals	Contents (mg/g of adrenal cortex, M±S.E.)	
			Ascorbic acid	Cholesterol
Control	—	10	3.2±0.28	36.3±5.37
Americanin A	100	10	2.3±0.17* (28.0)	22.9±2.68* (36.9)
	300	9	2.1±0.26** (34.4)	21.3±2.26* (41.3)
Aspirin	500	11	1.7±0.13** (46.9)	18.8±4.15* (48.2)

Significantly different from the control group; *p<0.05, **p<0.01, ***p<0.001.
The figures in parentheses indicate inhibition percentage.

iments as summarized in Table VII. However, hydrocortisone at a dose of 50mg/kg showed potent inhibitory activities in these experiments.

Effect on Ascorbic acid and Cholesterol Contents in Adrenal Cortex in Rats

The result on the ascorbic acid and cholesterol contents 3.5 hr after the administration of the compound and aspirin are summarized in Table VIII. The contents of ascorbic acid and cholesterol in control adrenal cortex were 3.2 and 36.3 mg/g of adrenal cortex, respectively. At a dose of 100mg/kg of the compound, the contents of ascorbic acid and cholesterol were significantly decreased by 28.0% and 36.9%, respectively. Much more reduction was observed at a dose of 300mg/kg, *i.e.* 34.4% in ascorbi acid and 41.3% in cholesterol. Aspirin at dose of 500mg/kg showed also a significant r duction.

Effects on Serum Glucose and Chlesterol Conten in the Rat

In the sera of the rats treated as mentione above, the glucose and cholesterol conten were shown no changes as summarized i Table IX.

Effect on the Stomach of Fasted Rats

The result is shown in Table X. Americani A at the doses of 100 and 300mg/kg whe given to the fasted rats did not produce an irritation as well as ulcerative symptom and

Table IX: The effect of americanin A and aspirin on the serum glucose and cholesterol contents in rats.

Treatment	Dose (mg/kg, p.o.)	No. of animals	Contents (mg/100 ml, M±S.E.)	
			Glucose	Cholesterol
Control	—	5	165.7±14.53	45.0±2.76
Americanin A	100	5	153.3±10.14	47.2±5.34
	300	5	156.3± 9.01	53.8±4.84
Aspirin	500	5	151.8±11.83	42.2±5.55

Table X: The influence of americanin A and aspirin on gastric ulceration of rats.

Compound	Dose (mg/kg, p.o.)	No. of animals	Ulcer index Mean±S.E. (mm^2)
Control	—	7	0
Americanin A	100	7	0
	300	6	0
	900	6	0[a]
Aspirin	200	7	6.7±5.4

a) Vasodilation in stomach was observed in 3 animals.

Table XI: Acute toxicity of americanin A.

Animal	No. of animals	Administration route	MLD (mg/kg)
Mouse	6	p.o.	4000
	6	i.p.	3000
Rat	4	p.o.	4000
	4	i.p.	3500

the dose as much as 900mg/kg, only vasodilation in stomach of 3 animals was observed. However, aspirin at the dose of 200mg/kg produced ulceration on the glandular portion in 6 among 7 animals of which the ulcer index was 6.7.

Acute Toxicity

The compound has very low LD_{50} values as shown in Table XI. The LD_{50} values in mice were estimated to be more than 4000 and 3000mg/kg in oral and intraperitoneal routes, respectively, and those in rats were more than 4000 and 3500mg/kg in oral and intraperitoneal route, respectively. No characteristic symptoms could not be found during 72 hr after administration of the compound.

DISCUSSION AND CONCLUSION

The present study demonstrated that americanin A had effectiveness in preventing the development of artificially induced inflammatory responses in rat using both of acute and chronic experimental procedures.

Di Rosa *et al.*[11] and Holsapple *et al.*[12] reported that carrageenin induced edema in rats was developed in two phases, *i.e.*, the early phase which is mediated by histamine and serotonin, and the late phase which is mediated by kinins and prostaglandins. The action of the compound was exhibited in both phases in edema. This fact is thought that the compound might have an inhibitory activity against some of these inflammatory mediators.

Its anticarrageenin activity is very strong and is similarly potent comparable to that of hydrocortisone. Americanin A is also active in inhibiting the formation of granulation tissue as well as accumulation of body fluid in granuloma pouch, and also ameliorated the immunological inflammatory response as measured in adjuvant arthritis in animals. However, this compound is less potent than mefenamic acid.

The compound had no influences on the weights of the organs, such as thymus, spleen and adrenals, following prolonged administration. However, hydrocortisone produced untoward effect shown by atrophy of adrenals and thymus as demonstrated by Adams *et al.*[13] and Itoga *et al.*[14] In our experiment, mefenamic acid did not show any influences on the weight of thymus, spleen and adrenals. This result is in agreement with the report by Tsurumi *et al.*[15]

In adrenalectomized animals, the compound was confirmed to be ineffective in carrageenin edema. These findings suggest that its antiedematous activity might be dependent on stimulation of the pituitary-adrenal axis. Furthermore, this fact is supported from the result that the contents of ascorbic acid and cholesterol in adrenal cortex were decreased following administration of the compound. Like indomethacin and glycyrrhetinic acid,[16] this compound was able to prevent the emigration of leucocytes into the pouch.

It is well known that most of the nonsteroidal antiinflammatory drugs produced gastric ulceration as well as irritation in rats. However, it is of interest that this compound did not produce gastric ulcer at the dose level o positive antiedematous activity, and its acute toxicity is very weak. It was demonstrated that this compound had neither analygesic action nor antipyretic action.[2]

In conclusion, americanin A is a new nonsteroidal antiinflammatory compound without producing gastric ulcer in animals and its activity might be revealed through stimulation of the pituitary-adrenal axis.

ACKNOWLEDGEMENT

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