Comparison of allergenic components and biopotency in whole body extracts of wild and laboratory reared American cockroaches, *Periplaneta americana*

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Summary

Background and objective: **Most allergen extracts/vaccines used today in clinical practice are derived from natural allergen sources. Therefore, their allergic components may vary as these are prone to natural variation. This study aims to compare the allergenic components and biological potency of crude extracts from wild and laboratory reared American cockroaches.**

Methods: **Crude extracts of male and female of wild and laboratory reared American CR, were prepared by the same method. Their allergenic components were evaluated by *in vit*ro assays such as protein contents, protein profiles, quantification of major allergens (Per a 1 and Per a 9) and IgE Inhibition ELISA assay.**

Results and Discussion: **There was no statistically significant difference between the protein contents and the concentrations of Per a 1 in the crude extracts from both groups. However, the Per a 9 levels in extracts of wild CR were significantly higher than those from the extracts of laboratory reared CR. The protein patterns of the extracts of laboratory reared CR exhibited more consistency in the number of bands with higher intensity than those of wild CR. Pooled extracts of laboratory reared CR could inhibit IgE binding to that of wild CR up to 78%. The endotoxin content of extracts of laboratory reared CR were ten times less than those of the the wild CR. We have successfully determined the allergenic potency of the extracts of laboratory reared CR versus those of the wild CRs by *in vitro* assays. Further studies should be performed to determine the biological potency of CR extracts by *in vivo* assays for clinical application.**

Conclusion: **Our finding indicates that the laboratory reared CR would be the better source of material in vaccine production than the wild CR. *(Asian Pac J Allergy Immunol 2012;30:231-8)***

Key words: *American cockroach, Periplaneta americana, wild cockroach, laboratory reared cockroach, Per a 1, Per a 9.*

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Introduction

The prevalence of allergic diseases including atopic dermatitis, allergic conjunctivitis, allergic rhinitis and asthma, has increased considerably all over the world. Approximately 25-40% of the global population, predominantly in Western countries, is affected and the problem is expanding to newly developed countries.[1] The allergic ailments are caused mainly, if not solely, by high production of allergen-specific IgE antibodies elicited commonly by indoor allergens.[2] Among the indoor allergens, cockroach (CR) derived proteins are regarded as the second most important allergen followed house dust

mite (HDM) allergens.[3] The incidence of CR allergy is rising and is found in at least 30-40% of atopic patients.[4]

In Thailand, 44-60% of allergic patients have positive skin prick tests to whole body extract of American CR, *Periplaneta americana* which is the predominant CR species in this country.[5-7] The CR allergens may be derived from any part of their bodies, as well as their ootheca, excretions and secretions. The total mass of their allergic proteins range from 6-120 kDa.[8,9] To date, six American CR allergens have been characterized, including Per a 1, Per a 3 (aryl phosphorin-hemocyanin), Per a 6 (troponin-C), Per a 7 (tropomyosin), Per a 9 (arginine kinase), and Per a 10 (serine protease).[9] Among them, the Per a 1.0105 (AY259514), which is one of the Per a 1 variants, and Per a 9 (arginine kinase) generate IgE in the sera of all CR allergic Thai patients, implying a major allergenic role in this population.[10-12]

Current management of allergic diseases involves allergen avoidance, symptom alleviation by using pharmacological agents and immune deviation by means of immunotherapy.[13] The therapeutic allergy vaccines given either subcutaneously (*i.e.*, subcutaneous immunotherapy or SCIT) or mucosally (intranasally or sublingually) have been shown to have therapeutic effectiveness for patients with IgE-mediated reaction to *Hymenoptera* venom,[14] allergic rhinitis,[15] and atopic bronchial asthma.[16]

