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# IMMUNOREACTIVITY FOR CANATOXIN AND CONCANAVALIN A AMONG PROTEINS OF LEGUMINOUS SEEDS*

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**Key Word Index**—Lectins; toxic protein; leguminous seeds; immunoreactivity; concanavalin A; canatoxin.

**Abstract**—The jack bean (*Canavalia ensiformis*) is the natural source of concanavalin A and also of canatoxin, a recently described neurotoxic protein. Among some other examples, the seeds of the euphorbiaceous plant *Ricinus communis* contain ricin, a toxic monovalent lectin, and also an agglutinin which are closely related molecules sharing a common polypeptide subunit. Thus, seeds containing two distinct and related proteins, an atoxic lectin and a highly toxic protein which behaves as an monovalent lectin or a hemilectin, seem to be widespread. In this paper we describe the simultaneous presence of both toxic proteins and lectins in the seed extracts of 13 out of 16 different leguminous plants examined. Immunodifusion studies with anticanatoxin and anticoncanavalin A IgG antibodies indicated that proteins structurally resembling canatoxin were preserved in almost all the leguminous seeds extract except in peanut and *R. communis*. Proteins immunogically related to concanavalin A were detected only for the *Canavalia* genus despite the ubiquitous presence of lectins in seeds. The data suggest that canatoxin-like proteins were conservatively preserved, indicating that this toxic protein as well as other toxic hemilectins may play an important physiological role in plants.

## INTRODUCTION

The presence of lectins and toxic proteins in vegetables, particularly in the seeds of leguminous plants, has been known for several decades [1–5]. The lectins, carbohydrate-specific binding proteins, have been throughly investigated while a hemagglutinating activity has been described for almost 800 seed extracts [6]. In some seeds the lectin(s) present in the crude extract have been isolated and purified. By contrast, the toxic proteins which are usually associated with the lectins in the same seed have received little attention and few of them have been isolated. This fact led to the misleading idea that lectins may display toxic activity [cf. 7]. Among the toxic proteins found in seeds, the first one to be isolated and identified as a distinct protein was ricin, found in *Ricinus communis* (castor beans) together with a galactose-specific lectin [8, 9]. Other toxic proteins which were separated from their lectin counterpart in the same seeds include abrin (*Abrus precatorius* [10]), moddecin (*Adenia diggitata* [11]), and mormodin (*Mormodica charantia* [12]). Recently, we isolated from *Canavalia ensiformis* (jack bean) seeds a potent neurotoxic and lethal protein named canatoxin (CNTX [13]), which is distinct from concanavalin A (Con A), the well known lectin present in these seeds [13].

There has been described a close structural relationship between the lectin and the toxic protein of some seeds. 2D-electrophoresis of ricin's tryptic fragments, for instance, revealed a pattern that is very similar to that obtained for *Ricinus* agglutinin [9]. In addition, extensive amino acid sequence homology was found for these proteins which seem also to share a common subunit [9, 14]. A close relationship between ricin and *Ricinus* agglutinin can also be detected by immunodiffusion tests, since a partial spur between the two proteins is formed with antiricin or antilectin sera, indicating the presence of common antigenic determinants [15].

On the other hand, lectins from different sources also bear some degree of molecular similarity. Extensive homology of amino acid sequence exist among the lectins from *C. ensiformis* (Con A), *Lens culinaris* and *Pisum sativum* [16, 17], despite the differences in carbohydrate-binding-specificity shown by the three lectins. These facts strongly suggest a common ancestral gene coding for both lectins and toxic proteins either when they occur in the same seed or in seeds from different species. It is also suggestive that both type of proteins may occur in the seeds in a higher than supposed frequency and that they may play an important, though yet unknown, physiological role, in plants [17].

We have been investigating the concomitant presence of both toxic and hemagglutinating activities in leguminous plants [18, 19]. In the present paper, we analysed the extracts from 16 different seeds of the Leguminoseae and one of the Euphorbiaceae for the simultaneous presence and immunological cross-reactivity of toxic proteins and lectins.

