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Studies on Crude Drugs Effective on Visceral Larva Migrans. III.[1] The Bursting Activity of Tannins on Dog Roundworm Larva

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Tannins, both condensed and hydrolyzable, were found to cause bursting of the second-stage larvae of dog roundworm (*Toxocara canis*), when combined with an appropriate larvicidal compound such as decanoic acid or tetradecanol. This bursting activity of tannins increased with increase of the degree of condensation for condensed tannins and with increase of the proportion of phenolic moieties for hydrolyzable tannins. Since tannins are not larvicidal by themselves, the appearance of this bursting activity requires the coexistence of larvicides. The activity was strongly induced by larvicides with a polar functional group such as an acid or an alcohol, but weakly induced by those with a less polar group such as an amide. Tannins were also found to enhance the killing activity of acidic larvicides. These facts suggested the practical effectiveness of the combination of tannins and an appropriate anthelmintic for the treatment of parasitic diseases.

Keywords——tannin; condensed tannin; hydrolyzable tannin; bursting activity; anthelmintic; larvicide; *Toxocara canis*; structure–activity relationship; synergistic effect

In a previous investigation,[2] we reported that the 50% acetone extract of betel nuts (*Areca catechu*) showed strong larvicidal activity against the larvae of dog roundworm (*Toxocara canis*), a common pathogenic parasite in visceral larva migrans,[3] with bursting of the worms. The active principles were separated into two fractions; one causes only killing of the worms and the other produces bursting of the worms when combined with the former but has no killing activity. The former fraction was identified as a mixture of fatty acids (the killing activity of those of C_{10} to C_{13} chain length was greatest), and the latter principle(s) was suggested to be tannins. Figure 1 shows a burst larva of *T. canis* after treatment with a combination of decanoic acid and the tannin-rich fraction (fr. E) of *A. catechu*.[2] The cuticle of the larva is torn along the lateral alae and the intestine is protruding.

Tannins occur widely in the plant kingdom and are usually found as a mixture of complex polyphenolic compounds. However, recent developments in isolation techniques and spectroscopic analyses have made it possible to isolate many tannins in pure states and to determine their structures.[4] Their biological activities are also under intensive investigation.[5]

In this paper, we report the bursting activity of some purified tannins and related

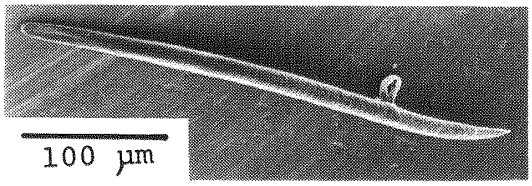


Fig. 1. Electron Microscopic Picture of a "Burst" Larva of *T. canis* Treated with the Combination of Decanoic Acid and Tannin-Rich Fraction (fr. E) Obtained from *A. catechu*

compounds against the second-stage larva of dog roundworm, *T. canis*, and discuss their structure–activity relationship.

Materials and Methods

Materials——Chemicals not otherwise described were purchased from Nakarai Chemicals Ltd. Gallic acid was obtained from Kanto Chemical Co. Epicatechin and tannic acid (from Chinese gallotannin) were purchased from Wako Pure Chemical Industries Ltd. 1-Tetradecanoylpyrrolidine and 1-tetradecanoylmorpholine were prepared by the Schotten–Baumann procedure from pyrrolidine and morpholine, respectively.

1-Tetradecanoylpyrrolidine: Colorless needles, mp 34.5—36 °C. IR(KBr): 1630 cm^{-1}.

1-Tetradecanoylmorpholine: Colorless needles, mp 33—34 °C. IR($CHCl_3$): 1629 cm^{-1}.