Extracts from natural allergenic sources have been used for over 100 years as diagnostic materials for allergic patient screening and as therapeutic vaccines.[17] For CR allergy, whole body extracts of wild CR, which represent the actual environmental allergens to which the patients had been sensitized, are used. However, their allergenic components vary from batch-to-batch according to their food and place of habitation. We envisaged that extracts of laboratory reared CR should be a better standardized, convenient and safe source of the CR allergens for both diagnostic and therapeutic purposes. Recently, *in vitro* methods, such as the determination of major allergen content using ELISA, has been increasingly used to define the specific potency of allergen extracts.[18] Thus in this study, the protein content, protein profiles, amounts of major allergens, namely Per a 1 and Per a 9, endotoxin levels and *Mycoplasma* contamination in whole body extracts of wild and laboratory reared American CR were compared in order to validate the surrogate potential of the latter for use as a source material for allergenic extracts. In addition, the differences between male and female CR extracts were investigated.

Methods

American CR allergic patients and their sera

This study received ethical approval from Siriraj Ethical Committee (COA No. Si 231/2011). Blood samples were taken from 10 consenting adult patients attending the Allergy Clinic of the Department of Oto-rhino-laryngology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, who were skin prick test positive to crude *P. americana* extracts. Individual sera were collected and kept at -20°C before use.

Preparation of P. americana whole body extracts

Whole body extracts of wild and laboratory reared American CR, namely, wCR and lCR, respectively, were prepared as described previously.[12] To prepare wCR extracts, 5 each of adult female and male *P. americana* (entomologically identified) were caught from houses in the Bangkok metropolitan area. The supernatants (designated fwCR1-fwCR5 for female extracts and mwCR1-mwCR5 for male extracts) were collected separately and the protein content of each extract was determined using Bradford reagents[19] (Bio-Rad, Hercules, CA, USA). Extracts of 5 each of male and female laboratory reared CR of relatively similar body weights to the wild CR, were similarly prepared and designated flCR1-flCR5 and mlCR1-mlCR5 for female and male extracts, respectively.

Preparation of recombinant Per a 1.0105 and mouse monoclonal antibody (MAb) to Per a 1

Recombinant Per a 1.0105 was prepared as described previously with modification.[10] The recombinant protein was used as an immunogen to immunize BALB/c mice for production of hybridoma secreting Per a 1 specific monoclonal antibody (MAb) as described previously.[11]

Preparations of native Per a 1 and Per a 9

Native Per a 1 and Per a 9 (*P. americana* arginine kinase) were prepared from the wild American CR whole body extract by affinity chromatography.[11,12] Briefly purified specific monoclonal antibody to Per a 1 and Per a 9 were coupled separately to Sepharose CL4B resins (Pharmacia, Uppsala, Sweden) according to the instruction manual. The CR extract was mixed and rotated with affi-gels at 4ºC overnight. The gels

were allowed to set by gravitation, the supernatant was discarded and the resins were washed several times with phosphate buffered saline (PBS) to remove unbound proteins. Each affi-resin preparation was packed into a column. The bound Per a 1 and Per a 9 were eluted out from the respective columns with 0.1 M glycine-HCl buffer, pH 3.0. One ml fractions were collected into tubes containing two drops of 1 M Tris-HCl pH 8.0. Fractions with optical density at absorbance 280 nm (OD_{280nm}) were pooled and dialyzed against distilled water at 4ºC overnight. These preparations were used as standard allergens for quantification of the Per a 1 and Per a 9 contents in the fwCR1-fwCR5, mwCR1-mwCR5, flCR1-flCR5 and mlCR1-mlCR5 and the respective pools by sandwich-ELISA.

SDS-PAGE analysis

SDS-PAGE was carried out as described previously.[12] Briefly, freshly prepared CR extracts, *i.e.*, fwCR1-fwCR5, mwCR1-mwCR5, flCR1-flCR5 and mlCR1-mlCR5 (equal amounts of total protein) were separated individually in 12% SDS-polyacrylamide gel slabs cast in a Mini-PROTEAN 3 Cell (Bio-Rad, USA). The separated proteins in each gel were stained by using Coomassie Brilliant Blue G-250 (CBB) dye to visualize directly the protein bands.