## RESULTS

The protein-rich fractions obtained from the extracts from 17 different seeds (16 leguminous and one euphorbi-

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aceous plant) were analysed for the presence of both toxic and hemagglutinating activities. Lethality induced by the non-dialysable and thermolabile fraction was found in 13 seed extracts (Table 1). Mice injected with purified canatoxin or with any of the three species of *Canavalia* seeds extracts showed a similar pattern of tonic-clonic convulsions before death [13]. Convulsions were also seen in the mice receiving *Glycina max* (soybean) and *Cajanus indicus* (guando) extracts. None of the other lethal extracts produced this type of neurotoxicity. The seeds contained different contents of toxic proteins (Table 1). Interestingly, the three most potent extracts were also the ones producing convulsions before death of the animals.

Hemagglutinating activity for at least one type of erythrocyte was found for all the extracts except that of *Cajanus indicus* seeds (Table 2). Most of the extracts agglutinated all types of cells tested. However, the lectin(s)

Table 1. Thermolabile and non-dialysable toxic activity of leguminous seeds extracts

| Seed | Toxicity | Convulsions | $LD_{50}$* | $LD_{50}$† g seed |
|---|---|---|---|---|
| *Canavalia ensiformis* | + | + | 0.35 | 264.0 |
| *C. gladiata* | + | + | ND | ND |
| *C. obtusifolia* | + | + | ND | ND |
| *Cajanus indicus* | + | + | 0.90 | 70.7 |
| *G. max* | + | + | 4.20 | 30.6 |
| *V. unguiculata* | + | − | 4.10 | 29.6 |
| *P. sativum* | + | − | 5.00 | 18.6 |
| *P. vulgaris* | + | − | 5.20 | 4.4 |
| *D. lablad* | + | − | 5.50 | 32.5 |
| *L. culinaris* | + | − | 30.00 | 14.0 |
| *Abrus precatorius* | + | − | ND | ND |
| *A. paronira* | + | − | ND | ND |
| *L. luteus* | + | − | ND | ND |
| *Cicer arietinum* | −‡ | | | |
| *P. lunatus* | − | | | |
| *Arachis hipogea* | − | | | |

*$LD_{50}$ is expressed as the amount of protein (mg) injected per 10 g of mouse body weight (see Methods).
†Data indicate the amount of $LD_{50}$ units present in one g of seed.
‡No deaths under the experimental conditions described.
ND Not determined.

Table 2. Presence of lectins in seed extracts of leguminous plants

| Seed | Positive hemagglutination | |
|---|---|---|
| | Normal cells | Trypsinized cells |
| *Canavalia ensiformis* | All cells* | All cells |
| *C. gladiata* | Rb | |
| *C. obtusifolia* | Rb | |
| *Cajanus indicus* | None of the cells | None of the cells |
| *G. max* | Rb, M | Rb, M |
| *V. unguiculata* | None of the cells | Only G |
| *P. sativum* | H, Rb, M | H, M |
| *P. vulgaris* | H, Rt | |
| *D. lablad* | H, Rb, M | H |
| *L. culinaris* | H, Rt | H, Rt |
| *Abrus precatorius* | Rb, Rt, G | |
| *A. paronira* | Rb, Rt | |
| *L. luteus* | None of the cells | Only Rt |
| *Cicer arietinum* | None of the cells | Only Rt |
| *P. lunatus* | H | H, Rt |
| *Arachis hipogea* | Rb | |

*Erythocytes were from rabbit (Rb), rat (Rt), guinea-pig (G), mouse (M) and human of A, B, and O types (H).

present in the extracts of *Vigna unguiculata* agglutinated only trypsinized guinea-pig red cells while *Lupinus luteus* and *Cicer arietinum* extracts reacted only with trypsinized rat erythrocytes.

Immunodifusion tests were carried out using purified anti-CNTX and anti-Con A antibodies. As illustrated in Fig. 1A, there is no common antigenic determinants between the two proteins from *Canavalia ensiformis* seeds since no reaction was seen between anti-CNTX and Con A, and between anti-Con A and CNTX. On the other hand, when the crude extracts of the two other *Canavalia* species were tested against either anti-CNTX or anti-Con A antibodies, immunodifusion patterns were obtained showing total identity (Fig. 1B) among the lectins or the toxic proteins present in the seeds of the three species of *Canavalia*. While our purified CNTX [13, 21] elicited the production of a monospecific antibody, the IgG fraction similarly obtained from rabbits immunized with commercial Con A gave two precipitate lines (Fig. 1B, left) whenever the same Con A antigen or the *Canavalia* seeds crude extracts were tested. Depending on the duration of the immunodifusion run (48 hr or longer), a third faint precipitin line could be seen (Fig. 2A, left).

