



Biopotency and identification of allergenic proteins in *Periplaneta americana* extract for clinical applications

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Abstract

Commercial cockroach extracts for diagnosis and therapy show batch-to-batch variation. This study aimed to standardize *Periplaneta americana* extract based on major IgE binding components using hypersensitive patients' sera. Extracts were prepared in phosphate buffered saline (PBS) or NH_4HCO_3 , from freeze-dried or 37 °C dried material and compared with commercial extracts by immunobiochemical methods. Cockroach positive patients' sera were collected after intradermal tests and specific IgE enzyme linked immunosorbent assay (ELISA). Allergenic proteins were identified by western-blotting and potency of extracts determined by ELISA-inhibition. Adult *P. americana* extract from freeze dried source material in PBS (PA extract) resolved into 45 protein bands and showed 22 IgE binding components with pooled patients' sera. It required 9–12 ng self-proteins for 50% ELISA-inhibition. Individual patients' sera identified 23, 28, 35, 38, 40, 49, 72, 78 and 97 kDa as major IgE binding components in PA extract. Nymph extract exhibited similar potency and protein profile to PA extract with 72 and 78 kDa proteins present in high intensities. Commercial extracts exhibited only 6–11 IgE reactive bands compared to PA extract and required 40 folds or more protein for 50% ELISA-inhibition. PA extract from freeze-dried source material seems a potent allergen preparation with 9-major IgE binding components. It can be referred to upgrade the quality of commercial extracts exhibiting low potencies due to poor quality source material, inadequate extraction procedures and improper storage.

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1. Introduction

Insects play an important role in the aetiology of allergic bronchial asthma. Infestation of homes, places of work and accumulation of their allergens in house dust account for widespread allergy to insects. Unlike other insects, cockroaches provoke clinical reactions as contactant, injectant, ingestant and inhalant allergens [1]. Cockroach infested dwellings are contaminated by their feces, vomitus and debris. Studies

have established a causal relationship between cockroach allergy and asthma [2] and cockroach sensitivity in low socio-economic group [3]. Association of cockroaches with asthma has been reported from Taiwan, Japan, Thailand, Singapore, India, Korea, Costa Rica, Puerto Rico, South Africa, Europe, Brazil and Poland [4–6]. Recent epidemiological studies show an increase in prevalence of asthma world over [7]. Studies in India also recorded significant increase in prevalence of asthma (8–11%) than reported earlier <1% [8,9].

Previously proteins of 12–45 kDa and 72–90 kDa were detected as important allergens from *Periplaneta americana* [10–13]. Further, three major allergens viz. Per a 1, Per a 3 and Per a 7 have been cloned, expressed and purified

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[13–15]. But whole body cockroach and other insect extracts are still used in diagnosis and therapy of allergy. *P. americana* (American cockroach) is most common in Indian household as high temperature and humidity favor the proliferation of cockroach populations [4]. Sensitization to cockroach allergens in India has been shown earlier by skin tests and radio allergosorbent test (RAST) [16,17].

It is recommended to standardize allergen extracts for diagnosis and therapy on the basis of major allergen content [18,19]. Considering the possibility of differences in sensitization pattern among countries and or individuals, the present study was aimed to identify the major IgE binding components of *P. americana* by immunoblot using hypersensitive patients' sera from this subcontinent. The source material and extraction of cockroach antigens were standardized to obtain an optimum allergen extract. Biopotency of nymph and adult stage extracts was evaluated with commercial cockroach extracts by immunobiochemical methods. In addition, Per a 1 was demonstrated in the PA extract (adult *P. americana* extract from freeze dried source material in PBS) using antibodies raised in mice against this major allergen.

2. Material and methods

2.1. Preparation of cockroach extract

Mixed culture of male and female cockroaches were freeze-dried or dried at 37 °C, ground to a fine powder and defatted with diethyl ether at 4 °C. The antigens were extracted from cockroach powder 1:50(w/v) in 0.1 M phosphate buffered saline (PBS), pH 7.2 or 125 mM NH₄HCO₃ buffer, pH 8.0 containing 5 mM EDTA and 1 mM phenyl methyl sulfonyl fluoride (PMSF), by continuous stirring for 8 h at 4 °C. The supernatant collected after centrifugation at 13,000 rpm for 1 h at 4 °C was dialyzed against distilled water. The adult and nymph cockroach extracts were prepared separately using the same procedure. Also *P. americana* allergen extracts were procured from 3 different manufacturers licensed to market allergen extracts in India. All the extracts were lyophilized, stored at –20 °C and their protein concentration determined by modified Lowry's method [20].