Sandwich ELISA for quantification of Per a 1 and Per a 9 in CR extracts

The concentrations of Per a 1 and Per a 9 in individual CR extracts and their respective pools were determined by sandwich-ELISA (MAb-allergen-PAb) as described previously.[11] Wells of ELISA plates (Corning, USA) were filled with 100 µl of carbonate-bicarbonate buffer, pH 9.6 containing 4 µg of either MAb to Per a 1 or Per a 9 and the plate was kept at 37ºC in a moisture chamber overnight. Individual wells were washed with washing buffer (phosphate buffered saline containing 0.05 % Tween-20; PBS-T) and blocked with 1% bovine serum albumin (BSA) in PBS. After washing away the excess blocking reagent with the washing buffer, 100 µl of *P. americana* extract diluted 1:2,000, 1:4,000 and 1:8,000 in PBS-T were added appropriately to the wells and incubated at 37°C for 30 minutes. After washing as above, 100 µl of 1:1,000 rabbit polyclonal antibodies to American CR extract were added to each well and the plate was re-incubated at 37°C for 1 hour. The wells were washed and 100 µl of goat anti-rabbit immunoglobulin-HRP conjugate (Santa Cruz, USA) diluted 1:1,000 were added, incubated, washed and HRP substrate was added. The OD of the content of each well was measured at 492 nm with an ELISA reader (Multiscan *EX*, Lab systems, Helsinki, Finland) against the blank (a well to which PBS-T was added instead of the CR extract). A series of different amounts of Per a 1 and Per a 9 were included in the same ELISA plate as standard allergens. The amount of Per a 1 and Per a 9 in CR extracts was calculated from the respective standard curves constructed by plotting the amounts of Per a 1 and Per a 9 standards against the read-out sandwich ELISA OD_{492nm}.

Endotoxin quantification and Mycoplasma detection

Pools of small equal aliquots of fwCR1-fwCR5, mwCR1-mwCR5, flCR1-flCR5 and mlCR1-mlCR5 were prepared. The endotoxin content of individual pools was determined by using a *Limulus* amebocyte lysate (LAL) test kit (Biolasco, Taiwan). The preparations were also checked for *Mycoplasma* contamination using RT-PCR (Minerva-biolabs, Germany).

IgE ELISA Inhibition assay

IgE inhibition ELISA assay was performed in triplicate to verify the potency of the CR allergen in pooled extracts from both groups as described previously with modification.[10] Briefly, pooled CR extracts (2 µg/well) from wild American CR were coated to microtiter plate (Costar, USA) and incubated overnight at 37^0C. After washing 3 times, the wells were blocked with 1% BSA in PBS at 37^0C for 1 hour. All wells were washed and then incubated with a 1:10 dilution of pooled serum from 10 allergic patients, previously incubated with graded amounts of pooled extracts from laboratory reared American CR (2.0; 1.5; 1.0; 0.5;0 µg/well). The wells were incubated at 37^0C for 2 hours and 100 µl of biotin labeled anti-human IgE (diluted 1:5000 (Zymed Laboratory, South San Francisco, CA), streptavidin-peroxidase conjugated (diluted 1:5000; Dakopatts, Glostrup, Denmark) and ABTS; 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; were used as a detection system with washing between each step.

Statistical analysis

SPSS version 18 computer programs were used for calculating the medians, standard deviations and ranges of the body weights of the CR, protein contents, and concentrations of Per a 1 and Per a 9 in individual CR extracts. The Mann-Whitney U test

and the Wilcoxon W rank sum test were used to compare parameters between the wild and laboratory reared American CR groups. *P*-values of < 0.05 was taken as significant differences.

Result

In this study the body weights of the CR varied from 0.92-1.64 g; nevertheless, there were no significant difference between the fw-, mw-, fl- and ml- CR groups, although fw- and fl- CR were generally heavier than their male counterparts (Figure 1 A).