Anti-CNTX and anti-Con A antibodies were tested against the extracts or ethanol-treated fractions of the other seeds. It was seen that anti-Con A antibodies did not react with any of the other extracts or fractions even after concentration. On the other hand, anti-CNTX IgG gave immunodifusion patterns showing partial identity (Fig. 2) with almost all the seed extracts tested, including *Cicer arietinum* and *Phaseolus lunatus* extracts, which showed no toxicity when injected (i.p.) into mice (Table 1). The extracts of peanut (*Arachis hipogea*) and that of the castor bean (*Ricinus communis*, an euphorbiaceous plant) gave no immunoprecipitate lines when tested against anti-CNTX. Fig. 2 illustrates typical patterns of immunoprecipitates obtained for some of the seeds tested. The partial identity patterns were similar for all the anti-CNTX positive seed extracts. The radial lines seen in Fig. 2C (arrows) for Con A and *Lens culinaris* extracts, as well as for several other seeds including *Phaseolus lunatus* (not shown), represent the non-immunological reaction between Con A and glycoproteins of the crude extracts tested as described by ref. [20] (Table 3).

## DISCUSSION

The results indicate that the occurrence of a toxic protein and a lectin in the same seed is widespread, particularly among the leguminous plants. In this study, 13 of the 16 seeds tested presented both hemagglutinating activity and a thermolabile non-dializable toxic principle. Under the experimental conditions employed, we were unable to detect the presence of lectins in *C. indicus* extract. Since we used only a few cell types for the hemagglutinating tests and considering that several lectins show restricted binding requirements the data cannot be interpreted as a definitive indication of the absence of lectins in the *C. indicus* seeds. The toxic activity found in all of the 14 seed extracts were thermolabile and non-dialysable. The extracts of *A. hipogea*, *P. lunatus*, *C. arietinum* were non-toxic to mice when administered by the intraperitoneal route even after a 10-fold concen-