2.2. Patient selection and collection of sera

One hundred forty consecutive cases referred to the Out Patient Department, V.P. Chest Institute, Delhi for allergy diagnosis were included in the present study during Jan–June 2004. The diagnosis of asthma was ascertained as per guidelines of the American thoracic society [21] and cases having any two of the symptoms such as sneezing, rhinorrhea, nasal blockage, post nasal drip, etc. for the last two years were diagnosed as rhinitis [22].

Intradermal (ID) tests were carried out with a battery of allergen extracts according to the history (exposure) of the patients. About 0.02 ml of each extract including *P. americana* (i.e. PA extract and one of the commercial extract) was injected into the forearm of the patient raising a bleb of

2–3 mm. The reactions were graded after 20 min on the basis of wheal size of positive control i.e. histamine diphosphate (100 µg/ml) [23]. Blood was collected from patients showing marked positive skin reactions (wheal size equal to positive control and or more), sera separated and stored at –70 °C. Informed consent of each patient was obtained for skin tests and blood collection. Normal human serum (NHS) was collected from 10 volunteers with negative skin reactivity to cockroach and other allergens.

2.3. SDS-PAGE analysis

P. americana extracts were subjected to SDS-PAGE on 10% resolving gel as described before [12]. One hundred microgram of each extract was loaded in separate wells, electrophoresed at 120 V and the gel stained with Coomassie brilliant blue.

2.4. Immunoblotting

SDS-PAGE separated proteins were electrophoretically transferred onto nitrocellulose membrane in Tris-glycine buffer containing methanol and probed with patients' sera (pooled/individual) as described before [24]. The membrane was cut into strips after blotting, blocked with 3% defatted milk and incubated overnight with 1:10 v/v diluted cockroach hypersensitive patients' sera at 4 °C. The strips were then probed with 1:1000 v/v diluted anti-human-IgE peroxidase (Sigma) for 3 h at 37 °C and IgE reactive bands were visualized with diaminobenzidine and hydrogen peroxide in acetate buffer. One of the strips was incubated with antibodies (polyclonal) against Per a 1 raised in mice (a kind gift from Dr. MD Chapman's laboratory) and the reactivity was detected with anti-mouse-IgG peroxidase. The reaction was stopped by washing the strips with distilled water.

2.5. Enzyme linked immunosorbent assay (ELISA)

Specific IgE to cockroach allergens were quantitated in patients' sera by ELISA as described before [24]. Briefly, microtiter plate coated with cockroach protein (1 µg/100 µl per well) in carbonate buffer, pH 9.6 was blocked with 3% defatted milk and incubated with patients' sera (1:10 v/v) at 4 °C overnight. After washing with PBST (PBS containing 0.2% Tween 20) and PBS, the plate was probed with 1:1000 v/v diluted anti-human-IgE peroxidase (Sigma). The color developed with o-phenylene diamine was read at 492 nm in an ELISA reader.

2.6. ELISA inhibition

Specific IgE inhibition assay was carried out to determine the potency of extracts. The microtiter plate was coated with crude cockroach protein (1 µg per well) and blocked with 3% defatted milk. The plate was incubated with a pre-incubated mixture of 1:10 v/v diluted cockroach hypersensitive pooled patients' sera and graded amounts of

cockroach extract, i.e. 0.1, 1, 10, 100, 1000 ng, as inhibitor in a 100 μ l volume. The remaining steps were the same as followed for ELISA.

3. Results

3.1. Total protein content and profile of extracts

Adult cockroach extracts prepared with freeze dried source material in PBS (PA extract) and NH_4HCO_3 yielded 150 mg and 100 mg protein/gram of cockroach powder, respectively. Whereas the PBS extract prepared with adult cockroaches dried at 37 °C yielded 75 mg protein/gram of cockroach powder. The protein content of the commercial extracts was highly variable, ranging from 60 to 96 mg protein/gram of cockroach powder. The nymph extract prepared with freeze dried source material in PBS yielded 200 mg protein/gram of nymph cockroach powder.