The median and range of protein concentrations, levels of Per a 1 and Per a 9 in the extracts of female and male wild and laboratory reared *Periplaneta americana* are shown in Table 1. The protein concentrations in the whole body extracts of individual CR were highly variable, ranging from 2.87-8.92 mg/ml (Tables 1). However, the protein content in the extracts of all 10 wild CR and laboratory reared CR were not significantly different (p = 0.82; Figure 1B).

Similar results were obtained for the concentrations of Per a 1 in extracts of fw-, mw-, fl- and ml- CR, which were not significantly different (Table 1). The concentrations of Per a 1 in pooled extracts of 10 wild CR and 10 laboratory reared CR were not different (p = 0.71; Table 1 and Figure 1C).

Although the concentrations of Per a 9 in extracts of females and males of wild CR were not different, these concentrations were significantly higher than those of their respective laboratory reared-counterparts (Table 1). The pooled extract of 10 wild CR also contained significantly higher Per a 9 concentration than the pooled extract from 10 laboratory reared CR (Table 1 and Figure 1 D).

The protein banding patterns of individual fw-, mw-, fl- and ml- CR, revealed by SDS-PAGE and CBB staining, are shown in Figure 2A-2E. The proteins ranged in molecular mass from < 10 to > 170 kDa. The numbers of protein bands in the extracts were not markedly different, although some components had much higher intensities. All CR extracts showed a high intensity of a protein doublet of ~ 40 kDa, while extracts of fl-CR had additional intense protein bands at ~ 80 kDa.

The endotoxin level in pooled extract from laboratory reared CR was only 863 EU/ml, while the pooled extract of wild CR contained 8,113 EU/ml. *Mycoplasma* was not found in any of the CR extracts. The results of IgE binding ELISA inhibition are shown in Figure 3. At a concentration of 2 µg the extract of laboratory reared American CR could compete for 78% of IgE binding of the wild CR extract.

Discussion

Allergenic extracts for diagnosis and treatment of allergic diseases, which are commonly derived from natural source materials, are complex mixtures of variable components.[20] Variability in the quality of the commercially available allergenic extracts has been reported repeatedly.[20-21] Careful standardization to control the variation and ensure consistency and reproducibility, as well as the safety and efficacy of the preparation, is a necessity for effective outcomes in allergic patient management.[21] Both commercial and in-house preparations of CR extracts currently

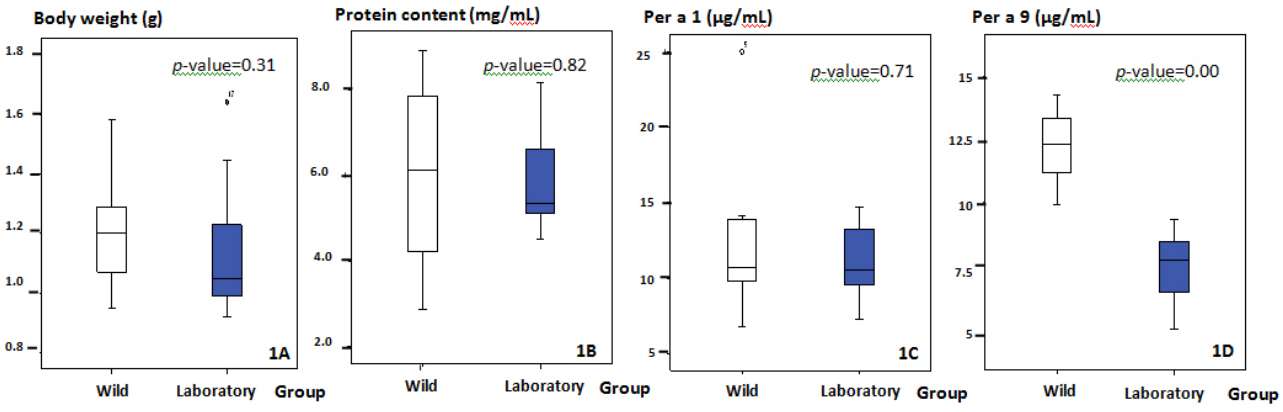

Figure 1. Means and 95% CI for body weight (1 A), protein content (1 B) and concentrations of Per a 1 (1 C) and Per a 9 (1 D) in the combined extracts of female and male wild and laboratory reared *Periplaneta americana*.