Condensed tannins and related compounds (**3**—**14**) were isolated from *Areca catechu*[6] and *Cinnamomum spp.*[7] Galloylglucoses (**16**—**28**) were isolated from peony root,[8] rhubarb,[9] and Chinese gallotannin.[10] Ellagitannins (**29**—**33**) were isolated from *Punica granatum*.[11]

Determination of Larvicidal Activity——Larvicidal activity against the larvae of dog roundworm was determined according to the method previously described.[2] For one assay, 20 second-stage larvae of *Toxocara canis* were incubated with the test solution in a Corning cell well at 37 °C for 24 h and the behavior of the larvae was observed under a microscope. All assays were done in duplicate. The larvicidal activity of test materials was evaluated in terms of the relative mobility (RM) value described in the previous paper.[2] A smaller RM value indicates stronger larvicidal activity, and when all larvae die, this value becomes 0. Minimal lethal concentration (MLC) was determined as the lowest concentration with an RM value of 0 after 24 h of incubation.

Evaluation of Bursting Activity——Tannins (100 μg/ml) were dissolved in 0.7% saline containing decanoic acid or other larvicidal compounds (100 μg/ml). Twenty larvae of *T. canis* were incubated in this test solution at 37 °C and the number of burst larvae was counted under a microscope after 24 h of incubation. All assays were done in duplicate. The percentage of burst larvae was calculated and used as a measure of bursting activity.

Results and Discussion

Larvicidal Activity of Tannins

First, the *in vitro* larvicidal activity of some purified tannins and related compounds against the larvae of *T. canis* was tested (Table I, II). Gallic acid showed no effect at a concentration of 200 μg/ml, although cinnamic and *p*-hydroxycinnamic acids had larvicidal activity (MLC: 0.1 and 1 mM, respectively).[1] Neither catechin nor epicatechin was larvicidal. Tannic acid showed only a weak killing effect at a concentration of 10 mg/ml (RM = 31). All other tannins tested showed little activity at a concentration of 200 μg/ml (RM = 54—100; after 24 h of incubation), although some of them were reported to have molluscicidal[12] and antiherpetic[13] activities.

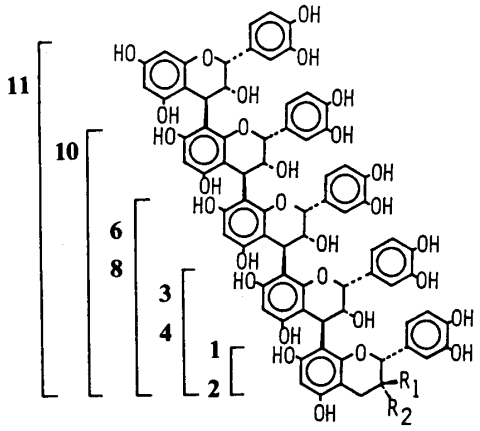
Thus, it was confirmed that tannins are not larvicidal by themselves.

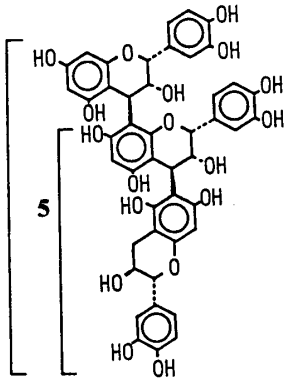
Bursting Activity of Condensed Tannins

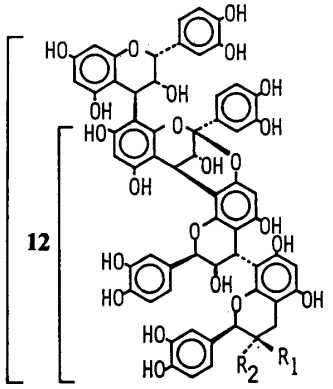
Although tannins are not larvicidal by themselves, they cause bursting when combined with a larvicidal compound such as decanoic acid. Table I shows the bursting activity of some condensed tannins at 100 μg/ml in the presence of 100 μg/ml of decanoic acid. Interestingly, the bursting activity of condensed tannins was found to be largely dependent on the degree of condensation of the monomers. The monomers, catechin and epicatechin, did not show the activity. Dimers (**3**—**4**) and trimers (**6**—**8**) were also almost inactive. However, the activity appeared for a tetramer (**10**) and a pentamer (**11**) which showed strong activity (64% and 61%, respectively), thus suggesting that the bursting activity of condensed tannins increases with increase of the degree of condensation. The polymer was completely insoluble in water, and it was inactive.