The PA and NH_4HCO_3 extracts of freeze dried adult cockroaches resolved into approximately 45 and 34 protein bands (MWs 10 to >97 kDa), respectively on SDS-PAGE (Fig. 1A). But the PBS extract of adult cockroaches dried at 37 °C separated into 25 slightly diffused bands with many high molecular weight bands missing (Fig. 1B, lane 2). SDS-PAGE profile of nymph extract prepared with freeze dried source material in PBS was similar to the PA extract (45 bands) with the exception of 72 and 78 kDa protein bands present in higher intensity in the nymph extract (Fig. 1B, lane 3). Probably, these proteins are over expressed in nymph stage of cockroach. The number (11–27) and intensity of protein bands were highly variable among the commercial cockroach extracts from different manufacturers (Fig. 5A).

3.2. Biopotency of extracts

Biopotency of the cockroach extracts was assessed by specific IgE ELISA inhibition. The PA and nymph extracts required 9–12 ng and 8.5 ng of self protein to achieve 50% inhibition of specific IgE binding in ELISA (Fig. 2). Whereas the PBS extract with source material dried at 37 °C and the commercial extracts required 400 ng and 450 to >1000 ng of self protein for 50% inhibition (Fig. 2).

The PA extract was better in terms of protein content, protein profile and allergenic potency compared to extracts prepared in NH_4HCO_3 or with source material dried at 37 °C or the commercial extracts (Table 1). Also, the nymph and adult extract (i.e. PA extract) were similar in terms of protein profile and biological potency (Table 1). Hence, the PA extract was selected for skin testing and used as standard extract for further immunobiochemical experiments.

3.3. Skin reactivity and specific IgE to *P. americana*

Of the 140 cases tested, 42 belonged to bronchial asthma, 41 to allergic rhinitis and 57 to asthma with associated rhinitis. The PA extract and the representative commercial extract showed marked positive skin reactions in 74 (74/140 i.e. 52.8%) and 20 (20/140 i.e. 14.3%) patients, respectively. The comparison of the results with PA extract showed significant differences ($p < 0.01$). The patients also elicited positive skin reactivity to some insect allergens (Housefly-60.7%; Moth-53.5%; Mosquito-52.1% and Rice-weevil 40%) followed by a few pollens (*Gynandropsis gynandra*-26.2%; *Penisetum typhoides*-24.3%; *Artemisia scoparia*-24.3% and *Imperata cylindrica*-23.3%) and fungal extracts (*Phoma hybernica*-20.4% and *Fusarium solani*-17.5%).

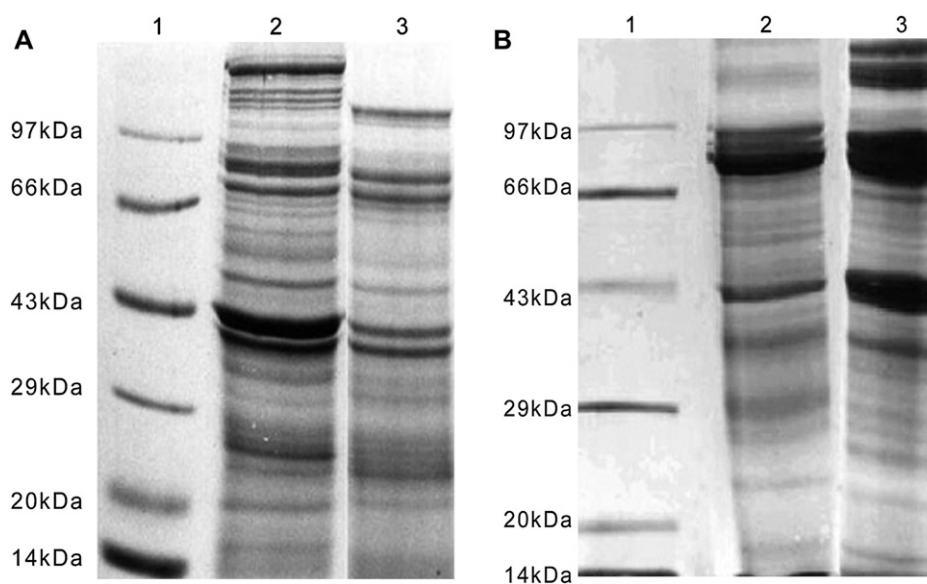


Fig. 1. SDS-PAGE (10%) profile of the whole body extracts of *P. americana*. CBB (Coomassie brilliant blue) was used for staining. (A) Lane 1- Molecular weight marker, Lane 2- Adult extract prepared in 0.1M phosphate buffered saline (PBS) with freeze-dried source material (PA extract) and Lane 3- Adult extract prepared in 125 mM ammonium bicarbonate buffer with freeze-dried source material. (B) Lane 1- Molecular weight marker, Lane 2- Adult extract prepared in PBS with source material dried at 37 °C and Lane 3- Nymph extract prepared in PBS with freeze-dried source material.