Table 1. Median (range) of protein content, Per a 1 and Per a 9 level in extracts of female and male wild and laboratory reared *Periplaneta americana.*

Type of CR	Protein content (mg/ml)			Major allergens (µg/ml)					
				Per a 1			Per a 9		
	Female and male	Female	Male	Female and male	Female	Male	Female and male	Female	Male
Wild *P. Americana* (n=10)	6.13 (2.87-8.92)	5.97 (2.87-8.19)	6.60 (4.22-8.92)	10.75 (6.79-24.99)	10.20 (9.06-14.12)	11.30 (6.79-24.99)	12.32[a] (9.89-14.27)	13.36[a] (9.89-14.27)	11.87[a] (10.25-12.49)
Laboratory reared *P. americana* (n=10)	5.35 (4.51-8.17)	6.25 (4.51-8.17)	5.31 (4.81-6.61)	10.59 (7.32-14.72)	10.88 (7.32-13.64)	10.31 (9.47-14.72)	7.71[b] (4.95-9.30)	8.44[b] (7.13-9.30)	6.41[b] (4.95-8.39)

[a,b] Entries with different superscripts are significantly different at $p < 0.05$ by Mann-Whitney analysis of variance for multiple comparisons

used in a variety of allergy clinics are prepared from wild CR. Standardization of the extracts is based only on the protein concentration and skin test results in patients. Measurement of the actual allergen quantities, in terms of individual allergen contents, and allergenic potency is not performed.[18] Hence, the amount of both major and minor allergenic components present in different batches of extracts vary consequently causing inaccurate potency and unreliability in the allergic patient screening, disease severity monitoring and vaccine dosing in immunotherapy. Thus, the selection of source material is the most important prerequisite for preparation of allergenic extracts.[18,22] In the present study, we compared the overall quality of *P. americana* extracts prepared from wild CR with the extracts derived from the more convenient source material, *i.e.,* laboratory reared CR.

Usually, the free-living American CR is a scavenger that feeds on decaying organic matter and a variety of foods, particularly fermenting foods. Differences in their environmental habitat would also affect their allergic components.[22] Patterson and Slater demonstrated that commercial cockroach extracts are highly variable in protein content, number of protein bands and potency.[23] In this study there was no significant difference in the protein content of the extracts from both wild and laboratory reared CR groups, implying that the laboratory reared CR can be used as a source material for allergenic extract instead of the wild CR. Apart from the protein content determination, the SDS-PAGE and CBB staining, which were performed to check the composition and activity of the allergen extracts, the protein profiles of extracts among the laboratory reared-CR were less variable than those from wild CR (Figure 2).

The level of Per a 1.0105 in the extracts from both groups of CR was also not statistically significantly different (Table 1 and Figure 1 C), indicating that the extracts from laboratory reared CR should contain comparable specific amount of Per a 1 to those in the extracts from wild CR.