The bursting activity was also dependent on the type of condensation, *i.e.* the shape of the compounds. Though the dimers and trimers containing only C4–C8 linkage (**3**—**4**, **6**—**8**) were inactive, those which include a C4–C6 linkage (**5** and **9**) showed appreciable activity, suggesting that tannins condensed between C4 and C6 of the monomers are more active than those condensed between C4 and C8.

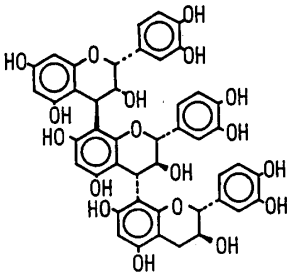
TABLE I. Bursting Activity of Condensed Tannins







	R_1	R_2
1:	OH	H
2:	H	OH
3:	OH	H
4:	H	OH
6:	H	OH
8:	OH	H
10:	OH	H
11:	OH	H



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	R_1	R_2
12:	OH	H
13:	OH	H
14:	H	OH

Compound	Degree of condensation	Type of linkage	RM[a]	Burst %[b]
Catechin (**1**)	1		99	0
Epicatechin (**2**)	1		100	0
Procyanidin B-1 (**3**)	2	C4–C8	97	0
Procyanidin B-2 (**4**)	2	C4–C8	—	0
Procyanidin B-7 (**5**)	2	C4–C6	98	17
Procyanidin C-1 (**6**)	3	C4–C8	—	0
Arecatannin C_1 (**7**)	3	C4–C8	96	0
Arecatannin A_1 (**8**)	3	C4–C8	90	4
Arecatannin B_1 (**9**)	3	C4–C8, C4–C6	90	45
Arecatannin A_2 (**10**)	4	C4–C8	81	64
Arecatannin A_3 (**11**)	5	C4–C8	100	61
fr. E[c]			78	34
Cinnamtannin B_1 (**12**)	3	C4–C8, C2–O7	95	34
Cinnamtannin B_2 (**13**)	4	C4–C8, C2–O7	99	38
Cinnamtannin D_2 (**14**)	4	C4–C8, C2–O7	100	54

a) Larvicidal activity was determined at 200 μg/ml and given by the RM value after 24 h of incubation. *b*) Burst % was determined at 100 μg/ml of tannins with 100 μg/ml of decanoic acid. Under these conditions, the RM value was 0 for all runs. *c*) See previous paper (ref. 2).

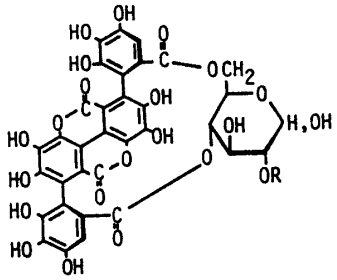
Cinnamtannins B_1 (**12**), B_2 (**13**) , and D_2 (**14**), which have an additional ether linkage between C2–O7, were also significantly active.

From the above results, it is apparent that the bursting activity of fr. E obtained from *A. catechu*[2] is ascribable to the above catechin and/or epicatechin oligomers.

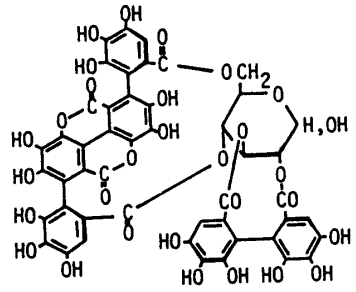
Bursting Activity of Hydrolyzable Tannins

Hydrolyzable tannins also exhibited bursting activity when applied with decanoic acid,

TABLE II. Bursting Activity of Hydrolyzable Tannins



29: R=H
30: R=galloyl



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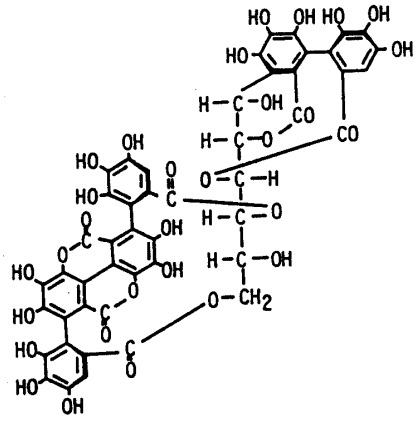