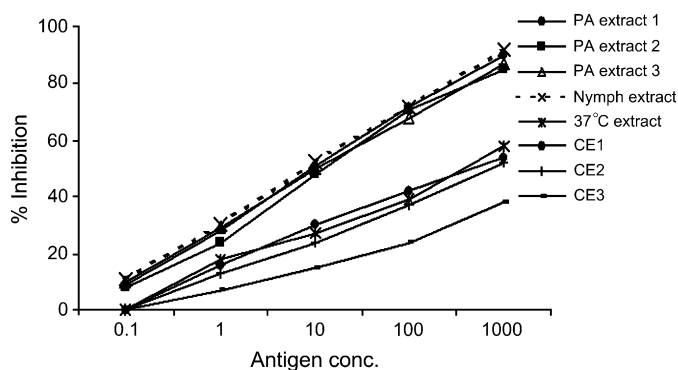


Fig. 2. ELISA inhibition: ELISA inhibition of three batches of PA extract (PA extract 1, PA extract 2, PA extract 3) wherein 1 μ g/well of PA extract 1 was coated in microtiter plate and incubated with a mixture of cockroach hypersensitive pooled patients' sera (1:10 v/v dilution) and 0.1, 1, 10, 100 & 1000 ng of PA extract 1, PA extract 2 and PA extract 3 taken separately as inhibitors; ELISA inhibition of nymph extract prepared in PBS with freeze dried source material (nymph extract); ELISA inhibition of adult extract prepared in PBS with source material dried at 37 °C (37 °C extract) and ELISA inhibition of 3 commercial extracts (CE 1, CE2, CE3).

Specific IgE to *P. americana* in sera ($n = 70$) of cockroach positive patients ranged from ODs 0.190 to 1.24. Twenty sera samples (ODs 0.398–1.24) with OD's >5 times of negative control (OD 0.075) were taken for immunoblotting. Specific IgE values with nymph extract in these 20 cockroach hypersensitive patients' sera (O.D's. 0.375–1.29) were similar to that of the PA extract (Fig. 3).

3.4. Allergenic proteins of *P. americana*

Immunoblot analysis of the PA extract with pooled patients' sera showed 22 IgE reactive proteins (Fig. 4A). Normal human sera used as a negative control did not show IgE reactivity. Immunoblots with individual patients' sera ($n = 20$) demonstrated diversity in the IgE binding patterns (Fig. 4B). Proteins of 38, 40, 72, 78 and 97 kDa reacted to 100%; 23, 35 and 49 kDa to 95% and 28 kDa to 80% of the cockroach positive patients' sera, and therefore, designated as the major IgE binding components. The other protein components such as 26, 22, 90, 83, 45 and 54 kDa reacted with 70%, 65%,

65%, 60%, 55% and 40% of patients' sera; the 31, 44, 51 and 115 kDa proteins reacted with 25% and the 60, 66 and 135 kDa reacted with 10%, 15% and 5% of the patients' samples, respectively. The Per a 1 antibodies bound to 26 and 40 kDa allergenic proteins in the PA extract on immunoblotting (data not shown).

The adult cockroach extract prepared in NH_4HCO_3 demonstrated 15 IgE binding bands on probing with pooled patients' sera (Table 1). The PBS extract with source material dried at 37 °C revealed 12 slightly diffused IgE binding bands with pooled patients' sera (Table 1). The IgE binding patterns and intensity of IgE reactive bands varied (6 to 11 allergenic bands) among commercial extracts (Fig. 5B) demonstrating substantial difference with the PA extract. Nymph extract demonstrated 22 IgE reactive bands, similar to the adult extract (PA extract) with pooled patients' sera. However, 72 and 78 kDa bands in the nymph extract showed a higher intensity of IgE binding (Fig. 4A).

4. Discussion

Cockroach extracts used currently for diagnosis of allergy are standardized using w/v basis and skin tests on patients, which do not measure the true quality of extract in terms of individual allergen content and allergenic potency. Commercial extracts for diagnosis and therapy are highly variable in potency and presence of major allergenic components. These discrepancies affect both outcome of diagnostic testing and the administration of allergen immunotherapy [25]. The present study was undertaken to obtain a consistent allergen preparation of *P. americana* from indigenous source material representing both major and minor allergens.