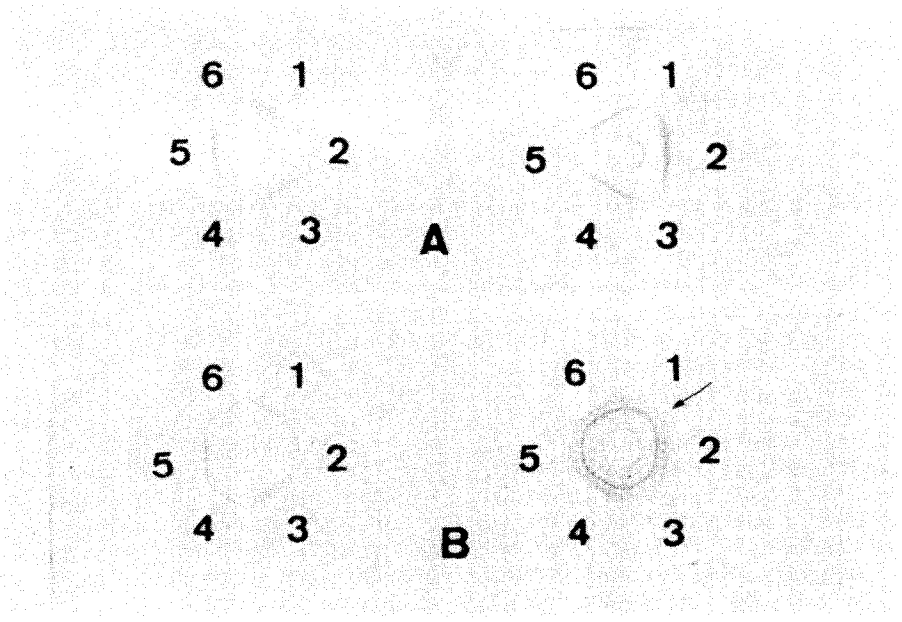


Fig. 1. Immunodifusion pattern of CNTX and Con A tested against anti-CNTX and anti-Con A antibodies. Aliquots of 10 $\mu$l of the fractions were applied to each well. After running for 24–36 hr, the gels were washed, dried and stained as described in Experimental. A: Left: central well, anticanatoxin IgG; 1, 3 and 5, canatoxin; 2, 4 and 6, concanavalin A; Right: central well, anticoncanavalin A IgG; 1, 3 and 5, canatoxin; 2, 4 and 6, concanavalin A. B: Left: central well, anticanatoxin IgG; 1, 3 and 5, canatoxin; 2, 4 and 6, *C. gladiata* crude extract; Right: central well, anticoncanavalin A IgG; 1, 3 and 5, concanavalin A; 2, 4 and 6, *C. gladiata* crude extract. The arrow indicates the second precipitin line observed in the immunoreaction between concanavalin A and its antibodies.

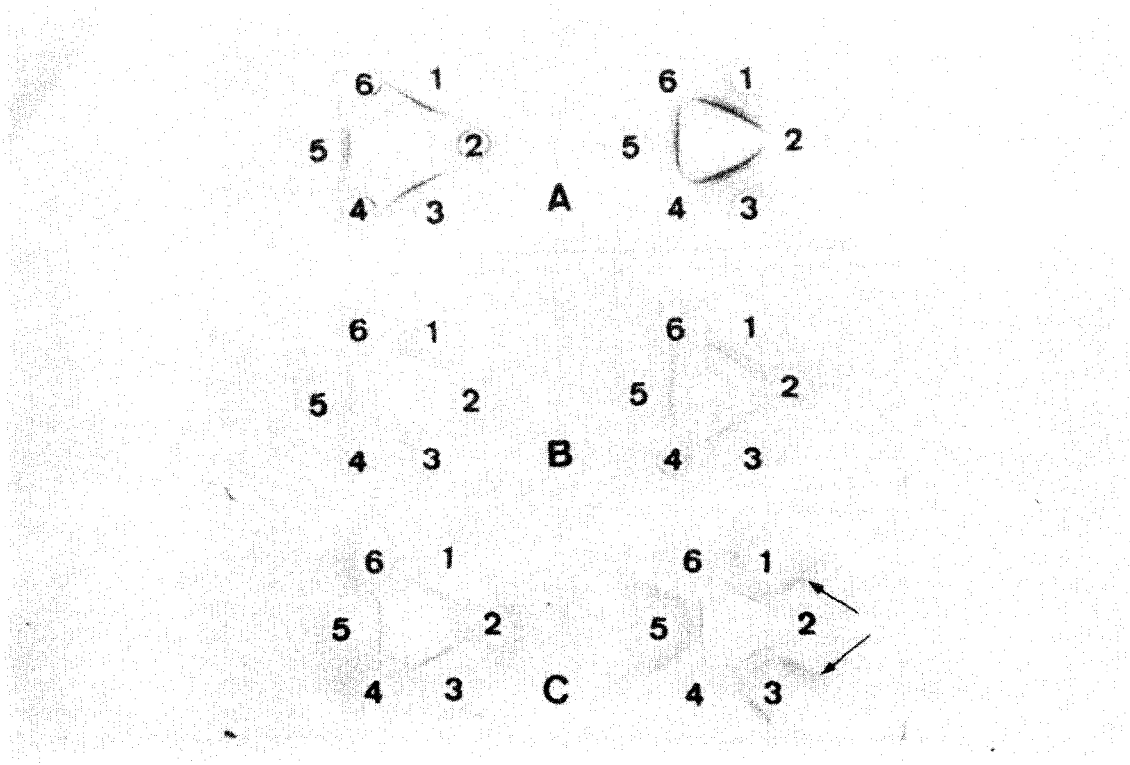


Fig. 2. Immunodifusion patterns of canatoxin, concanavalin A, *G. max*, *C. arietinum* and *L. culinaris* crude extracts tested against anticanatoxin and anticoncanavalin A antibodies. Aliquots of 10 $\mu$l of the fractions were applied to each well. After running the gels for 24–36 hr, the gels were washed, dried and stained as described in Experimental. In A: central well, anticanatoxin IgG (left), anticoncanavalin A (right side); 1, 3 and 5, canatoxin (left), concanavalin A (right); 2, 4 and 6, *G. max* crude extract (both sides). In B: central wells and wells 1, 3 and 5 were the same; 2, 4 and 6, *C. arietinum* crude extract (both sides). In C: central wells and wells 1, 3 and 5 were the same; 2, 4 and 6, *L. culinaris* crude extract (both sides). The arrows indicate the radial lines formed in the non-immunereaction between concanavalin A and carbohydrates-containing materials in the *L. culinaris* extract.

tration of the crude preparations or ethanol-treated fractions.