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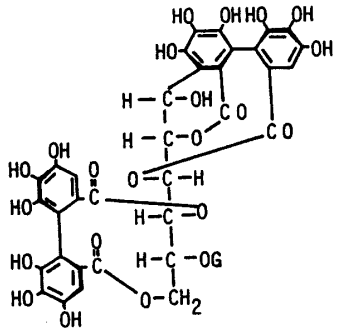

33: G=galloyl

Compound	RM	Burst %	Compound	RM	Burst %
Gallic acid (**15**)	100	0	Hepta- (**25**)	—	68
Galloylglucoses			Octa- (**26**)	—	70
1-Mono- (**16**)	—	0	Nona- (**27**)	—	84
6-Mono- (**17**)	—	0	Deca- (**28**)	—	90
1,6-Di- (**18**)	—	0	Tannic acid	85	83
1,2,6-Tri- (**19**)	—	0	Punica tannins		
1,4,6-Tri- (**20**)	—	0	Punicalin (**29**)	94	9
1,2,3,6-Tetra- (**21**)	—	20	2-*O*-Galloylpunicalin (**30**)	96	33
1,2,4,6-Tetra- (**22**)	—	24	Punicalagin (**31**)	54	60
1,2,3,4,6-Penta- (**23**)	—	36	Punicacortein C (**32**)	98	68
Hexa- (**24**)	—	62	Casuarinin (**33**)	95	68

and the activity was again found to be dependent on the number of phenolic component(s). Table II shows the bursting activity of some hydrolyzable tannins and related compounds.

Gallic acid did not show any activity. All regioisomers of mono-, di-, and tri-*O*-galloylglucose tested (**16**—**20**) were also inactive. The bursting activity appeared for tetra-*O*-gallate and increased with increase of the number of galloyl groups. Thus tetra-*O*-galloylglucoses (**21**, **22**) and penta-*O*-galloylglucose (**23**) were moderately active and hexa- to deca-*O*-galloylglucoses (**24**—**28**) were strongly active (62—90%); the latter compounds have at least one depside linkage in the molecule. Tannic acid, a mixture of oligogalloylglucoses (mainly hepta to nona), also showed strong bursting activity as expected.

Next, the bursting activities of some ellagitannins purified from *Punica granatum*, a famous anthelmintic in Chinese medicine, were also tested. Punicalin (**29**), which carries one gallagyl group, showed only weak activity. Introduction of a galloyl group at C2–O– or a

hexahydroxydiphenoyl (HHDP) group at C2,3–O– again enhanced the activity moderately or strongly. Thus 2-*O*-galloylpunicalin (**30**) and punicalagin (**31**) were moderately and strongly active, respectively.

The bursting activities of punicacortein C (**32**) and casuarinin (**33**) were comparable, both being fairly strong, suggesting that the contribution of a gallagyl group to the bursting activity may be comparable to that of an HHDP plus a galloyl groups.

Bursting Activity of Tannic Acid in Combination with Other Larvicides

The bursting of worms is observed only when the worms are dead, indicating the coexistence of a larvicidal compound to be necessary. We therefore tested the effect of several larvicides with various functional groups, taking tannic acid as a representative of tannins; the compounds tested were decanoic acid, cinnamic acid[1] (the functional group is acidic and very polar), tetradecanol (neutral, polar), and 1-tetradecanoyl-pyrrolidine and -morpholine[14] (neutral, weakly polar).

Table III indicates that the bursting caused by tannic acid occurs not only in combination with decanoic acid but also with other larvicidal compounds, though the activity changes depending on the nature of the larvicide. The bursting by tannic acid was very significant when combined with the acidic (decanoic acid, cinnamic acid) and alcoholic (tetradecanol) larvicides, but was very weak with the amidic larvicides (1-tetradecanoyl-pyrrolidine and -morpholine), although the larvicidal activity of the latter compounds is very strong. Hence, the bursting activity of tannic acid seems to have no correlation to the activity (MLC) of the coexisting larvicides.