Prevalence of positive skin reactivity to cockroach allergens has been reported from USA (17% to 41%), Taiwan (55%), Brazil (55%) and Poland (24.3%) [5,6,10]. In present study, marked positive skin reactivity to *P. americana* was observed in 53% cases comparable to the reports from Taiwan and Brazil. But, previous reports from India show cockroach sensitization in the range of 8–26% by skin tests [16,17]. High incidence of skin reactivity in the present study may be due to the use of potent cockroach allergen extract. Variation in skin reactivity may occur due to variable allergen content of the extracts used for

Table 1
Comparison of different *P. americana* extracts

Allergen extract	Protein content (mg/gm of cockroach powder)	SDS-PAGE bands	IgE reactive bands	Allergenic potency (ng protein for 50% inhibition)
NH_4HCO_3 (freeze-dried, adult)	100	34	15	—
PBS (PA extract)	150	45	22	9–12
PBS (freeze-dried, nymph)	200	45	22	8.5
PBS (dried at 37 °C, adult)	75	25	12	400
Commercial extract 1	90	22	9	750
Commercial extract 2	96	27	11	450
Commercial extract 3	50	11	6	>1000

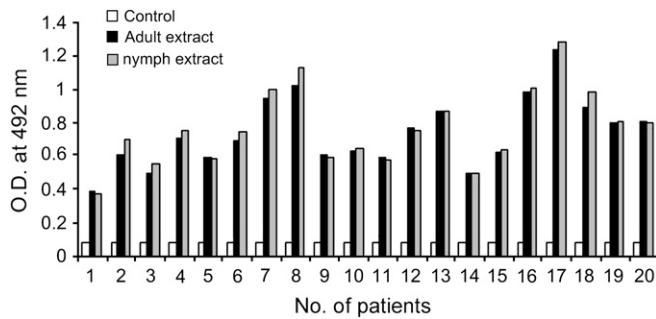


Fig. 3. Specific IgE values estimated by ELISA with adult (PA extract) and nymph stage cockroach extract using hypersensitive patients' sera. Absorbance was read at 492 nm; Control – normal human sera.

testing, increase in sensitivity, change in life style of people with greater exposure to indoor allergens, etc.

Earlier studies showed 28 protein bands on SDS-PAGE [10] and 29–50 antigen-precipitin bands in *P. americana* by crossed immunoelectrophoresis. Patterson and Slater [26] demonstrated that commercial cockroach extracts are highly variable in protein content, number of protein bands and potency. This discrepancy may be attributed to differences in source material processing, extraction buffer used, storage conditions, etc. In India, no cockroach reference extract is available. The PA extract (used as a standard in the present study) demonstrated significantly higher protein content, number of protein bands (45), allergenic bands (22) and potency as compared to the commercial extracts (Table 1).

In previous studies *P. americana* allergens were identified by analyzing fractionated extracts [10,27,28]. Wu et al. [13] detected five proteins of 28, 32, 45, 72 and 78 kDa as major

allergens with a prevalence of >80%. In present study, 9 major IgE binding components of 23, 28, 35, 38, 40, 49, 72, 78 and 97 kDa were recognized by immunoblotting with individual patients' sera. The 72 and 78 kDa proteins showed homology to arylphorin (Per a 3) an insect storage protein [29]. While 38 kDa major allergenic protein reacting with 100% of our patients correlates with Per a 7 [15]. Proteins of 26, 31, 45, 51 kDa identified here can be designated as Per a 1, which shows variations in molecular weights [14]. Thus, PA extract contains allergens as identified in previous studies and also establishes the importance of few more allergenic components (23, 35, 40, 49 and 97 kDa). The disparity in IgE reactivity of present extract and the extracts used in previous investigations may be due to variability of allergenic components among extracts and also the serum sources [12]. Both the nymph and adult stages of *P. americana* abundant in the Indian household demonstrated similar protein profile and IgE binding and hence, either can be used for allergy diagnosis by skin testing and RAST/ELISA.