The quantity of Per a 9 (arginine kinase) in crude extracts of laboratory reared CR in this study was significantly lower than that of in wild CR. This might be due to the diversity of food consumed and environments inhabited, as cited before. It is well established that arginine kinase is important not only for generation of ATP but also for muscle contraction and motility.[24] Furthermore, the arginine kinase content in living organisms is affected by dietary nutrients and some compounds such as lutein, flavonoid, sugar, polysaccharides etc.[25] The laboratory reared CR live in a limited area in a laboratory-container and feed on rat chow the composition of which is fixed according to the set up formula. By contrast wild CR searching for their own food, and have the chance to consume a variety of foodstuffs. This might be the explanation for the higher arginine kinase level in wild CR. In regards to U.S. FDA standards, the variability of the total allergenic activity within the same species is allowed to be 50–200%[26] whereas the Nordic Council set greater range: from 30–300%.[27] The median concentration of Per a 9 in the extracts from CR reared in the laboratory was approximately 50-70% of the Per a 9 obtained from the extracts from

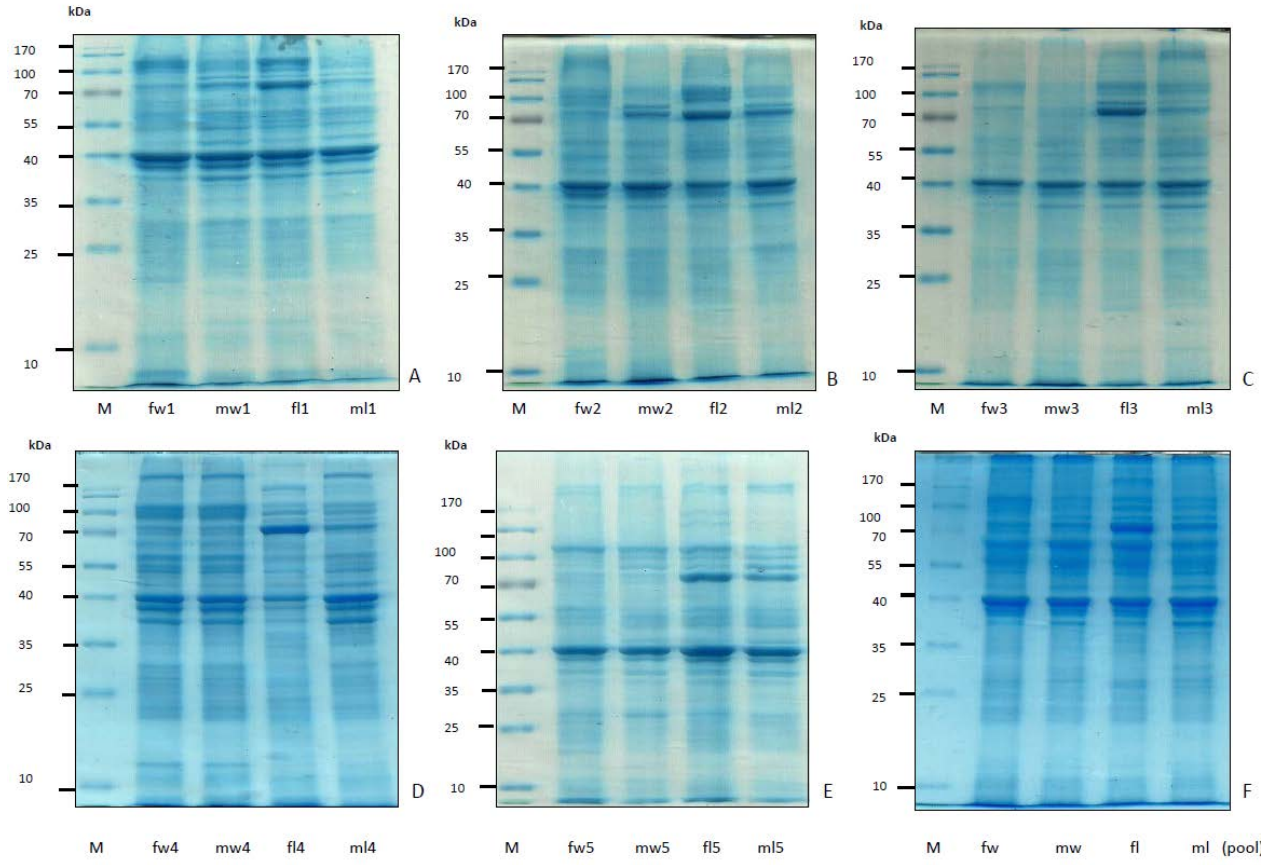

Figure 2. SDS-PAGE patterns (A-E) of crude extracts from individual female and male wild and laboratory reared *Periplaneta americana* stained with Coomassie brilliant blue. Lane M: standard protein markers, fw: crude extracts from wild female CR, mw: crude extracts from wild male CR, fl: crude extracts from laboratory reared female CR, ml: crude extracts from laboratory reared male CR, respectively. SDS-PAGE analysis (F) is the pooled extracts of female and male wild and laboratory reared CR.

wild CR. Compared to those reference limits, the deviation of detected Per a 9 level in the extracts from both groups is within an acceptable range.

In addition to the comparison of individual CR extracts from both groups, pooled extracts of the wild CR and laboratory reared CR were also compared with regard to endotoxin levels, *Mycoplasma* contamination and the IgE ELISA Inhibition assay. Importantly, naturally sourced materials usually contain some impurities such as endotoxin. This toxin is derived from the cell wall components of gram-negative bacteria and are known to induce inflammatory responses in human cells, subsequently affecting allergic diseases. It has been documented that endotoxins are ubiquitous in our environment and 300-18,000 ng/g of dust of endotoxin can be detected even in a normal indoor environment.