Several seeds used in this study are known sources of lectins, some of which have already been purified and physico-chemically characterized. It is clear from the results presented in this paper that the toxic proteins are widely distributed among the seeds of the Leguminoscae family, and that these proteins are concomitantly found associated with the lectins. Abrin (from the leguminous *A. precatorius*) and ricin (from the euphorbiaceous *R. communis*) are throughly investigated toxic proteins [8–10]. On the other hand, CNTX and Con A, both present in *C. ensiformis* seeds, were successfully separated from each other by our group in 1981 [13]. Other seeds, such as *G. max* [3, 4] and *P. vulgaris* [4, 5] are also known to contain toxic proteins. However in these seeds separation of toxic and hemagglutinating activities were not achieved and it is questioned whether both biological properties reside in the same or in distinct molecules.

In the euphorbiaceous plant *R. communis* it was found that its lectin and the toxic protein ricin are closely related as shown by immunodifusion, where a partial spur could be seen either with antiricin or with antilectin sera [15]. Actually ricin and *Ricinus* agglutinin (specific for D-galactose) have extensive homology and share a common subunit [9, 15]. Thus the toxic protein ricin behaves as a monovalent lectin with carbohydrate-specific binding properties which overlap that of *Ricinus* agglutinin. By contrast CNTX and Con A, despite the fact that both proteins are present in *C. ensiformis*, do not share common antigenic determinants as no cross-immunoreactivity with anti-CNTX or anti-Con A antibodies could be detected. Although there is no data available on the amino acid sequence of canatoxin, we demonstrated that this toxic protein is also a monovalent lectin which binds gangliosides but not monosacharides [21], similar to the binding specificity of other neurotoxic hemilectins such as tetanus and botulinum toxins [22]. Considering the difference in carbohydrate specificity and the absence of immunological cross-reactivity between CNTX and Con A, there seems to be a poor, if any, structural relationships between these two proteins.

Our results show that anti-Con A antibodies recognize only the lectins present in seeds of *Canavalia* spp., indicating that they are immunologically identical to Con A. Galbraith and Goldstein [20] found no immunological relationship between *P. lunatus* isolectins and Con A. This has been confirmed by our results. Surprisingly, no

Table 3. Comparison of the crossed immunoreactivity and the presence of toxic proteins and lectins among extracts of the leguminous seeds

| Seed | Presence of | | Crossed immunoreactivity | |
|---|---|---|---|---|
| | Toxic proteins | Lectins | Anti-canatoxin | Anti-con A |
| *Canavalia ensiformis* | + | + | Total | Total |
| *C. gladiata* | + | + | Total | Total |
| *C. obtusifolia* | + | + | Total | Total |
| *Cajanus indicus* | + | – | Partial | No reaction |
| *G. max* | + | + | Partial | No reaction |
| *V. unguiculata* | + | + | Partial | No reaction |
| *P. sativum* | + | + | Partial | No reaction |
| *P. vulgaris* | + | + | Partial | No reaction |
| *D. lablad* | + | + | — | — |
| *L. culinaris* | + | + | Partial | No reaction |
| *Abrus precatorius* | + | + | Partial | No reaction |
| *A. pavonira* | + | + | Partial | No reaction |
| *L. luteus* | + | + | Partial | No reaction |
| *Cicer arietinum* | N.D. | + | Partial | No reaction |
| *P. lunatus* | N.D. | + | Partial | No reaction |
| *Arachis hipogea* | N.D. | + | No reaction | No reaction |
| *R. communis** | Yes | Yes | No reaction | No reaction |

N.D. Not detected in the experimental conditions described.
*An euphorbiaceous plant, included for comparison.

crossed immunoreactivity to Con A was detected for the other seed extracts or fractions, despite the extensive amino acid homologies described for the lectins present in jack bean, peanut, soybean, red kidney bean and lentil [16, 17]. Altogether, the data suggest that there are no biologically dominant antigenic determinants present in the Con A molecule and in the other lectins that were conserved in the course of evolution. By contrast, proteins immunologically resembling canatoxin seem to be largely conserved in the leguminous plants, indicating that the toxic monovalent lectins such as canatoxin, abrin, mormodin, moddecin or ricin may play an important role in plant physiology. Our data also suggest that the presence of toxic monovalent lectins, or hemilectins, could be a valuable tool for vegetable taxonomy as evolutionary selection has directed its distribution among plant seeds.