It is well known that tannins bind to proteins to form water-insoluble complexes and this binding is affected by the pH of the medium.[15] Therefore, if we assume that the bursting is caused by the interaction between tannins and cuticle proteins, is the additional role of an

TABLE III. Effect of Larvicidal Compounds on the Bursting Activity of Tannic Acid

Larvicidal compound	MLC (μM)	Burst %	
		Tannic acid	Tannic acid + AcOH
Decanoic acid	250	83	79
Cinnamic acid	100	72	56
Tetradecanol	20	88	74
1-Tetradecanoylpyrrolidine	8	7	8
1-Tetradecanoylmorpholine	8	3	8

Larvae of *T. canis* were incubated in the test solution containing tannic acid (100 μg/ml) and various larvicidal compounds (100 μg/ml) in the presence (100 μg/ml) or absence of acetic acid. Under these conditions the medium pH values were as follows: decanoic acid, 5.5; tannic acid, 5.4; acetic acid, 4.1; tannic acid + decanoic acid, 4.7; tannic acid + decanoic acid + acetic acid, 4.2. The MLC and the burst activity were determined after 24 h of incubation.

TABLE IV. Effect of Tannic Acid on the Larvicidal Activity of Decanoic Acid (RM Value)

Decanoic acid concentration (μM)	250	200	150	100	50
Without tannic acid	0	48	68	76	90
With tannic acid	0	0	0	3	64
(burst %)	(93)	(58)	(10)	(0)	(0)

Larvae of *T. canis* were incubated in the test solution containing decanoic acid with or without tannic acid (100 μg/ml) for 24 h. RM value and burst % were determined after 24 h of incubation.

acidic larvicide such as decanoic acid to change the pH of the medium to allow optimal interaction? In order to check this possibility, the bursting activity of tannic acid with various larvicides was examined in the presence of acetic acid. Acetic acid itself is neither larvicidal (MLC > 1 mM) nor burst-inducing in combination with tannic acid (burst 0%). Table III also indicates that acetic acid does not produce any enhancement of the bursting activity, but instead slightly reduces the activity for acidic and alcoholic larvicides.

From the present results, although the mechanism of the burst is still unclear, we can say that a larvicidal compound is necessary for the bursting activity of tannins and that a polar functional group in the larvicidal compound plays an important role in the activity.

Synergistic Effect of Tannins with Acidic Larvicides

Besides the strong bursting activity, tannic acid was found to increase the larvicidal activity of decanoic acid. Table IV shows the larvicidal activity of decanoic acid with or without tannic acid. The MLC of decanoic acid itself was 250 μM. However, when 100 μg/ml of tannic acid was added, the activity was greatly enhanced, resulting in a decrease of the MLC to about 100 μM. Such an enhancement of the larvicidal activity by tannic acid was also observed for cinnamic acid (the MLC decreased to about two-thirds of the original one). On the other hand, the larvicidal activity of neither decanol nor 1-tetradecanoylpyrrolidine was affected by the addition of tannic acid.

It is interesting that the above synergistic effect of tannins was observed only for acidic larvicides, though the reason for this is not yet clear.

Conclusion

The above results may be summarized as follows.

1) Both condensed and hydrolyzable tannins show bursting activity when combined with an appropriate larvicidal compound such as decanoic acid, cinnamic acid, or tetradecanol. 2) The bursting activity of tannins increased roughly proportionally with increase of the degree of condensation for condensed tannins and with increase of the proportion of phenolic moieties for hydrolyzable tannins. 3) The bursting activity of tannins was strong with an acidic or alcoholic larvicide, but weak with an amidic larvicide. 4) This activity was scarcely affected by change of the pH of the medium. 5) Tannins enhanced the larvicidal activity of an acidic compound but did not affect that of a neutral compound.

Although the mechanisms of these activities are not clear at present, the results suggest that a combination of tannins with an appropriate anthelmintic may have great practical value for the treatment of parasitic diseases, since the synergistic action of tannins and an anthelmintic is expected not only to damage the worms irreversibly, but also, in some instances, to reduce markedly the required amount of the anthelmintic.

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