Recently few major allergens from *P. americana* and *Blattella germanica* were characterized as insect storage protein [29], tropomyosin [15], aspartic protease [30] and glutathione-S-transferase [31]. Such immunodominant allergens are being explored for diagnosis and therapy of cockroach allergy [32]. In conclusion the present study demonstrates an increase in cockroach sensitization in atopic subjects. Immunoblotting identified 9 major allergens in adult cockroach extract (PA extract) comparable to nymph stage extract. *P. americana* allergen preparation (PA extract) developed here can be taken as in house reference standard for comparing future batches of extract relevant to management of allergy.

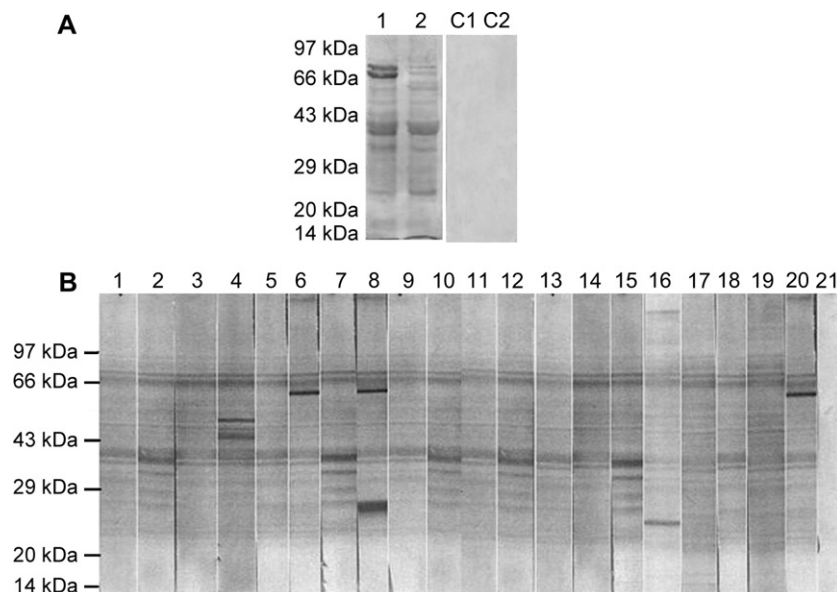


Fig. 4. (A) Immunoblot profiles of *P. americana* extracts with pooled patients' sera. Lane 1- Nymph extract prepared in PBS with freeze-dried source material, Lane 2- PA extract, Lanes C1 and C2- Nymph and PA extract probed with normal human sera respectively. (B) Immunoblots of PA extract with individual ($n = 20$) hypersensitive patients' sera (Lanes 1–20) shows IgE reactivity to cockroach allergens and Lane 21 probed with normal human sera.

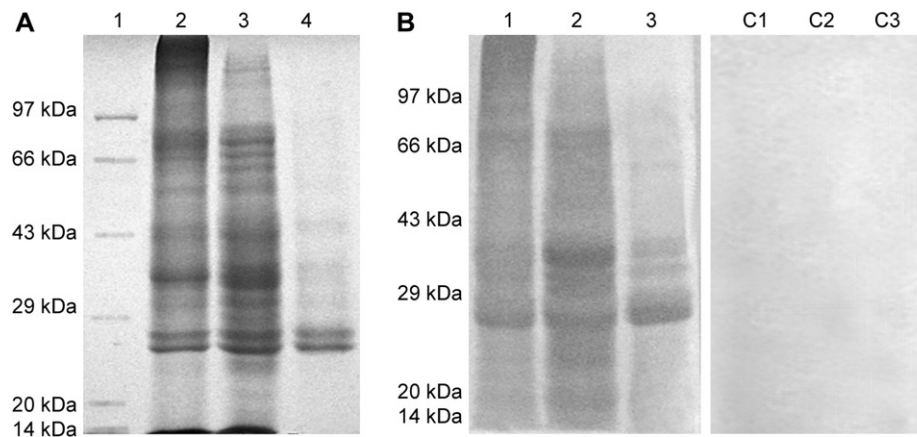


Fig. 5. (A) SDS-PAGE (10%) profile of commercial extracts of *P. americana*. Lane 1- Molecular weight marker, Lane 2- Commercial extract 1, Lane 3- Commercial extract 2 and Lane 4- Commercial extract 3. (B) Immunoblot profiles of commercial extracts of *P. americana* with pooled patients' sera. Lane 1- Commercial extract 1, Lane 2- Commercial extract 2, Lane 3- Commercial extract 3, Lanes C1, C2 and C3- Commercial extract 1, Commercial extract 2 and Commercial extract 3 probed with normal human sera respectively.

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