## EXPERIMENTAL

*Leguminous seeds* *G. max* (soybean) *V. unguiculata* (feijao fradinho) *P. sativum* (pea), *P. vulgaris* (black bean), *P. lunatus* (lima bean), *D. Lablad* (mangalo), *L. culinaris* (lentil), *A. hipoqea* (peanut), *C. indicus* (guando). *L. luteus* (lupin) and *C. arietinum* (chick pea) were obtained from local stores. Seeds of *A. precatorius* (jequiriti bean) and of the euphorbiaceae *R. communis* (castor bean) were obtained from 'macumba' (brazilian black magic) stores. Jack bean (*C. ensiformis*) and *C. gladiata* seeds were grown by ourselves. *C. obtusifolia* and *A. pavonira* (carolina) seeds were kind gifts from Dr Jose Salim, Universidade Federal de Vicosa, MG, and Dr Jose Xavier Filho, Universidade Federal do Ceara, CE, Brazil, respectively. Highly purified and stabilized canatoxin was prepared from *C. ensiformis* seeds according to refs 13 and 21. Concanavalin A (type IV) was purchased from Sigma Chemical Co., St. Louis, USA, or prepared by ourselves [13].

*Seed extracts.* Powdered seeds or whole seeds were homogenized in a Waring blender after swelling for 2–4 hr in buffered solns (10% w/v). The buffers used for extraction were 10 mM sodium phosphate, pH 7.3, or 25 mM Tris–HCl, pH 7.5. Starch-like material was removed by centrifugation. When cloudy supernatants were obtained after centrifugation, pre-chilled EtOH was slowly added to a final concentration of 30%. The clear crude extracts or the EtOH-treated supernatants were then dialysed twice against 10 vols of the extracting buffer. When necessary, the extracts or the EtOH-treated fractions were freeze-dried.

*Toxic activity.* Recorded as death of the animals tested within 24 hr after i.p. administration of 0.1–50.0 mg protein/10 g of mouse body weight of the dialysed and concentrated extracts. $LD_{50}$ were determined according to the method of ref. [23]. The toxic extracts were tested again after heat denaturation (100° for 15 min).

*Hemagglutinating activity.* Determined by incubation of serial dilutions of the seed extracts with different types of erythrocytes in microtitration plates [24]. Briefly, 25 μl of the diluted extract were mixed with 25 μl of a 2% washed erythrocyte suspension in phosphate-buffered saline containing 0.25% bovine serum albumin. Fresh erythrocytes were obtained from rabbit, rat, mouse, guinea-pig and human (pool of A, B and O types). Trypsinized erythrocytes were prepared by incubation of the cells with 0.25% trypsin at 37° for 1 hr and then extensively washed in isotonic phosphate-buffered saline.

*Double immunodifusion tests* [25]. Performed using 1% agarose gels in 20 mM Na-Pi–150 mM NaCl, pH 7.0, containing 0.02% $NaN_3$. After running 36–48 hr at room temp. in a humid chamber, the gels were washed several times in saline and destilled $H_2O$, dried and then stained with Coomassie Blue.

*Antibodies* specific for CNTX or Con A were raised in rabbits by intradermal dorsal injection (20–30 microspots) of the highly purified proteins (100 μg) emulsified in Freund's complete adjuvant. Monospecific IgG's were obtained by fractionation of the immunesera at 0–0.33 $(NH_4)_2SO_4$ saturation. The IgG-enriched fraction were then applied to a DEAE-cellulose column equilibrated with 20 mM Na-Pi buffer, pH 6.0. The columns were then

percolated with 3 vols of the equilibrating buffer. The non-adsorbed proteins contained the purified anti-CNTX or anti-Con A IgG fractions. The solns of purified polyclonal IgG's for use in the immunodiffusion tests were adjusted to 5 mg/ml.

*Protein concentration* of the extracts was determined by reaction with Coomassie Blue R-250 [26].

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