[28] Therefore, the endotoxin level in biological products like allergenic extracts is considered to be one of the important specifications.[29] The level of endotoxin content in the pooled extract of wild CR was ten times higher than that of laboratory reared CR. This might be due to the fact that CR reared in the laboratory have a low risk of exposure to infectious and toxic materials. This finding indicates that the extract from wild CR has the potential risk of having high levels of contamination with endotoxin. Besides, cultures or cultivated plants and animals are recommended for use as an allergen source in order to control the contamination by microbes and such impurities.[18,30] Thus, the laboratory reared CR are a more appropriate source of materials for allergenic extract.

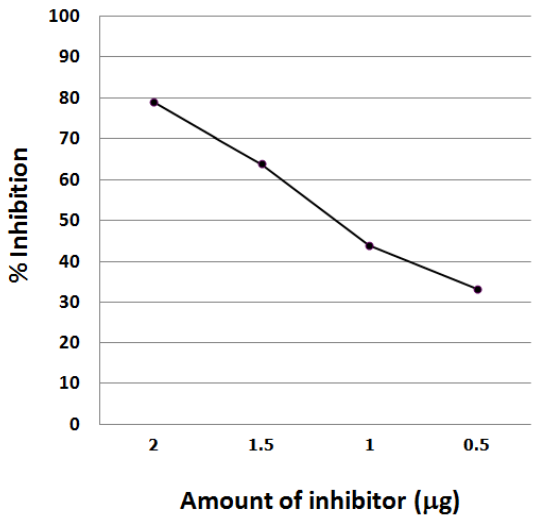

Figure 3. Inhibition of IgE binding to crude extracts of wild CR by crude extracts of laboratory reared CR.

Our findings indicate that pooled extracts of laboratory reared CR were able to inhibit up to 78% of IgE binding to pooled extracts from wild CR. The missing 22% of IgE inhibition could be due to the significantly lower level of Per a 9 in the laboratory reared CR extracts and the biological variation of allergenic components, especially the minor allergens in each group and even in individual CR.

As mentioned, the variability of allergenic components might be affected mainly by the source materials. To reduce this variability, the laboratory reared CR should be considered to be a proper source material for vaccine production instead of the wild CR. We have successfully determined the allergenic potency of the extracts of laboratory reared CR versus those of wild CR by *in vitro* assays. Therefore, a clinical study should be performed to determine the biological potency of CR extracts with the hope that a standard allergenic potency of CR extracts can be set up and used universally.

In conclusion, the present study demonstrates that the laboratory reared CR would be a better source material than wild CR for vaccine production.

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